UCH-FC
DOC-B
M828
C.1

Amplificación y caracterización *in vitro* de cepas de priones

Tesis
entregada a la
Universidad de Chile
en cumplimiento parcial de los requisitos
para optar al grado de
Doctor en Ciencias con mención en Biología

Facultad de Ciencias
por

Rodrigo Francisco Morales Loyola

Junio, 2009

Director de Tesis: Dr. Claudio Soto

FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

INFORME DE APROBACIÓN

TESIS DE DOCTORADO

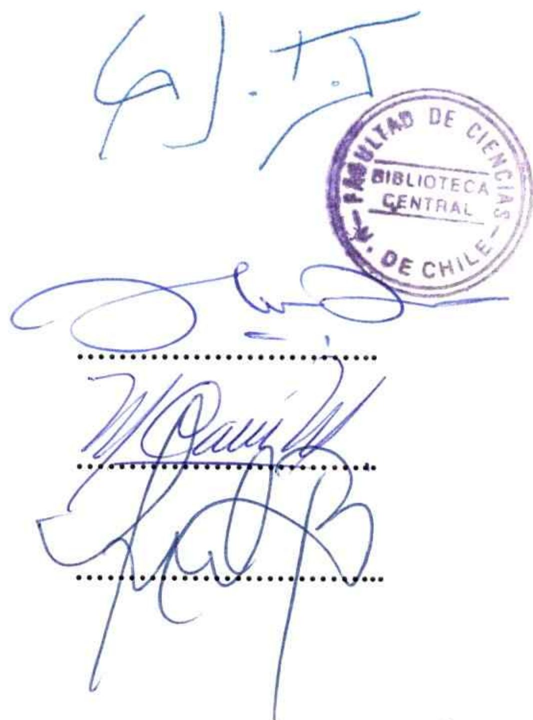
Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato

Rodrigo Francisco Morales Loyola

ha sido aprobada por la Comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Biología, en el exámen de Defensa de Tesis rendido el día diecinueve de junio de 2009.

Director de Tesis:

Dr. Claudio Soto



Comisión de Evaluación de la Tesis

Dr. Carlos Jerez ....

Dra. Margarita Carú ....

Dr. Christian González ....

A mis padres, mi norte en cada paso.

FACULTAD DE CIENCIAS
BIBLIOTECA
CENTRAL
U. DE CHILE

Llegué al mundo el día 13 de Agosto del año 1980, en momentos de gran agitación política y social para nuestra patria. Fui nacido y criado en la ciudad de Santiago de Chile. Mis estudios básicos los realicé en la comuna de Puente Alto, donde he vivido gran parte de mi vida. Mis estudios secundarios los realicé en el Instituto Nacional, lugar en el que aprendí muchas cosas que aún guían mis pasos, incluyendo el amor a mi país. Durante la estancia en esta institución fue que desarrollé mi interés por la bioquímica, carrera a la que ingresé en el año de 1999, en la Universidad de Chile. Esta época preciosa estuvo marcada por muchas cosas, donde además de la parte académica debo mencionar el trabajo en el Centro de Estudiantes, los amigos que ahí cultivé y el contacto que tuve con la disciplina del kendo.

Terminando mi licenciatura vine a probar suerte a tierras texanas donde mi mentor, el Dr. Claudio Soto, me dio la oportunidad de trabajar en un tema que siempre me apasionó mucho, los priones. Con el correr del tiempo fui también ingresando al estudio de la enfermedad de Alzheimer. Afortunadamente, fui bien recibido en un grupo líder en esta área. La mayor parte de mi tesis doctoral fue realizada en Galveston, Texas, mi primer destino en tierras estadounidenses. Lamentablemente, el huracán Ike en Septiembre del año 2008 nos obligó a cambiar nuestra ubicación física a Houston, donde actualmente me encuentro.

Agradecimientos.

FACULTAD DE CIENCIAS
BIBLIOTECA
CENTRAL
U. DE CHILE

Chile y resto del mundo:

En ciencia, a momentos las cosas son gratas pero la gran mayoría del tiempo más bien desaniman. La gente viene y va, pero la familia ha sido siempre un pilar sólido que me ha apoyado durante todo este tiempo. Gran parte de los valores fundamentales que me han formado se los debo a ellos. Gracias a mis padres por eso y a mis hermanas Pamela y Catalina, a Hugo y a mis sobrinos (Javiera y Matías) por su constante apoyo y preocupación.

En Chile, podría nombrar a mucha gente que constantemente me ha estado tirando buenas vibras, siempre pacientes en mis desahogos existenciales. Si los nombrara a todos, no me quedaría espacio para escribir mis palabras de aprecio. Entre ellos quiero hacer especial mención a Jaminton y Viviana quienes constantemente me ayudaron a desenredar muchas veces madejas de ideas mal hiladas.

Fuera de Chile quiero agradecer a grandes amigos que he conocido gracias a la ciencia o en alguno de mis extravíos por el mundo. Gracias especiales a Anita (Argentina) por sus constantes ánimos, a Sergio (España) por su buena onda y por sus consejos edificantes en ciencia, y a Alejandra (Venezuela) por todo su apoyo.

Texas:

Aunque en la cancha tratamos de ser un equipo ofensivo (3-1-2-3) eso se nos da bastante mejor en el laboratorio. En todo caso, en ambos frentes, nadie nos puede culpar de falta de empuje.

En primer lugar quiero agradecer al Dr. Claudio Soto, nuestro volante de creación (?), por inicialmente creer y apostar en mí y por seguir haciéndolo. Además de todo su apoyo en el desarrollo de esta tesis quiero agradecer el soporte que me ha dado para crecer y desarrollarme de una forma integral desde el punto de vista profesional.

A nuestro arquero, Baian Chen. A él gracias por todas sus ganas y por el entusiasmo que ha mostrado siempre en el trabajo. A Marcelo Barría mis agradecimientos son variados. Su apoyo dentro y fuera del laboratorio ha sido siempre algo importante que espero cultivar por mucho tiempo más. En resumen, gracias Marcelo por tu amistad. A Manuel Camacho, mis especiales agradecimientos por darse el tiempo de dar la primera ronda de corrección a este escrito.

En nuestro mediocampo y delantera también mis agradecimientos a Dennisse por su amistad durante todos estos años, a Aki por sus sugerencias en el laboratorio y a Rodrigo, Nata y Abhi por su constante buena onda.

Mis recuerdos a Lisbell Estrada por su apoyo en el comienzo de mi estadía en Texas, al Dr. Joaquín Castilla por su instrucción y apoyo en el inicio de muchos de los proyectos presentados en esta tesis, a Cristián Lasagna por discusión y sugerencias en muchos de los experimentos realizados y a Sylvia Magallan por su paciencia.

Protein Misfolding Disorders
Laboratory - 2009

Resumen.

El mal plegamiento y la agregación de proteínas es la principal característica de un grupo de enfermedades denominadas Desórdenes de Conformación Proteica (DCP). Los DCP incluyen a las Encefalopatías Espongiformes Transmisibles (EET) y las enfermedades de Alzheimer (EA) y de Parkinson, entre muchas otras. Diversos hallazgos demuestran que el único componente infeccioso en las EET corresponde a la forma anómalamente plegada de la proteína prion (denominada PrP^{Sc}). Se ha establecido que los priones infecciosos, como otros micro-organismos convencionales, exhiben variaciones de cepa. Las cepas de priones se pueden diferenciar por sus características particulares *in vivo* e *in vitro*, las que incluyen a los signos clínicos, diferencias en los períodos de incubación, los patrones de lesión cerebral y las propiedades bioquímicas de la PrP^{Sc}. En este trabajo hemos demostrado que las características de las cepas de priones pueden ser replicadas *in vitro* utilizando la técnica denominada *Protein Misfolding Cyclic Amplification* (*PMCA*). Nuestros resultados muestran la amplificación *in vitro* de varias cepas de priones establecidas en ratón. Interesantemente, los nuevos priones generados mantienen las propiedades bioquímicas y biológicas de su fuente original. La amplificación *in vitro* de cepas de priones fue expandida a modelos de ratones $sinc^{s7}$ y $sinc^{p7}$, polimórficos para la proteína del prion, como también cruzando barreras de especies (propagación inter-especie de priones). También hemos demostrado que dos DCP como la EA y las EET pueden interactuar al nivel de los agregados proteicos formados por las proteínas anómalamente plegadas. Adicionalmente, nosotros demostramos que los agregados de Aβ en la EA pueden ser transmitidos *in vivo* en un fenómeno similar al que ocurre con la propagación de priones en las EET. Nuestros resultados sugieren que las cepas de priones se pueden mantener al replicar a la PrP^{Sc} *in vitro* sugiriendo que sus características se encuentran exclusivamente en su estructura. Por último, nuestros resultados sugieren que la agregación de Aβ puede ser inducida por la adición exógena de tejidos “infectados” de pacientes afectados por la EA de forma similar a como ocurre en las EET y que un DCP puede ser un factor de riesgo para el desarrollo de un segundo DCP.

FACULTAD DE CIENCIAS
BIBLIOTECA CENTRAL
U. DE CHILE

Abstract.

Misfolding and aggregation of proteins is the main feature of a group of diseases, termed Protein Misfolding Disorders (PMDs). PMDs include Transmissible Spongiform Encephalopaties (TSEs), Alzheimer's (AD) and Parkinson's diseases, among many others. Compelling evidences show that the only component of the infectious agent in TSEs is the misfolded form of the prion protein (termed PrP^{Sc}). It is well established that the prion infectious agent, like conventional micro-organisms, exhibit strain variation. Prion strains can be differentiated by their particular *in vivo* and *in vitro* characteristics, including clinical signs, differences in incubation period, pattern of brain lesions and biochemical properties of PrP^{Sc}. Here it is demonstrated that prion strains characteristics can be replicated *in vitro* by Protein Misfolding Cyclic Amplification (PMCA). Our results show *in vitro* amplification of various established mouse prion strains. Interestingly, the newly generated prions maintain the biochemical and biological features of their original sources. Furthermore, i*n vitro* replication of prion strains was expanded to $sinc^{s7}$ and $sinc^{p7}$ polymorphic mice models as well as crossing species barriers (interspecies propagation). We also show that two PMDs such as AD and TSEs can interact at the level of the misfolded protein aggregates. In addition we demonstrated that Aβ aggregates in AD can be transmitted *in vivo* by a similar phenomenon of prion propagation as occurring in TSEs. Our results suggest that the strain characteristics of prions can be maintained by *in vitro* replication of PrP^{Sc} and therefore, that strain phenomenon is enciphered exclusively on the prion structure. In addition, our results suggest that Aβ aggregation can be induced by exogenous addition of "infected" tissues from AD patients in a similar way as occurring for TSEs and that one PMDs could be a risk factor for the development of a second one.

FACULTAD DE CIENCIAS
BIBLIOTECA CENTRAL
DE CHILE

ÍNDICE.

Sección I: Introducción 16
I.1. Introducción a los Desórdenes de Conformación Proteica (DCP) 16
I.2. La Enfermedad de Alzheimer 18
I.3. Las Encefalopatías Espongiformes Transmisibles, enfermedades producidas por priones o prionopatías 21
I.4. Cepas de priones, polimorfismos y el fenómenos de barrera de especie en las Encefalopatías Espongiformes transmisibles 29
I.5. Interacción de distintos agregados proteicos en los Desórdenes de Conformación Proteica y el fenómeno de nucleación heteróloga 35
I.6. La infectividad intrínseca de los agregados amiloides. El caso de la Enfermedad de Alzheimer 37
I.7. Estado actual del estudio de los Desórdenes de Conformación Proteica. Diagnóstico y terapia 39

Sección II: Hipótesis 40

Sección III: Objetivos 41
III.1. Objetivo general 41
III.2. Objetivos específicos 41

Sección IV: Resultados 42
IV.1. Replicación de cepas de priones en sistemas libres de células 42
IV.2. Validación del fenómeno de barrera de especies en las EET utilizando sistemas in vitro 45
IV.3. Cruces de barrera por replicación de PrP^{Sc} *in vitro* generan nuevas cepas de priones 47
IV.4. Cruces *in vitro* de PrP^{Sc} utilizando sistemas polimórficos 50
IV.5. Nucleación heteróloga de amiloides 51
IV.6. Proyecciones: mecanismos infectivos de los amiloides 53

Sección V: Discusión 57

Sección VI: Bibliografía 63

FACULTAD DE CIENCIAS
BIBLIOTECA CENTRAL
U. DE CHILE

SECCIÓN I: INTRODUCCIÓN.

1. Introducción a los Desórdenes de Conformación Proteica (DCP).

Características generales de los Desórdenes Conformacionales Proteicos y su impacto en la sociedad

El ADN es la herramienta encargada de almacenar la información necesaria para llevar a cabo los procesos biológicos necesarios para la vida. Sin embargo, esta información lineal no es utilizada hasta que es expresada tridimensionalmente por su producto de relevancia biológica final, las proteínas. Para que esta información sea correctamente utilizada, las proteínas deben adquirir un plegamiento adecuado que depende de la secuencia de ésta y de la maquinaria accesoria de plegamiento presente en la célula. Procesos anormales de plegamiento pueden resultar en la pérdida de la función normal de la proteína o en una ganancia de efectos tóxicos, lo cual podría desembocar en eventos patológicos en ambos casos [1-3]. En muchos casos, ciertos procesos de plegamientos anómalos (conocidos en lengua inglesa como *misfolding*) pueden resultar en la agregación de ciertas proteínas en tejidos específicos [1]. Como consecuencia de estos eventos muchas condiciones patológicas se han identificado y denominado Desórdenes de Conformación Proteica (DCP). Los DCP incluyen muchas enfermedades como las enfermedades de Alzheimer (EA), Parkinson (EP), Hungtingon (EH), la diabetes tipo-2 y las Encefalopatías Espongiformes Transmisibles (EET) o enfermedades producidas por priones, entre otras [1].

Se ha sugerido que la deposición de agregados proteicos en estas enfermedades es responsable de los daños tisulares y de los consecuentes cuadros clínicos generados. El tipo de daño que se produce en la gran mayoría de estas enfermedades es dramático, afectando de gran manera la calidad de vida del paciente y del entorno familiar en el cual vive. La degeneración progresiva manifestada en estas enfermedades arrastra lamentables consecuencias desde el punto de vista económico y social. Estas secuelas aumentan con la progresión de la enfermedad, repercutiendo no solo en el paciente sino también en su familia y seres cercanos [4]. Una lamentable excepción se da en el caso de las prionopatías humanas donde la etapa clínica de la enfermedad dura aproximadamente 6 meses, con un rápido deterioramiento del individuo seguido de su muerte [5]. Estudios epidemiológicos estiman que el número de pacientes afectados por DCP aumentará considerablemente en los próximos años [6]. Si tomamos en cuenta estos pronósticos y el hecho de que en este momento no existen claras estrategias diagnósticas o de terapia para estas enfermedades, un oscuro escenario se augura para el futuro. Por estas razones es muy importante conocer las bases moleculares que gobiernan el desarrollo de estas enfermedades. Con el conocimiento y las herramientas adecuadas se podría, eventualmente, frenar el aumento en el número de víctimas afectadas por estas patologías.

En cuento a la etiología de estas enfermedades, estas se han asociado principalmente a un origen esporádico, es decir, su génesis es desconocida. En términos generales se especula que la agregación proteica en estos casos está favorecida hacia la conformación patogénica. Actualmente existen muchas hipótesis que explican este fenómeno, pero en general se atribuye que el desplazamiento del equilibrio a la forma mal plegada de la proteína se debe a un funcionamiento anómalo de la maquinaria de plegamiento y degradación proteica, entre otros (apéndice 1). Estos procesos se exacerban con la edad, lo cual explica el porqué estas enfermedades tienden a aparecer en etapas tardías de la vida [6;7]. La segunda preferencia en la etiología de estas enfermedades proviene de la mutación en las proteínas involucradas, lo cual

favorece su deposición. Estas mutaciones usualmente resultan en una herencia dominante de la enfermedad [8-16]. Finalmente, podemos encontrar un origen infeccioso. En la actualidad esta propiedad solo se ha atribuido sola las EET [17].

Bases moleculares de los Desórdenes de Conformación Proteica: el modelo de nucleación-polimerización de proteinas amiloidogénicas.

A pesar que las enfermedades en este grupo difieren fuertemente en sus características clínicas, los tejidos afectados y la composición de los depósitos proteicos, todas ellas poseen mecanismos moleculares similares que explican su origen y progresión. Aunque los agregados proteicos en estas enfermedades se componen de diferentes elementos, los tipos de estructuras que ellos generan, desde el punto de vista morfológico y bioquímico, son bastante similares. Este tipo de agregados han sido indistintivamente denominados como amiloides. Desde el punto de vista estructural, las proteínas presentes en este tipo de estructuras son ricas en plegamiento del tipo β, lo cual aumenta la hidrofobicidad de las estructuras y favorece la agregación en estructuras específicas denominadas β-entrecruzadas [1;18]. Esta nueva disposición conformacional confiere a las proteínas resistencia a proteólisis, denaturación y a mecanismos generales de eliminación proteica en la célula. Los factores celulares que pueden guiar los procesos de mal plegamiento proteico han sido parcialmente identificados. Entre ellos, quizás el más importante está relacionado con las diferencias en la secuencia proteica. Sustituciones puntuales se han descrito como promotoras de una conformación amiloidogénica, lo cual puede favorecer la agregación y la subsecuente progresión patológica [19].

Los procesos de agregación proteica en estas patologías no son azarosos, sino más bien procesos lentos y organizados. Estos eventos han buscado una explicación en muchos modelos moleculares. Entre ellos, el más ampliamente aceptado es el modelo de nucleación-polimerización [20]. Brevemente, el modelo de nucleación-polimerización propone dos fases en la cinética de agregación proteica: una inicial y lenta dictada por los procesos de *misfolding* iniciales que resultarán en los primeros "núcleos" o "semillas" de polimerización, seguidos de una etapa rápida correspondiente a la acumulación exponencial de nuevas unidades a los agregados previamente formados (apéndice 2). Una vez que el proceso de agregación se ha establecido, estas semillas generan una población dinámica de oligómeros, lo cual subsecuentemente desemboca en la formación de fibras amiloidogénicas y en algunos casos deposición en forma de placas (apéndice 1 y [21]). La distribución dinámica de estas estructuras es característica para cada enfermedad y de la variante conformacional adquirida (como se observa para distintas cepas de priones (apéndice 2). Estos distintos agregados se han asociado con el inicio y la progresión de las distintas enfermedades de este grupo [21]. Ensayos *in vitro* utilizando distintos colorantes como Rojo Congo y Tioflavina T (específicos para estructuras amiloides) han demostrando que la fase de nucleación en estos procesos es altamente dependiente de la concentración inicial de la proteína [22].

A pesar de la relación existente entre este tipo de agregados y el desarrollo de la enfermedad, los mecanismos que llevan al daño celular típico de estas patologías es aún fuente de discusión. Evidencia experimental reciente, sugiere que oligómeros solubles, en la ruta de conversión a estructuras poliméricas más complejas, serían las especies más tóxicas en estas enfermedades [23]. En base a estos resultados se ha postulado que grandes agregados compuestos de fibras y placas amiloides podrían actuar como un mecanismo de defensa al atrapar estas partículas tóxicas y encapsularlas en los tejidos involucrados.

Del gran conjunto de enfermedades en este grupo hay dos interesantes que deseo destacar: la enfermedad de Alzheimer (EA) y las Encefalopatías Espongiformes Transmisibles (EET). La primera es el tipo de demencia más común a nivel mundial, mientras que la segunda ha sido destacada por sus singulares mecanismos de transmisión y por la epidemia de Encefalopatía Espongiforme Bovina en la década de los 90 del siglo pasado. El estudio de los mecanismos moleculares y patológicos de estas enfermedades fueron los que ocuparon mi trabajo de tesis y son los que en cierto detalle me ocuparé a continuación.

2. La Enfermedad de Alzheimer

Datos epidemiológicos de la enfermedad de Alzheimer.

Estudios epidemiológicos indican que el aumento en el número de individuos afectados por demencia está directamente relacionado con la edad, sin discriminar en grupos sociales, culturales o raciales. Los pronósticos actuales sugieren que en el año 2050 el número de individuos sobre 60 años alcanzaría la cifra de dos billones de personas, por lo que la demencia sería un grave problema de salud pública. Las demencias más comunes entre adultos mayores son la EA, la EP, y la demencia fronto-temporal. Considerando estos datos y la vejez progresiva que la población está adquiriendo se estima que para el año 2040 el número de personas que estarán afectadas por algún tipo de demencia incremente de 8.1 millones en la actualidad a mas de 24 millones [6].

Entre todos los tipos de demencia presentes en el adulto mayor, la EA es el tipo de demencia más común. Ésta se caracteriza principalmente por una disminución en las capacidades cognitivas y motoras en los pacientes afectados. La etapa clínica en esta enfermedad es larga y costosa en términos de terapia y cuidados, especialmente en etapas avanzadas, cercanas a la muerte del individuo [24]. Gran parte de los eventos degenerativos que se observan en estas enfermedades son causados por los mecanismos patológicos descritos en el siguiente punto.

Características patológicas de la enfermedad de Alzheimer.

Las principales características patológicas en esta enfermedad fueron por primera vez descritas en 1907 por Alois Alzheimer [25]. La enfermedad de Alzheimer presenta muchas características patológicas entre ellas, la principal es la agregación progresiva del péptido beta-amiloide (Aβ) en el cerebro de los pacientes afectados [26]. Agregados extracelulares de esta proteína son típicos en esta enfermedad, los que pueden ser encontrados tanto en la forma de placas altamente reactivas a tioflavina S (placas maduras) como sin reactividad a ella (placas difusas) [21]. Las deposiciones de este péptido pueden ser variadas, pero principalmente se localizan en la corteza cerebral, hipocampo y amigdala [27]. Los agregados de Aβ han sido descritos como los principales causantes de los efectos patológicos en estas enfermedades. Es por este motivo que las principales terapias para atacar este síndrome actualmente apuntan al bloqueo de la producción y agregación de esta proteína [28].

Otra característica importante encontrada en los pacientes afectados por esta enfermedad incluye la agregación intracelular de ovillos neurofibrilares de la proteína tau hiperfosforilada [29]. La proteína tau es una proteína normalmente asociada con la organización del citoesqueleto, pero su agregación ha sido descrita en diversos eventos patológicos neurodegenerativos además de la EA [29-31]. Los agregados de esta proteína han sido

principalmente descritos en células corticales o piramidales, aunque su ubicación puede cambiar de acuerdo al síndrome que el paciente presente [29]. En suma se ha demostrado que estos agregados son capaces de interactuar con agregados de Aβ en modelos animales, sugiriendo que una interacción entre ambas estructuras podría favorecer la progresión de la enfermedad [32;33].

Los agregados de Aβ y Tau presentes en la EA se han descritos como tóxicos en distintos modelos, tanto *in vitro* como *in vivo* . Una de las consecuencias de estas agregados en el cerebro es la inducción de inflamación [40;41], aunque se ha visto que las acumulaciones de estas proteínas pueden ser tóxicas directamente. Actualmente no es del todo claro como estos agregados inducen daño. Además de la inducción de muerte celular asociada a estos agregados se ha visto que ellos son capaces de producir defectos en la actividad cerebral (como defectos sinápticos) lo que sería la causa de los signos clínicos manifestados [42]. Los signos clínicos principalmente encontrados comienzan con problemas en la memoria del individuo, los cuales se incrementan a medida que la enfermedad avanza con déficits de la memoria a largo plazo, anomia, afasia, agnosia y apraxia. Finalmente, un debilitamiento producto de la enfermedad lleva al individuo a un deterioro crónico que finalizan con su muerte [28].

Mecanismos de plegamiento anómalo y agregación de Aβ

Como se mencionó con anterioridad, la agregación de Aβ es el principal evento asociado al desarrollo patológico de esta condición. Es por este motivo que en este punto me enfocaré principalmente en los mecanismos de agregación de este péptido.

Aβ asociado a la EA está compuesto por una cadena peptídica de entre 37 y 43 aminoácidos, aunque la población mayoritaria de agregados en los pacientes afectados está constituída por agregados de 40 ($A\beta_{40}$) y 42 ($A\beta_{42}$) residuos [44]. Aunque ambas proteínas son capaces de formar estructuras amiloidogénicas, estudios *in vitro* han demostrado que $A\beta_{42}$ presenta esta cualidad en mayor grado [45]. Ambos péptidos se generan a partir de la proteína precursora de amiloide (PPA), la cual es una proteína de membrana con funciones aun no del todo claras. La PPA puede sufrir un procesamiento proteolítico por tres distintas enzimas, denominadas α-, β- y γ- secretasas. La combinación de los cortes proteolíticos de están enzimas pueden dar cuenta de péptidos diversos, siendo solo la combinación entre β- y γ- secretasas la que produce el péptido que ocupa nuestro interés [46].

Como se ha descrito para el modelo de nucleación-polimerización, la agregación de Aβ es dependiente de la concentración inicial de esta molécula. Evidencia de esto lo podemos observar en individuos que padecen de síndrome de Down quienes poseen una trisomía en el cromosoma 21, el cual es portador del gen que codifica a la PPA. Por lo tanto, estos individuos generan una cantidad mayor de Aβ en comparación con individuos con una dosis génica normal, lo que resulta en la agregación de Aβ en etapas tempranas de la vida [47]. Desde otro punto de vista, se ha descrito que mutaciones en la PPA o en las secretasas (que resulten en un aumento de la producción de Aβ) son capaces de favorecer la deposición de placas seniles en la gran mayoría de los casos [28;31]. Además, muchas de las mutaciones en Aβ son capaces de estabilizar una conformación amiloidogénica. De esta forma la generación de la patología se favorece sin una necesaria sobreproducción del péptido en cuestión. En base a esta información se han descrito muchos modelos animales de la EA. En el siguiente punto mencionaremos algunos de ellos.

Modelos animales de la enfermedad de Alzheimer basados en la agregación de Aβ

Un modelo animal ideal debe imitar ciertas características establecidas de la enfermedad en estudio. Entre ellas, el modelo debe representar la etiología, patofisiología, sintomatología y respuesta a terapia tal como se observaría en humanos. Sin embargo, la gran mayoría de los modelos animales se enfocan solamente en aspectos específicos de la enfermedad. Han sido descritos pocos modelos que naturalmente presentan deposición de Aβ en el cerebro han sido descritos. Entre ellos podemos destacar cerdos de Guinea (*Cavia porcellus*), perros (*Canis lupus familiaris*,) y primates (*Macaca mulatta*) [41]. Los últimos son un excelente modelo para estos estudios debido a las similitudes anatómicas y genéticas con el ser humano. Sin embargo su uso es limitado debido al gran costo que significa el mantener este tipo de animales de experimentación. Por este motivo, se han buscado distintas alternativas en roedores para satisfacer de una forma controlada la agregación de Aβ en sistemas vivos.

Una primera aproximación experimental a la EA en sistemas vivos involucró la inyección de agregados de Aβ en ratones y ratas [42]. Este tipo de ensayos permitía alojar depósitos de Aβ similares a una placa en el cerebro de estos roedores y de esta forma evaluar en cierta medida los efectos que este tipo de estructuras podía tener en un sistema vivo [33]. Estos modelos también se utilizaron para evaluar el efecto de algunas drogas diseñadas para desestabilizar este tipo de arreglos proteicos [42]. Con el desarrollo de modelos transgénicos ha sido posible analizar el efecto de distintas mutaciones en la PPA (o en las enzimas que procesan a la PPA) y evaluar su contribución en el desarrollo de las placas seniles. El primer modelo reportado fue el denominado PDAPP [43], seguidos posteriormente por los tg2576 [44] y APP23 [45], entre otros [28;34]. Entre ellos es importante destacar al llamado tg2576 ya que es probablemente el modelo animal más comúnmente utilizado en el estudio de la EA [44]. Este modelo expresa la PPA humana con una mutación en el aminoácido 670. Esto resulta en un mayor procesamiento por parte de la β-secretasa, incrementando los niveles de Aβ en el cerebro del animal. La deposición de Aβ en estos animales comienza a los 10 meses de edad como placas difusas, las cuales se vuelven densas y reactivas a tioflavina S a los 12 meses de edad. En resumen, este modelo presenta claros niveles de Aβ humana en tejidos periféricos y fluidos biológicos, cantidad que aumenta a medida que el animal envejece. Sumado a estas características se ha descrito un decaimiento sostenido en las propiedades cognitivas de estos animales, descritas principalmente a través de ensayos como el *Water Maze* de Morris, *Fear Conditioning*, entre otros [44;46]. Este modelo animal ha sido extensivamente utilizado para ensayar nuevas estrategias terapéuticas, estudiar la dinámica de agregación de Aβ e identificar mecanismos moleculares de degeneración en esta patología. Sin embargo, este modelo carece de una importante propiedad de la EA como lo es la muerte neuronal. Otros modelos animales desarrollados con distintas mutaciones en la PPA, Tau o secretasas, han sido exitosos en replicar muchas de las características patológicas de la EA pero ninguno da cuenta de un desarrollo cabal de la enfermedad [28].

Otro modelo animal descrito, aunque no tan extensivamente usado es el modelo denominado HuAPPwt, modelo que sobre-expresa la PPA humana (sin mutaciones). Esta sobre-expresión resulta en la presencia de Aβ soluble en el cerebro de estos animales pero sin señales de deposición [47]. La sola presencia de Aβ (probablemente en forma de oligómeros) en estos animales es suficiente para producir una disminución en los niveles de sinaptofisina, proteína marcadora de conexiones sinápticas entre neuronas, reafirmando la naturaleza tóxica de esta molécula.

Estrategias de diagnóstico y terapia en la actualidad

Actualmente, las principales estrategias de terapia y diagnóstico para la EA se basan en frenar la deposición de Aβ en el cerebro en los individuos afectados [48-50]. Entre las estrategias de diagnóstico en la EA, la más clásica incluye seguir el deterioro de las características cognitivas, de aprendizaje y de memoria en los modelos de estudio. Otras estrategias involucran la medición de distintos metabolitos y/o activaciones de cascadas transduccionales en el cerebro [51]. Entre estas moléculas, el seguimiento de Aβ (en sus distintas etapas de agregación) es nuevamente una herramienta recurrentemente utilizada. Recientemente se ha descrito la presencia de Aβ en distintos fluidos biológicos como líquido céfalo-raquídeo (LCR) y sangre, lo cual ha dado prometedoras proyecciones para el futuro [52].

Las terapias enfocadas a prevenir el mal plegamiento y agregación de proteínas no solo se ven como prometedoras en aliviar los síntomas de la enfermedad sino que además como instrumentos que puedan revertir del todo la condición patológica. Actualmente, las terapias enfocadas a todos los DCP son solo paliativas. Varias estrategias se han desarrollado para atacar los procesos de mal plegamiento y agregación proteica. Algunas de ellas consisten en atacar directamente las proteínas anómalamente plegadas, las cuales pueden actuar como núcleos de agregación. Otras estrategias, consisten en inhibir el proceso de mal plegamiento en sí. La primera podría permitir la eliminación de semillas putativas, mientras que la segunda podría inhibir la formación de unidades convertidas *de novo*. Ambas aproximaciones podrían ayudar al funcionamiento del sistema de eliminación de proteínas mal plegadas, resultando en una depleción total de las estructuras asociadas con la enfermedad. Las estrategias de terapia actualmente seguidas para la EA involucran principalmente la búsqueda de pequeñas moléculas inhibitorias de estructura β en proteínas [42;49;53], vacunas [50;54], y el uso de "anticuerpos conformacionales" [55]. En la primera son importantes de destacar los *β-sheet breakers*, los cuales corresponden a pequeñas moléculas que inhiben la agregación proteica [42;53]. La búsqueda de análogos y péptido-miméticos a estas moléculas en librerías de compuestos químicos es foco de muchos grupos de investigación en la actualidad [48;49]. Entre la estrategia de las vacunas podemos resaltar los ensayos realizados por Elan Pharmaceuticals, donde una vacuna basada en péptidos sintéticos de Aβ resultó muy prometedora en modelos animales [54]. Desafortunadamente, una fracción de los pacientes involucrados en estudios clínicos posteriores desarrolló meningoencefalitis, resultando en la muerte de algunos individuos de este estudio [56;57]. Actualmente se están evaluando otras aproximaciones que presenten una respuesta inmune controlada pero suficientemente efectiva. Finalmente, el reciente desarrollo de anticuerpos que apuntan específicamente a la conformación mal plegada de la proteína, independiente de su secuencia, resultan atractivos como una posible terapia universal para todas las enfermedades en este grupo. Sin embargo, nuevas hipótesis que apuntan a un origen infeccioso de la EA (apéndice 4), plantean un escenario bastante sombrío en el futuro.

3. Las Encefalopatías Espongiformes Transmisibles, enfermedades producidas por priones o prionopatías

Características epidemiológicas de las prionopatías

Las EET son enfermedades raras y fatales que afectan de forma natural tanto a animales como a humanos. Entre ellas podemos destacar en humanos a las enfermedades de Creutzfeldt-

Jakob (ECJ), el insomnio fatal familiar (IFF), el síndrome de Gerstmann-Straussler-Scheinker (GSS), entre otros. En animales son de importancia el mencionar el scrapie en cabras y ovejas, la encefalopatía espongiforme bovina (EEB) y la enfermedad debilitante crónica (EDC) de ciervos [5;58].

Las enfermedades producidas por priones son enfermedades de baja incidencia. Aún así, su etiología tripartita (infecciosa, hereditaria y esporádica) y los mecanismos de transmisión y progresión que presentan han atraído bastante la atención de la comunidad científica y médica. Estudios clínicos indican que la gran mayoría de las EET corresponden a casos esporádicos (90%), mientras que los casos hereditarios (7%) y transmisibles (3%) son bastante escasos [17]. Entre todos los DCP, las EET son las únicas definitivamente descritas como transmisibles.

La transmisibilidad de estas enfermedades fue por primera vez reportada en 1937, cuando ciertas preparaciones de vacunas obtenidas a partir de cerebro de ovejas se utilizaron para inocular otros ovinos [59]. En años posteriores se describió que derivados de cadáveres (hormona de crecimiento o córneas) destinadas a trasplantes fueron capaces de transmitir la enfermedad a individuos sanos [5;17;60;61]. Sorprendentemente, el uso de instrumental quirúrgico en pacientes afectados es capaz de transmitir la enfermedad incluso luego de ser autoclavado [62].

Lo que finalmente dio a los priones su reconocimiento a nivel mundial fue la epidemia de EEB (o enfermedad de las vacas locas) en la década de los años 90 del siglo pasado [63;64]. El lazo establecido entre esta enfermedad y el desarrollo de la nueva variante de la enfermedad de Creutzfeldt Jakob (vECJ) puso en alarma a las autoridades sanitarias de todo el mundo, principalmente en Europa [65;66]. Los largos períodos de incubación que caracterizan a las EET llevan a pensar que la vECJ está en un proceso de incubación en la población, y que el pico en el número de casos positivos está aún por venir.

De importancia considerable es lo que ocurre en la industria ganadera cuando se consideran los casos de EEB y scrapie en bovinos y ovinos, respectivamente. La industria ganadera en los países afectados sufre considerables pérdidas cada vez que un caso de EEB es diagnosticado. Aun cuando se han hecho muchos esfuerzos para erradicar esta enfermedad su presencia real y el fantasma de la infectividad que la sigue siempre están presentes. Por otro lado, el scrapie es una enfermedad que se ha mostrado como muy persistente y difícil de erradicar. Muchas variantes de esta enfermedad han sido aisladas, tanto por la gran cantidad de casos que se presentan como por la gran variedad de ovejas que afectan [67]. Otro caso importante de mencionar es la EDC de ciervos, la que afecta a una gran proporción de los ciervos salvajes principalmente en los Estados Unidos y Canadá, [68]. Interesantemente, los casos de ciervos naturalmente afectados siguen en aumento, por lo que muchos grupos de investigación han dedicado sus esfuerzos a elucidar tanto los mecanismos de transmisión presentes en estas enfermedades como las posibles consecuencias de transmisión inter-especies que eventualmente podrían existir entre esta especie y el ser humano.

Como en el caso de la EA, la falta de terapias y métodos diagnósticos exitosos proyectan un escenario bastante oscuro si ocurriera una epidemia de EET.

La patología de las Encefalopatías Espongiformes Transmisibles

Entre las características más importantes en este grupo de enfermedades se incluyen los largos períodos de incubación y la falta de inmunogenicidad que presentan. Inicialmente se pensó que el agente infeccioso en estas enfermedades correspondía a un lentivirus [69] y la falta

de inmugenicidad fue atribuida la producción de compuestos propios del organismo receptor. Diferente al caso de la EA, las EET poseen una etapa sintomatológica corta. Usualmente entre los primeros síntomas (que incluyen depresión, problemas cognitivos y/o problemas motores) se observa una progresión bastante rápida de los signos clínicos. El período de tiempo entre los primeros signos clínicos y la muerte del individuo está en el rango de 6 a 18 meses [5].

Las características clínicas de esta enfermedad se pueden asociar claramente con el daño presente en el cerebro, mientras que otros órganos periféricos no se ven directamente afectados. Como su nombre formal lo refiere, la principal característica de las Encefalopatías Espongiformes Transmisibles es la degeneración espongiforme del cerebro. Este tipo de lesiones se encuentra en distintas regiones del cerebro de acuerdo a la naturaleza del agente infeccioso (lo cual se tratará en secciones siguientes). Otra característica importante en estas enfermedades es la acumulación en el cerebro de una forma anómalamente plegada de la proteína prion o PrP

Tabla 1. Índice de abreviaturas.

PrP	Abreviatura general dada a la proteína prion
PrP^C	La proteína prion fisiológica, normalmente expresada. Esta proteína posee un puente disulfuro, un tallo de glicosilfosfatidilinositol (GPI) y dos sitios putativos de glicosilación. Esta proteína posee una alta proporción de estructura a-helical.
PrP^{Sc}	Es una isoforma de PrP^C la cual es rica en estructura ß. El término PrP^{Sc} está ligado a infectividad, independiente de otras características que este agente infeccioso pueda poseer.
$PrP^{27\text{-}30}$	El fragmento de digestión proteolítica (PK) de la PrP^{Sc}. Su nombre se debe a la movilidad electroforética específica del segmento proteico resistente a proteólisis (forma diglicosilada).
PrP^{res}	PrP resistente a proteasas, la cual puede corresponder a $PrP^{27\text{-}30}$ o a otras formas de PrP resistentes a proteasas no ligadas a infectividad.
$sPrP^{Sc}$	PrP^{Sc} sensible a proteasas.
rPrP	PrP recombinante (producida en *Escherichia coli*, carente de GPI y glicosilaciones
EET	Encefalopatías Espongiformes Transmisibles
DCP	Desórdenes de Conformación Proteica
EA	Enfermedad de Alzheimer
EP	Enfermedad de Parkinson
EH	Enfermedad de Hungtington
ADN	Ácido Desoxirribonucleico
PPA	Proteína Precursora del Péptido ß-amiloide
Aß	Péptido ß-amiloide o Amiloide ß

(tabla 1). La agregación del prion en estas enfermedades recuerda a lo que es la agregación de Aβ en la EA. A diferencia de la EA, en las EET se puede encontrar una gran variedad de agregados, los cuales varían de acuerdo al síndrome en cuestión (apéndice 3). La variedad de agregados de PrP que se pueden encontrar fluctúan entre placas densas, agregados difusos y en algunos casos ausencia de agregados visibles por técnicas convencionales como inmunohistoquímica o *histoblotting* [11;70]. Otra característica interesante de analizar es la inflamación e inducción de cascadas de señalización específicas que se producen en el cerebro de

los individuos afectados, la cual emula a lo que ocurre en la EA y que lleva a pensar que mecanismos de toxicidad similares podrían estar actuando en ambos casos (apéndice 5 y [1;71]).

¿Qué es el prion?

Como se mencionó en el punto anterior, una de las principales características patológicas de las EET corresponde a la acumulación de una proteína constitutivamente expresada en el cerebro, la cual se ha denominado prion (PrP). El agente infeccioso se genera a partir de una proteína normal y ubicuamente expresada denominada PrP^C (tabla 1). Aunque PrP^C está presente en la gran mayoría de los tejidos, su ubicación principal está asociada al sistema nervioso central (SNC), mayoritariamente a las balsas lipídicas de la membrana neuronal [71]. Esta proteína, que tiene un peso molecular de aproximadamente 36KDa y está compuesta de 230 aminoácidos (el cual puede variar entre especies), posee dos sitios putativos de glicosilación en las posiciones 181 y 197 [17]. Como consecuencia, esta proteína puede presentarse en sus formas diglicosilada, monoglicosilada y sin glicosilar. Esta proteína posee además un puente disúlfuro en su estructura, lo que juega un papel importante en su plegamiento normal y conversión patogénica. En condiciones normales, esta proteína es principalmente rica en estructura α, lo que estructuralmente diferencia a la forma asociada a la enfermedad que contiene una mayor proporción de estructura β [72]. La función de esta proteína en condiciones fisiológicas aún no es clara, y más aún, podemos decir controversial. Entre los roles fisiológicos que se le han atribuido podemos citar funciones como receptora de membrana, transductora, como agente pro- o anti-apoptótico, como reguladora de la homeostasis de cobre, involucrada en memoria, reguladora de stress oxidativo [5;73-75], y recientemente identificada como receptor de oligómeros de Aβ [76], entre otras. En contraste a todos estos reportes, estudios en ratones genéticamente modificados que carecen de PrP^C muestran que su viabilidad y desarrollo es normal en todas las etapas de la vida [77].

PrP^{Sc}, la versión anómalamente plegada del prion ha sido descrita como el principal, si no el único agente infeccioso asociado a este grupo de enfermedades [17]. Una evidencia directa apoyando la frase anterior la podemos encontrar en las prionopatías hereditarias [5]. En estos casos, mutaciones dentro de la PrP favorecen la conformación amiloidogénica como se ha demostrado tanto en estudios *in vivo* como *in vitro* [78;79].

La teoría del prion fue propuesta inicialmente por Griffith en el año 1967 [80]. Como era de esperar para la época, el demostrar que la PrP anómalamente plegada era la parte mayoritaria, si no exclusiva de la infectividad fue muy discutida. Los característicos y prolongados períodos de incubación y la variación de cepa identificada en estas enfermedades hicieron creer por mucho tiempo que el agente infeccioso consistía de un lentivirus [69]. Muchos estudios enfocados en aislar material genético exógeno asociado a estas enfermedades fracasó, y la identidad del agente infeccioso permaneció incógnita por mucho tiempo. El tratamiento de muestras infecciosas con diversas sustancias o métodos disruptores de ácidos nucleicos resultó nulo en bloquear las características infectivas de la muestra en cuestión. En la misma línea de experimentos, el uso de agentes desnaturalizantes de proteínas como urea o detergentes fueron capaces de bloquear parcial o completamente la actividad infecciosa de las muestras analizadas [81;82]. Estudios realizados por Stanley Prusiner demostrando la inutilidad de tratamientos convencionales para inactivar ácidos nucleicos como UV y ADNasas entre otros, dieron un gran empuje a esta teoría [17;83]. La identificación de una proteína que modificaba el desarrollo patológico tanto en el huésped como en la muestra infecciosa dio un nuevo empuje a la hipótesis

inicialmente postulada por Griffith [84]. Estudios de infectividad utilizando muestras purificadas de PrP^{Sc} demostraron que la transmisión de las EET co-existía con los niveles de esta proteína de una forma dependiente de su concentración [17]. Estudios posteriores principalmente liderados por Charles Weissmann en ratones transgénicos que carecían de la PrP o que la sobre expresaban demostraron que la capacidad de transmitir la enfermedad era altamente dependiente de la expresión de PrP en el huésped [77;85]. Con todos estos avances, es importante señalar que la teoría del prion es aun controvertida. Con el propósito de fortalecer los postulados de la teoría del prion podemos mencionar los estudios realizados por David Kocisko y Byron Caughey en el cual se evaluó la conversión de PrP^{C} a PrP^{Sc} utilizando componentes purificados [86]. Este ensayo, denominado *cell free conversion assay* demostró que la combinación entre PrP^{C} y PrP^{Sc} era suficiente para formar nuevas unidades de $PrP^{27\text{-}30}$ (tabla 1). A pesar del gran apoyo de esta técnica en la comprensión de los mecanismos de conversión del prion, los bajos niveles de conversión que presentaba (1 molécula de PrP^{C} convertida por cada 50 de PrP^{Sc}) no permitieron evaluar otras características importantísimas, como por ejemplo, la capacidad infectiva de las nuevas partículas generadas. Una técnica mas reciente desarrollada en nuestro laboratorio y denominada *Protein Misfolding Cyclic Amplification (PMCA)* ha permitido evaluar distintas características del proceso de conversión de priones, apoyando y confirmando convincentemente la gran mayoría de los postulados de la hipótesis de infectividad proteica (lo cual será tratado en secciones posteriores).

Estudios posteriores utilizando cultivos celulares, modelos animales y estudios epidemiológicos apoyan sustancialmente que el agente infeccioso en estas enfermedades está constituido exclusivamente por proteínas [17;71]. La transmisibilidad en estas enfermedades radica principalmente en la capacidad de oligómeros de PrP^{Sc} en catalizar la agregación de nuevas unidades tal como se postula en el modelo de nucleación-polimerización [20]. Si tomamos en cuenta que los procesos de deposición de proteínas anómalamente plegadas son la causa de la enfermedad, ésto podría dar cuenta de la aceleración de procesos patogénicos que en la ausencia de estas semillas ocurrirían mucho mas tarde en la vida o que quizás nunca ocurrirían en el período de vida normal del individuo. En el caso de las vECJ o iECJ (ECJ iatrogénica) la infectividad transmitida por núcleos de agregación pre-establecidos se puede explica bastante bien teniendo en cuenta que individuos más jóvenes en comparación a pacientes de sCJD han sido identificados.

Terapia y diagnóstico en las prionopatías

La naturaleza particular del prion, agente causante de las EET hace que las estrategias a utilizar para su diagnostico y terapia deban ser también particulares. Nuevas aproximaciones que bloqueen al agente infeccioso, al agente a partir del cual es generado en el huésped (PrP^{C}) o a los mecanismos implicados en su propagación son a las que actualmente se apuesta. Actualmente existen dos principales estrategias de terapia para estas enfermedades: el bloqueo del proceso de mal plegamiento de la PrP y la inmunización contra formas anómalamente plegadas de esta proteína. Para la primera, muchas moléculas se han descrito las que a pesar de su efectividad no han sido suficientes para eliminar completamente la progresión de la patología en modelos animales ([87-91] y apéndice 1). Siguiendo las estrategias de inmunización podemos encontrar efectos interesantes prolongando significativamente la etapa sintomática de la enfermedad [92-94]. Entre los reportes existentes hasta ahora es interesante el mencionar una estrategia que involucra la utilización de cepas atenuadas de *Salmonella* que han sido modificadas para

expresar PrP. Utilizando este vehículo se ha descrito una atenuación de los efectos de la enfermedad aunque sin eliminarla completamente [95]. Otras estrategias terapéuticas se han enfocado en combatir los efectos de señalización celular generados en el cerebro de pacientes y animales de experimentación [96]. La gran dificultad a la que estas estrategias se enfrentan es el rápido declinamiento y la corta etapa clínica que estas enfermedades producen, lo que finalmente imposibilita un tratamiento efectivo. En esta situación, tratamientos en etapas tempranas de la agregación del prion serían óptimos, y es por este motivo que aproximaciones diagnósticas en la larga y silente etapa de incubación son una prioridad en la actualidad.

Desde el punto de vista clínico, los síntomas manifestados en pacientes generalmente aparecen en etapas avanzadas de la enfermedad cuando ya no es posible detener su avance. La búsqueda de marcadores moleculares en tejidos y fluidos biológicos en pacientes y animales de experimentación es la principal estrategia diagnóstica siendo evaluada en este momento por diversos grupos de investigación (apéndice 2). Debido a su disponibilidad y baja invasividad la búsqueda de marcadores moleculares en distintos fluidos como orina, sangre y LCR es bastante atractiva (apéndice 2, 6 y [97]). Al ser las prionopatías enfermedades principalmente dirigidas al SNC no es de sorprender que el principal candidato para buscar marcadores sea el LCR. En muestras de este fluido muchas proteínas han sido relacionadas con las prionopatías, entre las que podemos mencionar a la PPA, Aβ, Tau, 14-3-3 y recientemente GRP58, entre otras [97]. Actualmente la principal desventaja en la utilización de estos métodos es la inespecificidad que estos marcadores presentan, ya sea por su participación en otras patologías (como es el caso de Aβ) o su liberación como consecuencia de daño a la barrera hemato-encefálica, entre otras razones. Con respecto a la especificidad molecular como método diagnóstico, entre todos los metabolitos en búsqueda o por encontrar el más específico es PrP^{Sc}. Con el propósito de detectar esta molécula se han planteado diversas metodologías, entre las que podemos contar el diseño de metodologías de detección ultra sensibles en la proteína del prion (como anticuerpos y ligandos específicos y con gran afinidad), concentración del material infeccioso, o en el mejor de los casos, amplificación de la PrP^{Sc} de manera análoga a lo que se realiza con el ADN de patógenos utilizando la reacción en cadena de la polimerasa. De todas estas estrategias, la más exitosa ha sido la *PMCA* que hemos creado y optimizado en nuestro laboratorio (apéndice 7). A continuación, daremos una visión general acerca de las ventajas de esta técnica tanto en su aproximación diagnostica como la ayuda que nos puede dar en el entendimiento de estas raras y complejas enfermedades.

Protein Misfolding Cyclic Amplification (PMCA)

Esta técnica, se basa principalmente en los principios del modelo de nucleación-polimerizacion y fue descrita por primera vez por Saborio y cols. en el año 2001 [98]. En aquel estudio se describió por primera vez una amplificación a gran escala de PrP^{res} (tabla 1) en el tubo de ensayo. Esta técnica utiliza ciclos de incubación-sonicación, en los cuales homogenizados de cerebros de animales sanos, fuentes de la PrP^{C} "sustrato" de la reacción, son mezclados con el material infeccioso (PrP^{Sc}) a analizar. La reacción de amplificación ocurre tras el reclutamiento de nuevas unidades anómalamente plegadas en los extremos de los núcleos de agregación de los cuales está compuesto el material infeccioso inicial. Con el propósito de generar nuevos núcleos de agregación en este proceso, la mezcla de reacción es sometida a pulsos de sonicación que desestabilizan los agregados generados permitiendo la formación de nuevas "semillas" con extremos reactivos disponibles que acelerarán el *misfolding* y reclutamiento de partículas

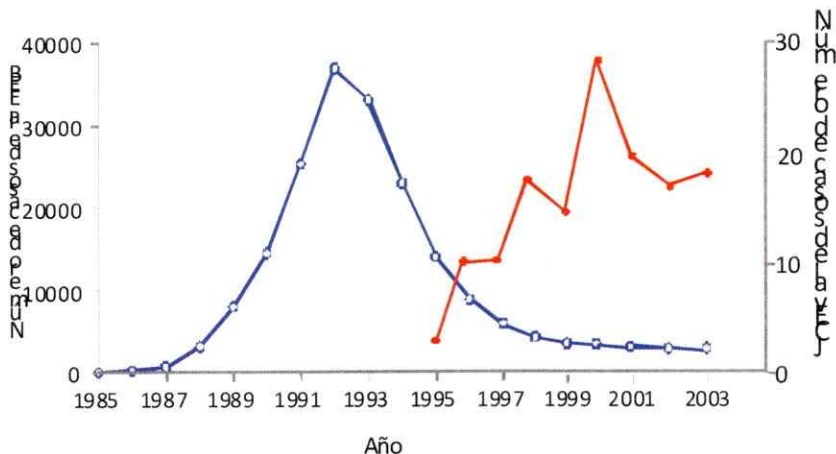
adicionales de PrP^{C} (apéndice 7). Estos procesos cíclicos de incubación y sonicación permiten un crecimiento exponencial de las partículas infecciosas, acelerando considerablemente procesos que pueden tomar años o décadas en animales. Modificaciones posteriores han permitido automatizar esta técnica, utilizando sonicadores programables y plataformas de sonicación [99]. Esto actualmente permite el uso de la *PMCA* a gran escala, lo cual resulta promisorio desde el punto de vista clínico e investigación permitiendo un análisis masivo de muestras.

Uno de los principales legados de esta técnica ha sido el gran apoyo que ha suministrado para validar la teoría de infectividad producida solo por proteínas. Resultados por *PMCA* apuntando en esta dirección fueron por primera vez publicados el año 2005 por nuestro grupo de investigación utilizando un modelo experimental de hámster y la cepa 263K de priones como material infeccioso [99]. En este reporte se describió la amplificación seriada de priones por *PMCA* (*sPMCA*) de la cual se tomó ventaja con el fin de diluir completamente el material infeccioso inicialmente suministrado y obtener PrP^{Sc} únicamente generada *in vitro*. En resumen, luego de cada ronda de *PMCA* una alícuota del material resultante era diluida en homogenizado fresco de animales sanos (apéndice 7). De acuerdo a nuestros cálculos, tras una dilución de 10^{-15} con respecto al material original no existirían moléculas de PrP^{Sc} derivadas de cerebro y todas las partículas infecciosas presentes en el tubo de ensayo corresponderían a material generado *in vitro*. Es importante señalar que reportes previos describen que la última dilución infecciosa para priones 263K corresponde a una dilución de 10^{-9} con respecto al cerebro de animales terminalmente enfermos, infectividad que se manifiesta con largos períodos de incubación y con una tasa de éxito incompleta [99]. Brevemente, este reporte demostró tras análisis bioquímicos del material generado *in vitro* que las características de la PrP^{Sc} generada en el tubo de ensayo eran indistinguibles del material original a partir del cual se habían generado. Consecuentemente, una vez que este material fue inoculado en hámsteres sanos se vio que las características infecciosas de las preparaciones generadas *in vitro* eran bastante similares a lo que se había observado con el material original. Estos resultados, como se ha mencionado anteriormente apoyan de una forma importantísima a la teoría que da cuenta de la PrP^{Sc} como el principal, si no el único agente infeccioso en estas enfermedades.

Debido a la rapidez demostrada en la producción de PrP^{Sc} utilizando esta técnica, sus aplicaciones son potencialmente importantes desde el punto de vista diagnóstico, lo que ha sido utilizado para la evaluación en diversas aplicaciones biotecnológicas (apéndice 11). Se ha descrito que ciertas modificaciones a la *PMCA* permiten identificar mínimas cantidades de proteína infecciosa, lo que de acuerdo a nuestras estimaciones serían del orden de 28 unidades monoméricas [100]. Esto visto desde el punto de vista de la dinámica del agente infeccioso correspondería a una única partícula infecciosa [101]. Con estos niveles de detección nuestro grupo ha logrado exitosamente la detección de PrP^{Sc} por primera vez en sangre de animales experimentales [102], inclusive en la etapa pre-sintomática de la enfermedad [103]. Esta información dio un esperanzador vuelco a la búsqueda de métodos diagnósticos, principalmente en humanos, donde se ha descrito que la transfusión de sangre desde individuos asintomáticos, incubando la vECJ, ha sido capaz de transmitir la enfermedad a individuos sanos [104]. Expandiendo estos resultados hemos recientemente descrito la presencia de priones infecciosos en orina de hámsteres infectados (apéndice 6) donde previa estimación por ensayos de infectividad y extrapolación utilizando nuestro ensayo *in vitro* dan cuenta de los reducidos niveles de la proteína infecciosa en este fluido en comparación con sangre.

Sumando a todas las ventajas que la *PMCA* nos puede dar como método diagnóstico, hemos recientemente descrito que muchos otros mecanismos que involucran la replicación de priones pueden ser resueltos utilizando esta técnica. Muchos de ellos, como los fenómenos de cepa y de transmisión inter-especie, fueron estudiados en esta tesis y sus resultados serán evaluados en secciones siguientes.

Figura 1. Incidencia de la EEB y la vECJ.
Número de casos reportados de la EEB (azul) y de la vECJ (rojo). Nótese la diferencia de la escala en cada uno de los casos.



Proyecciones acerca del estado actual de las prionopatías.

En los últimos años bastante información, alarmante en ciertos casos, ha sido recopilada para este grupo de enfermedades. Por ejemplo podemos destacar la epidemia de la EEB que afectó principalmente a Europa y que hemos mencionado anteriormente. A este respecto es importante el notar que el número de casos de la vECJ reportados no se relaciona con el número cabezas de ganado afectadas con la EEB, ni con los períodos de incubación que actualmente se han predicho. Como se describirá en puntos posteriores, la transmisión inter-especie de priones infecciosos es un fenómeno que se caracteriza por los largos períodos de incubación que se observan. Los picos de incidencia para la EEB y la vECJ descritos hasta este momento son de tan solo 8 años, lo que para un proceso de transmisibilidad inter-especie por ruta oral es bastante corto (figura 1). Si a esto sumamos la información de que ciertos casos pre-sintomáticos de la vECJ fueron capaces de ser transmitidos a personas sanas vía transfusión sanguínea [104] podríamos estar actualmente en un escenario bastante preocupante desde el punto de vista epidemiológico.

Otro importante tema en las EET hace mención a la variación de cepas que este agente infeccioso presenta (apéndice 3 y [105]). La variación de cepa en priones depende de muchos factores entre los cuales podemos mencionar la infectividad inter-especie, la infectividad inter-polimórfica, y la generación espontánea de estos agentes, entre otras (apéndice 3). El fenómeno de variación de cepas de priones, el cual ocupa una parte importante de este trabajo de tesis doctoral se describe en el punto a continuación.

4. Cepas, polimorfismos y el fenómeno de barrera de especies en las Encefalopatías Espongiformes Transmisibles.

Introducción a las cepas de priones. Bases moleculares y características patológicas.

Entre las características particulares de los priones está su capacidad para desarrollar variantes o cepas ("fenómeno de cepas de priones") [105]. Mucho antes de que la teoría del prion fuera establecida se observó que el material responsable de transmitir las EET era capaz de producir una gran variedad de signos clínicos en animales, lo cual fue atribuido a distintas variantes, o cepas infectivas [106]. Tras la identificación de la PrP^{Sc} se descubrió que las características bioquímicas de esta molécula podían variar de acuerdo al síndrome que se presentara [67;107;108]. A su vez, las características propias de cada una de estas variantes infecciosas podían mantenerse al infectar experimentalmente animales de la misma especie. Haciendo una analogía con otros agentes infecciosos, estas variantes han sido denominadas cepas (para una revisión ver el apéndice 3).

Una definición clásica de cepas hace mención a la variación genética o subtipo del agente infeccioso responsable de cada enfermedad. Este concepto, válido en virología y bacteriología no puede ser extendido cabalmente al campo de los priones. En etapas tempranas del estudio de estas enfermedades, la existencia de cepas de priones fue algo muy discutido, e incluso perjudicial para la teoría del prion. Con el paso de tiempo y la recolección de datos experimentales y clínicos muchas hipótesis para explicar estos procesos han sido postuladas. Entre ellas, se ha propuesto que los diferentes fenotipos encontrados en los animales afectados radican en diferencias en la información genética alojada por la PrP^{Sc} (apéndice 3). Por otro lado se ha postulado que ciertos co-factores compuestos por pequeños ácidos nucleicos serían los responsables de la variación de cepa [109]. Sin embargo, la hipótesis más aceptada en la actualidad explica que las principales diferencias entre las cepas de priones radican en las conformaciones alternativas que la PrP^{Sc} pueda adquirir, las cuales serían estables y propagables [110;111].

La primera evidencia acerca de la existencia de cepas de priones fue descrita en 1961 por Pattison y Millson en caprinos afectados por scrapie [106]. En este estudio, cabras afectadas con la misma mezcla de agente infeccioso de scarpie presentaron dos distintos fenotipos clínicos denominados por los autores como *scratching* (rascador) y *drowsy* (aletargado) de acuerdo a la manifestación clínica presentada en cada caso. Las diferencias entre estos agentes infecciosos fueron atribuidas a diferencias genéticas en el huésped. La evidencia actual apoya esta hipótesis, principalmente luego de aislar el gen que codifica para la proteína prion e identificar sus variaciones polimórficas [67].

Las cepas de priones pueden ser clasificadas de acuerdo a diversos parámetros. Los períodos de incubación, perfiles de daño cerebral y signos clínicos están entre los más ampliamente descritos (apéndice 3). En algunos casos, estudios histopatológicos han mostrado diferencias sustantivas en el cerebro de animales de una misma especie afectados con distintas cepas de priones [112]. Estas diferencias radican principalmente en la distribución e intensidad de vacuolización y PrP acumulada en distintas zonas del cerebro. Para caracterizar estas propiedades, sistemas bien estandarizados de patrones de vacuolizacion han sido descritos [112;113]. Utilizando este tipo de aproximaciones muchas cepas de priones con similares períodos de incubación y manifestación clínica han sido diferenciadas. De forma análoga, los

perfiles de deposición de PrP^{Sc} han sido útiles en el seguimiento del origen de ciertos materiales infecciosos, tal como ha ocurrido en el caso de la vECJ y la identificación de la EEB como su fuente [114]. Como fue inicialmente descrito por Pattison y Millson, los signos clínicos pueden ser la primera evidencia para mostrar diferencias entre cepas. Por ejemplo, en prionopatías humanas problemas motores, demencia, ataxia, depresión e insomnio son solo algunos de una larga lista de signos clínicos que pueden aparecer con mayor o menor intensidad dependiendo de la cepa de prion [115]. En otros animales las características clínicas pueden ser diametralmente opuestas como ocurre para las cepas *Drowsy* (DY) y *Hyper* (HY) de hámsteres [116]. Desafortunadamente, las diferencias en la manifestación clínica de la enfermedad no siempre pueden ser aplicadas a la distinción de cepas. En ratones, por ejemplo, muchas de las variantes del material infeccioso presentan una manifestación clínica similar, la cual incluye ataxia, pelo erizado y joroba; signos generales de debilitamiento en el animal (apéndice 8). Sin embargo, estudios posteriores utilizando sistemas que miden cambios en distintos parámetros del comportamiento animal han demostrado declinamientos diferenciales en cepas consideradas con signos clínicos equivalentes [117;118].

En suma a sus diferencias *in vivo*, cada cepa de PrP^{Sc} posee características bioquímicas particulares. Entre ellas podemos nombrar los patrones de glicosilación, sedimentación y resistencia a la denaturación o digestión proteolítica que la PrP^{res} puede presentar (apéndice 3). Una de las evidencias más claras que establece diferencias conformacionales entre distintas cepas de priones puede ser visualizada en el patrón de movilidad electroforética de estas moléculas luego de tratamiento con proteinasa K (PK) [66;116]. La resistencia a proteasas del prion es parcial, donde un segmento involucrando la región C-terminal (comenzando desde aproximadamente el aminoácido 90) resiste condiciones drásticas de digestión proteolítica [17]. Las diferencias de movilidad electroforética observadas en distintas cepas de priones luego de este tipo de tratamiento sugiere que distintos segmentos de la PrP^{Sc} son protegidos de acuerdo al plegamiento que la proteína adquiera [119-121]. Otros estudios utilizando *Fourier Transform Infrared Spectroscopy* (FTIR) [122], inmunoensayos dependientes de conformación [123], diferencias en la afinidad a unir cobre [124] y ensayos de microscopía de fuerza atómica en polímeros de priones sintéticos [125] han apoyado fuertemente la hipótesis de que distintas cepas de priones poseen conformaciones espaciales variadas. Sin embargo, la prueba definitiva acerca de la naturaleza conformacional de estos agregados esta aún ausente.

Transmisión inter-especies de cepas de priones. Barreras de transmisión y generación de nuevas cepas.

La principal fuente de variación de cepas de priones proviene de la transmisión inter-especie de este agente infeccioso. Con respecto a esta afirmación es importante destacar que cada cepa de PrP^{Sc} tiene la habilidad de infectar un número específico de especies animales, aunque siempre con cierto grado variable de dificultad. Este fenómeno, conocido como "barrera de especies" se manifiesta como una prolongación en los períodos de incubación cuando los priones de cierta especie son utilizados para infectar a una segunda (apéndice 3). Transmisiones inter-especie de priones infecciosos pueden dar como resultado situaciones variadas. En suma a la prolongación en los períodos de incubación anteriormente mencionados, es importante señalar la amplia distribución temporal que el material infeccioso toma para generar signos clínicos y muerte en los animales infectados de la nueva especie. En algunos casos, la incidencia de infectividad no afecta al total de animales inoculados, muchos de ellos llegando al punto normal de su vida con infecciones sub-clínicas [126;127]. En otras situaciones se observa que los

priones inoculados son completamente rechazados en la especie receptora como es el caso del conejo, especie que ha resultado negativa a la recepción de PrP^{Sc} de una gran variedad de especies. En este último caso podemos hablar de la presencia de barreras de especie absolutas [128;129].

Una vez que la transmisión de priones ha sido exitosa podemos claramente ver que el material infeccioso generado en la nueva especie reduce sus periodos de incubación al ser transmitido subsecuentemente en la misma especie. Luego de pases sucesivos de infectividad se puede observar una estabilización en el tiempo necesario para producir la enfermedad lo que va acompañado de una estabilización de las propiedades bioquímicas y patogénicas presentadas por la nueva PrP^{Sc}. Esta evidencia nos permite analizar la generación de nuevas cepas de priones en dos etapas: la primera comprendida por una adaptación del material infeccioso seguida de una segunda etapa de estabilización (apéndice 3 y [116;130;131]). Sorprendentemente, muchas transmisiones inter-especie de PrP^{Sc} han resultado en la generación de más de una variante infecciosa en el mismo huésped [110;119;130-132]. Las diferencias en la secuencia aminoacídica del prion traen como consecuencia distintas conformaciones espaciales en el agente infeccioso y la PrP^{C} receptora. Esto podría explicar la dificultad (o incompatibilidad en ciertos casos) traducida como "barrera de especies" y la diversidad de agentes infecciosos que se pueden generar.

La propagación inter-especie de priones entre ganado bovino y el ser humano es probablemente la más conocida desde el punto de vista de salud pública. Actualmente es altamente aceptado por la comunidad científica que el consumo de derivados proveniente de bóvidos afectados por la EEB es la causa de la vECJ, siendo el "mal de las vacas locas" la única prionopatía animal transmitida al humano. En un escenario opuesto, el consumo de derivados de ovinos o caprinos potencialmente infectados con scrapie en diversas partes del mundo nunca ha sido asociado con un aumento en la incidencia de EET en la población humana. Apoyando estos datos epidemiológicos, experimentos de transmisibilidad de scrapie utilizando ratones transgénicos que expresan PrP quiméricas de humano y ratón muestran una barrera de especie muy potente entre este material infeccioso y la proteína prion humana [133]. Actualmente mucha evidencia apunta a que la EEB fue originalmente producida al alimentar al ganado bovino con material contaminado con scrapie, indicando que la priones infecciosos en bovinos podrían ser un intermediario conformacional entre la PrP^{Sc} ovina y la PrP^{C} humana [134].

El uso de modelos animales para el estudio de la EETs.

Probablemente la mejor forma de estudiar el fenómeno de variación de cepa es utilizando animales de experimentación, principalmente roedores [135]. Entre las especies más utilizadas podemos contar al ratón (*mus musculus*) donde más de 20 cepas de priones fenotípicamente distintas se han aislado [136]. Muchas de estas cepas han tenido su origen en distintas fuentes de scrapie [137;138], EEB [130], ECJ [139], entre otras. Pases seriados del agente infeccioso en una especie con un fondo genético constante son necesarios para estabilizar y definir a una nueva cepa de prion.

Priones infecciosos de ratón obtenidos a partir de scrapie de diversas fuentes han resultado en cepas como Rocky Mountains Laboratory (RML), ME7, 139A y 79A, las que muestran patrones electroforéticos similares luego de tratamiento estándar PK: movilidad electroforética de aproximadamente 21 KDa para la forma sin glicosilar y presentando a la forma monoglicosilada como la más abundante (apéndice 8). A pesar de estas similitudes, estas cepas

pueden ser diferenciadas por los períodos de incubación o el patrón de lesión que producen en el cerebro. Otras cepas de ratón se han generado a partir de la EEB o la ECJ, originando a las cepas denominadas 301C o Fukuoka, respectivamente [130;139]. La transmisión y manifestación clínica de la EEB en ratones C57BL6 (la principal cepa de ratones utilizada en este tipo de estudios) puede ir acompañada de dos distintos fenotipos: uno presentando una forma de la PrP^{Sc} resistente a PK (PrP^{27-30}, tabla 1) y otra en la cual esta forma está ausente. Este fenotipo se mantiene luego de dos pases de infectividad *in vivo* en la misma especie hasta que finalmente solo el fenotipo resistente a proteasas es el que prevalece [130]. La presencia de material infeccioso sensible a proteasas (denominado como $sPrP^{Sc}$ – tabla 1) ha sido también descrita en algunas prionopatías humanas [11] e incluso como parte importante de material infeccioso resistente a PK (apéndice 12 y [140]).

En contraste con las características bioquímicas e infectivas de las cepas derivadas de scrapie podemos mencionar a la cepa 301C. Entre ellas podemos observar que tras una inoculación intra-cerebral de priones RML en ratones se observa una manifestación clínica avanzada a aproximadamente 150 días posteriores a la inoculación (d.p.i.), mientras que la cepa 301C lo hace a aproximadamente 200 días (apéndice 8). Inoculación de estas mismas cepas por la ruta intra-peritoneal presentan una diferencia aún más clara, con períodos de incubación cercanos a los 200 y 300 días, respectivamente (datos no publicados). Además de estas diferencias, podemos encontrar que ambas cepas producen vacuolización en distintas áreas del cerebro (apéndice 8). Sumando a sus diferencias patológicas, ambas cepas presentan claras diferencias bioquímicas, principalmente en su perfil electroforético. Como fue mencionado con anterioridad la movilidad electroforética de la banda sin glicosilar para la cepa RML es de aproximadamente 21 KDa luego de tratamiento con PK. Otra característica importante de RML es la preferencia que este prion infeccioso tiene para reclutar la forma monoglicosilada de la PrP. En contraste, la cepa 301C muestra un patrón de movilidad electroforética de 19KDa para la forma sin glicosilar y la distribución de sus glicoformas favorece a la forma diglicosilada, lo que sugiere la presencia de variaciones conformacionales entre ambos agentes infecciosos (apéndice 8).

La razón de como distintas conformaciones en la PrP^{Sc} pueden producir variedades estables del agente infeccioso en el huésped es aún tema de debate. En este ámbito, un fenómeno interesante lo podemos encontrar en el aislamiento de diferentes cepas de PrP^{Sc} a partir de la inoculación de un único extracto patógeno. Probablemente el caso más representativo está dado por las cepas DY y HY, generadas a partir de la transmisión de la Encefalopatía Transmisible del Visón (ETV) en hámsteres sirios (*Mesocricetus auratus*). Esta transmisión inter-especie de priones presenta el comportamiento clásico: el primer cruce de infectividad caracterizado por largos periodos de incubación seguidos de una adaptación del material infeccioso en la misma especie [110]. Sorprendentemente, esta adaptación resultó en dos distintos grupos, con signos clínicos diferentes: el primero (DY) presentó periodos de incubación de aproximadamente 150 días y fue clínicamente asociado con letargia, mientras que el segundo (HY) presentó períodos de incubación considerablemente cortos (60 d.p.i.) e hiperactividad. Sumado a estas características, análisis histopatológicos de los cerebros de estos animales mostraron diferencias tanto en la deposición de la PrP como en los patrones de vacuolización [116]. Como fue descrito para 301C y RML, DY y HY presentaron claras diferencias en sus movilidades electroforéticas posterior al tratamiento con PK. La banda sin glicosilar de la cepa DY mostró una movilidad electroforética de aproximadamente 19KDa, mientras que HY lo hizo a 21 KDa [110]. Análisis estructurales por *FTIR* realizados a preparaciones purificadas han arrojado diferencias

estructurales entre ambos agentes infecciosos [122]. Otra interesante diferencia bioquímica entre ambas cepas yace en la resistencia a PK que ambas presentan, siendo DY la más sensible a la actividad de esta proteasa [119]. Todas estas diferencias tanto bioquímicas como biológicas hacen de DY y HY un hermoso modelo de la variación de cepas en estas enfermedades.

Rol de los polimorfismos en la PrP con respecto a la infectividad.

Los polimorfismos en la PrP y sus efectos en la variación de cepa han sido indirectamente descritos desde hace mucho tiempo, antes que la teoría del prion fuera propuesta y aceptada. Diferencias patológicas han sido extensivamente descritas en ovinos, caprinos y ratones de experimentación. Los fenotipos *drowsy* y *scratchy* en ovejas anteriormente mencionados [106] pueden actualmente ser atribuidos a diferencias polimórficas en el huésped. La identificación del "gen modulador de los períodos de incubación de scrapie" (en inglés *scrapie incubation period gen* o *sinc*) y sus diferencias polimórficas fueron un excelente avance en el estudio de cepas de priones [141]. En ratones, se han descrito dos polimorfismos, denominados $sinc^{s7}$ o $sinc^{p7}$. Posteriormente, fue descrito que el gen *sinc* era equivalente con el gen del prion [107;108] y que los polimorfismos encontrados resultaban en diferencias en la secuencia en las posiciones 108 y 189 de la PrP [108]. La transmisión de PrP^{Sc} proveniente de ovejas, cabras y bovinos en ambos grupos polimórficos de ratones resultó en el aislamiento de una amplia diversidad de cepas de priones [142]. Una vez que los períodos de incubación se hicieron estables en la nueva especie, los priones nuevamente generados fueron ensayados en el grupo polimórfico opuesto. Estos ensayos interesantemente mostraron que polimorfismos en la PrP pueden generar prolongaciones en los periodos de incubación de forma similar a lo observado para la transmisión inter-especie de PrP^{Sc} [143]. Otro paralelo con las transmisiones inter-especies puede encontrarse en el hecho de que los cruces inter-polimórficos de infectividad pueden generar nuevas cepas de priones [142]. Todas estas características sugieren que la transmisión inter-polimórfica de PrP^{Sc} es capaz de promover la diversidad de cepas de priones.

Análisis de los animales presentando periodos de incubacion "cortos" y "largos" (inyectados con cepas de priones generadas en $sinc^{s7}$ o $sinc^{p7}$) revelaron diferencias en la secuencia de prnp [107]. Estos hallazgos fueron un gran apoyo para la teoría del prion debido a que tal cual como se describiera para el fenómeno de barrera de especies, diferencias en la secuencia del prion eran capaces de afectar significativamente la transmisión de un agente infeccioso determinado. De acuerdo a una nueva nomenclatura estos grupos polimórficos han sido designados como $prnp^a$ y $prnp^b$ respectivamente para los grupos $sinc^{s7}$ y $sinc^{p7}$ [108]. Recientemente, un nuevo grupo polimórfico ha sido identificado en ratones, el cual ha sido denominado como $prnp^c$ [144].

Polimorfismos en la prnp han sido descritos en muchas otras especies. En ovejas por ejemplo, la mezcla de muchas variedades de estos animales, realizada con el fin de optimizar la calidad y la productividad del ganado, ha generado numerosas combinaciones polimórficas en *prnp*. Sin embargo, principalmente cinco alelos de prnp están significativamente presentes dando un total de 15 posibles genotipos, cada uno de los cuales es capaz de favorecer o rechazar la selección de una determinada cepa de scrapie. Estas cinco variables corresponden a ARQ, ARR, AHQ, ARH y VRQ; cada aminoácido estando presente en las posiciones 136, 154 y 171 de la proteína del prion [67].

En humanos, la existencia de polimorfismos en la posición 129 de la PrP, donde ATG o GTG puede resultar en una metionina (M) o en una valina (V), ha sido descrita clínica y experimentalmente como muy importante con respecto a la susceptibilidad de ciertas cepas

infecciosas de priones [5]. Individuos M y V homocigotos son mayoritarios en la ECJ, mientras que los casos heterocigotos representan solo una baja proporción [145;146]. Alrededor del 40% de la población es homocigótica para M, sin embargo, el 78%, 50% y 100% de los pacientes afectados por la ECJ esporádica (sECJ), iECJ y vECJ respectivamente presentan M en la posición 129 de la PrP [147-150]. Esta evidencia sugiere que M en la posición 129 es un factor de riesgo con respecto a la conversión de PrP^{C} a PrP^{Sc}. Como en ratones y ovinos, la presencia de polimorfismos en la PrP humana puede modular las características patológicas y bioquímicas del material infeccioso. Actualmente, parámetros como la duración y severidad de la enfermedad, signos clínicos y patrón de vacuolización y deposición de PrP^{Sc} en el cerebro han sido altamente asociados con cada una de las variables polimórficas [151;152]. Un ejemplo lo podemos ver en pacientes M homocigotos donde se ha descrito un fenotipo más agresivo de la enfermedad, caracterizado por una etapa clínica corta (4.5 meses) en comparación con pacientes heterocigotos (16.9 meses) y V homocigotos (14.3 meses) [152]. Otro ejemplo muy didáctico de estos polimorfismos corresponden a mutaciones en la posición 178 de la PrP (D178N). Esta mutación puede manifestarse clínicamente como ECJ o IFF de acuerdo a la presencia de M o V en la posición 129 de esta proteína [153].

Las transmisiones inter-especies e inter-polimórficas de priones conjugadas en el modelo de nucleación-polimerización.

Como se ha discutido en secciones anteriores, la adición de núcleos pre-formados reduce los tiempos requeridos para agregar proteínas *in vitro*. Adicionalmente se ha descrito que la adición de núcleos amiloidogénicos heterólogos es capaz de generar un fenómeno similar, aunque en distinto grado cuando se comparan con núcleos formados por la proteína original. Un ejemplo lo encontramos en la nucleación de lisozima de huevo con una serie de agregados proteicos, donde se ha descrito que la habilidad de agregar a la proteína soluble es dependiente de la secuencia aminoacídica [154]. Estudios *in vivo* han demostrado que fibras de sistemas no mamíferos pueden favorecer la agregación de la proteína amiloide-A (AA) en modelos animales de amiloidosis generada por AA [155]. Muchos otros reportes mostrando la co-existencia de diferentes DCP en el mismo tejido apoyan la existencia de nucleación heteróloga como un mecanismo patológico. Esta información sugiere que un DCP podría ser capaz de influenciar el desarrollo de un segundo DCP (apéndice 1).

El hecho que la nucleación de proteínas con secuencia distinta tenga una menor influencia que la alcanzada con núcleos homólogos es de cierta forma equivalente a lo descrito con respecto a los fenómenos de transmisión inter-especie e inter-polimórficas en las EET. Este mecanismo se presenta como una explicación coherente frente a la baja proporción de animales infectados y a los largos períodos de incubación típicamente observados en la transmisión primaria de priones de una especie a otra. Experimentos recientes han demostrado que el fenómeno de infectividad inter-especies puede ser reproducido utilizando ratones transgénicos que expresan la PrP de diferentes especies [135], lo cual apoya la hipótesis de que la secuencia es el factor más importante en este fenómeno. Hasta ahora ningún otro elemento ha sido asociado como un potencial modulador de la nucleación heteróloga de PrP^{Sc} entre especies.

5. Interacción de distintos agregados en los Desórdenes Conformacionales Proteicos y el fenómeno de nucleación heteróloga

El estudio de la interacción putativa entre DCP a nivel proteico no es importante solo desde el punto de vista científico, sino también es de interés en salud pública. Una mejor comprensión de este fenómeno podría explicar el origen y prevalencia de muchas enfermedades en este grupo.

Evidencia epidemiológica de nucleación heteróloga

Probablemente el ejemplo más alarmante de la interacción entre DCP proviene de la asociación encontrada entre la EA y la diabetes tipo-2, enfermedades que están caracterizadas por la deposición de Aβ y amilina, respectivamente. Estudios clínicos han demostrado que un alto porcentaje de pacientes afectados por la EA son también positivos para diabetes tipo-2. Sin embargo, el mismo reporte sugiere que la deposición de Aβ no es un factor de riesgo para la aparición de diabetes tipo-2, indicando que este fenómeno podría ser unidireccional [156].

Otro ejemplo clínico ha sido descrito en pacientes afectados por la EP y la EA, donde claramente se ha descubierto la co-existencia de Aβ y α-sinucleína en las mismas estructuras amiloidogénicas [157]. En este caso, y a diferencia del caso anteriormente explicado, las características patológicas de ambas enfermedades co-existen en el cerebro, incrementando las posibilidades de que los agregados proteicos puedan interaccionar entre sí. Estudios recientes han demostrado que esta interacción es un hecho y que pacientes clínicamente diagnosticados con la EA presentan una mayor acumulación de α-sinucleína en sus cerebros en comparación con individuos sanos de la misma edad.

El fenómeno de co-existencia de amiloides ha sido descrito para otras combinaciones de proteínas amiloidiogénicas. Entre ellas, la presencia simultánea de Aβ y priones ha sido extensivamente documentada en pacientes afectados con la sECJ, GSS y la EA (apéndice 1). En algunos casos de la EA, la acumulación de PrP junto con Aβ ocurre en la forma de placas difusas, no reactivas a Tioflavina S. Estos agregados son fácilmente eliminados con tratamiento con PK sugiriendo que esta acumulación corresponde exclusivamente a PrP^{C} o a $sPrP^{Sc}$ (tabla 1) [158;159]. Estos hallazgos son similares a lo observado en ratones doble transgénicos que sobre-expresan a la PPA con las mutaciones sueca (K670N/M671L) y de Indiana (V171F/V717G) (las cuales están asociadas a una fuerte deposición de Aβ) y la PrP de hámster [160]. Sin embargo, debido a la alta densidad de PrP en estas áreas, la posibilidad de formación de PrP^{Sc} *de novo* debería ser considerada. En este aspecto es muy importante el recordar que muchos casos de EET han sido asociados con $sPrP^{Sc}$.

Por otro lado, la co-existencia de PrP y Aβ en pacientes afectados con la sECJ y GSS ha sido también identificada (apéndice 1). Un estudio reciente ha mostrado que sorprendentemente un sub-grupo de pacientes afectados con la sECJ posee altos niveles de $A\beta_{42}$ en sus cerebros. Como consecuencia, placas de Aβ han sido encontradas en el cerebro de estos individuos mientras que los pacientes que poseen concentraciones menores de esta proteína poseen un menor de depósitos de Aβ o no poseen en lo absoluto. Interesantemente, los pacientes que poseen altos niveles de $A\beta_{42}$ presentan menores niveles de acumulación de PrP^{Sc} [161]. Estos datos pueden ser explicados de dos formas distintas. La primera explicación es que la presencia de Aβ inhibe el mal plegamiento y acumulación de PrP^{Sc}. La segunda es que parte de las moléculas de PrP^{Sc} sean utilizadas para inducir el mal plegamiento y agregación de Aβ en lugar

de aumentar la de PrP. La presencia de ambos agregados proteicos podría resultar en eventos tóxicos sinérgicos, gatillando la manifestación clínica de la EET sin una acumulación extensiva de PrP^{Sc}. Tomando en cuenta las características específicas de ambas enfermedades existe la posibilidad de que las características clínicas de la EA pudiesen camuflar a las de la sECJ. Desde otro punto de vista, la formación de PrP^{Sc} (producida por ejemplo por una exposición infecciosa) podría estimular la agregación de Aβ, lo cual finalmente desencadenaría una patología clínica asociada a la EA en lugar de una asociada a prionopatías. Un escenario interesante podríamos encontrar al estudiar la incidencia de la EA y otros DCP en países con una alta exposición a la EEB (como por ejemplo el Reino Unido o Francia). Esta evidencia y las hipótesis que surgen de ella urgen por nuevos experimentos en modelos animales que puedan responder apropiadamente las interrogantes en este importante tema.

Evidencia in vitro

La mejor forma de probar la hipótesis de nucleación heteróloga de amiloides es realizando ensayos de agregación *in vitro*. Estudios clásicos en esta dirección muestran que la adición de núcleos preformados compuestos de proteínas mal plegadas es capaz de reducir la extensión de la fase de nucleación en proteínas amiloidogénicas. Adicionalmente, otros estudios han mostrado que la extensión de la fase de nucleación puede ser reducida luego de la adición de núcleos proteicos heterólogos. Este efecto de nucleación heteróloga puede ser distinto de acuerdo al par proteína-proteína que se esté estudiando (apéndice 1).

Un ejemplo lo podemos ver en el caso de la interacción entre agregados amiloidogénicos de-amilina y Aβ [162]. De forma interesante, podemos asociar el efecto de estos agregados proteicos con lo anteriormente descrito para la posible interacción entre la EA y la diabetes tipo-2 [156]. Ensayos de agregación muestran que los agregados de Aβ son muy buenos promotores para la deposición de amilina, mientras que estructuras amiloides fibrilares o globulares de amilina tienen muy bajo efecto o son completamente ineficaces reclutando unidades monoméricas de Aβ. Estos resultados son bastantes interesantes si consideramos los datos clínicos descritos con anterioridad donde la interacción entre la EA y la diabetes tipo-2 parece funcionar solo en una dirección.

Otro ejemplo importante proviene de la nucleación heteróloga entre agregados de α-sinucleína y Aβ [157]. Los datos experimentales en este caso van aun mas allá, explicando posibles asociaciones entre ambas proteínas para formar oligómeros tóxicos. Resumidamente, este estudio postula que ambas proteína podrían formar estructuras anulares capaces de generar poros en la membrana plasmática. Se ha hipotetizado que estas estructuras podrían producir un desbalance iónico entre los espacios intra- y extra-celulares, resultando así en la muerte neuronal característica de estos desórdenes.

Al juntar estos ensayos con otros a realizar *in vitro* o *in silico* podríamos mejorar enormemente nuestro conocimiento acerca de este importante tema. Sin embargo, la caracterización de la interacción entre DCP es necesaria en modelos animales donde efectos multifactoriales como inflamación, eliminación de proteínas y cascadas de señal intra-celulares podrían contribuir dramáticamente al desarrollo y potenciación de la enfermedad.

Estudios en modelos animales

La mejor forma de analizar si dos DCP pueden interaccionar es utilizando modelos animales de ambas enfermedades. Los modelos animales permiten analizar muchas características patológicas de la enfermedad de forma controlada y en distintas etapas. Afortunadamente, muchos modelos que nos aproximan a estas enfermedades están disponibles actualmente. Utilizando esta aproximación algunos pares de enfermedades han sido estudiados. Entre ellos podemos citar los realizados para evaluar la interacción entre α-sinucleína y Aβ [157]. Los resultados en estos estudios indican que Aβ aumenta los niveles de acumulación de α-sinucleína en el cerebro de animales transgénicos. Experimentos similares se han realizado para evaluar la posible asociación entre agregados de Aβ y Tau, evento que podría acelerar las características patológicas de la EA. En este caso se ha visto que la deposición de Tau aumenta ante la presencia de Aβ, no así en el caso opuesto [32;33].

Considerando los hallazgos que sugieren la co-existencia entre la EA y la sECJ, el estudio de una posible interacción entre ambas enfermedades es muy importante. Como ha sido sugerido por Debatin y cols. [161], el hecho de que el fenotipo de la EA pueda enmascarar algunos casos de sECJ hace este tópico aun más relevante. Debido a que Aβ y PrP^{Sc} tienen mecanismos de agregación similares y están localizados en el mismo tejido (cerebro) y localización sub-celular (espacio extra-celular) se podría esperar que ambos agregados proteicos pudiesen interactuar. Sin embargo, estudios recientes sugieren que la PrP^{C} podría tener un rol protector en la EA inhibiendo la actividad de la β-secretasa [163] estimulan aún más estudios adicionales en esta área. Las características estables de la acumulación de Aβ en animales transgénicos y los periodos constantes de incubación de priones en ratones deberían hacer de este tipo de estudios fáciles de seguir.

6. La infectividad intrínseca de los agregados amiloides. El caso de la Enfermedad de Alzheimer.

Los mecanismos de polimerización y los intermediaros estructurales que se generan en el proceso son comunes en todo proceso de agregación de amiloides. Los procesos por los cuales los amiloides son formados y replicados son de hecho bastante similares a los identificados en la replicación priónica descrita *in vivo*. De hecho, el modelo de nucleación-polimerización proporciona una excelente explicación a la naturaleza infecciosa de los priones y sugiere que los eventos de agregación proteica en los DCP tienen la habilidad inherente de ser transmisibles (apéndice 4 y [164]). La infectividad de los oligómeros formados por PrP^{Sc} yace en su capacidad de reclutar unidades solubles de PrP^{C}. La aceleración en la formación de amiloides al inocular núcleos preformados al sistema ha sido convincentemente demostrada *in vitro* para muchas proteínas implicadas en procesos patológicos [20]. Extrapolando estos hechos a situaciones *in vivo*, la administración correcta de estructuras pre-agregadas y estables podría acelerar substancialmente el mal plegamiento, acumulación y deposición de agregados proteicos tóxicos en tejidos. Si consideramos que la agregación y deposición de estas estructuras es la causa de la enfermedad podemos pensar que la administración exógena de agregados proteicos causaría una aceleración de procesos patológicos que en la ausencia de estos núcleos ocurrirían mucho más tarde en la vida o no ocurrirían nunca durante el período de vida del individuo.

Considerando el conocimiento que se ha obtenido durante años de investigación en las EET, es bastante probable que la infección producida por proteínas anómalamente plegadas

produjera períodos de incubación considerablemente largos antes de que la enfermedad se manifestara clínicamente. Es por este mismo motivo que un posible origen infeccioso para estas enfermedades sería difícil de identificar. El reconocimiento de las EET como infecciosas fue posible principalmente debido a un par de eventos fortuitos: el uso de materiales contaminados para tratar ganado ovino en la década de 1930 [59] y el descubrimiento de la transmisión del Kuru por rituales caníbales [165]. Además, la rara prevalencia de las EET hace fácil su identificación. Sin embargo, en enfermedades más frecuentes como la EA, la EP o la diabetes tipo-2, asociar la aparición de la enfermedad con un posible evento infectivo que pudo ocurrir décadas antes sería muy difícil. Las similitudes mecanísticas entre las EET y otros DCP han llevado a muchos investigadores a analizar esta posibilidad. Como resultado de estos estudios mucha información a favor y en contra de la transmisibilidad de este tipo de desordenes ha sido generada.

Entre los DCP, la búsqueda de una posible transmisión de la EA ha sido analizada debido a la alta prevalencia de este desorden. La idea de que la EA pueda ser infecciosa ha estado presente por mucho tiempo. Las primeras aproximaciones para probar esta hipótesis consistieron en la inoculación de homogenizados de cerebros de pacientes afectados con la EA en primates no humanos. Los resultados en estos experimentos fueron contradictorios, algunos resultando positivos y otros negativos [166;167]. Una razón posible para explicar estos datos la podemos encontrar en el bajo número de primates utilizados en estos experimentos. Adicionalmente, un posible efecto de "barrera de especies" similar al descrito en las EET podría también ser responsable de estos resultados. Sin embargo, el número limitado de animales utilizados y la falta de conocimiento acerca de la transmisión inter-especie de priones en aquel momento sugiere que estos estudios deben ser repetidos utilizando aproximaciones experimentales óptimas.

Con el desarrollo de animales transgénicos que expresan la PPA humana (y que presentan algunas de las características neuropatologías, bioquímicas y clínicas de la EA) la búsqueda de un posible origen infeccioso para la EA tomó un nuevo aliento. Recientemente, Kane y cols. han mostrado que la inoculación de homogenizados de cerebros de pacientes afectados con la EA pueden acelerar la deposición de Aβ en ratones tg2576 [168]. En estos estudios, la inyección de preparaciones derivadas de la EA aceleró el proceso de deposición 5 meses luego de la inoculación, pero solo en el hemisferio cerebral inoculado. Luego de 12 meses, los animales experimentales presentaron acumulación de Aβ en ambos hemisferios aunque la deposición era notoriamente mayoritaria en el hemisferio inoculado. Un estudio posterior mostro que la actividad "transmisible" de homogenizados de cerebros asociados a la EA se perdían luego de ser sometidos a inmunodepleción de Aβ, desnaturalización proteica o inmunización contra Aβ [169]. Sin embargo, debido a que los modelos animales utilizados espontáneamente generan una deposición de Aβ en alguna etapa de la vida, con estos experimentos no es posible afirmar que los agregados presentes en los homogenizados derivados de la EA sean "infecciosos". En este caso solo podemos hablar de la aceleración de un fenómeno que ocurriría posteriormente en la vida del animal debido a la manipulación genética a la que han sido sometidos.

Fenómenos similares se han encontrado para otras enfermedades como la amiloidosis sistémica reactiva o amilidosis senil de ratones [170;171]. En ambos casos, fenómenos de transmisión de amiloides, similares a los encontrados para las EET han sido descritos. Interesantemente, algunos de estos casos han sido descritos como transmisibles vía ruta oral, e incluso variaciones similares al fenómeno de cepas encontrado en las EET han sido reportados [172;173]. En resumen, las grandes similitudes de transmisión entre otras amiloidosis y las EET

obligan a realizar estudios más fino con respecto a estas enfermedades, identificando posibles mecanismos de transmisión patológicos con el propósito de identificarlos y prevenirlos.

7. Estado actual del estudio de los Desórdenes Conformacionales Proteicos: Diagnostico y terapia.

Métodos diagnósticos en etapas pre-sintomáticas y curas definitivas son actualmente inexistentes para todos de los DCP tratados anteriormente. En el caso de las enfermedades producidas por priones, la identificación de la enfermedad, incluso en etapas tempranas de manifestación clínica, hace inútil todo intento de terapia debido a la rápida progresión que los pacientes sufren. En este momento la única forma definitiva de diagnosticar estas enfermedades es por análisis post-mortem de tejido cerebral. Es importante señalar que los casos existentes de infectividad vía transfusión sanguínea han sido a través de pacientes incubando la enfermedad, sin ninguna evidencia clínica de prionopatía asociada al momento de donar sangre [104]. Actualmente, el desarrollo de técnicas como la *PMCA* anteriormente explicada abren nuevas esperanzas de tener, a corto plazo, un sistema de detección para el diagnostico de estas enfermedades [174;175].

Agravando el problema existente tenemos al controversial fenómeno de variación de cepas de priones y trasmisiones inter-especies (apéndice 3). Como ya he mencionado, la generación de nuevas cepas de priones podría tener consecuencias impredecibles para el ser humano en cuanto a la falta de entendimiento con respecto a las leyes que dictan la transmisión entre especies (y entre cepas). La posibilidad de que un ilimitado número de cepas de priones pueda ser generado postula que un mayor control, tanto diagnóstico como de evaluación de la propagación entre especies, deba ser considerado.

Sumado a este fenómeno se encuentra la posible interacción e influencia que ciertos agregados proteicos tendrían en la acumulación de otros, lo cual indicaría que un DCP podría ser un factor de riesgo para la aparición de un segundo DCP (apéndice 1). La alta incidencia de enfermedades "esporádicas" en los DCP hace de este tema un interesante tópico por analizar. Siguiendo esta lógica, y considerando una posible naturaleza infecciosa en los agregados amiloides (apéndice 4), estudios minuciosos en esta dirección deben ser tomados en cuenta. Por los motivos anteriormente mencionados, mi estudio de tesis doctoral se ha enfocado en explicar parcialmente estos procesos.

SECCIÓN II: HIPÓTESIS.

El agente infeccioso responsable de las EET está compuesto exclusivamente por una proteína y las cepas de priones son determinadas por las características conformacionales y bioquímicas de ésta. Toda la información requerida para propagar priones, mantener las características de cepa y cruzar las barreras de infectividad entre especies está contenida y depende de la estructura de la PrP^{Sc}, la cual puede ser reproducida *in vitro* usando la tecnología de la *PMCA*. Adicionalmente, siguiendo el mecanismo propuesto para el modelo de nucleación-polimerización de amiloides, postulamos la interacción de agregados proteicos en los DCP y que una enfermedad de este grupo puede ser un factor de riesgo para un segundo DCP. Además, postulamos que otras enfermedades de este grupo, como la enfermedad de Alzheimer, pueden seguir una ruta de transmisibilidad similar a lo que ocurre en las Encefalopatías Espongiformes Transmisibles.

SECCIÓN III: OBJETIVOS.

1. Objetivo General

Estudiar las bases moleculares responsables de la generación de cepas de priones y barrera de especies, ensayando *in vitro* la propagación de cepas de priones, el cruce de barrera de especies y la generación de nuevas cepas de priones. Además se estudiará la posible interacción entre distintos DCP y la inefectividad intrínseca de estos agregados en modelos animales de la enfermedad de Alzheimer.

2. Objetivos específicos

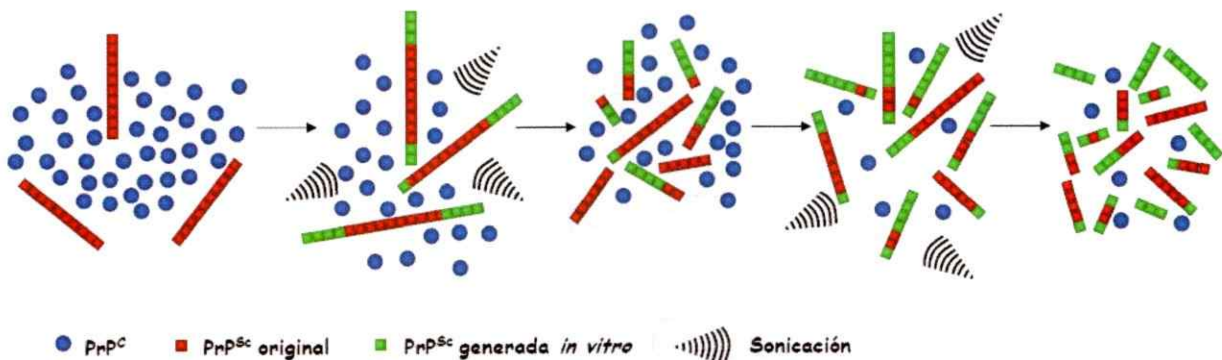
- Propagar *in vitro* distintas cepas de priones de ratón, estudiando sus características bioquímicas *in vitro* y sus propiedades infectivas *in vivo*.
- Reproducir *in vitro* el fenómeno de barrera de especies en las EET y utilizar los estudios de replicación de priones *in vitro* para evaluar el grado de dificultad en la transmisión de priones entre distintas especies y la generación de nuevas cepas de priones.
- Estudiar el efecto de polimorfismos en la PrP con respecto a la propagación de cepas de priones.
- Analizar la interacción de PrP^{Sc} y Aβ en modelos animales de la Encefalopatías Espongiformes Transmisibles y de la enfermedad de Alzheimer.
- Transmitir características patológicas de la enfermedad de Alzheimer inoculando material derivado de pacientes afectados por esta patología en animales transgénicos.

SECCIÓN IV: RESULTADOS

1. Replicación de cepas de priones en sistemas libres de células

El primer punto de estudio en mi trabajo de tesis doctoral involucró la replicación de distintas cepas de priones en sistemas libres de células. La idea de este primer punto es la de proporcionar evidencia sustancial que indique que la replicación *in vitro* de priones mantiene las características bioquímicas e infecciosas de la PrP^{Sc} inicialmente utilizada como inóculo. Teniendo en cuenta que las características propias de cada cepa de prion radican en su disposición tri-dimensoional, resultados positivos en este experimento apoyarían la hipótesis de diversidad conformacional entre cepas de priones. Además, este punto será la base que nos permitirá validar a la *PMCA* como una técnica útil en el análisis de materiales infecciosos y desentrañar los mecanismos involucrados en su generación y diversidad.

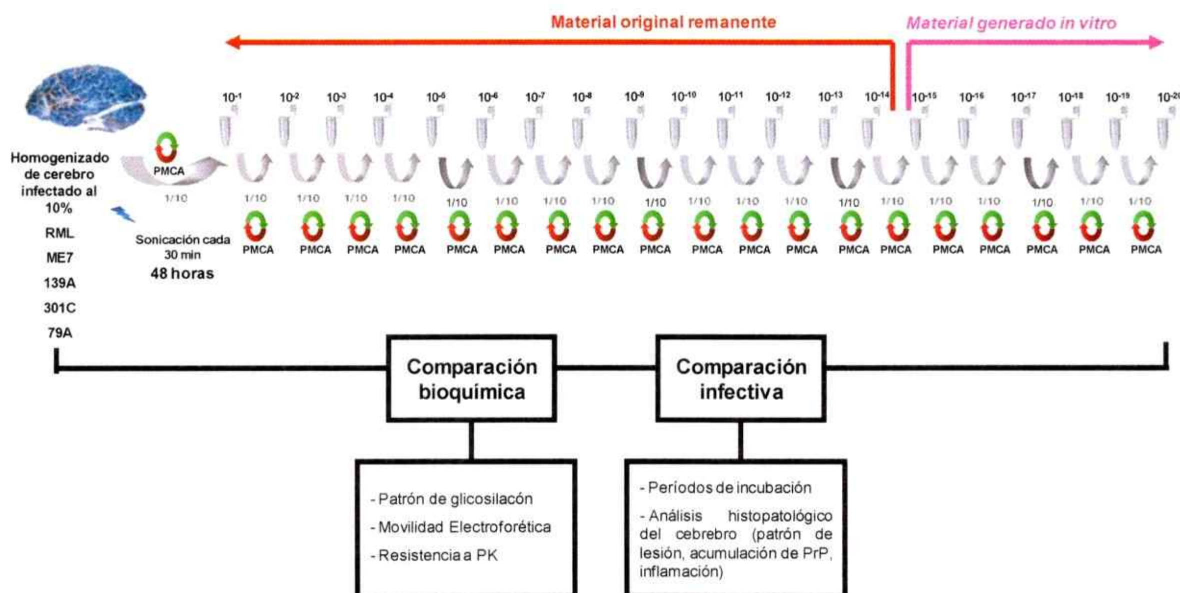
Figura 2. Representación esquemática del proceso de *Protein Misfolding Cyclic Amplification* (*PMCA*). Homogenizados de cerebros sanos (fuente de PrP^C o sustrato) se mezclan con homogenizados de tejidos infectados (fuente de PrP^{Sc} o inóculo) y se someten a ciclos de incubación/sonicación. La incubación de sustratos e inóculos permite la conversión de PrP^C a PrP^{Sc}, fomentándose su acumulación (fibrilar). El proceso de sonicación rompe las fibras generadas, resultando así en la producción de nuevas unidades infecciosas.



Con el propósito de validar nuestra hipótesis en este punto utilizamos cinco cepas de ratón, las cuales comparten propiedades bioquímicas y patológicas similares. Cuatro de estas cepas, denominadas RML, ME7, 139A y 79A han sido derivadas de distintas fuentes de scrapie. Ellas presentan periodos de incubación y características bioquímicas similares, donde principalmente podemos destacar el patrón de movilidad electroforético (aproximadamente 21 KDa para la forma sin glicosilar) y su enriquecimiento en la forma mono-glicosilada. La principal diferencia entre ellas radica en el patrón de vacuolización que generan en el cerebro de los animales infectados. La quinta cepa utilizada en este estudio corresponde a la cepa 301C, la cual fue originada a partir de la EEB. Ella presenta claras diferencias en las características patológicas (períodos de incubación y vacuolización) y bioquímicas (19 KDa para la forma sin glicosilar y principalmente di-glicosilada) en comparación con las cepas adaptadas de scrapie. Con el propósito de propagar estas distintas cepas de PrP^{Sc}, utilizamos la técnica denominada *PMCA*. Haciendo un rápido recuento de lo anteriormente mencionado, esta técnica utiliza homogenizados de cerebros de animales sanos y afectados de EET como sustratos (fuente de PrP^C) e inóculos (fuente de PrP^{Sc}), respectivamente (apéndice 7). En este proceso, la PrP^{Sc} oligomérica (semillas o núcleos de agregación) es amplificada *in vitro* a expensas de la PrP^C de

los cerebros sanos utilizando ciclos de incubación-sonicación. El modelo de la figura 2 describe los procesos involucrados en la *PMCA*. Con el fin de incrementar el nivel de amplificación hemos introducido el concepto de la *PMCA* seriada, en la cual varias rondas de aproximadamente 100 ciclos cada una son realizadas (figura 3 y [99]). El proceso consiste en agregar nuevo sustrato entre cada ronda, mediante dilución del material amplificado en homogenizado de cerebro normal. La idea de realizar pases seriados en nuestro estudio es diluir el material original de forma tal que se trabaje finalmente solo con el material generado *in vitro*. De esta forma los estudios bioquímicos e infectivos a realizar con el material obtenido *in vitro* no serán afectados por el inóculo original. Información adicional con respecto a la estrategia experimental utilizada se explica en la sección *Materials and Methods* del Apéndice 8.

<u>Figura 3.</u> **Amplificación seriada en el proceso de *PMCA*.** Homogenizados de cerebros de ratones infectados con distintas cepas de priones se mezclan con homogenizados de cerebros sanos. Luego de una ronda de *PMCA*, el material resultante se diluyó nuevamente en sustrato fresco (ver texto). El proceso se repetió hasta obtener una dilución 10^{-20} con respecto al material infeccioso original. Se realizaron análisis bioquímicos e infectivos del material obtenido en pase 20, comparándolo en cada caso con la PrP^{Sc} original.

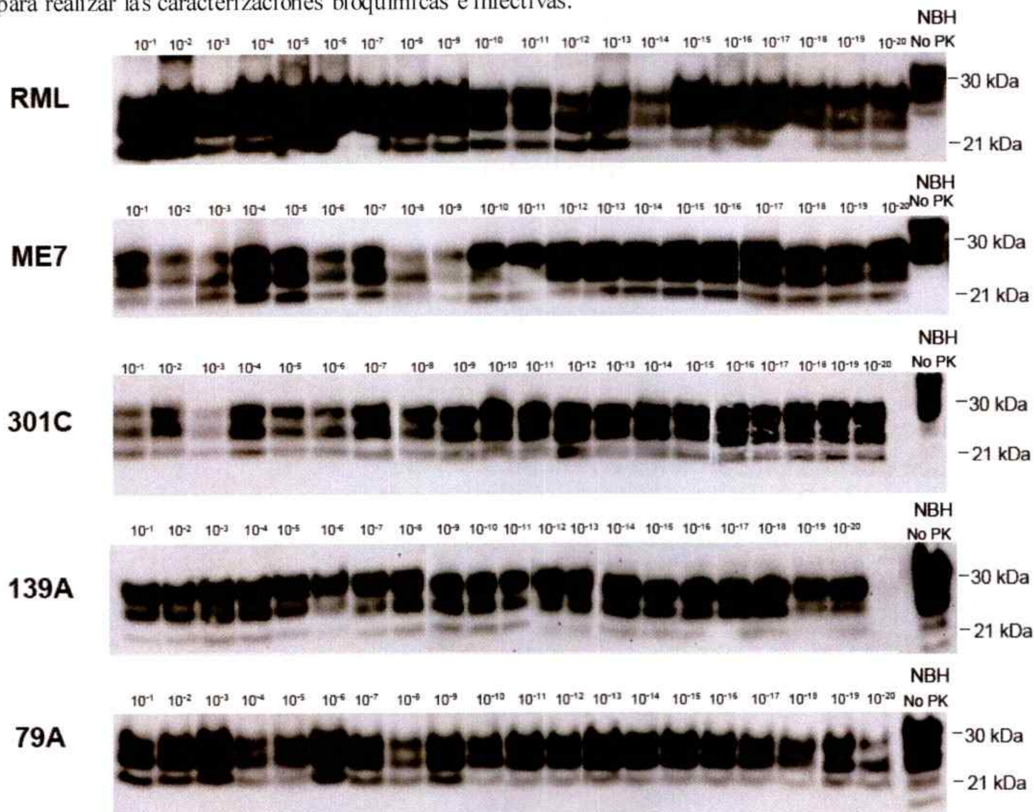


Las cepas que se utilizarán en la primera parte de nuestro estudio son RML, ME7, 139A y 79A, originadas a partir de diversas fuentes de scrapie; y 301C, cepa adaptada de la EEB. El agente infeccioso en todas estas cepas contiene la misma secuencia aminoacídica, variando solamente la fuente original de PrP^{Sc} a partir de la cual se generaron. Como se ha mencionado anteriormente, estas cepas pueden ser distinguidas tras analizar las características bioquímicas e infecciosas de cada una. Para demostrar que las características de cada cepa se mantienen después de su amplificación por *PMCA*, se analizarán distintas propiedades de los priones generados *in vitro*, comparándolos con la PrP^{Sc} original (figuras 3 y 4).

Luego de amplificar el material infeccioso durante veinte rondas de *PMCA* (con lo que nos aseguramos que nuestro material está compuesto solo por priones generados *in vitro*), analizamos algunas de sus propiedades bioquímicas, como movilidades electroforéticas luego de digestión con PK y patrones de glicosilación. Nuestros resultados muestran claramente que estas características se mantienen luego de su paso por nuestro sistema de amplificación *in vitro*, lo que sugiere que las propiedades bioquímicas de cada cepa de prion pueden ser reproducidas por

la *PMCA* (apéndice 8). Adicionalmente se realizó un proceso similar para cuatro cepas de priones humanas (trabajo realizado por el señor Marcelo Barría). Las figura 1c y 1d del apéndice 8 muestran que estas características son claramente propagadas por la *PMCA*, confirmando nuestros resultados vistos en distintas cepas de ratón.

Figura 4. Amplificación seriada de distintas cepas de priones de ratón utilizando *PMCA*.
Cada muestra amplificada se digirió con PK por 1h a 37ºC. NBH representa homogenizado de cerebros sanos (sin tratamiento con PK) el que es utilizado para comparar movilidades electroforéticas. El producto obtenido en la dilución 10^{-20} se utilizó para realizar las caracterizaciones bioquímicas e infectivas.



Posteriormente, analizamos las propiedades infectivas de la $PrP^{27\text{-}30}$ generada luego de veinte pases seriados de *PMCA* (libre de inóculo original) en ratones C57BL. Los resultados en este caso arrojaron períodos de incubación comparables a los producidos por priones infecciosos derivados de cerebros de animales infectados (apéndice 8). Para comprobar nuestros resultados realizamos un segundo pase de infectividad del material infeccioso generado en *PMCA* nuevamente en ratones C57BL. Los resultados obtenidos confirmaron nuestros hallazgos anteriores al resultar en similitudes interesantes con el material original y con el primer pase de infectividad utilizando a las cepas propagadas *in vitro* (apéndice 8). Esta información sugiere que las características infectivas del material generado eran las mismas que las presentadas por el inóculo original.

Adicionalmente, estudiamos la generación de características patológicas en los animales inoculados con el material generado *in vitro*. Claramente se observó que el material generado por *PMCA* producía características histopatológicas clásicas en el cerebro de los animales infectados, como vacuolización, inflamación y acumulación de PrP en distintas zonas del cerebro (apéndice

8). Yendo más lejos en el análisis de estas propiedades realizamos una comparación del patrón de vacuolización de cada una de estas cepas, comparando lo producido por el material generado *in vitro* con su fuente inicial. Nuevamente, los resultados en este caso fueron positivos, mostrando que las características histopatológicas de cada inóculo eran mantenidas luego de su amplificación *in vitro* (apéndice 8).

Finalmente se estudiaron las características bioquímicas de la PrP^{res} generada en el cerebro de los animales inoculados con el material generado por *PMCA* y se compararon con las cepas originales para confirmar, una vez más, que el material generado *in vitro* puede ser fielmente replicado nuevamente en el cerebro de animales sanos. En la figura 5 del apéndice 8 se puede observar que las características bioquímicas estudiadas en la figura 1 del mismo apéndice 8 se mantuvieron. Un análisis adicional consistió en revisar la resistencia específica a PK que este material podía generar. En la figura 6 del apéndice 8 se ven de forma representativa los datos para las cepas 139A, 79A y 301C. Nuevamente similitudes importantes fueron encontradas en cada par de muestras estudiadas. Es importante el señalar que datos similares a los encontrados aquí se han visto para la cepa HY de hámster (Morales, R., Castilla, J. y Soto, C.; datos no publicados).

Estos resultados confirman la hipótesis de que las características de cada cepa de prion son altamente dependientes del plegamiento que la proteína infecciosa pueda adquirir. Además hemos demostrado que la *PMCA* es capaz de replicar fidedignamente la conformación tridimensional de estas proteínas en un sistema libre de células. Estos resultados son un sólido apoyo a la teoría de infectividad proteica en las EET.

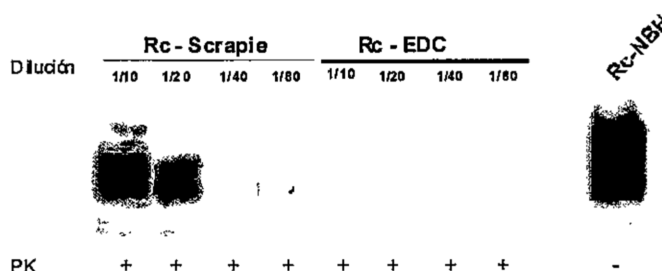


Figura 5. PrP^{Sc} generada in vivo en mapaches tras inocular material infeccioso proveniente de scrapie y de la EDC. Diluciones seriadas a partir de homogenizado de cerebros al 10% utilizando como disolvente homogenizados de cerebros de mapaches sanos al 10% (preparado como está descrito en la sección *Materials and Methods* del apéndice 8). Rc-NBH corresponde a muestras de mapaches sanos sin tratamiento con PK como marcador de movilidad electroforética.

2. Validación del fenómeno de barrera de Especie en las EET utilizando sistemas *in vitro*

Una de las principales fuentes de variación de cepa en las EET es la transmisión inter-especie de priones infecciosos. Este tema es de suma importancia debido a la epidemia de EEB en la década de los 90 del siglo pasado y la posterior identificación de la vECJ como consecuencia.

En la sección I de este escrito se ha introducido el fenómeno de transmisión inter-especie de priones. Una vez que una infección inter-especie de priones ha sido realizada podemos encontrar barreras de especie que son fáciles de cruzar, otras intermedias y otras muy difíciles. Un bello ejemplo de este fenómeno lo hallamos en el mapache (*Procyon lotor*), especie que ha sido identificada como posible receptora de priones silvestres. Ejemplificando las distintas posibilidades que pueden resultar de una infección inter-especie podemos encontrar en mapaches una barrera fácil de cruzar en los priones infecciosos de visón (Encefalopatía Transmisible del

Visón- ETV), lo que se refleja en períodos de incubación de aproximadamente seis meses. Una barrera intermedia está ejemplificada con scrapie, la cual resulta en manifestación clínica solo luego de dos años posterior a su inoculación. Finalmente, una barrera de especie absoluta está dada por priones de la EDC de ciervos, donde los mapaches no presentan ninguna manifestación clínica luego de muchos años posterior a su inoculación [176;177].

Tomando estos componentes, y reemplazando el material de ETV por scrapie adaptado en mapache (donde no esperamos una barrera de especie), evaluamos *in vitro* la dificultad de convertir priones infecciosos de mapache utilizando la tecnología de la *PMCA* (para ver la carga de PrP^{27-30} (tabla 1) en estos inóculos (figura 5). Luego de 5 rondas de *PMCA* encontramos que el fenómeno encontrado *in vivo* fue reproducido en los tres casos (tabla 2). El material adaptado de scrapie en mapache fue capaz de darnos señal en todas las rondas de *PMCA*, lo cual evidencia que estos priones no presentan una barrera alguna en su replicación. El material de scrapie solo presentó señal de amplificación en los pases cuarto y quinto de *PMCA* sugiriendo una dificultad inicial en su replicación, la cual en rondas sucesivas es superada. Finalmente, el material de EDC no presentó señal en ninguno de los cinco pases de *PMCA* realizados. Controles negativos en este experimento (homogenizado de cerebros de mapaches sin infectar) no presentaron ninguna amplificación. Con el propósito de enriquecer el sistema en presencia de posibles especies de PrP^{Sc} en muestras de mapaches inoculados con la EDC, se realizaron ensayos utilizando cerebros de mapaches inoculados con este agente como sustrato, EDC como inoculo, y pases subsecuentes utilizando el mismo sustrato en cada caso. Resultados negativos en este ensayo nuevamente demostraron la alta resistencia de priones de EDC en cambiar la conformación de la PrP^{C} de mapache. Estos datos dan cuenta de que el fenómeno de transmisión inter-especie es reproducido *in vitro* y nos sugiere que un sistema *in vitro* de infectividad inter-especie puede ser estandarizado. Estos hallazgos podrían tener un fuerte impacto en salud pública e industria veterinaria.

Tabla 2. Análisis de replicación de PrP^{Sc} de distintas fuentes en sustratos de mapaches.
La amplificación se llevó a cabo utilizando 96 ciclos de PMCA en cada ronda. Los inóculos utilizados corresponden a Scrapie adaptado en mapache (Rc-Sc), Scrapie y CWD (EDC). Como control se utilizaron los mismos ciclos de PMCA en homogenizados de cerebros sanos sin inóculo (NBH – *Normal Brain Homogenate*)

Grupos	PMCA 1	PMCA 2	PMCA 3	PMCA 4	**PMCA 5**
Rc-Sc	+	+	+	+	**+**
Scrapie	-	-	-	+	**+**
CWD	-	-	-	-	**-**
NBH	-	-	-	-	**-**

Debido a la alta sensibilidad en detección que la *PMCA* ha mostrado, hemos tratado de forzar el sistema para evaluar la posible generación de priones infecciosos en mapaches utilizando EDC como inóculo. La forma de forzar este sistema se realizó concentrando el material proveniente de mapaches infectados con la EDC (y que no mostraban signos clínicos),

seguido de una resuspensión del pellet en homogenizado de cerebro de mapaches sanos. Sorprendentemente este ensayo resultó en la generación de un material resistente a proteasas, similar al asociado regularmente a priones infecciosos (figura 6). Todos los controles adicionales en este experimento proponen que la presencia de este material podría corresponder a especies priónicas infecciosas. Estos resultados sugieren que forzando la *PMCA* podemos cruzar barreras de especies consideradas actualmente como absolutas y que nuevas cepas de priones podrían ser generadas como consecuencia.

Para concluir este punto deseo mencionar que estos resultados nos muestran que la *PMCA* puede ser reproducida en modelos clásicos de experimentación y en cualquier sistema vivo que pueda ser afectado por estas enfermedades. Además, estos datos nos dan una clara evidencia de que el fenómeno de transmisión inter-especies puede ser reproducido *in vitro*.

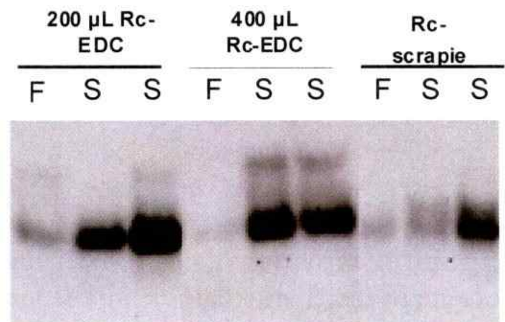


Figura 6. Amplificación de material priónico derivado de la EDC en homogenizado de cerebros de mapaches. 200 µL y 400 µL de homogenizado de cerebro de mapaches inoculados con material infeccioso derivado de la EDC (Rc-EDC) se concentraron por ultracentrifugación a 100000g por una hora a 4 C. Los sedimentos resultantes se resuspendieron en 100 µL de homogenizado de cerebros de mapaches sanos y se sometieron a 96 ciclos de *PMCA*. El material generado se trató con PK y fue analizado por Western blot. Rc-scrapie corresponde al control de amplificación, donde 10 µL de scrapie adaptado en mapache generado *in vitro* fueron mezclados con 90 µL de homogenizado de cerebros de mapaches sanos. S: Material cometido a PMCA. F: Material congelado sin someter a PMCA (control de amplificación).

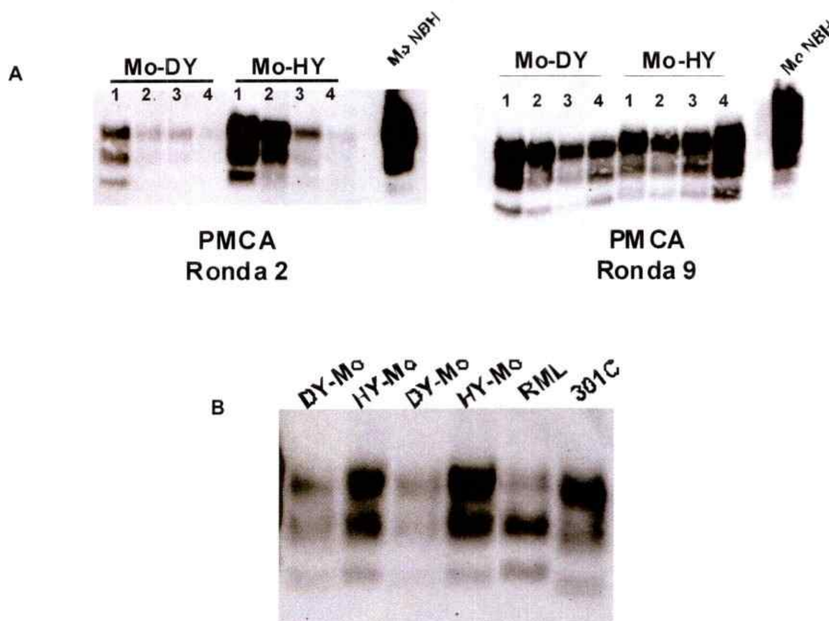
3. Cruces de barrera por replicación de PrP^{Sc} *in vitro* generan nuevas cepas de priones

La información obtenida en el punto anterior nos lleva a explorar el fenómeno de variación de cepa de una forma más controlada. Para ello utilizamos como modelo animales de experimentación clásicos para estas enfermedades, como lo son ratones y hámsteres. El análisis de estas especies nos da dos ventajas. Primero, que la infección inter-especie entre ellos está bien documentada y segundo que la diferencia en la secuencia aminoacídica en sus PrPs nos permite realizar inmuno-análisis diferenciados. El trabajo presentado en los dos siguientes párrafos representa un trabajo en conjunto en el cual participaron el Dr. Joaquín Castilla, la Dra. Paula Saá, la señorita Dennisse González-Romero, el señor Jorge De Castro y el Dr. Claudio Soto (apéndice 9).

Se ha hipotetizado que las diferencias en infectividad entre distintas especies se deben principalmente a la diferente secuencia aminoacídica que el prion pueda tener. Esto es relativo, ya que en muchos casos similitudes en la secuencia del prion de distintas especies presentan grandes diferencias con respecto a la infectividad y a las cepas que pueden propagar. Un claro ejemplo lo podemos ver en el caso de infecciones polimórficas en ratones y las distintas susceptibilidades a enfermedades priónicas en humanos de acuerdo al residuo aminoacídico presente en la posición 129. Las barreras de infectividad descritas entre ratones y hámsteres se pueden clasificar como fuertes o absolutas. Con respecto a la cepa de ratón RML, nuestro sistema de amplificación *in vitro* puede amplificar este material sin problemas a una dilución de

1:1000 con respecto al material infeccioso original cuando se utiliza homogenizado de cerebro de ratones sanos como sustrato. Utilizando las mismas condiciones, y reemplazando los priones RML por 263K (una estable cepa de priones de hámster) la amplificación a esta dilución es nula (apéndice 9). Como fue desarrollado en el punto anterior, para romper esta barrera debemos forzar el sistema, por ejemplo, incrementando la cantidad inicial de priones infecciosos. Como podemos observar en la figura 1 del apéndice 9, la barrera de infectividad inter-especie puede ser rota utilizando esta estrategia. Tomando ventaja del anticuerpo 3F4 que reconoce específicamente priones de hámster, podemos verificar que los priones generados corresponden exclusivamente a PrP de esta especie (apéndice 9). De forma interesante observamos un proceso de adaptación en el material infeccioso, similar al que se observa *in vivo*. Nuestros resultados muestran que tras una ronda de *PMCA* en la nueva especie el perfil electroforético es similar al que encontramos para priones de ratón, patrón que luego se pierde y se hace similar al perfil clásicamente encontrado para priones de hámsteres (apéndice 9). Sorprendentemente encontramos que algunas de las características patológicas e infectivas de los priones generados luego de 20 rondas de *PMCA* eran distintas a cualquier otra cepa de priones conocida, sugiriendo que estos priones correspondían a una nueva cepa de estos agentes infecciosos (apéndice 9).

Figura 7. Perfil bioquímico de las cepas de hámsteres Hyper y Drowsy luego de su adaptación *in vitro* en sustrato de ratón.
(A) Resultados de la amplificación de Hyper y Drowsy en la segunda y novena ronda de *PMCA*. MoNBH corresponde a homogenizado de cerebros sanos sin tratamiento con PK, el cual nos sirve como control de movilidad electroforética para PrP^{C}. Cada ensayo fue realizado en cuadruplicado. (B) PrP^{Sc} generada tras la inoculación en ratones sanos del material generado *in vitro* luego de 15 rondas de *PMCA*. Como controles de movilidad electroforética y de perfil de glicosilación se utilizaron las cepas 301C y RML. Todas las muestras en B fueron pre-tratadas con PK.



Una tendencia similar en los resultados se encontró luego de que priones 263K de hámsteres fueran ensayados en *PMCA* utilizando homogenizados de cerebros de ratones sanos como sustrato. En este caso, los períodos de incubación fueron medidos en distintas rondas de *PMCA* con el propósito de analizar el fenómeno de adaptación de la nueva cepa de forma más cuidadosa. En este caso, observamos que en el primer pase de *PMCA* los períodos de incubación

fueron muy extendidos y además que el número de animales afectados nunca alcanzó al 100%. En subsecuentes rondas de *PMCA* observamos que los períodos de incubación se redujeron, siendo todos los animales inoculados afectados por signos clínicos (apéndice 9). Como en el cruce entre priones de ratón en hámsteres anteriormente descrito, las características patológicas e infectivas en este ensayo sugieren el aislamiento de una nueva cepa de priones (apéndice 9). Estos resultados nos sugieren nuevamente que el fenómeno de transmisión inter-especie se reproduce *in vitro* utilizando la técnica denominada *PMCA*. Estos resultados apoyan una vez más que las características de cepa están dictadas por la conformación de la PrP^{Sc}. Además, podemos ver que utilizando esta aproximación podemos analizar la extensión de la barrera de infectividad entre pares específicos de especies. Finalmente, pero no menos importante, la *PMCA* nos permite crear nuevas cepas de priones y evaluar el origen de algunas naturalmente generadas.

Además, hemos realizado otro estudio de transmisión inter-especies utilizando las cepas DY y HY de hámsteres utilizando PrP^C de ratón como sustrato. Los resultados de la amplificación nos muestran que los nuevos priones generados en sustrato de ratón en distintas rondas de *PMCA* poseen propiedades de movilidad electroforéticas similares a las de las cepas DY y HY (figura 7). Estudios de infectividad para ambas cepas nos muestran que no existen diferencias significativas en los períodos de incubación para los materiales generados (figura 7). Actualmente, la única cepa de ratón en nuestro laboratorio con una movilidad electroforética "rápida" (19KDa) para la banda sin glicosilar es la cepa 301C, la cual presenta períodos de incubación que van en el rango de 180-200 días. Los períodos de incubación en priones derivados de DY (DYMo), sumados a su perfil electroforético (figura 7) sugieren la presencia de una nueva cepa de priones infecciosos. La cepa derivada de HY (HYMo) nos da un período de incubación similar a muchas cepas de priones derivadas de scrapie y que presentan la misma movilidad electroforética. Sorprendentemente, el análisis del patrón de glicosilación en el material generado es distinto a las cepas anteriormente mencionadas, ya que mientras que ellas presentan preferencialmente una banda monoglicosilada, los priones en HYMo son principalmente diglicosilados, lo cual es una característica típica de los priones de hámsteres (figura 7). Estos datos una vez más confirman que la utilización de *PMCA* involucrando especies distintas puede resultar en la generación de nuevas cepas de priones, lo que puede ser muy útil en el estudio de la dinámica infecciosa en estas patologías y, posiblemente, en la búsqueda de nuevas terapias para estas enfermedades.

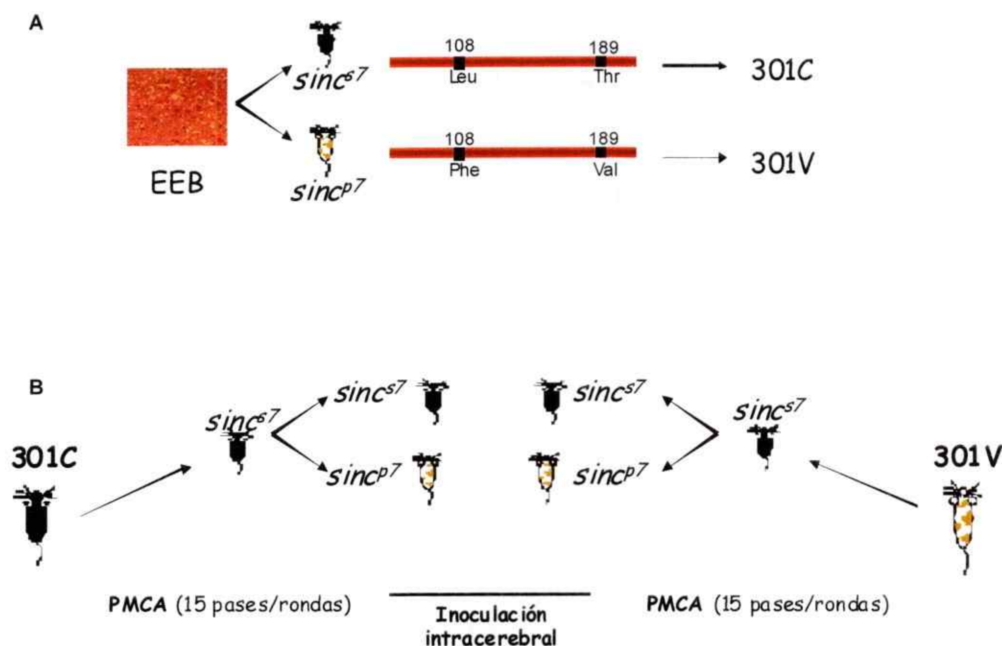
Tabla 3. Períodos de incubación de priones 301C y 301V amplificados en sustrato *sinc^{s7}*.
Los datos representan el promedio de los períodos de incubación de los animales infectados error estándar.

Prion Strain	*sinc^{s7}*	*sinc^{p7}*	PMCA C57 (*sinc^{s7}*)	
			sinc^{s7}	sinc^{p7}
301C	189,0 ± 1,7	398,3 ± 15,2	180,6 ± 2,3	392,25 ± 17,2
301V	305,8 ± 1,4	156,6 ± 4,5	204,0 ± 0,9	406,0 ± 12,8

4. **Cruces *in vitro* de PrP^{Sc} utilizando sistemas polimórficos**

Como hemos visto, alguna de las fuentes en la generación de distintas cepas de priones incluye la nucleación utilizando proteínas de distinta secuencia. Por este motivo hemos analizado la presencia de barreras de infectividad en la misma especie, pero utilizando individuos que naturalmente presentan diferencias en la secuencia aminoacídica del prion. Como modelos experimentales hemos utilizado a las cepas de ratones C57Bl e I/LnJ, las cuales son representativas de los grupos que denominaremos $sinc^{s7}$ y $sinc^{p7}$, respectivamente (variaciones en las posiciones 108 y 189 en PrP respectivamente, figura 8). Los agentes infecciosos utilizados en este grupo de experimentos incluyen a las cepas infectivas 301C y 301V, generadas en ratones $sinc^{s7}$ y $sinc^{p7}$, respectivamente. La idea de este trabajo fue realizar cruces del material infeccioso *in vitro* e *in vivo* en los contextos genéticos opuestos, viendo las características de los materiales infecciosos generados (figura 8).

Figura 8. Polimorfismos en la proteína del prion y cruces de infectividad inter-polimórficos.
(A) Infección intra-cerebral de la EEB en ratones de los grupos $sinc^{s7}$ y $sinc^{p7}$ dan como resultado la aparición de las cepas 301C y 301V, las que presentan diferentes características bioquímicas estables y particulares en cada grupo polimórfico. La figura muestra las sustituciones polimórficas en el prion, para cada grupo (Phe-Fenilalanina; Leu-Leucina; Thr-Treonina; Val-Valina). (B) Resumen de los ensayos de amplificación por *PMCA* e infectividad desarrollados para cada cepa de priones. La amplificación por *PMCA* y los ensayos de infectividad se realizaron como se describe en la sección *Materials and Methods* del apéndice 8.



Los datos obtenidos confirman la existencia de una barrera de infectividad inter-polimórfica en la replicación de priones. Realizando pases sucesivos a bajas diluciones del material infeccioso, se ha conseguido romper la gran mayoría de los cruces polimórficos *in vitro*. Los experimentos a presentar en esta sección confirman aquellos que describen la propagación de cepas de priones utilizando la *PMCA*. Sorprendentemente, una vez que los priones 301V son amplificados en el grupo polimórfico opuesto, podemos ver que el patrón de ellos cambia, sugiriendo una posible mutación de la cepa con características aproximadas a la cepa 301C (tabla 3). Los resultados de infectividad en este experimento nos confirman que utilizando el sustrato

correspondiente en la amplificación por *PMCA*, las características de las cepas utilizadas se mantienen. Luego de tomar las cepas 301C y 301V y realizar amplificación *in vitro* en sustrato *sinc*^{s7}, podemos ver que los períodos de incubación se aproximan a los de la cepa 301C tras ensayos de infectividad en ambos grupos polimórficos (tabla 3). Estos datos son interesantes ya que sugieren la mantención de una cierta "memoria conformacional" del inóculo original (EEB), que pueden ser utilizada para recuperar priones 301C a partir de 301V. Estos resultados nos sugieren que los priones infecciosos, además de tener arreglos conformacionales particulares que dan cuenta de sus características bioquímicas y biológicas propias, pueden poseer cierta plasticidad que permita recuperar su "memoria conformacional", la cual estaría dictada por el agente infeccioso original. Esta información es de suma importancia en salud pública, ya que nos permitiría conocer el origen de ciertos priones y su expansión, además de conocer la posible patogenicidad que el material infeccioso puede tener para los seres humanos u otras especies animales importantes desde el punto de vista económico.

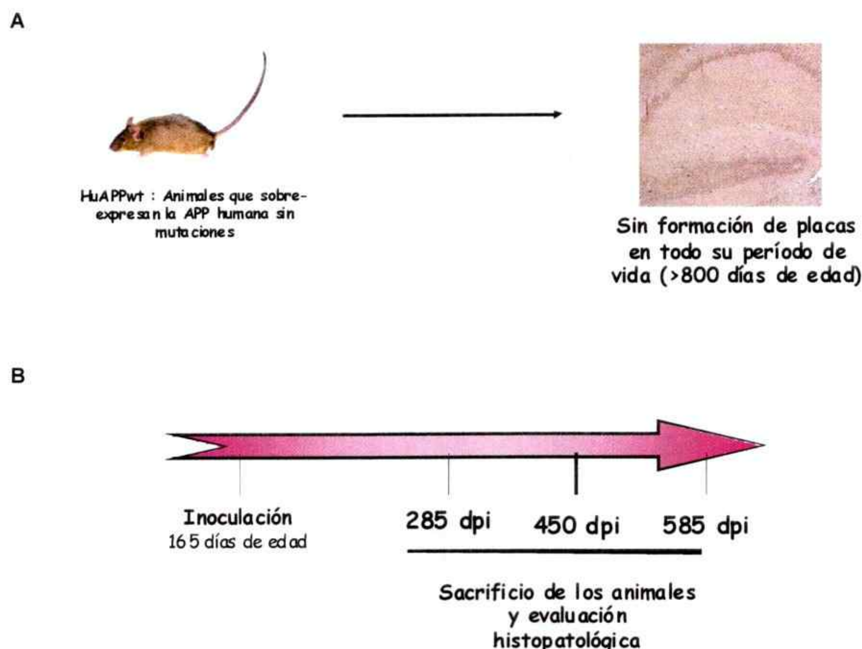
5. Nucleación heteróloga de amiloides

Luego de analizar los puntos involucrados en infecciones inter-especies e inter-polimórficas, podemos entender que las prolongaciones en los períodos de incubación y las dificultades en la replicación en sustratos heterólogos se deben principalmente a diferencias en la secuencia de la proteína (las que son traducidas en diferencias en su estructura). Estos fenómenos pueden ser considerados como "cruces de nucleación", donde una especie amiloidogénica es capaz de favorecer la nucleación de una proteína distinta (apéndice 1). Entre ellas, la EA y las EET son importantes de analizar debido a la similitud en los mecanismos moleculares que ambas presentan y la extensiva evidencia clínica que apunta a su co-existencia (apéndice 1). Para analizar este fenómeno de una forma controlada, escogimos el modelo animal tg2576. Estos ratones, modelos de la EA, generan espontáneamente placas de Aβ a los 12 meses de vida. Estos animales fueron infectados con priones RML a distintos tiempos (45 y 365 días de edad). Nuestros resultados de infectividad muestran claramente que los períodos de incubación de priones son menores en animales transgénicos de la EA en comparación con controles silvestres inoculados con el mismo stock del agente infeccioso. Sorprendentemente, los análisis muestran una dependencia con la edad de los animales, donde los períodos de incubación fueron significativamente menores para los animales inoculados a un año de edad en comparación con los de 45 días (apéndice 10).

Análisis histopatológico del cerebro de los animales utilizados en este estudio muestran que las características patológicas de ambas enfermedades co-existen en el cerebro de los animales transgénicos infectados, tal como se revela en la co-existencia de vacuolización y deposición de PrP^{res} (típicas de las prionopatías), acumulación de Aβ (típica de la EA) e inflamación (clásica de ambas patologías) (apéndice 10). De forma interesante, la carga de Aβ resultó ser significativamente mayor en los animales inyectados con RML en comparación con los controles tratados con tampón salino (apéndice 10). Estos controles apoyan fuertemente nuestros hallazgos debido a que animales silvestres inoculados con RML desarrollaron características clásicas de priones sin patología de Aβ. Lo contrario fue observado para ratones tg2576 sin inocular con este material infeccioso (apéndice 10). Sorprendentemente, 2 de 8 ratones tg2576 inoculados a los 45 días de edad presentaron acumulación difusa de Aβ (no reactiva a tioflavina S), etapa de su vida en la cual no se espera una acumulación visible de estos agregados en una situación normal (apéndice 10).

Analizando más a fondo las características patológicas de la cepa RML en ratones tg2576 realizamos un estudio del patrón de lesión generado en estos animales (apéndice 10). Nuestras observaciones no arrojaron ninguna diferencia significativa entre los animales transgénicos de ambos grupos y los animales silvestres. Estos datos sugieren que las características patogénicas de RML no cambian luego de replicar el material infeccioso en el nuevo huésped. Para confirmar estos resultados realizamos un segundo pase de infectividad utilizando la PrP^{Sc} generada en animales tg2576 inoculados a los 365 días en ratones silvestres. Los resultados tanto de infectividad (períodos de incubación) como de patrones de lesión fueron similares a los obtenidos en animales silvestres controles inoculados con la fuente original de RML, afirmando nuestros resultados del primer pase que sugieren que las características de esta cepa se mantienen en el nuevo huésped (apéndice 10). Estos datos fueron apoyados adicionalmente por estudios en la PrP^{res} generada en estos animales, donde se vio que el patrón de glicosilación y la movilidad electroforética fue similar en todos los grupos de animales inoculados con priones infecciosos (apéndice 10).

Figura 9. Características histopatológicas del modelo huAPPwt.
(A) El modelo huAPPwt expresa la PPA humana sin ninguna mutación, lo que resulta en la producción de Aß soluble en el cerebro. Estos ratones no presentan deposición de Aß durante toda su vida, tal como se puede ver en esta sección de cerebro de un animal sacrificado sobre los 800 días de edad (detección con el anticuerpo 4G8 que reconoce a Aß). (B) Estrategia experimental de la inducción de agregación de Aß *in vivo*. Ratones huAPPwt se inocularon estereotácticamente en el hipocampo (ambos hemisferios) con 5 µL de las muestras descritas en la figura 10. Posteriormente, los animales se sacrificaron a distintos tiempos con el propósito de seguir la dinámica de acumulación de Aß y otras características patológicas en el cerebro.



Los resultados obtenidos anteriormente pueden ser explicados de distintas formas. Entre ellas podemos destacar una saturación de los mecanismos de plegamiento y de degradación de proteínas, cascadas transduccionales sinérgicas entre ambas enfermedades y como hemos mencionado anteriormente, mecanismos de nucleación heteróloga entre los agregados amiloides de ambas enfermedades. La figura 5 del apéndice 10 muestra que animales silvestres controles, inoculados con la misma cantidad y fuente del material infeccioso y sacrificados a distintos tiempos, presentaban cantidades inferiores o nulas de PrP^{res} en comparación con animales

transgénicos infectados y sacrificados a los mismos tiempos. Estos datos apoyan aún más los resultados anteriores, demostrando que el mal plegamiento y agregación del agente infeccioso en los animales transgénicos se ve aumentada. Adicionalmente, ensayos de nucleación *in vitro* muestran que los agregados de PrP^{Sc} pueden facilitar la deposición de Aβ, lo que a su vez sugiere que nuestros resultados *in vivo* son consecuencia, al menos en parte, de interacciones proteína-proteína entre Aβ y priones (figura 6a del apéndice 10, realizado por la señorita Lisbell Estrada). Adicionalmente la agregación de rPrP (tabla 1) en una forma resistente a proteasas es estimulada por Aβ agregada en una relación concentración-dependiente (figura 6b del apéndice 10, estudio realizado por el señor Rodrigo Díaz-Espinoza). Estos datos sugieren que las enfermedades que involucran agregados proteicos pueden interaccionar a este nivel, produciendo una potenciación en las condiciones patológicas de ambas.

6. Proyecciones: mecanismos infectivos de los amiloides

Los resultados presentados en el punto anterior nos guían a la hipótesis de una posible naturaleza infecciosa común para los amiloides. El modelo de nucleación – polimerización explica muy bien la cinética de agregación de estas proteínas, además de brindar un excelente soporte para entender los mecanismos de infectividad en las EET [20;164]. El alto número de individuos que desarrollan la forma esporádica de la EA y teniendo en cuenta que la principal molécula asociada con la EA es Aβ, hacen pensar acerca de un posible mecanismo transmisible para esta enfermedad. Para evaluar la hipótesis de infectividad de los amiloides, utilizamos un modelo transgénico que sobre-expresa Aβ humano, pero con la importante característica de que no resulta en la acumulación de este elemento durante toda la vida del animal (huAPPwt – [47] y figura 9a). Para probar nuestra hipótesis de que los agregados de Aβ pueden ser transmisibles, estos animales fueron inoculados con extractos (homogenizados) de cerebro de pacientes clínica e histopatológicamente diagnosticados con la EA. Como control se utilizó el homogenizado de cerebro de un infante de 163 días de edad (figura 10). El propósito de utilizar el cerebro de este individuo como control se debe a que agregados proteicos en el cerebro de personas seniles pueden estar presentes incluso sin la presencia de signos clínicos correspondientes a la enfermedad [6]. Debido a los resultados procedentes del punto anterior de esta tesis hemos considerado el no utilizar preparaciones de un individuo senil en nuestros experimentos con el propósito de evitar falsos positivos en nuestros resultados. Luego de inocular estos animales a los 165 días de edad, los sacrificamos a distintos tiempos (285, 450 y 585 d.p.i.) de forma tal de analizar la progresión de la acumulación de agregados de Aβ en el tiempo (figura 9b).

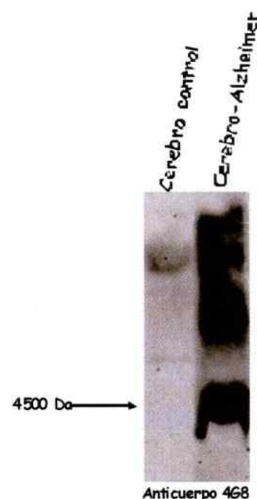


Figura 10. Inóculos utilizados para el experimento de nucleación inducida de Aβ *in vivo*.
Homogenizados de cerebro al 10% se prepararon en solución tampón salina de fosfato e inhibidores de proteasas. 20 µL de cada muestra se aplicaron en un gel de dodecilsulfato de sodio - poliacrilamida al 12% y se separaron por tamaño. Las proteínas se transfirieron posteriormente a una membrana de nitrocelulosa y se ensayaron por Western blot utilizando el anticuerpo 4G8.

Figura 11: Acumulación de Aβ en ratones transgénicos huAPPwt.

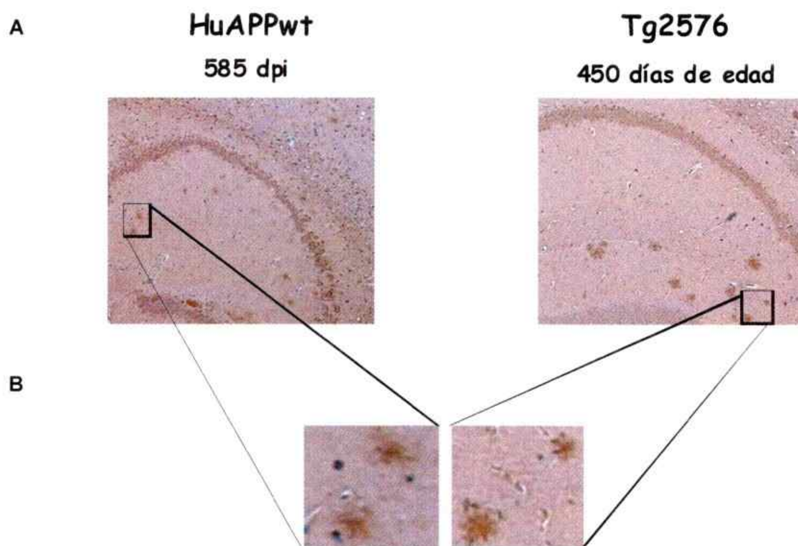
Ratones huAPPwt fueron inoculados con homogenizados de cerebros de pacientes que padecían de la EA e individuos que no sufrían desórdenes neurológicos. (A) Inmuno-histoquímica contra Aβ en cerebros de ratones huAPPwt inoculados con homogenizados controles y derivados de la EA y sacrificados a distintos tiempos. (B) Análisis semi-cuantitativo de los distintos grupos analizados en este experimento. dpi: días post-inoculación; NB: animales inoculados con homogenizados de cerebros controles; AB: animales inoculados con homogenizados de pacientes de la enfermedad de Alzheimer. Factor de amplificación en las fotos: 40.

Nuestros experimentos señalan claramente que la inoculación de homogenizados de cerebros de pacientes con la EA (los cuales presentan una gran acumulación de Aβ) es capaz de producir la deposición de esta proteína en los animales tratados, donde de forma interesante podemos ver que la acumulación aumenta con el tiempo. Nuestro grupos controles, inoculados con homogenizados de cerebro del individuo sano descrito anteriormente, no mostró ningún tipo de acumulación en ninguno de los grupos temporales analizados (figura 11). Yendo más allá con este análisis se realizó una semi-cuantificación (puntaje) de la agregación de Aβ (descrito en la leyenda de la figura 12) con el propósito de analizar la incidencia y la carga de agregados de Aβ en el cerebro de estos animales. En el gráfico de la figura 12 podemos ver nuevamente que en ninguno de los animales inoculados con cerebro controles presentaron ningún tipo de agregación, mientras que en los animales inoculados con extractos de Aβ se pudo apreciar que tanto el número de animales afectados como la cantidad de agregados en cada caso experimentó un incremento con el tiempo. Al realizar una comparación más en detalle acerca de la calidad de los agregados, observamos que los depósitos generados en los animales huAPPwt son similares a los que se generan en los animales tg2576 descritos en el punto anterior (figura 12). Estos resultados nos sugieren que el tipo de agregación inducida en forma exógena en ratones huAPPwt es similar a la generada espontáneamente en modelos establecidos de la EA.

Además, en el análisis de las características histopatológicas de estos animales podemos ver que otras características de la EA (como presencia de astrocitos reactivos y tinción de Aβ con tioflavina S) son reproducidas (figura 13). Es importante señalar que las características inflamatorias en estos cerebros es enmascarada en los grupos de más edad debido a la constante degeneración que se produce en estos animales a medida que la edad progresa. Por otro lado, la

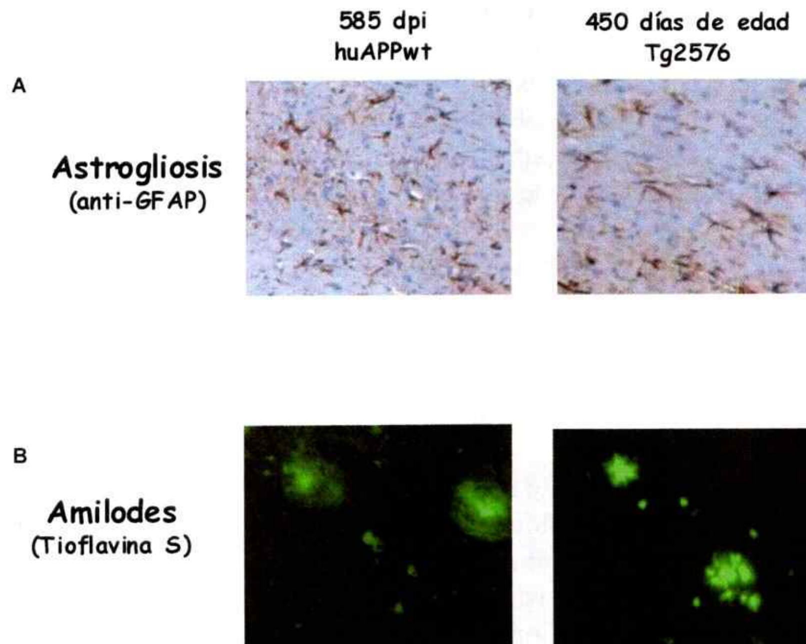
reactividad a tioflavina S fue positiva solamente en algunos de los agregados generados en el último grupo temporal, sugiriendo que en los primeros grupos experimentales la reactividad de Aβ se debe principalmente a placas inmaduras (lo que se observa en etapas tempranas de la patología - datos no mostrados).

Figura 12: Comparación de los depósitos de Aß en animales tg2576 y ratones huAPPwt inducidos con extractos de cerebros derivados de la EA. (A) Análisis inmunohistoquímico de la agregación de Aß (anticuerpo 4G8). Factor de amplificación en las fotos: 40. dpi: días post-inoculación. (B) Un análisis más cuidadoso de las estructuras generadas muestra similitudes morfológicas en los agregados de ambos grupos. Factor de amplificación en las fotos: 100.



Estos datos sugieren que efectivamente estas enfermedades pueden ser transmisibles tal como ocurre con las prionopatías y exigen que nuevos y rigurosos estudios se realicen en esta área con el fin de prevenir su ocurrencia en etapas tardías de la vida. Nuestra proyección es que evitando posibles "infecciones amiloidogénicas" se podría suprimir un gran número de casos de DCP o su posible interacción con otras enfermedades en este grupo.

Figura 13: Inflamación y reactividad a tioflavina S en ratones huAPPwt inducidos con extractos de cerebros de la EA. (A) Análisis inmunohistoquímico de inflamación cerebral utilizando el anticuerpo anti-GFAP que reconoce a la proteína acídica fibrilar de la glía. (B) Reactividad de tioflavina S con estructuras amiloidogénicas generadas por la agregación de Aß. Ambas características, típicas de la EA y convencionalmente reproducidas en el modelo tg2576, se identificaron en nuestros animales huAPPwt inducidos. dpi: días post-inoculación. Factor de amplificación de las fotos: 40 (a) y 100 (B).



SECCIÓN V: DISCUSIÓN

Los resultados analizados en las secciones anteriores (y apéndices correspondientes) nos llevan a diversas conclusiones, muchas de las cuales pueden tener muy importantes repercusiones desde el punto de vista científico, de salud pública e industria ganadera.

Las cepas de priones han sido uno de los temas más controversiales en el campo de las EET. Muchas hipótesis se han postulado con el propósito de explicar la diversidad de cepas en este agente infeccioso, donde variaciones patológicas considerables se han encontrado con una única secuencia en la proteína infecciosa (apéndice 3). Entre estas hipótesis se ha atribuido la existencia de distintos ácidos nucleicos que podrían estar asociados a la proteína infecciosa [109]. Sin embargo, se ha propuesto que el prion está exclusivamente compuesto de estructuras proteicas (específicamente de PrP^{Sc}). La evidencia posterior en este campo ha propuesto que las diferencias entre cepas de priones radican en los distintos arreglos conformaciones que la PrP^{Sc} pueda adquirir. Esta teoría ha sido apoyada por distintas aproximaciones experimentales en las cuales se postula que la principal diferencia entre cepas de priones radica en la estructura del prion, evento que puede ser dependiente de su composición aminoacídica o independiente de ella [111].

La primera parte experimental de esta tesis doctoral involucró el estudio del fenómeno de cepas de priones, analizando su propagación en sistemas *in vitro* libre de células. Para este propósito hemos utilizado la técnica denominada *PMCA*, la cual ha tenido un impacto muy importante en el estudio de las EET. En este punto demostramos la amplificación exitosa de cepas de priones usando este sistema. Sorprendentemente hemos visto que los productos resultantes de esta amplificación son capaces de mantener las características bioquímicas como se ha ensayado para distintas cepas de priones de ratón y de humanos (apéndice 8). Adicionalmente se analizaron las características infectivas producidas por el material infeccioso generado *in vitro*. Estos ensayos nos mostraron que estas propiedades también se mantuvieron (apéndice 8). Teniendo en cuenta que cada una de las muestras amplificadas fue replicada utilizando PrP^{C} proveniente de la misma fuente, estos resultados nos sugieren fuertemente que las características de cada cepa están codificadas exclusivamente en la estructura de la PrP^{Sc}. Estos hallazgos tienen una fuerte importancia desde el punto de vista científico debido a que los procesos de mal plegamiento inducidos por priones infecciosos preformados pueden ser acelerados en una forma considerable, manteniendo intactas las características intrínsecas del material infeccioso. La posible generación de grandes cantidades de PrP^{Sc} en el tubo de ensayo facilitaría enormemente el estudio de distintas cepas de priones desde el punto de vista funcional y estructural. Si sumamos el posible valor que la *PMCA* pueda tener como método diagnóstico es muy importante el saber que el material generado va a seguir manteniendo las características de la cepa original. Con esta herramienta en nuestras manos podríamos conocer (con la ayuda de otras cualidades bioquímicas como el patrón electroforético luego de tratamiento con PK) qué agente infeccioso está afectando específicamente a una población (humana o animal).

Otro fenómeno bastante polémico en las EET corresponde a los procesos de transmisión inter-especie de priones. Estos eventos son los más comúnmente asociados en la generación de nuevas cepas de PrP^{Sc} (apéndice 3), siendo factores de esta diversidad tanto el agente infeccioso utilizado como la proteína normalmente plegada presente en el huésped. Como ejemplo podemos tomar a la EEB, enfermedad que no solo ha sido transmitida a humanos sino también a felinos exóticos como tigres (*Panthera tigris*) y guepardos (*Acinonyx jubatus*), a varias especies de primates e incluso a gatos domésticos (*Felis catus*) [178-180]. La transmisión de la EEB a estas

distintas especies ha creado muchas variantes del agente infeccioso, cada una con características bioquímicas y biológicas particulares. De acuerdo a su origen, estás nuevas cepas podrían presentar cierto peligro en cuanto a una posible transmisión al ser humano. Otro ejemplo alarmante lo dicta la exitosa transmisión de la EEB a cerdos (*Sus domestica*) y a ratones transgénicos expresando PrP^C de esta misma especie. Debido al alto consumo de derivados porcinos el caso hipotético de una epidemia de "cerdos locos" podría incrementar los eventos de transmisión zoonótica de EET a la población humana. Afortunadamente, la transmisión de la EEB a esta especie ocurre solo en condiciones experimentales drásticas, las que no estarían ocurriendo en la naturaleza [181;182]. Aun más inquietante es la posible transmisión de la EEB en cabras y ovejas. Muchos estudios han mostrado que la posible transmisión a estas especies es posible, hecho que ocurre con relativa facilidad [183]. Los signos clínicos generados tras esta transmisión son bastantes similares a scrapie, evento que podría enmascarar o dificultar su diagnóstico. El origen bovino de esta nueva variante de scrapie hace posible que esta enfermedad pueda ser transmitida a humanos. Experimentos de transmisión utilizando homogenizados de cerebros de ovejas infectadas con la EEB en modelos transgénicos que expresen la PrP humana están siendo evaluados en muchos laboratorios.

Actualmente no existe forma de predecir la identidad que un nuevo agente infeccioso pueda adquirir tras infectar una nueva especie. Un ejemplo interesante está dado por la EDC que afecta a cérvidos salvajes y en cautiverio [68]. El origen de esta enfermedad, su diseminación y su potencial transmisión a seres humanos son aún desconocidos. Esto es preocupante considerando que la EDC se ha vuelto endémica en algunas regiones de los Estados Unidos y que el número de casos de ciervos infectados sigue en aumento. Se presume que un gran número de cazadores en los Estados Unidos han estado en contacto o han consumido carne de ciervos infectada con EDC, la que ha sido asociada con cantidades fácilmente detectables del material infeccioso. Estudios de transmisibilidad para esta enfermedad se han realizado en diversas especies con el propósito de predecir su transmisión. En estos estudios, una especial atención se ha prestado a animales carroñeros, los que estarían en contacto directo con cadáveres de ciervos muertos naturalmente infectados. Como fue anteriormente referido para el caso de la EEB, esto podría resultar en la generación de nuevas formas de EET con peligrosidad incierta con respecto al ser humano. Afortunadamente, resultados negativos se han obtenido en experimentos de transmisibilidad en mapaches expuestos a la EDC [177] o evidencia que asocie casos de la EDC con la ECJ u otras prionopatías humanas [184]. No obstante, experimentos de transmisibilidad inter-especie entre EDC y monos ardilla (*Saimiri sciureus*) han sido positivos [185]. La evidencia experimental y clínica actual sugiere que las barreras de especie presentes en la EDC son mayores que las observadas en la EEB. Aun así es necesario estar alerta con respecto a la transmisión de esta enfermedad a otras especies donde algún tipo de "intermediario conformacional" pueda ser generado, facilitando la infectividad a seres humanos.

En esta tesis doctoral hemos exitosamente reproducido *in vitro* el fenómeno de barrera de especie observado en modelos animales. La ventaja de nuestro sistema es que este proceso lo podemos realizar de una forma acelerada y con características similares a lo que estaría ocurriendo en sistemas superiores (apéndice 9). Primeramente hemos sido capaces de reproducir distintos niveles de barrera de especie utilizando como sustrato PrP^C de mapache (tabla 2). Esta especie ha sido previamente infectada con inóculos de distintas fuentes, arrojando distintos niveles de dificultad en la propagación de estos agentes infecciosos. Al evaluar estos inóculos *in vitro* observamos que la dificultad para convertir estos priones es similar a la vista *in vivo*, lo que fue evaluado en distintas rondas de *PMCA*. Además, hemos tratado de modificar nuestro ensayo

con el propósito de forzar el sistema y tratar de romper barreras consideradas absolutas *in vivo*. Concentrando el material infeccioso de la EDC, y resuspendiendo el pellet en sustrato de mapache, fuimos capaces de generar priones que tienen características bioquímicas similares a las encontradas en otros priones infecciosos (resistencia parcial a PK en un segmento de la proteína). La sensibilidad de este ensayo, sumada a la concentración del material infeccioso, sería suficiente para generar priones infecciosos donde naturalmente no es posible hacerlo. Estos resultados nos sugieren que un "índice de infectividad" puede ser aproximado utilizando esta técnica de replicación. La simplicidad de la *PMCA*, la posibilidad de realizarla a gran escala y los datos que involucran la mantención de las características de cada cepa en este sistema podrían nuevamente ser bastante útiles desde el punto de vista científico. Además, la posibilidad de analizar de forma rápida la transmisión de priones infecciosos específicos en distintas especies luce bastante atractiva desde para la industria ganadera y la salud pública.

Siguiendo nuestros estudios de transmisiones priónicas entre distintas especies hemos investigado la posibilidad de generar nuevas cepas de PrP^{Sc} en el tubo de ensayo. Con este propósito hemos analizado la barrera de especie existente entre dos especies de roedores experimentales extensivamente utilizados en el estudio de estas enfermedades (apéndice 9). Las cepas RML de ratones y 263K de hámsteres son probablemente las más utilizadas en el campo de las prionopatías. Estudios previos han demostrado una gran barrera de infectividad entre estas dos especies. Por este motivo hemos ensayado la conversión de cada una de estas cepas de priones en sustrato opuestos, analizando las características bioquímicas y patológicas de cada PrP^{Sc} generada. En resumen, estudios detallados para ambos cruces de barrera no solo mostraron una dificultad en la conversión tal como ocurre *in vivo*, sino que también se observaron procesos de adaptación bioquímica y patológica en los priones generados. Nuestros resultados dan una cuenta clara de que los nuevos priones generados corresponden a nuevas cepas del agente infeccioso. Esta información es bastante novedosa en el sentido de que por primera vez se ha descrito la generación de agentes infecciosos únicos en el tubo de ensayo. Interesantemente, la generación de estas nuevas cepas fue realizada solo por cambios inducidos *in vitro* en la conformación de la PrP^{Sc} y no por modificaciones constitutivas en la secuencia de la PrP. Esto nos abre nuevas puertas en el estudio de los priones al permitirnos analizar variantes conformacionales de la PrP^{Sc} y su dinámica replicativa en distintas especies.

Los resultados obtenidos anteriormente se expandieron utilizando otras cepas de hámster, específicamente a las cepas DY y HY. En este grupo de experimentos nuevamente observamos replicación de PrP^{Sc} utilizando sustrato de ratones sanos. El material infeccioso generado en este ensayo resultó en períodos de incubación similares a los observados para otras cepas de priones (figura 7 y apéndice 8). Estudios bioquímicos de los priones generados *in vitro* (y luego de su inoculación en ratones sanos) mostraron claras diferencias en las movilidades electroforéticas luego de tratamientos con PK. Esto ha sido atribuido a una cierta "memoria conformacional", herencia de los inóculos originales DY y HY. Estas diferencias siguieron la pauta esperada con respecto al templado original, donde los priones derivados de DY presentaron una movilidad electroforética mayor. Apoyando los análisis anteriores, ambas cepas de priones presentaron una distribución de las formas glicosiladas similar, congruente con lo observado para ambas cepas de priones de hámsteres.

Finalmente, y terminando con la sección de replicación de cepas de priones *in vitro*, hemos analizado la contribución de los polimorfismos en la replicación de la PrP^{Sc}. Con este propósito hemos tomado las cepas de priones 301C y 301V, las cuales han sido originalmente propagadas en distintos grupos polimórficos de ratones a partir de la EEB [136]. Como se ha

señalado con anterioridad ambas cepas presentan una cierta resistencia a infectar los grupos polimórficos opuestos. Como parte de este trabajo doctoral hemos propagado ambas cepas de priones utilizando como sustrato la PrP^C de animales *sinc*^{s7} (donde la cepa 301C ha sido generada) y realizando estudios de infectividad en ambos grupos polimórficos. Nuestros grupos controles (polimorfismos homólogos en el agente infeccioso, sustrato de *PMCA* y especie receptora) mostraron alta sensibilidad con respecto a la infectividad (bajos períodos de incubación) mientras que la inoculación de PrP^{Sc} en el grupo polimórfico opuesto resultó en una dificultad considerable de esta característica (tabla 3). Luego de su amplificación en sustrato *sinc*^{s7}, los priones 301C no presentaron diferencias en los períodos de incubación en comparación con los priones originales. Los períodos de incubación en el grupo polimórfico opuesto no arrojaron diferencias significativas con respecto al cruce realizado con el homogenizado infeccioso original. Para el caso de los priones 301V se vio de forma interesante que tras amplificar este material en sustrato *sinc*^{s7} (e inocular en animales del mismo grupo polimórfico) se encontraron períodos de incubación bastante similares a los que ocurren para los priones 301C. Todos estos datos nos confirman que diferencias polimórficas en la proteína del prion son capaces de resultar en una dificultad de replicar el material infeccioso, ergo dificultando la aparición de la enfermedad. Además, nuestros resultados proponen nuevamente que cierta "memoria conformacional" esté presente en estos priones. Esto es bastante atractivo si tomamos en cuenta la posibilidad de seguir la dinámica de ciertas variantes de PrP^{Sc} en distintas especies.

Los procesos estudiados con anterioridad engloban un fenómeno muy amplio, de vital importancia en el campo de los priones. Dos de las principales fuentes de variación de cepa han sido analizadas en este ensayo, como son la generación de nuevas cepas de PrP^{Sc} por transmisiones inter-especie o variación inter-polimórfica. La mayor parte del trabajo discutido en estos puntos ha sido realizada a través de procesos replicativos *in vitro*, los cuales pueden acelerar estos procesos considerablemente. Para validar nuestro trabajo, comprobamos que las características de cada cepa son capaces de ser mantenidas utilizando estos procedimientos y que los procesos de replicación inter-especie pueden ser reproducidos de una forma equivalente a lo que ocurre *in vivo*. Los resultados obtenidos en las tres primeras secciones de esta tesis doctoral han sido de gran apoyo para la teoría del prion, y han dado un gran apoyo al estudio de estas patologías desde el punto de vista molecular y epidemiológico.

La agregación de PrP en las enfermedades producidas por priones sigue un mecanismo de nucleación-polimerización. Por otro lado, la resistencia a la transmisibilidad de priones en distintas especies radica principalmente en la secuencia aminoacídica que posee la PrP^C de la nueva especie receptora. Este hecho nos hace pensar en un fenómeno de nucleación heteróloga, donde podemos considerar al prion infectivo y al prion receptor como entidades amiloidogénicas distintas. Existe mucha evidencia clínica que indica que estructuras amiloidogénicas pueden co-existir (apéndice 1), lo cual ha sido apoyado por ensayos *in vitro* [157;162]. Además, el estudio de este fenómeno en modelos animales de algunos DCP ha sugerido que este mecanismo de interacción es importante en relación al origen y progresión de estas enfermedades. Con esta información, nos propusimos analizar la interacción entre estructuras amiloidegénicas involucradas en las EET (PrP^{Sc}) y la EA (Aβ). Con el propósito de validar nuestra hipótesis hemos inoculado ratones tg2576 intra-peritonealmente con la cepa RML de priones en dos distintas etapas de la vida (apéndice 10) con la idea de analizar la interacción entre ambas enfermedades y su potenciación en distintas etapas patológicas. Los animales fueron inoculados a los 365 días de edad (donde encontramos una extensiva acumulación de placas de Aβ en el

cerebro) y a los 45 días (donde la presencia de agregados es nula). Nuestros resultados arrojaron períodos de incubación considerablemente menores en estos animales en comparación con animales silvestres inoculados a la misma edad. Interesantemente, los animales inoculados a los 365 días de edad presentaron períodos de incubación significativamente menores en comparación con los animales inoculados a los 45 días de edad (apéndice 10). Estos datos, nos sugieren que la acumulación de Aβ en estos animales es capaz de acelerar considerablemente los procesos patológicos generados por los priones infecciosos inyectados. Estos datos, sumados al análisis de la acumulación de PrP^{res} a distintos tiempos en animales silvestres (apéndice 10), nos sugieren que el proceso de replicación de PrP^{Sc} es acelerado en etapas establecidas de deposición de Aβ, lo cual finalmente plantea una posible interacción entre ambas estructuras amiloidogénicas. Las características bioquímicas e infectivas de los nuevos priones generados no se vieron afectadas por el nuevo huésped, proponiendo que el proceso de mal plegamiento y agregación de PrP^{Sc} está siendo solamente siendo acelerado. Nuestros resultados no solo apuntan a una aceleración de la patología priónica, sino también en características asociadas a la EA. La deposición de agregados de Aβ en el cerebro de animales infectados con priones es significativamente mayor en comparación a animales controles sacrificados a la misma edad (apéndice 10). Sorprendentemente, una fracción de animales inoculados a los 45 días presentó deposición de esta proteína en etapas en las que normalmente no debería ocurrir (apéndice 10). El análisis de la interacción entre componentes proteicos purificados nos sugiere que la interacción entre las estructuras amiloidogénicas de priones y Aβ pueden potenciar la agregación de una forma bidireccional.

Finalmente, el último punto de mi tesis doctoral involucró el estudio de la putativa infectividad que las estructuras amiloides puedan poseer. Esto nuevamente va de la mano con la teoría de nucleación-polimerización donde se propone que la adición de agregados proteicos pre-formados reduce la etapa de nucleación en estos procesos. Aunque esto explica muy bien el fenómeno de transmisibilidad de priones *in vivo*, estos supuestos han sido pobremente explorados en otros DCP. Para probar esta posibilidad tomamos nuevamente como modelo a la EA. Para evaluar nuestra esta hipótesis de una manera apropiada es crítico usar el modelo animal adecuado. Por este motivo escogimos a los animales huAPPwt, los cuales expresan la PPA humana sin ninguna mutación [47]. Como consecuencia, estos animales no presentan deposición de Aβ en sus cerebros durante toda su vida (figura 9). Este modelo imita muy bien lo que ocurre en un ser humano normal y es por este motivo que lo hemos utilizado en nuestra propuesta experimental. Tras inocular homogenizados de cerebros de pacientes afectados con la EA pudimos observar una inducción en la agregación de Aβ la que aumenta en incidencia y cantidad con respecto al tiempo. Esto es similar a lo observado *in vivo* para las EET e *in vitro* para otras proteínas amiloidogénicas. Otras características patológicas clásicas de la enfermedad como lo son la inflamación cerebral y la tinción de estos agregados con tioflavina S sugieren que las características patológicas de esta enfermedad están siendo reproducidas. Estos datos apoyan fuertemente nuestros supuestos acerca de la naturaleza transmisible de la EA.

Los resultados obtenidos en los dos últimos puntos de esta tesis doctoral arrojaron importante información con respecto al posible origen y factores que pueden modular las enfermedades pertenecientes a este grupo. Es importante tener en cuenta que la interacción entre dos estructuras amiloidogénicas puede presentar cierta "barrera", tal como se ha visto en el fenómeno de infectividad de prionopatías entre distintas especies. En nuestro caso, nuestros resultados arrojaron una interacción bidireccional entre PrP^{Sc} y Aβ. Lo mismo se ha observado *in vitro* para el par α-sinucleína/Aβ [157]. Esto es distinto si consideramos a los pares tau-Aβ y

amilina-Aβ, donde estudios *in vivo* en *in vitro* han demostrado que este fenómeno puede ocurrir solo en una dirección [32;33;156;162]. Aún así, los mecanismos que modulan la interacción entre distintos agregados proteicos pueden ser un factor importantísimo en el origen y la progresión molecular, clínica y patológica de diversas enfermedades.

Por otro lado, el fenómeno de nucleación de amiloides y la capacidad intrínseca de estos agregados a ser infecciosos nos lleva a un escenario alarmante, donde nuevas posibilidades epidémicas deben ser exploradas. La existencia de transmisión vía transfusión sanguínea en las EET sugiere que mecanismos de transmisión similares podrían estar actuando en otras enfermedades manifestadas en etapas tardías de la vida. La presencia de agregados proteicos aún no identificados en animales podría ser una fuente adicional de adquisición de semillas amiloides que podrían gatillar procesos patológicos que en su ausencia no ocurrirían. Por otro lado, si consideramos la prolongada latencia presente en las EET, esperamos que otros DCP presenten un tiempo de incubación mayor, haciendo difícil la identificación de estas patologías como entidades infecciosas. Estos resultados abren nuevas puertas de investigación para este tipo de enfermedades y trae importantes repercusiones que involucran a la salud pública, donde nuevas mecanismos, orígenes y rutas para estas enfermedades deben ser explorados.

SECCIÓN VI: BIBLIOGRAFÍA

(1) Soto C: Unfolding the role of Protein Misfolding in Neurodegenerative Diseases. Nature Rev Neurosci 2003; 4:49-60.

(2) Carrell RW, Lomas DA: Conformational disease. Lancet 1997; 350:134-138.

(3) Dobson CM: Protein misfolding, evolution and disease. Trends in Biochemical Sciences 1999; 24(9):329-332.

(4) Engelborghs S, Maertens K, Nagels G, Vloeberghs E, Marien P, Symons A, Ketels V, Estercam S, Somers N, De Deyn PP: Neuropsychiatric symptoms of dementia: cross-sectional analysis from a prospective, longitudinal Belgian study. Int J Geriatr Psychiatry 2005; 20(11):1028-1037.

(5) Collinge J: Prion diseases of humans and animals: their causes and molecular basis. Annu Rev Neurosci 2001; 24:519-550.

(6) Brayne C: The elephant in the room - healthy brains in later life, epidemiology and public health. Nat Rev Neurosci 2007; 8(3):233-239.

(7) Thal DR, Del TK, Braak H: Neurodegeneration in normal brain aging and disease. Sci Aging Knowledge Environ 2004; 2004(23):e26.

(8) Delacourte A, Sergeant N, Champain D, Wattez A, Maurage CA, Lebert F, Pasquier F, David JP: Nonoverlapping but synergetic tau and APP pathologies in sporadic Alzheimer's disease. Neurology 2002; 59(3):398-407.

(9) Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, .: Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349(6311):704-706.

(10) Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L: A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1992; 1(5):345-347.

(11) Collinge J, Owen F, Poulter M, Leach M, Crow TJ, Rossor MN, Hardy J, Mullan MJ, Janota I, Lantos PL: Prion dementia without characteristic pathology. Lancet 1990; 336(8706):7-9.

(12) Medori R, Tritschler HJ, LeBlanc A, Villare F, Manetto V, Chen HY, Xue R, Leal S, Montagna P, Cortelli P, .: Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. N Engl J Med 1992; 326(7):444-449.

(13) The Huntington's Disease Collaborative Research Group (HDCRG): A novel gene containing a trinucleotide repeat that is unstable on Huntington's disease chromosomes. Cell 1993; 72:971-983.

(14) Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di IG, Golbe LI, Nussbaum RL: Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997; 276(5321):2045-2047.

(15) Hsiao K, Baker HF, Crow TJ, Poulter M, Owen F, Terwilliger JD, Westaway D, Ott J, Prusiner SB: Linkage of a prion protein missense variant to Gerstmann-Straussler syndrome. Nature 1989; 338(6213):342-345.

(16) Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, .: Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993; 362(6415):59-62.

(17) Prusiner SB: Prions. Proc Natl Acad Sci U S A 1998; 95(23):13363-13383.

(18) Blake CC, Serpell LC, Sunde M, Sandgren O, Lundgren E: A molecular model of the amyloid fibril. Ciba Found Symp 1996; 199:6-15.

(19) Hardy J, Gwinn-Hardy K: Genetic classification of primary neurodegenerative disease. Science 1998.

(20) Jarrett JT, Lansbury PT, Jr.: Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 1993; 73(6):1055-1058.

(21) Soto C: Protein misfolding and disease; protein refolding and therapy. FEBS Lett 2001; 498:204-207.

(22) Soto C, Estrada L, Castilla J: Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. Trends Biochem Sci 2006; 31(3):150-155.

(23) Haass C, Selkoe DJ, Haass C, Selkoe DJ: Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. [Review] [130 refs]. Nature Reviews Molecular Cell Biology 2007; 8(2):101-112.

(24) Brayne C, Gill C, Huppert FA, Barkley C, Gehlhaar E, Girling DM, O'Connor DW, Paykel ES: Incidence of clinically diagnosed subtypes of dementia in an elderly population. Cambridge Project for Later Life. Br J Psychiatry 1995; 167(2):255-262.

(25) Alzheimer A: Uber eine eigenartige Erkrankung der Hirnrinde. Allg Zeitschr Psych Gerichtl Med 1907; 64:146-148.

(26) Glenner GG, Wong CW: Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 1984; 120(3):885-890.

(27) Arnold SE, Hyman BT, Flory J, Damasio AR, Van Hoesen GW: The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. Cereb Cortex 1991; 1(1):103-116.

(28) Van DD, De Deyn PP: Drug discovery in dementia: the role of rodent models. Nat Rev Drug Discov 2006; 5(11):956-970.

(29) Lee VM, Goedert M, Trojanowski JQ: Neurodegenerative tauopathies. Annu Rev Neurosci 2001; 24:1121-1159.

(30) Goedert M, Jakes R, Crowther RA, Six J, Lubke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM: The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. Proc Natl Acad Sci U S A 1993; 90(11):5066-5070.

(31) Gotz J, Streffer JR, David D, Schild A, Hoerndli F, Pennanen L, Kurosinski P, Chen F: Transgenic animal models of Alzheimer's disease and related disorders: histopathology, behavior and therapy. Mol Psychiatry 2004; 9(7):664-683.

(32) Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E: Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 2001; 293(5534):1487-1491.

(33) Gotz J, Chen F, Van Dorpe J, Nitsch RM: Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. Science 2001; 293(5534):1491-1495.

(34) McGowan E, Eriksen J, Hutton M: A decade of modeling Alzheimer's disease in transgenic mice. Trends Genet 2006; 22(5):281-289.

(35) Gomez-Ramos A, az-Hernandez M, Cuadros R, Hernandez F, Avila J: Extracellular tau is toxic to neuronal cells. FEBS Lett 2006; 580(20):4842-4850.

(36) Dahlgren KN, Manelli AM, Stine WB, Jr., Baker LK, Krafft GA, LaDu MJ: Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. J Biol Chem 2002; 277(35):32046-32053.

(37) McGeer EG, McGeer PL: The importance of inflammatory mechanisms in Alzheimer disease. Exp Gerontol 1998; 33(5):371-378.

(38) Weninger SC, Yankner BA: Inflammation and Alzheimer disease: the good, the bad, and the ugly. Nat Med 2001; 7(5):527-528.

(39) Squire LR, Kandel ER: Memory. From mind to molecules. Scientific American Library, 2000.

(40) Mann DM: Cerebral amyloidosis, ageing and Alzheimer's disease; a contribution from studies on Down's syndrome. Neurobiol Aging 1989; 10(5):397-399.

(41) Sani S, Traul D, Klink A, Niaraki N, Gonzalo-Ruiz A, Wu CK, Geula C: Distribution, progression and chemical composition of cortical amyloid-beta deposits in aged rhesus monkeys: similarities to the human. Acta Neuropathol (Berl) 2003; 105(2):145-156.

(42) Soto C, Sigurdsson EM, Morelli L, Kumar RA, Castano EM, Frangione B: Beta-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy. Nat Med 1998; 4(7):822-826.

(43) Games D, Adams D, Alessandrini R, Barbour R, Berthelette P, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, .: Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 1995; 373(6514):523-527.

(44) Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G: Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 1996; 274(5284):99-102.

(45) Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Burki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B: Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 1997; 94(24):13287-13292.

(46) Kawarabayashi T, Younkin LH, Saido TC, Shoji M, Ashe KH, Younkin SG: Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J Neurosci 2001; 21(2):372-381.

(47) Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L: High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci 2000; 20(11):4050-4058.

(48) Wisniewski T, Sadowski M: Preventing beta-amyloid fibrillization and deposition: beta-sheet breakers and pathological chaperone inhibitors. BMC Neurosci 2008; 9 Suppl 2:S5.

(49) Soto C, Martin Z: Therapeutic strategies against protein misfolding in neurodegenerative diseases. Exp Op Drug Disc 2009; 4:71-84.

(50) Wisniewski T, Konietzko U: Amyloid-beta immunisation for Alzheimer's disease. Lancet Neurol 2008; 7(9):805-811.

(51) Stefani A, Sancesario G, Pierantozzi M, Leone G, Galati S, Hainsworth AH, Diomedi M: CSF biomarkers, impairment of cerebral hemodynamics and degree of cognitive decline in Alzheimer's and mixed dementia. J Neurol Sci 2009.

(52) Frankfort SV, Tulner LR, van Campen JP, Verbeek MM, Jansen RW, Beijnen JH: Amyloid beta protein and tau in cerebrospinal fluid and plasma as biomarkers for dementia: a review of recent literature. Curr Clin Pharmacol 2008; 3(2):123-131.

(53) Soto C, Kindy MS, Baumann M, Frangione B: Inhibition of Alzheimer's amyloidosis by peptides that prevent beta-sheet conformation. Biochem Biophys Res Commun 1996; 226(3):672-680.

(54) Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, Kholodenko D, Lee M, Liao Z, Lieberburg I, Motter R, Mutter L, Soriano F, Shopp G, Vasquez N, Vandevert C, Walker S, Wogulis M, Yednock T, Games D, Seubert P: Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 1999; 400(6740):173-177.

(55) Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG: Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 2003; 300(5618):486-489.

(56) Spinney L: Update on Elan vaccine for Alzheimer's disease. Lancet Neurol 2004; 3(1):5.

(57) Schenk D: Amyloid-beta immunotherapy for Alzheimer's disease: the end of the beginning. Nat Rev Neurosci 2002; 3(10):824-828.

(58) Sigurdson CJ, Miller MW: Other animal prion diseases. Br Med Bull 2003; 66:199-212.

(59) Cullie J, Chelle PL: Experimental transmission of trembling to the goat. Comptes Rendus des Seances de l'Academie des Sciences 1939; 208:1058-1160.

(60) Public Health Service Interagency Coordinating Comitee: Report on Human Growth Hormone and Creutzfeldt - Jakob Disease; 1997, pp 1-11.

(61) Brown P, Preece M, Brandel JP, Sato T, McShane L, Zerr I, Fletcher A, Will RG, Pocchiari M, Cashman NR, D'Aignaux JH, Cervenakova L, Fradkin J, Schonberger LB, Collins SJ: Iatrogenic Creutzfeldt-Jakob disease at the millennium. Neurology 2000; 55(8):1075-1081.

(62) Zobeley E, Flechsig E, Cozzio A, Enari M, Weissmann C: Infectivity of scrapie prions bound to a stainless steel surface. Mol Med 1999; 5(4):240-243.

(63) Collee JG, Bradley R: BSE: a decade on--Part I. Lancet 1997; 349(9052):636-641.

(64) Collee JG, Bradley R: BSE: a decade on--Part 2. Lancet 1997; 349(9053):715-721.

(65) Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ: Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature 1997; 389(6650):498-501.

(66) Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P: The same prion strain causes vCJD and BSE. Nature 1997; 389(6650):448-50, 526.

(67) Baylis M, Goldmann W: The genetics of scrapie in sheep and goats. Curr Mol Med 2004; 4(4):385-396.

(68) Sigurdson CJ, Aguzzi A: Chronic wasting disease. Biochim Biophys Acta 2006.

(69) Sigurdsson B: Maedi, a slow progressing pernumonia of sheep: An epizoological and pathological study. Br Vet J 1954; 110:255-258.

(70) Medori R, Montagna P, Tritschler HJ, LeBlanc A, Cortelli P, Tinuper P, Lugaresi E, Gambetti P: Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. Neurology 1992; 42(3 Pt 1):669-670.

(71) Aguzzi A, Sigurdson C, Heikenwaelder M: Molecular mechanisms of prion pathogenesis. Annu Rev Pathol 2008; 3:11-40.

(72) Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohen FE, .: Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A 1993; 90(23):10962-10966.

(73) Aguzzi A, Polymenidou M: Mammalian prion biology: one century of evolving concepts. Cell 2004; 116(2):313-327.

(74) Roucou X, Gains M, LeBlanc AC: Neuroprotective functions of prion protein. J Neurosci Res 2004; 75(2):153-161.

(75) Brown DR, Qin K, Herms JW, Madlung A, Manson J, Strome R, Fraser PE, Kruck T, von Bohlen A, Schulz-Schaeffer W, Giese A, Westaway D, Kretzschmar H: The cellular prion protein binds copper in vivo. Nature 1997; 390(6661):684-687.

(76) Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM: Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 2009; 457(7233):1128-1132.

(77) Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C: Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature 1992; 356(6370):577-582.

(78) Hsiao K, Scott M, Foster D, DeArmond SJ, Groth D, Serban H, Prusiner SB: Spontaneous neurodegeneration in transgenic mice with prion protein codon 101 proline----leucine substitution. Ann N Y Acad Sci 1991; 640:166-170.

(79) Kaneko K, Ball HL, Wille H, Zhang H, Groth D, Torchia M, Tremblay P, Safar J, Prusiner SB, DeArmond SJ, Baldwin MA, Cohen FE: A synthetic peptide initiates Gerstmann-

Straussler-Scheinker (GSS) disease in transgenic mice. J Mol Biol 2000; 295(4):997-1007.

(80) Griffith JS: Self-replication and scrapie. Nature 1967; 215(105):1043-1044.

(81) Kimberlin RH, Walker CA: Competition between strains of scrapie depends on the blocking agent being infectious. Intervirology 1985; 23(2):74-81.

(82) Millson GC, Hunter GD, Kimberlin RH: The physico-chemical nature of the scrapie agent. Front Biol 1976; 44:243-266.

(83) Gibbs CJ, Jr., Gajdusek DC, Latarjet R: Unusual resistance to ionizing radiation of the viruses of kuru, Creutzfeldt-Jakob disease, and scrapie. Proc Natl Acad Sci U S A 1978; 75(12):6268-6270.

(84) Dickinson AG, Meikle VM, Fraser H: Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. J Comp Pathol 1968; 78(3):293-299.

(85) Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C: Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J 1996; 15(6):1255-1264.

(86) Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B: Cell-free formation of protease-resistant prion protein. Nature 1994; 370(6489):471-474.

(87) Soto C, Kascsak RJ, Saborio GP, Aucouturier P, Wisniewski T, Prelli F, Kascsak R, Mendez E, Harris DA, Ironside J, Tagliavini F, Carp RI, Frangione B: Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides. Lancet 2000; 355(9199):192-197.

(88) Bach S, Tribouillard D, Talarek N, Desban N, Gug F, Galons H, Blondel M: A yeast-based assay to isolate drugs active against mammalian prions. Methods 2006; 39(1):72-77.

(89) Korth C, May BC, Cohen FE, Prusiner SB: Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. Proc Natl Acad Sci U S A 2001; 98(17):9836-9841.

(90) Ryou C, Legname G, Peretz D, Craig JC, Baldwin MA, Prusiner SB: Differential inhibition of prion propagation by enantiomers of quinacrine. Lab Invest 2003; 83(6):837-843.

(91) Vetrugno V, Di Bari MA, Nonno R, Puopolo M, D'Agostino C, Pocchiari M, Agrimi U: Oral Pravastatin prolongs survival time of scrapie-infected mice. J Gen Virol 2009.

(92) Sigurdsson EM, Wisniewski T: Promising developments in prion immunotherapy. Expert Rev Vaccines 2005; 4(5):607-610.

(93) Sigurdsson EM, Brown DR, Daniels M, Kascsak RJ, Kascsak R, Carp R, Meeker HC, Frangione B, Wisniewski T: Immunization delays the onset of prion disease in mice. Am J Pathol 2002; 161(1):13-17.

(94) Magri G, Clerici M, Dall'Ara P, Biasin M, Caramelli M, Casalone C, Giannino ML, Longhi R, Piacentini L, Bella SD, Gazzuola P, Martino PA, Bella SD, Pollera C, Puricelli M, Servida F, Crescio I, Boasso A, Ponti W, Poli G: Decrease in pathology and progression of scrapie after immunisation with synthetic prion protein peptides in hamsters. Vaccine 2005; 23(22):2862-2868.

(95) Goni F, Knudsen E, Schreiber F, Scholtzova H, Pankiewicz J, Carp R, Meeker HC, Rubenstein R, Brown DR, Sy MS, Chabalgoity JA, Sigurdsson EM, Wisniewski T: Mucosal vaccination delays or prevents prion infection via an oral route. Neuroscience 2005.

(96) Foster BA, Coffey HA, Morin MJ, Rastinejad F: Pharmacological rescue of mutant p53 conformation and function. Science 1999; 286(5449):2507-2510.

(97) Van Everbroeck B, Quoilin S, Boons J, Martin JJ, Cras P: A prospective study of CSF markers in 250 patients with possible Creutzfeldt-Jakob disease. J Neurol Neurosurg Psychiatry 2003; 74(9):1210-1214.

(98) Saborio GP, Permanne B, Soto C: Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 2001; 411(6839):810-813.

(99) Castilla J, Saá P, Hetz C, Soto C: In vitro generation of infectious scrapie prions. Cell 2005; 121(2):195-206.

(100) Saa P, Castilla J, Soto C: Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. J Biol Chem 2006; 281(46):35245-35252.

(101) Silveira JR, Raymond GJ, Hughson AG, Race RE, Sim VL, Hayes SF, Caughey B: The most infectious prion protein particles. Nature 2005; 437(7056):257-261.

(102) Castilla J, Saa P, Soto C: Detection of prions in blood. Nat Med 2005; 11:982-985.

(103) Saa P, Castilla J, Soto C: Presymptomatic detection of prions in blood. Science 2006; 313(5783):92-94.

(104) Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, Mackenzie J, Will RG: Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet 2004; 363(9407):417-421.

(105) Collinge J, Clarke AR: A general model of prion strains and their pathogenicity. Science 2007; 318(5852):930-936.

(106) Pattison IH, Millson GC: Scrapie produced experimentally in goats with special reference to the clinical syndrome. J Comp Pathol 1961; 71:101-109.

(107) Hunter N, Dann JC, Bennett AD, Somerville RA, McConnell I, Hope J: Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in Sinc congenic mice. J Gen Virol 1992; 73 (Pt 10):2751-2755.

(108) Westaway D, Goodman PA, Mirenda CA, McKinley MP, Carlson GA, Prusiner SB: Distinct prion proteins in short and long scrapie incubation period mice. Cell 1987; 51(4):651-662.

(109) Weissmann C: A 'unified theory' of prion propagation. Nature 1991; 352(6337):679-683.

(110) Bartz JC, Bessen RA, McKenzie D, Marsh RF, Aiken JM: Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. J Virol 2000; 74(12):5542-5547.

(111) Peretz D, Scott MR, Groth D, Williamson RA, Burton DR, Cohen FE, Prusiner SB: Strain-specified relative conformational stability of the scrapie prion protein. Protein Sci 2001; 10(4):854-863.

(112) Fraser H, Dickinson AG: The sequential development of the brain lesion of scrapie in three strains of mice. J Comp Pathol 1968; 78(3):301-311.

(113) Fraser H, Dickinson AG: Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation. J Comp Pathol 1973; 83(1):29-40.

(114) Will RG, Ironside JW, Hornlimann B, Zeidler M: Creutzfeldt-Jakob disease. Lancet 1996; 347(8993):65-66.

(115) Budka H, Aguzzi A, Brown P, Brucher JM, Bugiani O, Gullotta F, Haltia M, Hauw JJ, Ironside JW, Jellinger K, .: Neuropathological diagnostic criteria for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases). Brain Pathol 1995; 5(4):459-466.

(116) Bessen RA, Marsh RF: Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. J Gen Virol 1992; 73 (Pt 2):329-334.

(117) Dell'Omo G, Vannoni E, Vyssotski AL, Di Bari MA, Nonno R, Agrimi U, Lipp HP: Early behavioural changes in mice infected with BSE and scrapie: automated home cage monitoring reveals prion strain differences. Eur J Neurosci 2002; 16(4):735-742.

(118) Cunningham C, Deacon RM, Chan K, Boche D, Rawlins JN, Perry VH: Neuropathologically distinct prion strains give rise to similar temporal profiles of behavioral deficits. Neurobiol Dis 2005; 18(2):258-269.

(119) Bessen RA, Marsh RF: Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. J Virol 1992; 66(4):2096-2101.

(120) Collinge J, Sidle KC, Meads J, Ironside J, Hill AF: Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 1996; 383(6602):685-690.

(121) Parchi P, Castellani R, Capellari S, Ghetti B, Young K, Chen SG, Farlow M, Dickson DW, Sima AA, Trojanowski JQ, Petersen RB, Gambetti P: Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. Ann Neurol 1996; 39(6):767-778.

(122) Caughey B, Raymond GJ, Bessen RA: Strain-dependent differences in beta-sheet conformations of abnormal prion protein. J Biol Chem 1998; 273(48):32230-32235.

(123) Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB: Eight prion strains have PrP(Sc) molecules with different conformations. Nat Med 1998; 4(10):1157-1165.

(124) Wadsworth JD, Hill AF, Joiner S, Jackson GS, Clarke AR, Collinge J: Strain-specific prion-protein conformation determined by metal ions. Nat Cell Biol 1999; 1(1):55-59.

(125) Jones EM, Surewicz WK: Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids. Cell 2005; 121(1):63-72.

(126) Hill AF, Collinge J: Subclinical prion infection. Trends Microbiol 2003; 11(12):578-584.

(127) Hill AF, Collinge J: Subclinical prion infection in humans and animals. Br Med Bull 2003; 66:161-170.

(128) Barlow RM, Rennie JC: The fate of ME7 scrapie infection in rats, guinea-pigs and rabbits. Res Vet Sci 1976; 21(1):110-111.

(129) Vorberg I, Groschup MH, Pfaff E, Priola SA: Multiple amino acid residues within the rabbit prion protein inhibit formation of its abnormal isoform. J Virol 2003; 77(3):2003-2009.

(130) Lasmezas CI, Deslys JP, Robain O, Jaegly A, Beringue V, Peyrin JM, Fournier JG, Hauw JJ, Rossier J, Dormont D: Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science 1997; 275(5298):402-405.

(131) Kimberlin RH, Walker CA: Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. J Gen Virol 1978; 39(3):487-496.

(132) Lloyd SE, Linehan JM, Desbruslais M, Joiner S, Buckell J, Brandner S, Wadsworth JD, Collinge J: Characterization of two distinct prion strains derived from bovine spongiform encephalopathy transmissions to inbred mice. J Gen Virol 2004; 85(Pt 8):2471-2478.

(133) Gombojav A, Shimauchi I, Horiuchi M, Ishiguro N, Shinagawa M, Kitamoto T, Miyoshi I, Mohri S, Takata M: Susceptibility of transgenic mice expressing chimeric sheep, bovine and human PrP genes to sheep scrapie. J Vet Med Sci 2003; 65(3):341-347.

(134) Narang H: Origin and implications of bovine spongiform encephalopathy. Proc Soc Exp Biol Med 1996; 211(4):306-322.

(135) Groschup MH, Buschmann A: Rodent models for prion diseases. Vet Res 2008; 39(4):32.

(136) Bruce ME: Scrapie strain variation and mutation. Br Med Bull 1993; 49(4):822-838.

(137) Chandler RL: Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet 1961; 1:1378-1379.

(138) Bruce M, Chree A, McConnell I, Foster J, Pearson G, Fraser H: Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. Philos Trans R Soc Lond B Biol Sci 1994; 343(1306):405-411.

(139) Muramoto T, Kitamoto T, Tateishi J, Goto I: Successful transmission of Creutzfeldt-Jakob disease from human to mouse verified by prion protein accumulation in mouse brains. Brain Res 1992; 599(2):309-316.

(140) Cronier S, Gros N, Tattum MH, Jackson GS, Clarke AR, Collinge J, Wadsworth JD: Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin. Biochem J 2008; 416(2):297-305.

(141) Dickinson AG, Meikle VM: Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. Mol Gen Genet 1971; 112(1):73-79.

(142) Bruce ME, Fraser H, McBride PA, Scott JR, Dickinson AG: The basis of strain variation in scrapie; in: Prusiner S, Collinge J, Powell J, Anderton B, (eds): Prion diseases of humans and animals. Chichester, Ellis Horwood, 1992, pp 497-508.

(143) Bruce ME, McConnell I, Fraser H, Dickinson AG: The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. J Gen Virol 1991; 72 (Pt 3):595-603.

(144) Lloyd SE, Thompson SR, Beck JA, Linehan JM, Wadsworth JD, Brandner S, Collinge J, Fisher EM: Identification and characterization of a novel mouse prion gene allele. Mamm Genome 2004; 15(5):383-389.

(145) Collinge J, Palmer MS, Dryden AJ: Genetic predisposition to iatrogenic Creutzfeldt-Jakob disease. Lancet 1991; 337(8755):1441-1442.

(146) Palmer MS, Dryden AJ, Hughes JT, Collinge J: Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. Nature 1991; 352(6333):340-342.

(147) Hill AF, Joiner S, Wadsworth JD, Sidle KC, Bell JE, Budka H, Ironside JW, Collinge J: Molecular classification of sporadic Creutzfeldt-Jakob disease. Brain 2003; 126(Pt 6):1333-1346.

(148) Brandel JP, Preece M, Brown P, Croes E, Laplanche JL, Agid Y, Will R, Alperovitch A: Distribution of codon 129 genotype in human growth hormone-treated CJD patients in France and the UK. Lancet 2003; 362(9378):128-130.

(149) Bishop MT, Hart P, Aitchison L, Baybutt HN, Plinston C, Thomson V, Tuzi NL, Head MW, Ironside JW, Will RG, Manson JC: Predicting susceptibility and incubation time of human-to-human transmission of vCJD. Lancet Neurol 2006; 5(5):393-398.

(150) Collinge J, Beck J, Campbell T, Estibeiro K, Will RG: Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease. Lancet 1996; 348(9019):56.

(151) Hauw JJ, Sazdovitch V, Laplanche JL, Peoc'h K, Kopp N, Kemeny J, Privat N, Delasnerie-Laupretre N, Brandel JP, Deslys JP, Dormont D, Alperovitch A: Neuropathologic variants of sporadic Creutzfeldt-Jakob disease and codon 129 of PrP gene. Neurology 2000; 54(8):1641-1646.

(152) Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, Windl O, Zerr I, Budka H, Kopp N, Piccardo P, Poser S, Rojiani A, Streichemberger N, Julien J, Vital C, Ghetti B, Gambetti P, Kretzschmar H: Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. Ann Neurol 1999; 46(2):224-233.

(153) Goldfarb LG, Petersen RB, Tabaton M, Brown P, LeBlanc AC, Montagna P, Cortelli P, Julien J, Vital C, Pendelbury WW, .: Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. Science 1992; 258(5083):806-808.

(154) Krebs MR, Morozova-Roche LA, Daniel K, Robinson CV, Dobson CM: Observation of sequence specificity in the seeding of protein amyloid fibrils. Protein Sci 2004; 13(7):1933-1938.

(155) Lundmark K, Westermark GT, Olsen A, Westermark P: Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism. Proc Natl Acad Sci U S A 2005; 102(17):6098-6102.

(156) Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC: Increased risk of type 2 diabetes in Alzheimer disease. Diabetes 2004; 53(2):474-481.

(157) Tsigelny IF, Crews L, Desplats P, Shaked GM, Sharikov Y, Mizuno H, Spencer B, Rockenstein E, Trejo M, Platoshyn O, Yuan JX, Masliah E: Mechanisms of hybrid oligomer formation in the pathogenesis of combined Alzheimer's and Parkinson's diseases. PLoS ONE 2008; 3(9):e3135.

(158) Ferrer I, Blanco R, Carmona M, Puig B, Ribera R, Rey MJ, Ribalta T: Prion protein expression in senile plaques in Alzheimer's disease. Acta Neuropathol (Berl) 2001; 101(1):49-56.

(159) Leuba G, Saini K, Savioz A, Charnay Y: Early-onset familial Alzheimer disease with coexisting beta-amyloid and prion pathology. JAMA 2000; 283(13):1689-1691.

(160) Schwarze-Eicker K, Keyvani K, Gortz N, Westaway D, Sachser N, Paulus W: Prion protein (PrP(c)) promotes beta-amyloid plaque formation. Neurobiol Aging 2005; 26(8):1177-1182.

(161) Debatin L, Streffer J, Geissen M, Matschke J, Aguzzi A, Glatzel M: Association between deposition of beta-amyloid and pathological prion protein in sporadic Creutzfeldt-Jakob disease. Neurodegener Dis 2008; 5(6):347-354.

(162) O'Nuallain B, Williams AD, Westermark P, Wetzel R: Seeding specificity in amyloid growth induced by heterologous fibrils. J Biol Chem 2004; 279(17):17490-17499.

(163) Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, Manson JC, Baybutt HN, Turner AJ, Hooper NM: Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. Proc Natl Acad Sci U S A 2007; 104(26):11062-11067.

(164) Soto C, Estrada L, Castilla J: Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. Trends in Biochemical Sciences 2006; 31(3):150-155.

(165) Collinge J, Whitfield J, McKintosh E, Beck J, Mead S, Thomas DJ, Alpers MP: Kuru in the 21st century--an acquired human prion disease with very long incubation periods. Lancet 2006; 367(9528):2068-2074.

(166) Palazzi X, Switzer R, George C: Natural occurrence of amyloid-Abeta deposits in the brain of young common marmosets (Callithrix jacchus): a morphological and immunohistochemical evaluation. Vet Pathol 2006; 43(5):777-779.

(167) Goudsmit J, Morrow CH, Asher DM, Yanagihara RT, Masters CL, Gibbs CJ, Jr., Gajdusek DC: Evidence for and against the transmissibility of Alzheimer disease. Neurology 1980; 30(9):945-950.

(168) Kane MD, Lipinski WJ, Callahan MJ, Bian F, Durham RA, Schwarz RD, Roher AE, Walker LC: Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta -amyloid precursor protein-transgenic mice. J Neurosci 2000; 20(10):3606-3611.

(169) Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret JM, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, Jucker M: Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. Science 2006; 313(5794):1781-1784.

(170) Kisilevsky R, Boudreau L: Kinetics of amyloid deposition. I. The effects of amyloid-enhancing factor and splenectomy. Lab Invest 1983; 48(1):53-59.

(171) Xing Y, Higuchi K: Amyloid fibril proteins. Mech Ageing Dev 2002; 123(12):1625-1636.

(172) Xing Y, Nakamura A, Chiba T, Kogishi K, Matsushita T, Li F, Guo Z, Hosokawa M, Mori M, Higuchi K: Transmission of mouse senile amyloidosis. Lab Invest 2001; 81(4):493-499.

(173) Higuchi K, Naiki H, Kitagawa K, Kitado H, Kogishi K, Matsushita T, Takeda T: Apolipoprotein A-II gene and development of amyloidosis and senescence in a congenic strain of mice carrying amyloidogenic ApoA-II. Lab Invest 1995; 72(1):75-82.

(174) Supattapone S, Geoghegan JC, Rees JR: On the horizon: a blood test for prions. Trends Microbiol 2006.

(175) Soto C: Diagnosing prion diseases: needs, challenges and hopes. Nat Rev Microbiol 2004; 2(10):809-819.

(176) Hamir AN, Miller JM, O'Rourke KI, Bartz JC, Stack MJ, Chaplin MJ: Transmission of transmissible mink encephalopathy to raccoons (Procyon lotor) by intracerebral inoculation. J Vet Diagn Invest 2004; 16(1):57-63.

(177) Hamir AN, Miller JM, Cutlip RC, Stack MJ, Chaplin MJ, Jenny AL, Williams ES: Experimental inoculation of scrapie and chronic wasting disease agents in raccoons (Procyon lotor). Vet Rec 2003; 153(4):121-123.

(178) Kirkwood JK, Cunningham AA: Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. Vet Rec 1994; 135(13):296-303.

(179) Bons N, Mestre-Frances N, Belli P, Cathala F, Gajdusek DC, Brown P: Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. Proc Natl Acad Sci U S A 1999; 96(7):4046-4051.

(180) Pearson GR, Wyatt JM, Gruffydd-Jones TJ, Hope J, Chong A, Higgins RJ, Scott AC, Wells GA: Feline spongiform encephalopathy: fibril and PrP studies. Vet Rec 1992; 131(14):307-310.

(181) Wells GA, Hawkins SA, Austin AR, Ryder SJ, Done SH, Green RB, Dexter I, Dawson M, Kimberlin RH: Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. J Gen Virol 2003; 84(Pt 4):1021-1031.

(182) Castilla J, Gutierrez-Adan A, Brun A, Doyle D, Pintado B, Ramirez MA, Salguero FJ, Parra B, Diaz SS, Sanchez-Vizcaino JM, Rogers M, Torres JM: Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein. J Neurosci 2004; 24(21):5063-5069.

(183) Foster JD, Hope J, Fraser H: Transmission of bovine spongiform encephalopathy to sheep and goats. Vet Rec 1993; 133(14):339-341.

(184) MaWhinney S, Pape WJ, Forster JE, Anderson CA, Bosque P, Miller MW: Human prion disease and relative risk associated with chronic wasting disease. Emerg Infect Dis 2006; 12(10):1527-1535.

(185) Marsh RF, Kincaid AE, Bessen RA, Bartz JC: Interspecies Transmission of Chronic Wasting Disease Prions to Squirrel Monkeys (Saimiri sciureus). J Virol 2005; 79(21):13794-13796.

Apéndice 1

Cross Currents in Protein Misfolding Disorders: Interactions and Therapy.

Rodrigo Morales[1,2], Kristi M. Green[1] and Claudio Soto[1]*

[1] Protein Misfolding Disorders Laboratory, Department of Neurology, University of Texas Medical School at Houston, 6431 Fannin Street, Houston, Texas, 77030, USA.
[2] Facultad de Ciencias, Universidad de Chile, Santiago, Chile

* To whom correspondence should be addressed at Claudio.Soto@uth.tmc.edu.

Abstract

Protein Misfolding Disorders (PMDs) are a group of diseases characterized by the accumulation of abnormally folded proteins. Despite the wide range of proteins and tissues involved, PMDs share similar molecular and pathogenic mechanisms. Several epidemiological, clinical and experimental reports have described the co-existence of PMDs, suggesting a possible cross-talk between them. A better knowledge of the molecular basis of PMDs could have important implications for understanding the mechanism by which the diseases appear and progress and ultimately to develop novel strategies for treatment. Due to their similar molecular mechanisms, common therapeutic strategies could be applied for the diseases in this group.

Molecular basis of Protein Misfolding Disorders (PMDs)

For a protein, the amino acid sequence is the blue print that dictates the biologically active conformation. However, throughout the life of the protein there are many factors that lead to its unfolding and refolding, opening the door for a misfolding event to occur. This misfolding event can then lead to the aggregation of the misfolded protein into amyloids which then wreak havoc on the body, leading to a variety of debilitating diseases. Protein misfolding disorders (PMDs) are a group of diverse, fatal diseases resulting from the failure of a protein to fold into the correct conformation followed by its subsequent aggregation and deposition in tissues (1;2). This group of diseases comprises disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), transmissible spongiform encephalopathies (TSEs) and type II diabetes among many others (2).

Transmissible Spongiform Encephalopathies (TSEs)

TSEs, also known as prion diseases, consist of a group of fatal neurodegenerative disorders that are found in a variety of mammals. TSEs are the only PMDs known to be transmissible by infection and the infectious agent (termed prion) is most likely composed exclusively of the misfolded prion protein. During prion replication, the disease associated isoform of the prion protein (PrP^{Sc}) imposes its three dimensional structure on the normal cellular form of the protein (PrP^{C}) resulting in the exponential accumulation and aggregation of PrP^{Sc} (3;4). A remarkable feature of these diseases is the prolonged incubation period followed by a very rapid clinical phase that is invariably fatal. TSEs are unique diseases due to the tripartite epidemiological appearance (inherited, sporadic, and acquired), and while rare in humans, with a sporadic Creutzfeldt

Jacob Disease (sCJD) incidence of 1-2 cases per million persons (5), they are more common in animals. TSEs can occur naturally or as a direct result of the consumption of contaminated food. The recently described variant CJD (vCJD) resulted from the consumption of meat infected with the Bovine Spongiform Encephalopathy (BSE) agent (6-8).

Prions as other more conventional type of infectious agents have two important properties: prion strains and the species barrier phenomenon (9;10). Prion strains are characterized classically by their stable pathological and biochemical characteristics, including incubation time in susceptible animals, clinical symptoms, and by their lesion profile in the central nervous system (9). While it is not entirely clear how strain characteristics are encoded, most data indicates that the tertiary structure of PrP^{Sc} is responsible (11-13). However, recent evidence suggests that quaternary structure also likely has an influence on strain properties (14).

The species barrier phenomenon in TSEs describes the ability of prions from one species to cause disease in another (15;16). Initial passage of prions from one species to another can be associated with complete resistance or long incubation times with 100% attack rate or less followed by a large drop in the incubation period on second passage with all animals developing disease (17). A clear example can be seen in the difficulty of hamster prions to cause disease in wild type mice (18). However, expression of hamster PrP^{C} in transgenic mice was shown to abolish this resistance (19). Further experiments then demonstrated that the most important determinant of the species barrier is the degree of homology between PrP^{C} and PrP^{Sc} (20). Although this is thought to result in optimal prion transmission, prion strains have been found to overcome the influence of primary

structure (21). One example of this is BSE, which has been found to transmit to multiple species while having a primary structure that is not identical to PrP^{C} in the host (22-26). This demonstrates that the factors controlling prion transmission barriers likely work in combination rather than one factor dominating over the others.

Mechanisms of protein misfolding and seeded aggregation

In each PMD, protein aggregates are composed of a misfolded protein unique to the disease, such as amyloid β (Aβ) peptide in AD or PrP^{Sc} in TSEs. The misfolded protein aggregates in PMDs have similar characteristics, but vary in distribution and composition leading to different deposition profiles and pathologies (2). In addition, there are no sequence similarities between the various proteins that are implicated in these disorders (2). Protein aggregation is not a random process, but occurs slowly through an ordered mechanism that is the basis of the nucleation-dependent polymerization model (27;28), the most accepted model for protein aggregation in these maladies.

The nucleation-dependent polymerization model features a slow and thermodynamically unfavorable nucleation phase followed by a rapid elongation phase (28;29). In the nucleation phase, the rate-determining step is the formation of a stable seed or nucleus of polymerized protein (figure 1). This seed can then simultaneously bind to and convert multiple molecules of the normal protein generating a thread of aggregated small oligomers, which subsequently will lead to the formation of amyloid fibrils and in some cases, plaques (30) (figure 1). The dynamic distribution of this toxic species is characteristic for each disease but interestingly it is also dependent of the specific conformation that the proteins can acquire (i.e. as it is observed in different prion

strains) (2;9). These amyloid fibrils are thought to be associated with the onset and progression of the various PMDs (30). Amyloidogenic-specific dyes such as Thioflavin T and Congo Red can be used to observe the kinetics of fibril formation and have demonstrated that the length of the nucleation phase (or lag phase) and extension phase are highly dependent on the concentration of protein (2;30).

The infectious nature of prions can be rationalized by the seeding-nucleation model (28). While the lag phase is normally a slow process, this phase can be reduced or eliminated by the addition of a preformed seed or nucleus to serve as a template (31). This would mean that in the case of acquired prion diseases, such as vCJD or iatrogenic CJD (iCJD), a PrP^{Sc} seed is introduced into the body and used as a template to seed the polymerization of human PrP^{Sc} fibrils, reducing the lag phase and accelerating the elongation phase (Figure 1). This provides a plausible explanation for why vCJD appeared in much younger individuals, which is very different from the appearance of disease late in life that occurs with the sporadic and inherited TSEs in which there is no preformed seed introduced (6). However, in all forms of prion diseases, once the clinical phase ensues there is a very rapid fatal decline. Since, *in vitro* and *in vivo* evidence suggest that in all PMDs the process of protein misfolding and aggregation also follow a seeding-nucleation mechanism (29;32), it is possible that other PMDs might be transmissible by infectious proteins.

Interaction of PMDs: fact or chance?

The co-existence of various PMDs in the same individual has been extensively described (33-35). Moreover, the presence of two misfolded proteins has also been

described in the same amyloidogenic structure (see below). On this basis, it could be hypothesized that PMDs might interact at the protein level through a process termed heterologous seeding or cross-seeding, suggesting that one PMD could be an important risk factor for the development of a second one.

Cross-seeding

While it is well established that fibril formation is enhanced by adding preformed homologous or heterologous seeds, it has also been shown *in vitro* that sequence similarity could have an effect on seeding efficiency. By cross-seeding hen lysozyme with a series of proteins, it was demonstrated that differing sequences had a lower efficiency of seeding (36). *In vivo* studies revealed that non-mammalian protein fibrils can cross-seed amyloid A protein (AA) in a murine experimental AA amyloidosis model (37). In addition, several reports showing the co-existence of different amyloid pathologies in the same tissue, further support the existence of cross-seeding as a disease mechanism (34;35;38-42). This data suggests that one PMD may be capable of influencing the development of another.

The demonstration that seeding with proteins of differing sequences has a lower efficiency is reminiscent of the species barrier described earlier for TSEs. This could be a plausible explanation for the low attack rate and long incubation periods typically observed during the primary transmission of prions from one species to another (9;12;17;18). However, there are many other factors that could influence this phenomenon, such as the strain of the agent and the genetic background of the host (43;44). Nevertheless, recent experiments showing that the species barrier phenomenon is reproduced in transgenic mice expressing PrP from different species support the

hypothesis that its sequence is the most important factor responsible for this phenomenon (19;45-47). So far, no elements have been identified in order to predict a potential for cross-seeding between species.

Co-existence of PMDs

The study of a putative interaction of PMDs at the protein level is not only important from a scientific point of view, but also for public health. A better understanding of this phenomenon could explain the origin and prevalence of several PMDs. Next we will discuss epidemiological and experimental evidence suggesting a cross talk between PMDs.

Epidemiological evidence

Probably the clearest evidence for the interaction of PMDs comes from the link between AD and Diabetes type-2, characterized by Aβ and amylin deposition, respectively. Clinical studies had shown that a high percentage of patients affected with AD are also positive for diabetes type-2 (48). In 2004, Janson and co-workers showed that in a cohort of AD patients, 81% of them had either diabetes type-2 or Impaired Fasting Glucose (IFG) (48). In addition, and comparing with age matched non-demented individuals, AD patients have a higher incidence of islet amyloidosis than healthy individuals. However, this study described that Aβ deposition is not increased in patients affected with Diabetes type-2, suggesting that this phenomenon could be uni-directional. Other reports have been published supporting or contradicting this conclusion (49-54). However, the location of amyloid proteins in different tissues and the lack of reports showing the co-existence of both misfolded aggregates in the same organ cloud this issue.

More information from both patients and transgenic mouse models are needed in order to settle this topic properly.

Other clinical examples are found in patients affected with PD and AD where the co-existence of Aβ and α-synuclein has been described (33;55;56). In this case, pathological features of both diseases co-exist in the brain, increasing the probabilities that misfolded protein aggregates can interact. Recently, Tsigelni *et al.* showed that α-synuclein and Aβ directly interact in the brain of patients with Lewy Body Disease (LBD) (55). This report also describes patients clinically diagnosed with AD presenting higher accumulations of α-synuclein compared to healthy individuals. In addition, co-inmmunoprecipitation experiments using anti-Aβ antibodies showed that brains from LBD patients presented strong anti-Aβ immunoreactivity after analysis by Western blot (55).

The phenomenon of amyloids' co-existence has been described for several other amyloidogenic proteins. Among them, the simultaneous presence of Aβ and prions has been extensively documented in patients affected with sCJD, Gerstmann-Sträussler-Scheinker (GSS) disease and AD (34;35;39;57-60). In some cases of AD, accumulation of PrP within Aβ deposits occurs as diffuse plaques (57;60). These aggregates are easily eliminated after PK treatment on brain slides, suggesting that prion aggregates are composed in part or totally by PrP^{C} or by protease-sensitive PrP^{Sc}. These findings are similar to what is observed for double transgenic mice over-expressing APP with Swedish and Indiana mutations (associated with strong Aβ deposition) and hamster PrP (61). However, due to the high density of PrP in these areas, the possibility of *de novo* PrP^{Sc} generation should be considered. In addition, it is important to mention that many

cases of TSEs are associated with PK sensitive PrP^{Sc} (62-64), which could be mainly composed of oligomeric protease-sensitive species.

Several cases of sCJD and GSS patients with plaques composed of misfolded PrP and Aβ have been reported (34;35;39;58;59). Surprisingly, a recent study shows that a subgroup of sCJD patients show higher levels of $A\beta_{42}$ in their brains (58). As a consequence, Aβ plaques were found in the brain of these sCJD affected individuals. As expected, little or no senile plaques were identified in sCJD patients harboring lower levels of Aβ. Interestingly, sCJD patients harboring higher amounts of Aβ have lower levels of PrP^{Sc} accumulation. This data can be interpreted in two different ways. The first explanation is that the presence of Aβ inhibit misfolding and accumulation of PrP^{Sc}. The second one is that part of the PrP^{Sc} molecules are used to induce misfolding and aggregation of Aβ instead of further accumulation of PrP. The presence of both amyloidogenic species could have enhanced toxic effects, triggering clinical disease without substantial accumulation of PrP^{Sc}. Due to the specific characteristics of these disease, it is possible that some clinical and pathological features of AD may camouflage sCJD (58;65). In a more extreme case, it could be possible to imagine that PrP^{Sc} formation, produced for example by an infectious exposure may trigger Aβ misfolding and accumulation and lead to AD clinical disease, instead of CJD. Thus, it could be interesting to study whether in countries with high exposure to BSE (such as UK or France) there are an increased number of cases of AD or other neurodegenerative diseases associated to protein misfolding and aggregation. It would also be important to carry out more experiments in animal models to assess properly this important issue.

Evidence in vitro

The easiest way to test the hypothesis of cross-seeding is by *in vitro* aggregation assays. Classical seeding assays show that the addition of a preformed seed reduced the extent of the lag phase of aggregation in amyloidogenic proteins (27;29;66). In addition several reports show that the extent of the nucleation phase of a specific protein could be reduced after the addition of a heterologous seed (55;67). This cross-seeding effect can be different according to the protein/protein pair studied.

Subsequent to the clinical evidence described earlier in Diabetes type-2 and AD patients (48), *in vitro* seeding assays using amylin and Aβ were performed (67). Interestingly, there is some degree of sequence similarity between amylin and Aβ. If we add this information to the previously mentioned reports that link AD and Diabetes type-2 (48), it would be rational to look for a putative interaction between both proteins. Seeding assays show that Aβ amyloids are good seeds for amylin aggregation. However, the same report described that globular and fibrillar aggregates of amylin have inert or very low effects on Aβ aggregation (67). These results are interesting if we consider the clinical data reported by Janson *et al.*, where the interaction between Diabetes Type-2 in AD seems to work only in one direction (48).

Another important example of *in vitro* cross-seeding comes from studies involving α-synuclein and Aβ (55;68). The evidence in this case goes further, explaining a possible association between different oligomeric aggregates (55). Additionally, it is suggested that both proteins could form annular structures able to generate pores in the cell membrane (55). It is hypothesized that these structures could produce cationic

imbalance between intracellular and extracellular spaces, leading to cell death events observed in this disorders.

By coupling seeding assays with other *in vitro* and *in silico* techniques, we could enormously improve our knowledge regarding this important issue. However, a characterization of PMDs co-existence should be necessary in animal models where multi-factorial effects (e.g. inflammation, clearance and signaling cascades) could contribute dramatically to the outcome.

Studies in animal models

The best way to analyze if two PMDs can cross-talk is by using animal models. Animal models allow us to measure many pathological features in a controlled way and at different stages of the disease. Several reports studying PMDs interaction in mice models are available. Again, the Aβ/α-synuclein pair is one of the best studied cases (55;69). The results indicate that Aβ enhances alpha-synuclein accumulation and neuronal deficits in a double transgenic mouse model expressing both proteins (70). Immunoprecipitation assays demonstrated the co-existence of both proteins in the mouse brain (55).

The presence of Aβ aggregates and Tau neurofibrillary tangles is the major hallmark in AD. It has been proposed for a long time that a putative interaction between both proteins could enhance the clinical decline observed in AD patients. Two interesting pieces of evidence were supplied in 2001 by Lewis *et al.* (70) and Gotz *et al.* (71). These studies show that the simultaneous presence of Aβ and Tau enhanced neurofribillar accumulation in mouse models. In the first approach, researchers crossed Tg2576 mice with transgenic mice expressing a mutant form of Tau protein. They observed that

Tg2576 and double transgenic mice develop Aβ deposit at the same age, however neurofibrillar deposition was significantly enhanced compared to the single mutant Tau transgenic (70). Interestingly, the increase in Tau deposition was not correlated with areas rich in Aβ deposits. The second study consisted in the inoculation of pre-formed $A\beta_{42}$ fibrils in the brain of P301L Tau transgenic mice. As in the previous report, the presence of misfolded Aβ in the brain of these mice increased Tau deposition (71).

Considering the findings suggesting the co-existence of AD and sCJD, the study of a possible link between both diseases is important. As suggested by Debatin *et al.* (58), the fact that AD phenotype could mask some sCJD cases, make this issue even more relevant. Since Aβ and PrP^{Sc} have similar aggregation mechanisms and are located in the same tissue (brain) and subcellular location (extracellular space), it is likely that these misfolded protein may interact. However, recent findings suggest that PrP^{C} could be a protective molecule in AD via β-secretase inhibition (72) demonstrating the need for more studies in this topic. The stable characteristic of Aβ accumulation in transgenic mice (73-75) and the constant incubation periods for prions (13) should make these studies easy to follow.

Strategies for PMDs treatment.

It is well accepted that the misfolding process is the key event in the development of PMDs. Therefore, therapeutic strategies targeted to the prevention of misfolding not only look promising at alleviating the disease, but curing it as well.

Currently, therapies for most PMDs are just palliative. It is possible that attacking the central event in the disease (the misfolding and aggregation of the protein) could lead

to more effective disease-modifying therapies. In order to attack misfolding processes, different approaches have been followed. One approach is attack specifically abnormally folded proteins that can later on act as aggregation nuclei. Other strategy consists of inhibiting the misfolding process itself. The former may allow depletion of putative seeds, whereas the latter might inhibit the formation of newly converted units. Both approaches could help the endogenous protein clearance systems, resulting in the total depletion of disease associated structures.

β-sheet breakers small molecule inhibitors.

One approach that has been used to discover small chemical inhibitors of protein misfolding and aggregation has been the screening of large libraries of compounds using simple *in vitro* assays (76-78). The development of high throughput screening requires a robust, simple and relevant *in vitro* assay to monitor compound activity. In the case of the search for compounds capable to prevent or reverse protein misfolding and aggregation, development of a screening assay with these characteristics have been challenging, owing to the intrinsic variability of the interaction between misfolded aggregates and the heterogeneity of structures. Nevertheless, there have been many small chemical compounds that have been reported to inhibit protein misfolding and aggregation. Some of these compounds came from screening, but several others were identified serendipitously or based on epidemiological studies which have suggested they may be active. Some small molecules that that have been reported to prevent the misfolding and aggregation of proteins involved in PMDs include (a more comprehensive list of compounds, including the specific references, can be found in (76-79): Congo red and derivatives, curcumin and rosmarinic acid, small sulfonated anions, wine polyphenols

and tannic acid, melatonin, nicotine, estrogen, 1,2-(dimethoxymethano)fullerene, hexadecyl-*N*-methylpiperidinium bromide, benzofuran-based compounds, amphiphilic surfactants, such as di-C6-PC and di-C7-PC, the disaccharide trehalose, the anti-leprosy drugs dapsone and rifampicin, inositol, nordihydroguaiaretic acid, β-cyclodextrins, apomorphine and analogues, anthracyclines, thyroxine, diclofenac analogues, tetracyclines, quinacrine, branched polyamines, acridine and phenotiazine derivatives, flufenamic acid, N-substituted anthranilic acids, N-phenylphenoxazines, nitrophenols, porphyrins and phtalocyanines, 4'-iodo-4'-deoxy-doxorubicin, pentosane polysulphate, amphotericin B, (−)-5,8-dihydroxy-3R-methyl-2R-(dipropylamino)-1,2,3,4-tetrahydronapthalene, salvianolic acid B, suramin, and "chemical chaperones" (such as glycerol, dimethyl sulfoxide, trimethylamine-N-oxide) . Even though many molecules have been identified as strong inhibitors, the usefulness of these small molecules is compromised by their lack of specificity, toxicity and their unclear mechanism of action in most of the cases.

A strategy that has been more successful in identifying potential hit compounds is the rational development of specific inhibitors based on the use of short peptides targeting the protein region needed for protein-protein interaction (79;80). The approach consists of synthesizing short peptides combining a self-recognition motif with a ß-sheet disrupting element. The self-recognition domain is typically the region of the protein implicated in early misfolding and protein-protein interaction. As disrupting elements, different groups have used distinct strategies, including: the use of a bulky group (e.g cholyl) that sterically inhibit protein aggregation; N-methylations (or N-alkylations) to generate peptides having a blocking face; β-sheet breaker amino acids to disrupt beta-

sheet conformation; and addition of charged residues to reduce the hydrophobic interaction which trigger protein aggregation (79;80). The use of peptides as a therapy specifically struggle with three main problems, namely transport across biological membranes (i.e. the blood brain barrier and intestinal barrier), their rapid degradation in the body and generation of an immune response. However, these problems can be minimized by shortening the length of the peptide or by introducing chemical modifications (81).

Vaccines.

Several attempts for immunization against PMDs have been tried. Probably the best known is the one tried by Elan pharmaceuticals against Aβ deposition (82). The experimental strategy included synthetic $A\beta_{42}$ as immunogen in PDAPP transgenic mice, which spontaneously generate AD plaques with age. Results showed an almost complete prevention of Aβ aggregates and significant reduction of astrocytosis in mice immunized before plaque generation. Animals treated with the Aβ vaccine at late stages of Aβ deposition showed a significant reduction in amyloid burden compared to PBS treated controls. As expected, high levels of antibodies against Aβ were found in the blood of $A\beta_{42}$ treated mice. Unfortunately, clinical trials using this vaccine in humans were not successful since some of the patients involved in the trial developed meningeoencephalitis, leading in some of the cases to the death of the individuals (83;84). Currently, several approaches looking to reduce these adverse effects are under development. They include non-fibrillary Aβ derivatives and DNA immunization, among others (85-87).

Similar strategies targeting amyloid aggregates have been tried for α-synuclein in Parkinson's disease (88) and PrP in TSEs (89). For the latter we can mention the studies of Sigurdsson *et al.* (90) and Magri *et al.* (91), where the treatment with recombinant PrP or synthetic prion derived peptides increase the incubation periods of mice and hamsters inoculated intraperitoneally with the 139A and 263K prion strains, respectively. These results expand the concept applied for AD and confirm that immunization using sequence specific peptides can decrease clinical features of PMDs. Recently, an interesting approach using an attenuated *Salmonella* vaccine expressing mouse PrP showed promising results in mice infected with 139A prions (92).

Conformational antibodies.

Recent studies pioneered by the group of Charles Glabe showed the generation of antibodies specific to diverse misfolded aggregated forms of amyloidogenic proteins, including oligomers and fibrils (93). Surprisingly, these antibodies are able to recognize oligomeric or fibrillar species regardless of the sequence. The use of such antibodies may represent a universal treatment for all amyloidogenic proteins and has the additional advantage that it targets exclusively the structure that is thought to be the toxic one.

One of the main problems with using antibodies for treatment is their stability and delivery into the CNS. Improvements in their delivery and stability could increase the use of these molecules for the treatment of PMDs affecting the brain. However, this approach could be very interesting in treating peripheral amyloidoses such as diabetes type-2. Additionally, the use of these antibodies may contribute to increase our understanding of the amyloids biology and the nature of the toxic species.

Concluding Remarks and future directions

This article has focused on describing the common mechanisms associated to a variety of human diseases, collectively called PMDs. Compelling evidences have accumulated over the years to indicate that the key event in these diseases is the misfolding, aggregation and tissue accumulation of a protein. The available evidence suggests that these proteins misfold and aggregate by a seeding - nucleation mechanism. The formation of a stable seed, corresponding to an oligomer of the protein adopting the misfolded structure, is the key event in the process. The oligomeric seed is not only the most likely candidate for the toxic species, but also is capable to accelerate the further polymerization of the aggregates. As such, seeds have the intrinsic ability to propagate the misfolding and aggregation process. This provides a plausible model to explain the infectious nature of TSEs, which is so far the only PMD considered transmissible. Importantly the ability of seeds composed of one protein to nucleate the aggregation of a different protein, through a phenomenon termed cross-seeding, provides a mechanistic explanation for the relatively common co-existence of more than one PMD in the same patient. In this article we also discussed epidemiological and experimental evidence for the interaction of PMDs and their implications for public health.

These topics open new issues in public health. The interaction between PMDs and the effect that some of them can have as risk factors for more prevalent PMDs need to be considered. In addition, the information gathered so far suggests that all PMDs could have the inherent ability to be infectious. Considering the high incidence of sporadic cases in some PMDs, it is possible that a fraction of sporadic PMDs are originally generated by seeding or cross-seeding mechanisms. Therefore, disease propagation by

seeding and cross-seeding is an important topic that needs to be further addressed in the years to come. A better understanding of these mechanisms could help for rationale design of novel therapeutic and diagnostic methodologies targeting these disorders.

Acknowledgements

This work was supported in part by the NIH grant R01AG028821 and by funding from the Mitchell Foundation and CART Foundation.

References

1. Dobson,CM: Protein misfolding, evolution and disease. [Review] [35 refs]. *Trends Biochem Sci* 24:329-332, 1999

2. Soto,C: Unfolding the role of Protein Misfolding in Neurodegenerative Diseases. *Nature Rev Neurosci* 4:49-60, 2003

3. Collinge,J: Prion diseases of humans and animals: Their causes and molecular basis. *Annual Review of Neuroscience* 24:519-550, 2001

4. Prusiner,SB: Prions. *Proceedings of the National Academy of Sciences of the United States of America* 95:13363-13383, 1998

5. Wadsworth,JD, Collinge,J: Update on human prion disease. *Biochim Biophys Acta* 1772:598-609, 2007

6. Collinge,J: Variant Creutzfeldt-Jakob disease. *Lancet* 354:317-323, 1999

7. Hill,AF, Desbruslais,M, Joiner,S, Sidle,KC, Gowland,I, Collinge,J, Doey,LJ, Lantos,P: The same prion strain causes vCJD and BSE. *Nature* 389:448-50, 526, 1997

8. Bruce,ME, Will,RG, Ironside,JW, McConnell,I, Drummond,D, Suttie,A, McCardle,L, Chree,A, Hope,J, Birkett,C, Cousens,S, Fraser,H, Bostock,CJ: Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 389:498-501, 1997

9. Morales,R, Abid,K, Soto,C: The prion strain phenomenon: molecular basis and unprecedented features. *Biochim Biophys Acta* 1772:681-691, 2007

10. Hill,AF, Collinge,J: Prion strains and species barriers. *Contrib Microbiol* 11:33-49, 2004

11. Peretz,D, Scott,MR, Groth,D, Williamson,RA, Burton,DR, Cohen,FE, Prusiner,SB: Strain-specified relative conformational stability of the scrapie prion protein. *Protein Sci* 10:854-863, 2001

12. Bartz,JC, Bessen,RA, McKenzie,D, Marsh,RF, Aiken,JM: Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *J Virol* 74:5542-5547, 2000

13. Castilla,J, Morales,R, Saa,P, Barria,M, Gambetti,P, Soto,C: Cell-free propagation of prion strains. *EMBO J* 27:2557-2566, 2008

14. Sim,VL, Caughey,B: Ultrastructures and strain comparison of under-glycosylated scrapie prion fibrils. *Neurobiol Aging* 2008

15. Hill,AF, Collinge,J: Subclinical prion infection. *Trends Microbiol* 11:578-584, 2003

16. Pattison IH: Experiments with scrapie with special reference to the nature of the agent and the pathology of the disease. In *Slow, Latent and Temperate Virus Infections, NINDB Monograph 2*. Gajdusek CJ, Gibbs CJ, Alpers MP, Eds. Washington, DC, U.S. Goberment Printing Office, 1965, p. 249-257

17. Collinge,J, Clarke,AR: A general model of prion strains and their pathogenicity. *Science* 318:930-936, 2007

18. Race,R, Meade-White,K, Raines,A, Raymond,GJ, Caughey,B, Chesebro,B: Subclinical scrapie infection in a resistant species: persistence, replication, and adaptation of infectivity during four passages. *J Infect Dis* 186 Suppl 2:S166-S170, 2002

19. Scott,M, Foster,D, Mirenda,C, Serban,D, Coufal,F, Walchli,M, Torchia,M, Groth,D, Carlson,G, DeArmond,SJ, .: Transgenic mice expressing hamster prion

protein produce species-specific scrapie infectivity and amyloid plaques. *Cell* 59:847-857, 1989

20. Prusiner,SB, Scott,M, Foster,D, Pan,KM, Groth,D, Mirenda,C, Torchia,M, Yang,SL, Serban,D, Carlson,GA, .: Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* 63:673-686, 1990

21. Moore,RA, Vorberg,I, Priola,SA: Species barriers in prion diseases--brief review. *Arch Virol Suppl*187-202, 2005

22. Collee,JG, Bradley,R: BSE: a decade on--Part I. *Lancet* 349:636-641, 1997

23. Collee,JG, Bradley,R: BSE: a decade on--Part 2. *Lancet* 349:715-721, 1997

24. Kirkwood,JK, Cunningham,AA: Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. *Vet Rec* 135:296-303, 1994

25. Pearson,GR, Wyatt,JM, Gruffydd-Jones,TJ, Hope,J, Chong,A, Higgins,RJ, Scott,AC, Wells,GA: Feline spongiform encephalopathy: fibril and PrP studies. *Vet Rec* 131:307-310, 1992

26. Bons,N, Mestre-Frances,N, Belli,P, Cathala,F, Gajdusek,DC, Brown,P: Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. *Proc Natl Acad Sci U S A* 96:4046-4051, 1999

27. Jarrett,JT, Lansbury,PT, Jr.: Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell* 73:1055-1058, 1993

28. Kocisko,DA, Come,JH, Priola,SA, Chesebro,B, Raymond,GJ, Lansbury,PT, Caughey,B: Cell-free formation of protease-resistant prion protein. *Nature* 370:471-474, 1994

29. Soto,C, Estrada,L, Castilla,J: Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. *Trends Biochem Sci* 31:150-155, 2006

30. Soto,C: Protein misfolding and disease; protein refolding and therapy. *Febs Letters* 498:204-207, 2001

31. Jarrett,JT, Lansbury,PT, Jr.: Amyloid fibril formation requires a chemically discriminating nucleation event: studies of an amyloidogenic sequence from the bacterial protein OsmB. *Biochemistry* 31:12345-12352, 1992

32. Gajdusek,DC: Nucleation of Amyloidogenesis in Infections and Noninfectious Amyloidoses of Brain. *Slow Infections of the Central Nervous System* 724:173-190, 1994

33. Lippa,CF, Duda,JE, Grossman,M, Hurtig,HI, Aarsland,D, Boeve,BF, Brooks,DJ, Dickson,DW, Dubois,B, Emre,M, Fahn,S, Farmer,JM, Galasko,D, Galvin,JE, Goetz,CG, Growdon,JH, Gwinn-Hardy,KA, Hardy,J, Heutink,P, Iwatsubo,T, Kosaka,K, Lee,VM, Leverenz,JB, Masliah,E, McKeith,IG, Nussbaum,RL, Olanow,CW, Ravina,BM, Singleton,AB, Tanner,CM, Trojanowski,JQ, Wszolek,ZK: DLB and PDD boundary issues: diagnosis, treatment, molecular pathology, and biomarkers. *Neurology* 68:812-819, 2007

34. Hainfellner,JA, Wanschitz,J, Jellinger,K, Liberski,PP, Gullotta,F, Budka,H: Coexistence of Alzheimer-type neuropathology in Creutzfeldt-Jakob disease. *Acta Neuropathol (Berl)* 96:116-122, 1998

35. Tsuchiya,K, Yagishita,S, Ikeda,K, Sano,M, Taki,K, Hashimoto,K, Watabiki,S, Mizusawa,H: Coexistence of CJD and Alzheimer's disease: an autopsy case showing typical clinical features of CJD. *Neuropathology* 24:46-55, 2004

36. Krebs,MR, Morozova-Roche,LA, Daniel,K, Robinson,CV, Dobson,CM: Observation of sequence specificity in the seeding of protein amyloid fibrils. *Protein Sci* 13:1933-1938, 2004

37. Lundmark,K, Westermark,GT, Olsen,A, Westermark,P: Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism. *Proc Natl Acad Sci U S A* 102:6098-6102, 2005

38. Brown,P, Jannotta,F, Gibbs,CJ, Jr., Baron,H, Guiroy,DC, Gajdusek,DC: Coexistence of Creutzfeldt-Jakob disease and Alzheimer's disease in the same patient. *Neurology* 40:226-228, 1990

39. Miyazono,M, Kitamoto,T, Iwaki,T, Tateishi,J: Colocalization of prion protein and beta protein in the same amyloid plaques in patients with Gerstmann-Straussler syndrome. *Acta Neuropathol (Berl)* 83:333-339, 1992

40. Muramoto,T, Kitamoto,T, Koga,H, Tateishi,J: The coexistence of Alzheimer's disease and Creutzfeldt-Jakob disease in a patient with dementia of long duration. *Acta Neuropathol (Berl)* 84:686-689, 1992

41. Iida,T, Doh-Ura,K, Kawashima,T, Abe,H, Iwaki,T: An atypical case of sporadic Creutzfeldt-Jakob disease with Parkinson's disease. *Neuropathology* 21:294-297, 2001

42. Preusser,M, Strobel,T, Gelpi,E, Eiler,M, Broessner,G, Schmutzhard,E, Budka,H: Alzheimer-type neuropathology in a 28 year old patient with iatrogenic Creutzfeldt-Jakob disease after dural grafting. *J Neurol Neurosurg Psychiatry* 77:413-416, 2006

43. Bessen,RA, Marsh,RF: Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. *J Gen Virol* 73 (Pt 2):329-334, 1992

44. Stephenson,DA, Chiotti,K, Ebeling,C, Groth,D, DeArmond,SJ, Prusiner,SB, Carlson,GA: Quantitative trait loci affecting prion incubation time in mice. *Genomics* 69:47-53, 2000

45. Browning,SR, Mason,GL, Seward,T, Green,M, Eliason,GA, Mathiason,C, Miller,MW, Williams,ES, Hoover,E, Telling,GC: Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. *J Virol* 78:13345-13350, 2004

46. Groschup,MH, Buschmann,A: Rodent models for prion diseases. *Vet Res* 39:32, 2008

47. Windl,O, Buchholz,M, Neubauer,A, Schulz-Schaeffer,W, Groschup,M, Walter,S, Arendt,S, Neumann,M, Voss,AK, Kretzschmar,HA: Breaking an absolute species barrier: transgenic mice expressing the mink PrP gene are susceptible to transmissible mink encephalopathy. *J Virol* 79:14971-14975, 2005

48. Janson,J, Laedtke,T, Parisi,JE, O'Brien,P, Petersen,RC, Butler,PC: Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes* 53:474-481, 2004

49. Ott,A, Stolk,RP, van Harskamp,F, Pols,HA, Hofman,A, Breteler,MM: Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology* 53:1937-1942, 1999

50. Luchsinger,JA, Tang,MX, Stern,Y, Shea,S, Mayeux,R: Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort. *Am J Epidemiol* 154:635-641, 2001

51. Landin,K, Blennow,K, Wallin,A, Gottfries,CG: Low blood pressure and blood glucose levels in Alzheimer's disease. Evidence for a hypometabolic disorder? *J Intern Med* 233:357-363, 1993

52. Nielson,KA, Nolan,JH, Berchtold,NC, Sandman,CA, Mulnard,RA, Cotman,CW: Apolipoprotein-E genotyping of diabetic dementia patients: is diabetes rare in Alzheimer's disease? *J Am Geriatr Soc* 44:897-904, 1996

53. Kokmen,E, Beard,CM, Chandra,V, Offord,KP, Schoenberg,BS, Ballard,DJ: Clinical risk factors for Alzheimer's disease: a population-based case-control study. *Neurology* 41:1393-1397, 1991

54. Curb,JD, Rodriguez,BL, Abbott,RD, Petrovitch,H, Ross,GW, Masaki,KH, Foley,D, Blanchette,PL, Harris,T, Chen,R, White,LR: Longitudinal association of vascular and Alzheimer's dementias, diabetes, and glucose tolerance. *Neurology* 52:971-975, 1999

55. Tsigelny,IF, Crews,L, Desplats,P, Shaked,GM, Sharikov,Y, Mizuno,H, Spencer,B, Rockenstein,E, Trejo,M, Platoshyn,O, Yuan,JX, Masliah,E: Mechanisms of hybrid oligomer formation in the pathogenesis of combined Alzheimer's and Parkinson's diseases. *PLoS ONE* 3:e3135, 2008

56. McKeith,IG, Galasko,D, Kosaka,K, Perry,EK, Dickson,DW, Hansen,LA, Salmon,DP, Lowe,J, Mirra,SS, Byrne,EJ, Lennox,G, Quinn,NP, Edwardson,JA, Ince,PG, Bergeron,C, Burns,A, Miller,BL, Lovestone,S, Collerton,D, Jansen,EN, Ballard,C, De Vos,RA, Wilcock,GK, Jellinger,KA, Perry,RH: Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop. *Neurology* 47:1113-1124, 1996

57. Ferrer,I, Blanco,R, Carmona,M, Puig,B, Ribera,R, Rey,MJ, Ribalta,T: Prion protein expression in senile plaques in Alzheimer's disease. *Acta Neuropathol (Berl)* 101:49-56, 2001

58. Debatin,L, Streffer,J, Geissen,M, Matschke,J, Aguzzi,A, Glatzel,M: Association between deposition of beta-amyloid and pathological prion protein in sporadic Creutzfeldt-Jakob disease. *Neurodegener Dis* 5:347-354, 2008

59. Paquet,C, Privat,N, Kaci,R, Polivka,M, Dupont,O, Haik,S, Laplanche,JL, Hauw,JJ, Gray,F: Cerebral amyloid angiopathy with co-localization of prion

protein and beta-amyloid in an 85-year-old patient with sporadic Creutzfeldt-Jakob disease. *Acta Neuropathol* 116:567-573, 2008

60. Leuba,G, Saini,K, Savioz,A, Charnay,Y: Early-onset familial Alzheimer disease with coexisting beta-amyloid and prion pathology. *JAMA* 283:1689-1691, 2000

61. Schwarze-Eicker,K, Keyvani,K, Gortz,N, Westaway,D, Sachser,N, Paulus,W: Prion protein (PrP(c)) promotes beta-amyloid plaque formation. *Neurobiol Aging* 26:1177-1182, 2005

62. Lasmezas,CI, Deslys,JP, Robain,O, Jaegly,A, Beringue,V, Peyrin,JM, Fournier,JG, Hauw,JJ, Rossier,J, Dormont,D: Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science* 275:402-405, 1997

63. Manuelidis,L, Sklaviadis,T, Manuelidis,EE: Evidence suggesting that PrP is not the infectious agent in Creutzfeldt-Jakob disease. *EMBO J* 6:341-347, 1987

64. Collinge,J, Owen,F, Poulter,M, Leach,M, Crow,TJ, Rossor,MN, Hardy,J, Mullan,MJ, Janota,I, Lantos,PL: Prion dementia without characteristic pathology. *Lancet* 336:7-9, 1990

65. Manuelidis,EE, Manuelidis,L: A transmissible Creutzfeldt-Jakob disease-like agent is prevalent in the human population. *Proc Natl Acad Sci U S A* 90:7724-7728, 1993

66. Lomakin,A, Chung,DS, Benedek,GB, Kirschner,DA, Teplow,DB: On the nucleation and growth of amyloid beta-protein fibrils: detection of nuclei and quantitation of rate constants. *Proc Natl Acad Sci U S A* 93:1125-1129, 1996

67. O'Nuallain,B, Williams,AD, Westermark,P, Wetzel,R: Seeding specificity in amyloid growth induced by heterologous fibrils. *J Biol Chem* 279:17490-17499, 2004

68. Mandal,PK, Pettegrew,JW, Masliah,E, Hamilton,RL, Mandal,R: Interaction between Abeta peptide and alpha synuclein: molecular mechanisms in overlapping pathology of Alzheimer's and Parkinson's in dementia with Lewy body disease. *Neurochem Res* 31:1153-1162, 2006

69. Masliah,E, Rockenstein,E, Veinbergs,I, Sagara,Y, Mallory,M, Hashimoto,M, Mucke,L: beta-amyloid peptides enhance alpha-synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease and Parkinson's disease. *Proc Natl Acad Sci U S A* 98:12245-12250, 2001

70. Lewis,J, Dickson,DW, Lin,WL, Chisholm,L, Corral,A, Jones,G, Yen,SH, Sahara,N, Skipper,L, Yager,D, Eckman,C, Hardy,J, Hutton,M, McGowan,E:

Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* 293:1487-1491, 2001

71. Gotz,J, Chen,F, Van Dorpe,J, Nitsch,RM: Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. *Science* 293:1491-1495, 2001

72. Parkin,ET, Watt,NT, Hussain,I, Eckman,EA, Eckman,CB, Manson,JC, Baybutt,HN, Turner,AJ, Hooper,NM: Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A* 104:11062-11067, 2007

73. Hsiao,K, Chapman,P, Nilsen,S, Eckman,C, Harigaya,Y, Younkin,S, Yang,F, Cole,G: Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274:99-102, 1996

74. Van,DD, De Deyn,PP: Drug discovery in dementia: the role of rodent models. *Nat Rev Drug Discov* 5:956-970, 2006

75. Dodart,JC, May,P: Overview on rodent models of Alzheimer's disease. *Curr Protoc Neurosci* Chapter 9:Unit, 2005

76. De,LE, Giorgetti,S, Grossi,S, Merlini,G, Caccialanza,G, Bellotti,V: Pharmaceutical strategies against amyloidosis: old and new drugs in targeting a "protein misfolding disease". *Curr Med Chem* 11:1065-1084, 2004

77. Cohen,FE, Kelly,JW: Therapeutic approaches to protein-misfolding diseases. *Nature* 426:905-909, 2003

78. Soto,C, Martin,Z: Therapeutic strategies against protein misfolding in neurodegenerative diseases. *Exp Op Drug Disc* 4:71-84, 2009

79. Mason,JM, Kokkoni,N, Stott,K, Doig,AJ: Design strategies for anti-amyloid agents. *Curr Opin Struct Biol* 13:526-532, 2003

80. Estrada,LD, Soto,C: Peptide inhibitors of protein misfolding and aggregation. *Curr Pharm Des* 12:2557-2568, 2006

81. Adessi,C, Soto,C: Converting a peptide into a drug: Strategies to improve stability and bioavailability. *Curr Med Chem* 9:963-978, 2002

82. Schenk,D, Barbour,R, Dunn,W, Gordon,G, Grajeda,H, Guido,T, Hu,K, Huang,JP, Johnson-Wood,K, Khan,K, Kholodenko,D, Lee,M, Liao,ZM, Lieberburg,I, Motter,R, Mutter,L, Soriano,F, Shopp,G, Vasquez,N, Vandevert,C, Walker,S, Wogulis,M, Yednock,T, Games,D, Seubert,P: Immunization with amyloid-beta

attenuates Alzheimer disease-like pathology in the PDAPP mouse. *Nature* 400:173-177, 1999

83. Spinney,L: Update on Elan vaccine for Alzheimer's disease. *Lancet Neurol* 3:5, 2004

84. Schenk,D: Amyloid-beta immunotherapy for Alzheimer's disease: the end of the beginning. *Nature Reviews Neuroscience* 3:824-828, 2002

85. Sigurdsson,EM, Knudsen,E, Asuni,A, Fitzer-Attas,C, Sage,D, Quartermain,D, Goni,F, Frangione,B, Wisniewski,T: An attenuated immune response is sufficient to enhance cognition in an Alzheimer's disease mouse model immunized with amyloid-beta derivatives. *J Neurosci* 24:6277-6282, 2004

86. Schiltz,JG, Salzer,U, Mohajeri,MH, Franke,D, Heinrich,J, Pavlovic,J, Wollmer,MA, Nitsch,RM, Moelling,K: Antibodies from a DNA peptide vaccination decrease the brain amyloid burden in a mouse model of Alzheimer's disease. *J Mol Med* 82:706-714, 2004

87. Okura,Y, Matsumoto,Y: DNA vaccine therapy for Alzheimer's disease: present status and future direction. *Rejuvenation Res* 11:301-308, 2008

88. Masliah,E, Rockenstein,E, Adame,A, Alford,M, Crews,L, Hashimoto,M, Seubert,P, Lee,M, Goldstein,J, Chilcote,T, Games,D, Schenk,D: Effects of alpha-

synuclein immunization in a mouse model of Parkinson's disease. *Neuron* 46:857-868, 2005

89. Sigurdsson,EM, Wisniewski,T: Promising developments in prion immunotherapy. *Expert Rev Vaccines* 4:607-610, 2005

90. Sigurdsson,EM, Brown,DR, Daniels,M, Kascsak,RJ, Kascsak,R, Carp,R, Meeker,HC, Frangione,B, Wisniewski,T: Immunization delays the onset of prion disease in mice. *American Journal of Pathology* 161:13-17, 2002

91. Magri,G, Clerici,M, Dall'Ara,P, Biasin,M, Caramelli,M, Casalone,C, Giannino,ML, Longhi,R, Piacentini,L, Della Bella,S, Gazzuola,P, Martino,PA, Della Bella,S, Pollera,C, Puricelli,M, Servida,F, Crescio,I, Boasso,A, Ponti,W, Poli,G: Decrease in pathology and progression of scrapie after immunisation with synthetic prion protein peptides in hamsters. *Vaccine* 23:2862-2868, 2005

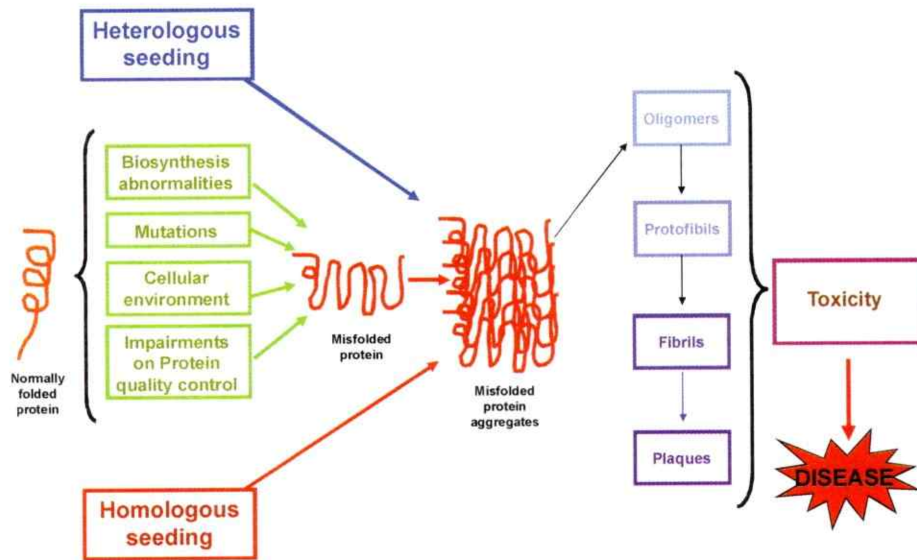
92. Goni,F, Knudsen,E, Schreiber,F, Scholtzova,H, Pankiewicz,J, Carp,R, Meeker,HC, Rubenstein,R, Brown,DR, Sy,MS, Chabalgoity,JA, Sigurdsson,EM, Wisniewski,T: Mucosal vaccination delays or prevents prion infection via an oral route. *Neuroscience* 2005

93. Kayed,R, Head,E, Thompson,JL, McIntire,TM, Milton,SC, Cotman,CW, Glabe,CG: Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300:486-489, 2003

Figure Legend

Figure 1: Seeding and cross-seeding mechanisms in Protein Misfolding Disorders. A variety of factors, including mutations, abnormal biosynthesis, changes on cellular environment or impairments on the quality control machinery, induce the initial misfolding of normally folded proteins. Misfolded proteins become stabilized upon oligomerization and formation of long fibrillar amyloid aggregates. Preformed seeds have been described to speed this process *in vitro* and *in vivo*. In addition, the presence of misfolded nuclei could enhance the aggregation of other amyloids, phenomenon known as cross-seeding.

Figure 1



Apéndice 2

10

Advances in Prion Detection

Rodrigo Morales, Dennisse González, Claudio Soto, and Joaquín Castilla

CONTENTS

10.1 Introduction 256
10.1.1 How Is a Normal Protein Converted to a TSE Agent? 257
10.1.2 From Food to Brain 258
10.1.3 Species Barrier Phenomenon 259
10.1.4 Animal TSEs as Zoonoses 259
10.2 Current Methods in Prion Detection 260
10.2.1 Needs for an Ultra-Sensitive Method for Prion Detection 260
10.2.2 In Vitro Assays for Prion Detection 262
10.2.2.1 Histopathological Study 262
10.2.2.2 Western Blot-Based Tests 262
10.2.2.3 ELISA-Based Tests 262
10.2.2.4 BSE and Scrapie Discriminatory Test 263
10.2.2.5 Conformational Assays 264
10.2.2.6 Spectroscopic Techniques: Multispectral Ultraviolet Fluoroscopy 266
10.2.2.7 Prion Concentration and Specific Ligands 266
10.2.3 Amplification Assays for Prion Detection 268
10.2.3.1 In Vitro Amplification 268
10.2.3.2 In Vivo Amplification 271
10.3 Concluding Remarks 273
Acknowledgments 275
References 275

10.1 Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal, transmissible neurodegenerative diseases affecting humans and animals (Table 10.1). They are usually characterized by the presence of protease-resistant prion protein (PrP^{res}), an abnormal, protease-resistant isoform of the normal host cell surface protein[1] denoted PrP^{C}. No amino acid sequence or posttranslational differences have been detected between PrP^{C} and its pathological form, PrP^{Sc}. The conversion of PrP^{C} into PrP^{Sc} involves a conformational change, whereby the α-helical content diminishes and the amount of β-sheet increases.[2] Depending on how they arise, TSEs can be classified as sporadic, hereditary, or infectious; most have been experimentally transmitted and, with some exceptions, the presence of PrP^{res} is related to their infectivity.[1,3–5] The etiology of spontaneous and familial TSE has been only described in humans and includes the Creutzfeldt–Jakob disease (CJD), the Gertsmann–Straüssler–Scheinker syndrome, and fatal familial insomnia.[6–12] It is generally accepted that misfolding of the cellular prion protein (PrP^{C}) leads to the build up in the brain of an insoluble, pathologic PrP isoform (PrP^{Sc}). PrP^{Sc} aggregation and accumulation in the brain produce spongiform brain degeneration (vacuolization), neuronal death, astrocytosis, and microglial proliferation.[2] Clinically, the disease is manifested mainly by rapidly progressive dementia, visual and motor impairments, and inexorable death.

TABLE 10.1

Prion Diseases, Hosts, and Mechanism of Infection

Disease	Host	Mechanism of Infection	Reference
Sporadic CJD	Human	Unknown	[128]
Sporadic fatal insomnia	Human	Unknown	[129]
Fatal familial insomnia	Human	Mutations in the *prnp* gene	[130]
Familial CJD	Human	Mutations in the *prnp* gene	[131]
Gerstmann–Straussler–Scheinker syndrome	Human	Mutations in the *prnp* gene	[132]
Iatrogenic CJD	Human	Acquired from human sources (growth hormone, corneal grafts, others).	[133]
Kuru	Human	Cannibalism	[134]
Variant CJD	Human	Ingestion of BSE-contaminated food	[45]
Scrapie	Sheep and goat	Unknown	[135]
Transmissible mink encephalopathy	Mink	Probably ingestion of TSE-contaminated food	[136]
Chronic wasting disease	Cervids	Unknown	[47]
Bovine spongiform encephalopathy (BSE) or mad cow disease	Cattle	Ingestion of TSE-contaminated food	[137]
Feline spongiform encephalopathy	Cats	Ingestion of BSE-contaminated food	[138]

Although prion diseases are rare in humans, the established link between a new variant form of CJD (vCJD) and the consumption of cattle meat contaminated by BSE have raised concern about a possible outbreak of a large epidemic in the human population.[13–16] Over the past few years, BSE has become a significant health problem affecting many countries, and it seems now apparent that vCJD can be iatrogenically transmitted from human to human by blood transfusion.[17–19] Exacerbating this state of affairs is the lack of a reliable test to identify individuals incubating the disease during the long and silent period from the onset of infection to the appearance of clinical symptoms.[20,21]

At the molecular level, the main difference between vCJD and other human prion diseases such as sporadic CJD (sCJD) is the accumulation of PrP^{Sc} in the lymphoreticular system.[22] Furthermore, 100% of the vCJD patients are methionine homozygous at codon 129 in the PrP gene (*prnp*), while this genotype represents only 48%–52% of the Caucasian population.[23,24] This distribution is not shared with other human prion diseases. Although it has been recently demonstrated that the other M129V and V129V genotypes could be infected by vCJD, at this moment, homozygosis at codon 129 seems one of the most important risk factors associated to developing prion diseases by the host.[25]

10.1.1 How Is a Normal Protein Converted to a TSE Agent?

The prion protein is a glycoprotein of around 210 amino acids coded by one unique gene (*prnp*) that appears attached to the outer plasmatic membrane by a glycosylphosphatidylinositol (GPI) anchor.[2] The protein is expressed in most of the mammalian cells including CNS, lymphoreticular tissues, and muscle.[2,26] Although its function is still unknown, some studies suggest that it could be participating in copper uptake, protection against oxidative stress, cell adhesion, modulation of apoptotic process, and modulation of neuronal excitability, among others.[27–31]

In spite of PrP^C and PrP^{Sc} sharing the same amino acid sequence, their biochemical and physiological properties differ. While the secondary structure of PrP^C is composed mainly of α-helix, PrP^{Sc} is highly enriched in β-sheet structure, resulting in the formation of a protein core resistant to proteases digestion, chemical agents, radiation, high temperature, and non-denaturing detergents.[2]

Aggregates of PrP^{Sc} form fibrilar structures.[32] The kinetics follows a nucleation–polymerization model with presumably two stages. The first step is a nucleation phase, which is rate limiting to form stable polymerization nucleus or seeds composed of monomeric PrP (Figure 10.1). Then, a fast elongation phase occurs in which the PrP^C interacts with PrP^{Sc} changing its conformation to a β-sheet rich structure. In TSE diseases the rate limiting phase could be bypassed for the addition of seeds from the environment (as PrP^{Sc} is contained in food, surgical procedures, blood transfusions, etc.), increasing the kinetics of the PrP^{Sc} formation.[33]

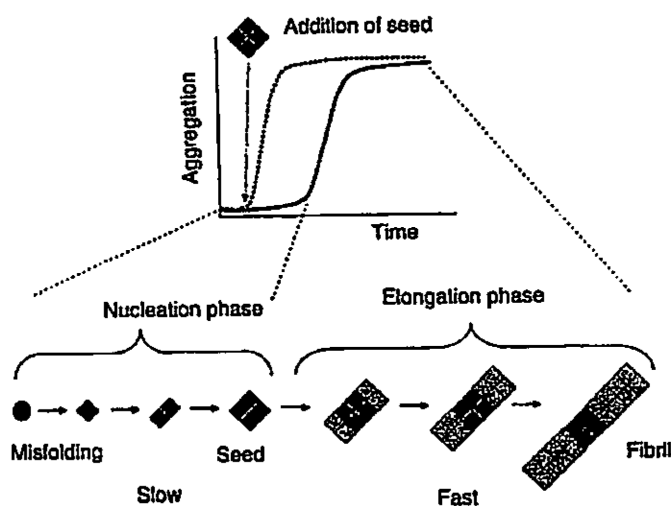


FIGURE 10.1
Nucleation-polymerization model for PrP^{Sc} formation. PrP^{Sc} fibrils formation follows a kinetics characteristic of polymerization-crystallization process. The first step is the limiting-rate reaction in which monomers of PrP^{C} are misfolded into a β-sheet rich structure and form a polymerization nucleus or seed (nucleation phase). This slow step can be accelerated for addition of preformed seed (dashed line). The second step is the elongation phase in which fibrils grow at the ends of preformed fibrils by recruitment of PrP^{C}.

10.1.2 From Food to Brain

How a protein maintains its infective properties across the digestive tract is astonishing. It is known experimentally that PrP^{Sc} is resistant to degradation by standard digestive enzymes. The result of its digestion is a protease-resistant fragment that remains infectious even at low pH characteristic of the stomach.[34] However, how prions can cross the intestinal epithelium is still unclear. Some evidence indicates that post-digested infectious fragments are complexed with ferritin (a protein highly present in meat) and is basolaterally translocated across epithelial cells.[35] Another plausible explanation would be that the infectious agent crosses the barrier through microfold (M) cells (specialized cells for transepithelial transport of macromolecules and particles) in an intact misfolded conformation.[36] These cells are part of Peyer's patches (lymphoid follicles localized in the epithelial layer of the small intestine). Then, the TSE agent is transported to the lymphoid tissue, where reside follicular dendritic cells (FDC) that maintain PrP^{Sc} and other proteins attached in the plasmatic membrane for long periods.[37] These properties of FDC and other immune cells are crucial for replication of the infectious agent.[38] Supporting evidence indicates that PrP^{C} binds to C1q (a complement protein) creating a complex capable of binding to the FDC membrane and other migratory immune cells as dendritic cells

or phagocytic cells such as macrophages. For this, C1q might participate in TSE agent uptake and spread along different and distant targets.[39]

Data from TSE-inoculated rodents indicate that the transfer from lymphoid tissue to CNS is through the enteric nervous system, both by sympathetic and parasympathetic nerves, i.e., splenic nerve and vagus nerve, respectively. This process is denominated neuroinvasion, where the TSE agent moves retrogradely until it reaches the brain, where it replicates and aggregates producing cellular damage and, as a consequence, typical clinical signs and death.[40]

10.1.3 Species Barrier Phenomenon

The species barrier phenomenon is defined as the difficulty associated with a specific prion to infect other species.[41] Although the molecular mechanisms involved in this intriguing phenomenon are not completely known, the conformational similarity between both host PrP^{C} and the TSE-causing agent (PrP^{Sc}) determines the strength of the barrier. Unfortunately, the conformation of PrP^{Sc} and PrP^{C} is not determined entirely by the PrP amino acid sequence. Thus, it has been described by many different PrP^{Sc} conformations, named prion strains, in the same host, and therefore with the same PrP amino acid sequence.[42] In other words, the same PrP^{C} could yield different PrP^{Sc} with many different conformations depending on which prion has been used to convert it. Interestingly, different prion strains might result in distinctive infective capabilities expressed by the incubation time of the TSE agent, brain lesion profile, and other biochemical parameters. The following examples could resume the importance of this phenomenon for public heath: (1) A natural scrapie agent from sheep does not infect humans but efficiently infects cattle, developing a bovine encephalopathy spongiform that is able to infect humans[43] and (2) while scrapie is a noninfectious disease for humans, the infectivity of the new ovine prion generated as a result of BSE inoculation in sheep remains still unknown. Recent experiments have revealed that both ovine prions show a non-distinguishable pathology in sheep, but they show differential biochemical characteristics.[44] Biological studies related to infectivity in humans are addressed. However, these studies will take several years; thus it is a priority to improve current prion detection tests in order to increase their sensitivity and also to differentiate between these two ovine prion strains, in order to avoid BSE-contamination food for human consumption.

10.1.4 Animal TSEs as Zoonoses

The most important outbreak of TSE for human public health arose in the late 1980s and early 1990s. The United Kingdom population was exposed to BSE-contaminated food and as a consequence more than 150 human deaths have been reported.[45]

Another TSE affecting animals for human consumption is currently in the spotlight. Chronic wasting disease (CWD), a prion disease that naturally affects deer (*Odocoileus* spp.) and Rocky Mountain elk (*Cervus elaphus nelsoni*) has been detected in the United States and Canada since late 1960s, both in free-ranging and captive animals.[46,47] In 2005, a first case of CWD in moose (*Alces alces*) was reported (unpublished data) and recently it has been published that infective prions can be found in preparations from semitendinosus/semimembranous muscles of CWD-affected mule deer[26], raising the question if venison-consuming people are at risk of developing some prion diseases, repeating the BSE episode in the United Kingdom. Nevertheless, there are no epidemiological links between CWD outbreak and vCJD or other sCJDs in United States. To address this question, several experimental approaches have been taken. Mice expressing human PrP inoculated with CWD-brain extract failed to develop prion diseases.[48] Furthermore, in vitro studies evidence a strong species barrier between cervid and human PrP.[49] However, two squirrel monkeys challenged intracerebrally with brain tissue from CWD-infected deer died due to prion disease after 31 and 34 months.[50] Unfortunately, the previous evidence data do not clarify if CWD-venison is an infectious source for humans, and more experiments should be done to clarify it.

One of the most intriguing features of prion diseases is the large incubation time, which depends on the prion strain and species, inoculation route, host, and dose of the TSE agent. This variability and the uncertain presence of prions in accessible fluids (blood, CSF, saliva, and urine) make an early clinical diagnosis of the disease difficult. In the present chapter, we review the latest advances in prion detection. These are principally focused on the improvement of high-throughput screening and sensitivity, trying to detect prions in preclinical phases and in more accessible tissues.

10.2 Current Methods in Prion Detection

10.2.1 Needs for an Ultra-Sensitive Method for Prion Detection

Currently no commercial assays available are accurate enough or highly sensitive for prion detection. However, five recent events in the prion field have increased the need for developing methods capable of detecting minute amounts of the prion infectious agent: (1) the diagnosis of three new cases of vCJD contracted by blood transfusion in the United Kingdom[18] and consequently the uncertainty about the real incidence of vCJD in the asymptomatic United Kingdom population, (2) the first case of BSE in sheep and its unknown ability to infect humans, (3) the detection of infectious prion agent in muscle from sheep[51] and cervid,[26,50] (4) the transmission of the CWD agent to nonhuman primates,[50] and (5) the detection of prions in other tissues and fluids, e.g., urine.[52] Although these new incidents are demanding more accurate and more sensitive detection methods, at present,

postmortem analysis must be performed in order to faithfully diagnose prion-related disorders. It is a priority in this field to improve the actual detection methods mainly to avoid TSE outbreak similar to the one in Europe. In other words, highly sensitive and pre-symptomatic detection methods are required to prevent TSE-contaminated food from entering the human food chain again.

The food industry has been seriously affected after the most recent and extensive outbreak of BSE in cattle, which occurred in Europe in the 1980s.[53] BSE has important implications for human health, and the consumption of BSE-contaminated meat or meat products has been linked to the advent of a new human TSE disease, the variant Creutzfeldt–Jakob disease.[13–16] The potential spread of BSE to other domestic animals such as sheep, cervids, and pigs has consequences for human health, and these populations of animals need to be monitored for signs of BSE infection. On the other hand, the high incidence of CWD in wild-ranging cervids in U.S. territory[54] and the actual data about the infectivity of these prions in primates are demanding the use of a more accurate detection method in edible samples.[55,56]

Both, strain and species barrier phenomena are complicating the investigations about prion infectivity in humans due to the singularity of its outstanding features as infectious proteins. These phenomena are responsible for what most of investigators in the field think: while scrapie is not infectious at all in the human being, a scrapie prion coming from the inoculation with the BSE agent may be infectious in spite of being pathologically indistinguishable from a natural case of scrapie. This concern is having a huge repercussion in the ovine food industry, as it happened in the past with cattle. Since the memory of prions after crossing species barriers is unpredictable we have to rule out infectivity in humans using animal models as bioassays. Hundreds of different prions have been tested for several years in animal models, principally in hamsters and mice. At present, other more sophisticated animal models have been generated to answer these questions. Unfortunately, there is not a way to predict how hazardous to humans a prion strain could be. However, as it will be described later, some detection techniques could be adapted in order to address this question.

Different strategies have been developed that focus on the improvement of sensitivity, specificity, and suitability for high throughput of current prion detection methods. They include (1) increasing the sensitivity by improving the affinity of the antibody to bind the PrP molecule, (2) increasing the concentration of the infectious material, (3) selective recognition of disease associated isoform of PrP, and (4) amplification of the infectious agent, among others. In the present chapter, we describe several detection methods for prions that are currently in use but also others, the most promising, which may probably be in use in a short-term future. Most of them will be used for the screening of "packaged" products, but also directly in presymptomatic livestock.

10.2.2 In Vitro Assays for Prion Detection

10.2.2.1 *Histopathological Study*

In spite of using an assay with a low sensitivity, histopathological studies are still an essential method for confirmatory studies and it was the first method used for prion diagnosis.[57] In fact, the name of TSE comes from the histopathological analysis of brain samples, where spongiform degeneration of the brain is observed. The current histopathological procedure does not differ very much from other histopathological studies used in other infectious diseases. The pathognomonic depositions of PrP^{Sc} (prion agent) in CNS and other lymphoreticular tissues and the spongiosis specific to CNS make this method highly precise. However, the limitations of this method are clear and the negative results obtained should be cautiously analyzed. For example, (1) samples obtained from carcasses after a long postmortem period are not useful for this assay, (2) it is frequent that positive cases are denoted negatives using this technique because not all brain areas show the same TSE characteristics, i.e., differential histopathological patterns of BSE and BASE (atypical BSE),[58] (3) other tissues different from CNS result in negatives most of the time depending on the prion strain evaluated, and (4) in asymptomatic phases the amount of prions and the lesions are undetectable. In addition, since this assay is time consuming and is not suitable for high-throughput studies, it is not useful in the food industry and is only appropriate for suspected livestock analysis.

10.2.2.2 *Western Blot-Based Tests*

10.2.2.2.1 *Prionics-Check Western Test*

This Western blot-based test takes advantage of the protease-resistant feature of PrP^{Sc}. The sample is treated by proteinase K digestion and is resolved in a SDS-PAGE gel. The PrP^{res} fragment (27–30 kDa for the diglycosylated band versus ~36 kDa of PrP^{C}) is detected using the 6H4 monoclonal antibody. The principal advantage of this technique is the possibility to see the pathognomonic sign unique to these diseases, i.e., the PrP^{res} signal, after PK digestion, making this method highly specific.[59] Meanwhile, this assay is less time consuming compared to other assays; it is also easy to perform and many samples can be analyzed in a relatively short period of time. The main disadvantage of this assay is its low sensitivity, an indispensable characteristic needed in the food industry.

10.2.2.3 *ELISA-Based Tests*

10.2.2.3.1 *Enfer Test*

This standard ELISA-based assay is one of the first methods for BSE prion detection approved by the European Union.[59] Although it was selected because of its sensitivity, which is higher than that of the Western blot, and the possibility to use it in a high throughput manner, the number of

false positives is still high. For this reason, the positive cases are usually confirmed using another Western blot-based assay.

10.2.2.3.2 CEA/BioRad Test

Although the false positives remain high in this assay, the principal advantage of this procedure is its sensitivity, much higher than other standard ELISA-based assays.[59] Thus, this DAS (double antibody sandwich) ELISA is capable of detecting brain samples to a dilution of up to $10^{-2.5}$, making it suitable for pre-diagnostic analysis. The immunoassay is based on the detection of the PK resistant core of PrP^{Sc} after denaturation and concentration processes, and is also used in high throughput procedures.

10.2.2.3.3 Prionics-Check LIA Test

This is one of the fastest assays for BSE prion detection.[60] However, this test does not show any other advantage in sensitivity or specificity when compared to Western blot assay or other ELISA-based assays. The method uses two monoclonal antibodies to bind the PrP^{Sc}. In the first step, the samples are incubated with one of the monoclonal antibodies and the mixture is transferred to another microtitre plate coated with the other monoclonal antibody. As in other ELISA-based tests, the high number of false positives is its main disadvantage.

10.2.2.3.4 Prionics PrioSTRIP System

This recent test based on the same principles as other STRIP-based immunoassays (a primary antibody is conjugated with blue latex beads that allow the recognition after the migration of the samples in the kit's strips) makes the BSE detection a very simple and friendly procedure. The principal advantage is its simplicity (more than 400 samples can be analyzed by one person in around 2 h), and high sensitivity and specificity close to 100%.[61] Unfortunately, its incomparable characteristics are restricted by the use of specific samples based on brain homogenates from obex.

10.2.2.4 BSE and Scrapie Discriminatory Test

As mentioned above, species barrier and prion strain phenomena could lead to the generation of many different infectious proteins. Each one could be a potential hazardous agent for humans. For this reason, the BSE transmission to sheep and the consequent generation of a new scrapie strain able to infect humans[62] is currently one of the most important concerns in the prion field. In addition, this concern is extended to the food industry since ovine and goat products are one of the most popular meat-products after cow, chicken, and pork.

Natural and experimental transmissions of BSE in sheep have revealed that the new sheep infected BSE (sBSE) strain presents several biochemical differences compared to natural or experimental cases of scrapie.[62,63] The most important feature is its *sui generis* electrophoretical motility in

SDS-PAGE gels compared with scrapie and BSE.[64] Thus, the electrophoretical motility of the unglycosylated form after PK digestion in scrapie is 18.1 kDa, whereas in sBSE it is 16.5 kDa.[64] Based on these differences, several discriminatory tests have been developed. The principal method is a standard Western blot using two very well-known antibodies: 6H4 and P4.[65] While the 6H4 monoclonal antibody is able to recognize PrP^{Sc} after PK digestion from both scrapie and sBSE cases, P4 is only able to detect PrP^{Sc} from scrapie but not sBSE.[63–65] This is possible due to the specific epitopes that each antibody is recognizing: the 6H4 epitope is located between the amino acids 148–156 of ovine PrP, while the P4 epitope is located in positions 94–99, critical in terms of PK digestion, and therefore, recognition.[64] Unfortunately, false positives could appear in the case of CH1641 sheep prion strain, due principally to its unique conformation compared to the rest of scrapie agents.[65]

The European Union guidelines command the use of primary molecular testing with a discriminatory immuno-blotting to all samples from clinical suspect cases. In case the presence of BSE cannot be excluded in ovine samples, the Community Reference Laboratory should carry out at least a second discriminatory immuno-blotting, a discriminatory immunocytochemistry, and a discriminatory ELISA. Finally, samples indicative for BSE by the three different methods and samples inconclusive should be further analyzed by a mouse bioassay for final confirmation.

10.2.2.5 Conformational Assays

10.2.2.5.1 Conformational-Dependent Immunoassay Test

The conformational-dependent immunoassay (CDI) is an original immunoassay that is based on the specific antibody binding to an epitope that is always available in PrP^C in standard conditions and becomes available in PrP^{Sc} only after denaturation.[66,67] The procedure requires splitting the sample into two aliquots that are treated or not with a denaturant agent. The rest of the procedure is standard for an ELISA-based technique. Although the first immunoassay based on these conformational-dependent properties was accepted in the United States for CWD prion detection, recently a new assay for BSE prion detection has been accepted by the European Union. Although the sensitivity of this assay is higher than other regular ELISAs, the general procedure is more complicated and requires more time than usual. Probably, the principal advantage of this method is its use in all kinds of atypical BSE and scrapie strains. Since the method is based on the conformational differences between PrP^C and PrP^{Sc}, we cannot rule out that noninfectious PrP^C, misfolded as a consequence of mutations in its amino acid sequence, causes false positives.

10.2.2.5.2 Multimer Detection System

The multimer detection system is a promising assay still under development and is based on the property of the misfolded proteins to generate

aggregates, as it happens with the PrP^{Sc} in prion diseases.[68] The method is based on a DAS-ELISA where two antibodies recognizing the same epitope are used. The fact that the antibodies used recognize the same epitope in any PrP yield a positive result when two or more PrP molecules are together, as in the case of aggregates of PrP^{Sc}. The proof of concept of this assay has been successfully established using recombinant protein, brain from infected hamsters, and blood from different sources. However, this procedure has an important disadvantage because other kinds of non-pathological aggregations are sources of false positives. On the other hand, the principle of this method could be applied to other protein aggregates as Aβ in Alzheimer disease or huntingtin in Huntington disease.

10.2.2.5.3 Misfolded Protein Diagnostic Assay

The misfolded protein diagnostic assay (MPD) technology developed by Adlyfe, and performed originally for prion blood detection, is based on the PrP misfolded isoform recognition by fluorescent peptides instead of standard antibodies.[69] When conformational changes occur in the fluorescent peptides after the binding to a misfolded PrP, slight differences in the fluorescence are detectable. This technique has been experimentally tested in blood from different animal models in preclinical and clinical cases from endemic and experimental prion diseases, including human, cattle, and sheep. However, more data about the specificity and sensitivity are needed.

10.2.2.5.4 Prionics Conformational Assay

In 1997, B. Oerch and coworkers developed the first antibody able to recognize specifically PrP^{Sc} but not PrP^{C}.[70] The principal advantage of this antibody, called 15B3, is the possibility to be used in immunoprecipitation and concentration procedures. Since it is specific for misfolded PrPs, it is also able to recognize mutant isoforms that have been described as non-pathological.[71] Currently, Prionics has developed a new assay based on this IgM monoclonal antibody that combines its specific characteristic with a regular ELISA test.[70] Although this system is still under development it would be necessary to know more details about its sensitivity and specificity, especially in other non-pathological forms of prion related diseases. However, this antibody is a great tool in prion studies where a pathological process is involved but PrP^{Sc} (as a protease resistant isoform) is not present.

10.2.2.5.5 Other Conformational Antibodies

Although still under development, other conformational antibodies have shown interesting results in the detection of specific prionopathies as the V5B2 monoclonal antibody, which is able to recognize specifically PrP^{Sc} in CJD.[72]

Another example also under development is the monoclonal antibody OCD4 that recognizes specifically DNA or DNA binding proteins, and is

able to capture PrP from brains affected by prion diseases in both humans and animals but not from unaffected controls.[73]

10.2.2.6 Spectroscopic Techniques: Multispectral Ultraviolet Fluoroscopy

Conceptually, multispectral ultraviolet fluoroscopy (MUFS) is a smart method that uses the conformational differences between PrP^C and PrP^{Sc} to detect them differentially by fluorescent emission after ultraviolet excitation.[74] The advantage of this assay is that no treatment to the sample is necessary and, in addition, it could allow distinguishing between diverse structures of PrP^{Sc} (prion strains). However, this technology shows a poor sensitivity and is completely impractical in current diagnosis systems.

10.2.2.6.1 *Confocal Dual-Color Fluorescence Correlation Spectroscopy*

In confocal dual-color fluorescence correlation spectroscopy (FCS), a sophisticated method, the sample is mixed with PrP specific fluorescent antibodies. Based on the assumption that the polymeric PrP^{Sc} shows more antibody binding sites versus the monomeric PrP^C, these slight differences of fluorescence after the antibody binding are detected using excitation by laser beams and a confocal microscope equipped with a single photon counter.[75] Although the technique is more sensitive than any Western blot or ELISA, it is not suitable for high-throughput approaches, being an excellent technique for dedicated laboratory studies.

10.2.2.6.2 *Fourier-Transformed Infrared Spectroscopy*

The Fourier-transformed infrared (FTIR) spectroscopy assay was used initially for the detection of PrP^{Sc} in blood samples.[76] The method is based on the differences in the infrared spectra shown by the different isoforms of PrP. The test shows very good and promising results in terms of sensitivity and specificity. However, its principal problem is the high experience required to interpret the result obtained. On the other hand, it seems to be difficult to perform as a large-scale diagnostic method.

10.2.2.7 Prion Concentration and Specific Ligands

10.2.2.7.1 *Sodium Phosphotungstic Acid*

After the discovery of sodium phosphotungstic acid (NaPTA) as a molecule able to precipitate PrP^{Sc} predominantly over PrP^C, many laboratories have included this technique as a standard procedure in prion detection.[66,67,77] However, although this procedure increases the sensitivity about 10 times when coupled to other techniques such as Western blot or ELISA, it is not a very practical technique for high-throughput screening. The method has been applied satisfactorily for prion detection in several tissues with very interesting results.[67,78,79] This technique can also be applied to big samples, including food, where the amount of prion can be undetectable using other

techniques. In general, this technique is a great alternative as pretreatment when an increase in sensitivity is required.

10.2.2.7.2 PrP^{Sc} Binding Peptides

It has been described how some peptides and proteins are able to bind specifically PrP^{Sc} but not PrP^{C}. Although plasminogen was one of the first examples published, likely due to its not total specificity for PrP^{Sc} binding, no tests have been developed at this moment using this approach.[80] However, further investigations have given way to the design of other synthetic peptides with similar properties.[81] The main advantage in the use of synthetic peptides is the possibility for modifications: fluorescent dye linked, enzyme activity linked, and coated by magnetic beads, among others. The biotechnology company Chiron is working in this direction, using a peptide that specifically binds PrP^{Sc} and is coated to magnetic beads.[82] This approach allows the concentration of PrP^{Sc}, which can then be used for further analysis using PrP specific antibodies. In unpublished results, the company has showed detection up to a 10^{-5} dilution of BSE infected brain homogenates, 1000-fold the standard limit for a regular Western blot or ELISA.[83]

This system is also suitable for high throughput, but more data are required about its specificity to be approved.

10.2.2.7.3 Seprion Ligand Platform

Polyanionic molecules are intriguing molecules able to bind PrP^{Sc} and disrupt the infectivity in cell cultures,[83] or even enhance amplification of infectious particles in vitro.[84] Microsense Biotechnologies has developed a system using these kinds of polyanionic polymers that are able to specifically capture rogue prion protein and avoid the need of proteinase K. This allows accelerating the detection process compared to conventional Western blot or ELISA. The analysis proposed by Microsense Biotechnologies takes around 4 h and is suitable for high throughput.[85] This system has been tested in spiked plasma using PrP^{CJD} from brain and spleen, and the detection level obtained was 330 infectious units (IU)/mL in brain spiked plasma and 10^2 IU/mL of spleen spiked plasma. Although the studies are not completed and more evidence are necessary for it to be approved as a regular assay, the preliminary results are very promising compared with other techniques on development.

10.2.2.7.4 Other Specific Ligands

A recent discovery has showed that the antibiotic streptomycin is able to bind specifically oligomeric PrP^{Sc}.[86] This property has been used in the study of 150 (52 negative controls and 98 CJD cases) CJD infected brain samples demonstrating 100% specificity and 100% sensitivity.[87] Since the streptomycin-PrP^{Sc} complex cannot be retained on an antibody coated microplate, it is necessary that its previous denaturalization be attached to

the microplate coupled to chemical ligands (calyx-arenes). The company Biomérieux has successfully developed this diagnostic assay in a high throughput manner.[87] Although this new approach is highly promising, more studies of specificity and sensitivity will have to be carried out using the final assay.

10.2.3 Amplification Assays for Prion Detection

In the methods described above for prion detection, different approaches based on the direct detection of PrP^{Sc} as the only validated surrogate marker for the disease have been shown. In all cases, the sensitivity and specificity were limited by the affinity of the ligand (antibody, peptide, polyanions, or other molecules) for the PrP^{Sc}. Most of them, in spite of presenting sufficient sensitivity for postmortem diagnosis are not sensitive enough for presymptomatic samples and, needless to say, to detect prions in other less infectious tissues that, unfortunately, are the most probable food contaminants. For this reason, other approaches are necessary to overcome the problem that most of the assays showed. In the same manner that PCR based on DNA amplification is the most used technique for infectious agents' detection, the amplification of prion proteins might be likely the unique way to increase the sensitivity enough to avoid TSE contaminated tissues from entering the human food chain. In this section, we review four different approaches based on in vitro or in vivo prion amplification and replication as methods for detection.

10.2.3.1 In Vitro Amplification

10.2.3.1.1 Cell-Free Conversion

Nucleation of the misfolded form of PrP and polymerization of the recruiting isoforms resulting from conversion are the responsible processes in the development of prion diseases. Misfolded protein nuclei formation is the key step of amyloid formation. As shown in Figure 10.1, the lag phase is usually long, but after nuclei formation, an exponential recruitment of misfolded protein occurs, triggering the disease. Cell-free conversion is based on this kind of kinetics, where PrP^{Sc} acts as a seed accelerating the misfolding conversion process.

As it happens in vivo, the cell-free conversion technique uses the PrP^C as the source for the generation of new PrP^{Sc}.[49,88,89] The PrP^C produced in the cells is radiolabeled using ^{35}S-methionine and separated by immunoprecipitation. The PrP^{Sc} acting as seed is purified by conventional methods and both highly purified components are mixed and incubated in the absence of any other component. To evaluate the amplification of the infectious material, radioactivity of the new PK resistant PrP is measured. To avoid detection of non-converted PrP, the observations are made using Western blot, where the shift in the electrophoretical motility between non-PK treated PrP^C and PK treated PrP^{Sc} is observed. Since the principle of this method is

based on the in vivo prion replication, the efficiency of the conversion occurring in vitro is very low and cannot be applied as a feasible detection method. In order to detect prion amplification, a dilution ratio of 50 parts of PrP^{Sc} versus one of PrP^{C} must be done. However, this in vitro procedure was the first technique able to mimic the prion replication and it has been an essential system for developing other more feasible approaches for prion diagnosis.

10.2.3.1.2 Protein Misfolding Cyclic Amplification

On the basis of the principle that it is better to imitate the nature than invent anything, the protein misfolding cyclic amplification (PMCA) technique was devised. In this technique, it is possible to simulate prion replication in the test tube in an accelerated mode.[90] PMCA is a cyclic process leading to accelerated prion replication.[90,91] Each cycle is composed of two steps (Figure 10.2A). During the first step, the sample, containing minute amounts

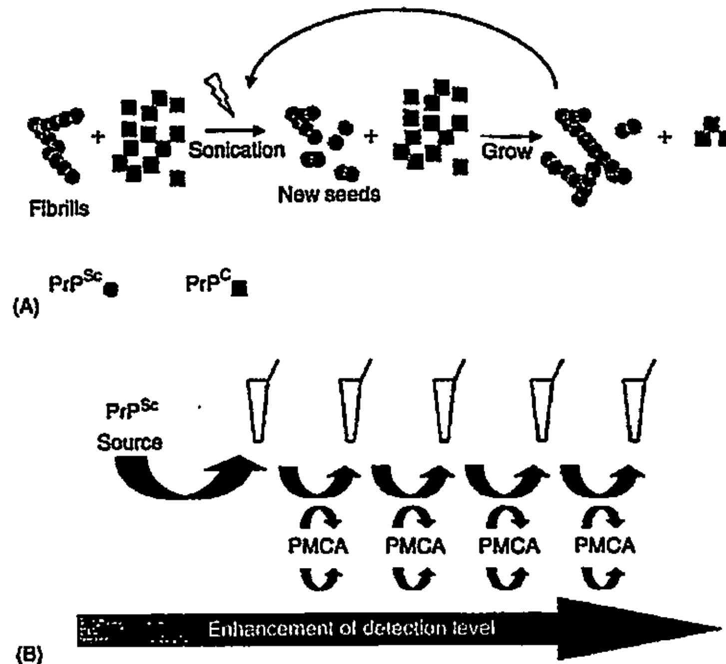


FIGURE 10.2
Protein misfolding cyclic amplification (PMCA) system. PMCA was developed to increase the total amount of PrP^{Sc} in a sample at the expense of PrP^{C}. (A) Samples are submitted to sonication in order to disrupt the PrP^{Sc} fibrils forming new seeds. Then the samples are submitted to incubation at 37°C allowing the conversion of PrP^{C} into PrP^{Sc}. Samples undergo multiple sonication–incubation cycles. (B) Serial passages of PMCA generated samples. PrP^{Sc}-containing material is diluted in normal brain homogenate as source of PrP^{C} and submitted to incubation–sonication cycles. Then the PMCA generated material is diluted in new aliquots of normal brain homogenate in order to "refresh" the source of PrP^{C}.

of PrP^{Sc} and a large excess of PrP^{C}, is incubated to induce formation of PrP^{Sc} aggregates. In the second step, the sample is sonicated in order to break down the aggregates, thus multiplying the number of growth sites for subsequent conversion. With each successive cycle, there is an exponential increase in the number of "seeds" and thus the conversion process is dramatically accelerated (Figure 10.2A).[90] The cyclic nature of the system permits the use of as many cycles as required to reach the amplification state needed for the detection of PrP^{Sc} in a given sample.

Reproduction of the process of prion conversion using PMCA represents a novel platform technology, which is likely to have a sustained impact in the field of prion biology. The ability to simulate this process in accelerated mode under controlled conditions in vitro provides an opportunity to examine many aspects of prion biology, which hitherto have been inaccessible to experimentation, i.e., (a) the molecular mechanism of species barrier and prion strains phenomena, (b) investigation of factors involved in the PrP^{C} to PrP^{Sc} conversion, (c) screening for inhibitors of prion propagation, and (d) diagnosis.

One of the most important applications of PMCA is in TSE diagnosis. As stated before, the biggest problem that biochemical tests have to overcome to detect PrP^{Sc} pre-symptomatically in tissues other than brain is the very low amount of PrP^{Sc} existing in them. PMCA offers the opportunity to enhance existing methods by amplifying the amount of PrP^{Sc} in the sample. The aim would be not only to detect prions in the brain in early presymptomatic cases, but also to generate a test to diagnose living animals and humans. For this purpose, a tissue other than brain is required and, in order to have an easier noninvasive method, detection of prions in body fluids such as urine or blood are the best options.

PMCA in its original mode was done by manual operation,[90] but recently an automated mode (aPMCA) has been developed in order to increase sensitivity, specificity, and throughput.[92] Automated PMCA overcomes one of the major drawbacks of manual PMCA, namely cross-contamination, since there is no direct contact between the sonicator probe and the sample. In addition, the sensitivity has been further increased by the introduction of a new concept involving serial rounds of amplification. This procedure is named serial automated PMCA (saPMCA) and is similar to the application of multiple rounds of PCR amplification to reach high sensitivity detection of DNA. Serial automated PMCA consists of successive rounds of aPMCA in which at each round the amplified sample is diluted into fresh substrate, as shown in Figure 10.2B. This approach is highly recommended when elevated levels of amplification are required, especially when working with samples containing minute initial amounts of PrP^{Sc}, such as blood, CSF, muscle, or peripheral non-lymphoid tissues. The rounds of saPMCA can be repeated as many times as needed to reach the detection threshold of Western blotting. Samples remaining negative after 10 rounds of saPMCA can be considered negative, because according to our experience, around 6–8 rounds of saPMCA can amplify the minimum amount of

material required for amplification (approximately 100 molecules of PrP^{Sc} monomers) (unpublished data).

Since the replication of prions from different species is based on the same phenomenon, this technique is applicable to all of them independent of the prion strain or species trying to be detected. This advantage permits using the same procedure for any TSE-contaminated sample. Another great advantage of this amplification-based technique is the possibility to couple it to other high-sensitive biochemical detection techniques. Combining the strategy of reproducing prions in vitro with any of the high-sensitive detection methods, the early diagnosis of TSE may be achieved.

10.2.3.2 *In Vivo Amplification*

10.2.3.2.1 *Cell Infectivity Assay*

Like in a prion infectious process in animals, culture cells have been used trying to mimic the in vivo prion replication phenomenon. Although, this assay may be used theoretically as a prion detection method, there are a lot of pitfalls that make the system not suitable for the detection of the most important prions. The principal problem is that the culture cells selectively replicate prions from just a few species.[93,94] This specificity can even be extended to just a few prion strains from the same species.[95] Thus, most of the neural cells used for mouse prion replication are able to replicate only RML or RML-like prion strains but not others.[96] Unfortunately, the in vitro replication using cells has only been described for some mouse prion strains, CWD (from mule deer),[97] and some scrapie prion strains from sheep;[98] but it is impossible at this moment to mimic the same phenomenon with BSE from cattle or other human prions, making this cell-based system inadequate to be used for practical prion detection. Why a good number of the cells used for this purpose are resistant to the infection with most prions remains still unknown. In fact, the susceptible cells have been usually generated after a dedicated clonation process, since most of the cells from a standard culture are resistant for prion replication. In spite of all the problems related to this system, the sensitivity obtained using these cells replicating some mouse prions has been as high as that of the most sensitive bioassay, being possible to detect dilution up to 1000 infectious units. In this case, the great advantage of this system in comparison to other bioassays is the possibility for robotization. In other words, if in the future the intriguing phenomenon of why just certain prions are able to be replicated in cell cultures is solved, and if the system works with the rest of the prions as CJD, BSE, and scrapie, among others, this method could be converted to one of the most interesting techniques for prion detection. And at that moment it might also be useful not only for research but also for routinary food screening.

10.2.3.2.2 *Bioassays*

Bioassay is defined as any assay that uses animals to detect an infectious agent. In the 1960s, although still unknown that the agent causing TSEs was

an infectious protein, the natural bioassays were crucial to study the transmissible character of the disease. Thus, samples from scrapie infected sheep were inoculated in different rodents (mouse, rat, hamster, guinea pig, gerbil, and bank vole) confirming its transmissibility.[99] In spite of the low efficiency of these first in vivo assays due to the species barrier between the ovine PrP^{Sc} and the PrP^{C} from rodent, they were essential tools for developing investigations in the prion field. Years later, other manageable animals (minks, raccoons, marmosets, and other primates) were used as bioassays in the detection of CWD, TME, Kuru, and CJD prions.[100–111] However, we had to wait until the British BSE-outbreak in order to see a tremendous progress in this field. The undutiful relationship between BSE in cattle and vCJD in human explained the concept of species barrier and triggered the developing of new bioassays based on transgenic (Tg) animals. As a consequence, in the last 15 years PrP Tg mice overexpressing PrP^{C} from different species (human,[112–115] bovine,[115–118] ovine,[119] porcine,[120] cervid,[121] and murine,[122] etc.) have been generated. The development of Tg mouse models carrying the PrP gene of species naturally affected by the TSE agents has provided the best way to overcome the difficulties in transmitting TSEs from their natural host species to laboratory animals. The incubation time in the mouse bioassay for a BSE isolate varies between 250 and 550 days depending on the mouse genetic background.[115–118] However, the bioassay based on transgenic mice expressing bovine PrP reduces the time to less than 200 days.[117]

Bioassays are playing an important role in the food industry, especially in the detection of prions from edible samples that give negative results using conventional immunological techniques. Hence, Tg mice expressing bovine PrP^{C} were challenged with various tissues from cattle with end-stage clinical BSE, and infectivity was found only in the central and peripheral nervous system and not in lymphatic tissues; the only exception was the Peyer's patches of the distal ileum, which most likely are the site of entry for BSE infectivity.[123] Another recent study based on transgenic mice expressing cervid PrP^{C} revealed the presence of infectious prions in skeletal muscles of CWD-infected deer, demonstrating that humans consuming or handling meat from CWD-infected deer are at risk of prion exposure.[26]

Other studies difficult to address in farm animals have been realized using more practical bioassays. Thus, the mother-to-offspring transmissibility study was done using transgenic mice expressing bovine PrP experimentally infected by intracerebral administration of BSE prions. In this assay, PrP^{Sc} was detected in brains of newborns from infected mothers only when mating had been allowed near to the clinical stage of the disease, when brain PrP^{Sc} deposition could be detected by Western blot analysis.[124] However, attempts to detect infectivity in milk after intracerebral inoculation in these Tg mice were unsuccessful, suggesting the involvement of other tissues as carriers of prion dissemination.

In the same manner that cattle were infected by TSE contaminated food, other species, principally captive animals in zoos, were also infected with

the same source of contamination. However, natural cases of TSE in swine have not been reported in spite of this species also being exposed to the same TSE contaminated food. Castilla et al. examined the barrier from cows to pigs using a new bioassay based on transgenic mice expressing the porcine prion protein gene (poTg).[120] Intracerebral inoculation with a high-titer BSE inoculum, but not low-titer BSE inoculum, caused clinical signs of BSE, consistent with a strong species-barrier. However, second passage with brain homogenates from low-titer inoculated mice produced detectable prion protein in new poTg mice. These results suggest that the barrier is not absolute and that pigs are also susceptible to develop a TSE with unpredictable infectivity for humans.

Although the transgenic mice described above are considered the best bioassays for TSE detection, recently, a new bioassay based on a wild rodent, the bank vole (*Clethrionomys glareolus*), is showing promising results in the detection of natural scrapie (unpublished results). The results obtained in the primary transmission with some strains of scrapie indicate that the bank vole is a species much more susceptible to the scrapie agent than wild-type mice and even more susceptible than bioassays based on ovine PrP Tg mice.

At this moment, it is a priority to develop efficient bioassays that help in the risk evaluation and control of scrapie and BSE in sheep. This will provide the European agro industry with an opportunity to discard products that might contain TSE-causing agents and would guarantee the production of safer sheep and beef products.

10.3 Concluding Remarks

In the present review we have summarized the principal advances in prion detection based on methods currently in use in diagnostic laboratories and also others that should be considered, since they are showing interesting characteristics or are promising techniques under development. In Table 10.2 are represented the most reliable techniques for prion detection ordered by sensitivity. With the exception of the PMCA method, still not commercially available, no method is accurate enough to ensure that prion contaminated edible samples are not entering the human food-chain daily. However, many other aspects should be taken into account in order to evaluate the true risk for humans. Prions are unconventional infectious agents with extraordinary physicochemical features that confer on them the property to resist the most common food treatments in use. Even though the amount of prions in the current edible tissues is very low, it is able to experimentally infect the animals used in some of the available bioassays via intracerebral inoculation. Still, most of the samples able to infect by intracerebral route would not be infectious by oral ingestion. But we cannot underestimate the accumulation effect until conclusive experiments have

TABLE 10.2
Comparison of the Sensitivity of Several Methods to Detect PrP^{Sc}

Assay	Maximum Dilution Detected[a]	Minimum PrP Quantity Detected[b]	Increase in Sensitivity[c]
Standard Western blot	3.0×10^3	4.0 ng	1
ELISA	2.5×10^4	0.5 ng	8
Phosphotungstic acid precipitation	1.5×10^5	80 pg	50
Conformation-dependent immunoassay[d]	2.0×10^5	150 pg	27
Animal assay	1.0×10^5	12 fg	330,000
Single PMCA[e]	7.0×10^5	1.6 pg	2,500
Double PMCA[e]	2.0×10^{10}	0.6 fg	6,500,000
Seven rounds PMCA[e]	1.0×10^{12}	1.3 ag	3,300,000,000

[a] The maximum dilution detected corresponds to the last dilution of 263 K scrapie brain in which PrP^{Sc} is detectable.
[b] The minimum quantity of PrP^{Sc} detected in a brain sample volume of 20 μL.
[c] The increase of sensitivity is expressed in relation to the standard western blot assay using 3F4 antibody.
[d] The data for the conformation-dependent immunoassay was taken from the literature, whereas all the others were experimentally calculated.
[e] The data for single and double PMCA correspond to the average obtained in three different experiments using 100 cycles in both first and second PMCA.

been done. Thus, it would be necessary to perform experiments that mimic more accurately an actual real scenario, especially now when we dispose off the bioassays and other techniques necessary to accomplish them. For example, it would be necessary to study in primate or human PrP Tg mice if a repetitive oral dose of BSE-contaminated muscle or other common edible BSE-contaminated tissues from cattle or sheep are able to infect. In other words, even if we can guarantee that prion from different species are entering the human food-chain we cannot assure that they should be considered a risk for humans.

On the other hand, the detection of prions in blood, as one of the most accessible tissue, in a pre-symptomatic phase of the disease would be the goal that all the assays have to reach. At this moment, the PMCA is the only technique that has shown positive results at that level,[125] but only in experimental models. The extrapolation of these results to cattle, sheep, cervid, and human might be one of the most important achievements in the prion field. Fortunately, other promising techniques under development are close to having enough sensitivity for pre-symptomatic studies in accessible fluids. The combination of amplification-based techniques as PMCA or cell-infectivity assays and other detection methods will be likely the most appropriate system in the future of prion detection.

In the meantime, the prion detection methods are being improved; the food industry should apply the established guidelines in order to diminish the prion contamination in the edible tissues. These guidelines have been

established according to the results obtained using the current bioassays and they determine the industrial procedures for food control. Thus, the guiding principles determine which animals should be analyzed in the abattoir and which products should be excluded from human consumption. Recently, three discoveries have substantially changed these guidelines: (1) the first natural case of BSE in goat,[126] (2) the detection of prion in muscle of CWD-infected cervid[26], and (3) the ectopic accumulation of PrP^{Sc} in organs subjected to an inflammation process.[127] The emergent scrapie strain in caprine that could be infectious for humans, the existence of the infectious agent in the most edible tissue (muscle) and the ectopic accumulation of prions in excretory organs as kidney and mammary gland that could unleash their presence in milk and urine are the new dares that the prion-detection methods should confront.

Acknowledgments

This work is partially supported by NIH grants AG0224642 and NS049173. We thank Arancha Desojo for helpful discussion of this manuscript.

References

1. Prusiner, S.B., Molecular biology of prion diseases, *Science*, 252, 1515–1522, 1991.
2. Prusiner, S.B., Prions, *Proc. Natl. Acad. Sci. U. S. A.*, 95, 13363–13383, 1998.
3. Cohen, F.E. and Prusiner, S.B., Pathologic conformations of prion proteins, *Annu. Rev. Biochem.*, 67, 793–819, 1998.
4. McKinley, M.P., Bolton, D.C., and Prusiner, S.B., A protease-resistant protein is a structural component of the scrapie prion, *Cell*, 35, 57–62, 1983.
5. Prusiner, S.B., Novel proteinaceous infectious particles cause scrapie, *Science*, 216, 136–144, 1982.
6. Beck, E., Daniel, P.M., Matthews, W.B., et al., Creutzfeldt–Jakob disease. The neuropathology of a transmission experiment, *Brain*, 92, 699–716, 1969.
7. Bendheim, P.E., The human spongiform encephalopathies, *Neurol. Clin.*, 2, 281298, 1984.
8. Collinge, J., Harding, A.E., Owen, F., et al., Diagnosis of Gerstmann-Straussler syndrome in familial dementia with prion protein gene analysis, *Lancet*, 2, 15–17, 1989.
9. Goldfarb, L.G., Petersen, R.B., Tabaton, M., et al., Fatal familial insomnia and familial Creutzfeldt–Jakob disease: Disease phenotype determined by a DNA polymorphism, *Science*, 258, 806–808, 1992.
10. Manetto, V., Medori, R., Cortelli, P., et al., Fatal familial insomnia: Clinical and pathologic study of five new cases, *Neurology*, 42, 312–319, 1992.
11. Medori, R., Tritschler, H.J., LeBlanc, A., et al., Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene, *N. Engl. J. Med.*, 326, 444–449, 1992.

12. Dormont, D., Unconventional transmissible agents or prions, *Rev. Prat.*, 44, 882–887, 1994.
13. Collinge, J., Sidle, K.C., Meads, J., et al., Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD, *Nature*, 383, 685–690, 1996.
14. Collinge, J. and Rossor, M., A new variant of prion disease, *Lancet*, 347, 916–917, 1996.
15. Will, R. and Zeidler, M., Diagnosing Creutzfeldt–Jakob disease, *BMJ*, 313, 833–834, 1996.
16. Scott, M.R., Will, R., Ironside, J., et al., Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans, *Proc. Natl. Acad. Sci. U. S. A.*, 96, 15137–15142, 1999.
17. Bradley, R. and Liberski, P.P., Bovine spongiform encephalopathy (BSE): The end of the beginning or the beginning of the end?, *Folia Neuropathol.*, 42 Suppl A, 55–68, 2004.
18. Llewelyn, C.A., Hewitt, P.E., Knight, R.S., et al., Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion, *Lancet*, 363, 417–421, 2004.
19. Peden, A.H., Head, M.W., Ritchie, D.L., et al., Preclinical vCJD after blood transfusion in a *PRNP* codon 129 heterozygous patient, *Lancet*, 264, 527–529, 2004.
20. Schiermeier, Q., Testing times for BSE, *Nature*, 409, 658–659, 2001.
21. Soto, C., Diagnosing prion diseases: Needs, challenges and hopes, *Nat. Rev. Microbiol.*, 2, 809–819, 2004.
22. Ironside, J.W., McCardle, L., Horsburgh, A., et al., Pathological diagnosis of variant Creutzfeldt–Jakob disease, *APMIS*, 110, 79–87, 2002.
23. Collinge, J., Beck, J., Campbell, T., et al., Prion protein gene analysis in new variant cases of Creutzfeldt–Jakob disease, *Lancet*, 348, 56, 1996.
24. Mitrova, E., Mayer, V., Jovankovicova, V., et al., Creutzfeldt–Jakob disease risk and PRNP codon 129 polymorphism: Necessity to revalue current data, *Eur. J. Neurol.*, 12, 998–1001, 2005.
25. Ironside, J.W., Variant Creutzfeldt–Jakob disease: Risk of transmission by blood transfusion and blood therapies, *Haemophilia.*, 12 Suppl 1, 8–15, 2006.
26. Angers, R.C., Browning, S.R., Seward, T.S., et al., Prions in skeletal muscles of deer with chronic wasting disease, *Science*, 317, 1117, 2006.
27. Lasmezas, C.I., Putative functions of PrP(C), *Br. Med. Bull.*, 66, 61–70, 2003.
28. Klamt, F., Dal Pizzol, F., Conte da Frota, M.L., Jr, et al., Imbalance of antioxidant defense in mice lacking cellular prion protein, *Free Radic. Biol. Med.*, 30, 1137–1144, 2001.
29. Graner, E., Mercadante, A.F., Zanata, S.M., et al., Cellular prion protein binds laminin and mediates neuritogenesis, *Brain Res. Mol. Brain Res.*, 76, 85–92, 2000.
30. Chiarini, L.B., Freitas, A.R., Zanata, S.M., et al., Cellular prion protein transduces neuroprotective signals, *EMBO J.*, 21, 3317–3326, 2002.
31. Mallucci, G.R., Ratte, S., Asante, E.A., et al., Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration, *EMBO J.*, 21, 202–210, 2002.
32. Jones, E.M. and Surewicz, W.K., Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids, *Cell*, 121, 6372, 2005.
33. Caughey, B., Prion protein conversions: Insight into mechanisms, TSE transmission barriers and strains, *Br. Med. Bull.*, 66, 109–120, 2003.

34. Martinsen, T.C., Taylor, D.M., Johnsen, R., et al., Gastric acidity protects mice against prion infection?, *Scand. J. Gastroenterol.*, 37, 497–500, 2002.
35. Mishra, R.S., Basu, S., Gu, Y., et al., Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells: Implications for species barrier in prion uptake from the intestine, *J. Neurosci.*, 24, 11280–11290, 2004.
36. Heppner, F.L., Christ, A.D., Klein, M.A., et al., Transepithelial prion transport by M cells, *Nat. Med.*, 7, 976–977, 2001.
37. Yoshida, K., van den Berg, T.K., and Dijkstra, C.D., Two functionally different follicular dendritic cells in secondary lymphoid follicles of mouse spleen, as revealed by CR1/2 and FcR gamma II-mediated immune-complex trapping, *Immunology*, 80, 34–39, 1993.
38. Oldstone, M.B., Race, R., Thomas, D., et al., Lymphotoxin-alpha- and lymphotoxin-beta-deficient mice differ in susceptibility to scrapie: Evidence against dendritic cell involvement in neuroinvasion, *J. Virol.*, 76, 4357–4363, 2002.
39. Blanquet-Grossard, F., Thielens, N.M., Vendrely, C., et al., Complement protein C1q recognizes a conformationally modified form of the prion protein, *Biochemistry*, 44, 4349–4356, 2005.
40. Mabbott, N.A. and MacPherson, G.G., Prions and their lethal journey to the brain, *Nat. Rev. Microbiol.*, 4, 201–211, 2006.
41. Moore, R.A., Vorberg, I., and Priola, S.A., Species barriers in prion diseases—Brief review, *Arch. Virol. Suppl*, 187–202, 2005.
42. Chen, S.G. and Gambetti, P., A journey through the species barrier, *Neuron*, 34, 854–856, 2002.
43. Houston, E.F. and Gravenor, M.B., Clinical signs in sheep experimentally infected with scrapie and BSE, *Vet. Rec.*, 152, 333–334, 2003.
44. Baron, T.G., Madec, J.Y., Calavas, D., et al., Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep, *Neurosci. Lett.*, 284, 175–178, 2000.
45. Hilton, D.A., Pathogenesis and prevalence of variant Creutzfeldt–Jakob disease, *J. Pathol.*, 208, 134–141, 2006.
46. Williams, E.S., Chronic wasting disease, *Vet. Pathol.*, 42, 530–549, 2005.
47. Belay, E.D., Maddox, R.A., Williams, E.S., et al., Chronic wasting disease and potential transmission to humans, *Emerg. Infect. Dis.*, 10, 977–984, 2004.
48. Kong, Q., Huang, S., Zou, W., et al., Chronic wasting disease of elk: Transmissibility to humans examined by transgenic mouse models, *J. Neurosci.*, 25, 7944–7949, 2005.
49. Raymond, G.J., Bossers, A., Raymond, L.D., et al., Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease, *EMBO J.*, 19, 4425–4430, 2000.
50. Marsh, R.F., Kincaid, A.E., Bessen, R.A., et al., Interspecies transmission of chronic wasting disease prions to squirrel monkeys (*Saimiri sciureus*), *J. Virol.*, 79, 13794–13796, 2005.
51. Andreoletti, O., Simon, S., Lacroux, C., et al., PrPSc accumulation in myocytes from sheep incubating natural scrapie, *Nat. Med.*, 10, 591–593, 2004.
52. Seeger, H., Heikenwalder, M., Zeller, N., et al., Coincident scrapie infection and nephritis lead to urinary prion excretion, *Science*, 310, 324–326, 2005.
53. Ammendrup, S., Summary review of the chronology of the major measures in relation to bovine spongiform encephalopathy (BSE) in the United Kingdom and the European Union, *Acta Vet. Scand. Suppl.*, 91, 19–20, 1999.

54. Miller, M.W. and Conner, M.M., Epidemiology of chronic wasting disease in free-ranging mule deer: Spatial, temporal, and demographic influences on observed prevalence patterns, *J. Wildl. Dis.*, 41, 275–290, 2005.
55. Belay, E.D., Gambetti, P., Schonberger, L.B., et al., Creutzfeldt–Jakob disease in unusually young patients who consumed venison, *Arch. Neurol.*, 58, 1673–1678, 2001.
56. Xie, Z., O'Rourke, K.I., Dong, Z., et al., Chronic wasting disease of elk and deer and Creutzfeldt–Jakob disease: Comparative analysis of the scrapie prion protein, *J. Biol. Chem.*, 2005.
57. Beck, E., Daniel, P.M., and Parry, H.B., Degeneration of the cerebellar and hypothalamoneurohypophysial systems in sheep with scrapie; and its relationship to human system degenerations, *Brain*, 87, 153–176, 1964.
58. Casalone, C., Zanusso, G., Acutis, P., et al., Identification of a second bovine amyloidotic spongiform encephalopathy: Molecular similarities with sporadic Creutzfeldt–Jakob disease, *Proc. Natl. Acad. Sci. U. S. A.*, 101, 3065–3070, 2004.
59. Moynagh, J. and Schimmel, H., Tests for BSE evaluated. Bovine spongiform encephalopathy, *Nature*, 400, 105, 1999.
60. Biffiger, K., Zwald, D., Kaufmann, L., et al., Validation of a luminescence immunoassay for the detection of PrP(Sc) in brain homogenate, *J. Virol. Methods*, 101, 79–84, 2002.
61. http://www.prionics.ch/prionics-e-diagnostics-priostrip-applicationlit.pdf
62. Foster, J.D., Hope, J., and Fraser, H., Transmission of bovine spongiform encephalopathy to sheep and goats, *Vet. Rec.*, 133, 339–341, 1993.
63. Stack, M.J., Balachandran, A., Chaplin, M., et al., The first Canadian indigenous case of bovine spongiform encephalopathy (BSE) has molecular characteristics for prion protein that are similar to those of BSE in the United Kingdom but differ from those of chronic wasting disease in captive elk and deer, *Can. Vet. J.*, 45, 825–830, 2004.
64. Thuring, C.M., Erkens, J.H., Jacobs, J.G., et al., Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein, *J. Clin. Microbiol.*, 42, 972–980, 2004.
65. Stack, M.J., Chaplin, M.J., and Clark, J., Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies, *Acta Neuropathol. (Berl)*, 104, 279–286, 2002.
66. Safar, J., Wille, H., Itri, V., et al., Eight prion strains have PrP(Sc) molecules with different conformations, *Nat. Med.*, 4, 1157–1165, 1998.
67. Safar, J.G., Scott, M., Monaghan, J., et al., Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice, *Nat. Biotechnol.*, 20, 1147–1150, 2002.
68. Seong, An., Detection of PrP^{Sc} in blood using newly developed multimer detection system, Presented in the *"10th International Transmissible Spongiform Encephalopathies meeting."* March 7–9 2006, Baltimore, MD, 2006.
69. Orser, C., The Misfolded Protein Diagnostic (MPD) Blood Assay. In *"10th Transmissible Spongiform Encephalopathies Meeting."* March 7–9, 2006. Baltimore, MD, 2006.
70. Korth, C., Stierli, B., Streit, P., et al., Prion (PrPSc)-specific epitope defined by a monoclonal antibody, *Nature*, 390, 74–77, 1997.

71. Nazor, K.E., Kuhn, F., Seward, T., et al., Immunodetection of disease-associated mutant PrP, which accelerates disease in GSS transgenic mice, *EMBO J.*, 24, 2472–2480, 2005.
72. Šerbec, V.Č., V, Bresjanac, M., Popović, M., Hartman, K.P., Galvani, V., Rupreht, R., Černilec, M., Vranac, T., Hafner, I., and Jerala, R., Monoclonal antibody against a peptide of human prion protein discriminates between Creutzfeldt-Jacob's disease-affected and normal brain tissue, *J. Biol. Chem.*, 279, 3694–3698, 2004.
73. Zou, W.Q., Zheng, J., Gray, D.M., et al., Antibody to DNA detects scrapie but not normal prion protein, *Proc. Natl. Acad. Sci. U. S. A.*, 101, 1380–1385, 2004.
74. Rubenstein, R., Gray, P.C., Wehlburg, C.M., et al., Detection and discrimination of PrPSc by multi-spectral ultraviolet fluorescence, *Biochem. Biophys. Res. Commun.*, 246, 100–106, 1998.
75. Bieschke, J., Giese, A., Schulz-Schaeffer, W., et al., Ultrasensitive detection of pathological prion protein aggregates by dual-color scanning for intensely fluorescent targets, *Proc. Natl. Acad. Sci. U. S. A.*, 97, 5468–5473, 2000.
76. Lasch, P., Schmitt, J., Beekes, M., et al., Antemortem identification of bovine spongiform encephalopathy from serum using infrared spectroscopy, *Anal. Chem.*, 75, 6673–6678, 2003.
77. Wadsworth, J.D., Joiner, S., Hill, A.F., et al., Tissue distribution of protease resistant prion protein in variant Creutzfeldt–Jakob disease using a highly sensitive immunoblotting assay, *Lancet*, 358, 171–180, 2001.
78. Collins, S.J., Lewis, V., Brazier, M.W., et al., Extended period of asymptomatic prion disease after low dose inoculation: Assessment of detection methods and implications for infection control, *Neurobiol. Dis.*, 20, 336–346, 2005.
79. Birkmann, E., Schafer, O., Weinmann, N., et al., Detection of prion particles in samples of BSE and scrapie by fluorescence correlation spectroscopy without proteinase K digestion, *Biol. Chem.*, 387, 95–102, 2006.
80. Fischer, M.B., Roeckl, C., Parizek, P., et al., Binding of disease-associated prion protein to plasminogen, *Nature*, 408, 479–483, 2000.
81. Rhie, A., Kirby, L., Sayer, N., et al., Characterization of 2'-fluoro-RNA aptamers that bind preferentially to disease-associated conformations of prion protein and inhibit conversion, *J. Biol. Chem.*, 278, 39697–39705, 2003.
82. Peretz, D., Towards development of a prion blood test, In "*10th Annual Transmissible Spongiform Encephalopathies Meeting*". March 7–9, 2006. Baltimore, MD.
83. Caughey, B. and Raymond, G.J., Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells, *J. Virol.*, 67, 643–650, 1993.
84. Deleault, N.R., Geoghegan, J.C., Nishina, K., et al., Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions, *J. Biol. Chem.*, 280, 26873–26879, 2005.
85. Wilson, S., An animal model evaluation and assay protocol for screening blood for vCJD, In "*10th Annual Transmissible Spongiform Encephalopathies Meeting*." March 7–9, 2006. Baltimore, MD, USA, 2006.
86. Moussa, A., Coleman, A.W., Bencsik, A., et al., Use of streptomycin for precipitation and detection of proteinase K resistant prion protein (PrPsc) in biological samples, *Chem. Commun. (Camb.)*, 973–975, 2006.
87. Perron, H., Immunoassay for the detection of resistant prion protein (prpres) in human plasma using an original combination of chemical ligands, In "*10th Annual Transmissible Spongiform Encephalopathies Meeting*". March 7–9, 2006. Baltimore, MD, U. S. A., 2006.

88. Kocisko, D.A., Come, J.H., Priola, S.A., et al., Cell-free formation of protease-resistant prion protein, *Nature*, 370, 471–474, 1994.
89. Bessen, R.A., Kocisko, D.A., Raymond, G.J., et al., Non-genetic propagation of strain-specific properties of scrapie prion protein, *Nature*, 375, 698–700, 1995.
90. Saborio, G.P., Permanne, B., and Soto, C., Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding, *Nature*, 411, 810–813, 2001.
91. Soto, C., Saborio, G.P., and Anderes, L., Cyclic amplification of protein misfolding: Application to prion-related disorders and beyond, *Trends Neurosci.*, 25, 390–394, 2002.
92. Castilla, J., Saá, P., Hetz, C., et al., In vitro generation of infectious scrapie prions, *Cell*, 121, 195–206, 2005.
93. Enari, M., Flechsig, E., and Weissmann, C., Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody, *Proc. Natl. Acad. Sci. U. S. A.*, 98, 9295–9299, 2001.
94. Bosque, P.J. and Prusiner, S.B., Cultured cell sublines highly susceptible to prion infection, *J. Virol.*, 74, 4377–4386, 2000.
95. Arima, K., Nishida, N., Sakaguchi, S., et al., Biological and biochemical characteristics of prion strains conserved in persistently infected cell cultures, *J. Virol.*, 79, 7104–7112, 2005.
96. Klohn, P.C., Stoltze, L., Flechsig, E., et al., A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions, *Proc. Natl. Acad. Sci. U. S. A.*, 100, 11666–11671, 2003.
97. Raymond, G.J., Olsen, E.A., Lee, K.S., et al., Inhibition of protease-resistant prion protein formation in a transformed deer cell line infected with chronic wasting disease, *J. Virol.*, 80, 596–604, 2006.
98. Archer, F., Bachelin, C., Andreoletti, O., et al., Cultured peripheral neuroglial cells are highly permissive to sheep prion infection, *J. Virol.*, 78, 482–490, 2004.
99. Chandler, R.L. and Turfrey, B.A., Inoculation of voles, Chinese hamsters, gerbils and guinea-pigs with scrapie brain material, *Res. Vet. Sci.*, 13, 219–224, 1972.
100. Gibbs, C.J., Jr., Amyx, H.L., Bacote, A., et al., Oral transmission of kuru, Creutzfeldt-Jakob disease, and scrapie to nonhuman primates, *J. Infect. Dis.*, 142, 205–208, 1980.
101. Tateishi, J., Ohta, M., Koga, M., et al., Transmission of chronic spongiform encephalopathy with kuru plaques from humans to small rodents, *Ann. Neurol.*, 5, 581–584, 1979.
102. Peterson, D.A., Wolfe, L.G., Deinhardt, F., et al., Transmission of kuru and Creutzfeldt–Jakob disease to marmoset monkeys, *Intervirology*, 2, 14–19, 1974.
103. Field, E.J., Transmission of kuru to mice, *Lancet*, 1, 981–982, 1968.
104. Gajdusek, D.C., Gibbs, C.J., and Alpers, M., Experimental transmission of a Kuru-like syndrome to chimpanzees, *Nature*, 209, 794–796, 1966.
105. Taylor, D.M., Dickinson, A.G., Fraser, H., et al., Evidence that transmissible mink encephalopathy agent is biologically inactive in mice, *Neuropathol. Appl. Neurobiol.*, 12, 207–215, 1986.
106. Kimberlin, R.H., Cole, S., and Walker, C.A., Transmissible mink encephalopathy (TME) in Chinese hamsters: Identification of two strains of TME and comparisons with scrapie, *Neuropathol. Appl. Neurobiol.*, 12, 197–206, 1986.
107. Miller, M.W. and Williams, E.S., Chronic wasting disease of cervids, *Curr. Top. Microbiol. Immunol.*, 284, 193–214, 2004.

108. Espana, C., Gajdusek, D.C., Gibbs, C.J., Jr., et al., Transmission of Creutzfeldt-Jakob disease to the stumptail macaque (Macaca arctoides), *Proc. Soc. Exp. Biol. Med.*, 149, 723–724, 1975.
109. Espana, C., Gajdusek, D.C., Gibbs, C.J., Jr., et al., Transmission of Creutzfeldt-Jakob disease to the patas monkey (Erythrocebus patas) with cytopathological changes in in vitro cultivated brain cells, *Intervirology*, 6, 150–155, 1975.
110. Tateishi, J., Koga, M., and Mori, R., Experimental transmission of Creutzfeldt-Jakob disease, *Acta Pathol. Jpn.*, 31, 943–951, 1981.
111. Tateishi, J., Doi, H., Sato, Y., et al., Experimental transmission of human subacute spongiform encephalopathy to small rodents. III. Further transmission from three patients and distribution patterns of lesions in mice, *Acta Neuropathol. (Berl)*, 53, 161–163, 1981.
112. Asante, E.A., Linehan, J.M., Desbruslais, M., et al., BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein, *EMBO J.*, 21, 6358–6366, 2002.
113. Collinge, J., Palmer, M.S., Sidle, K.C., et al., Unaltered susceptibility to BSE in transgenic mice expressing human prion protein, *Nature*, 378, 779–783, 1995.
114. Telling, G.C., Scott, M., Hsiao, K.K., et al., Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein, *Proc. Natl. Acad. Sci. U. S. A.*, 91, 9936–9940, 1994.
115. Scott, M.R., Peretz, D., Nguyen, H.O., et al., Transmission barriers for bovine, ovine, and human prions in transgenic mice, *J. Virol.*, 79, 5259–5271, 2005.
116. Scott, M.R., Safar, J., Telling, G., et al., Identification of a prion protein epitope modulating transmission of bovine spongiform encephalopathy prions to transgenic mice, *Proc. Natl. Acad. Sci. U. S. A.*, 94, 14279–14284, 1997.
117. Castilla, J., Gutierrez, A.A., Brun, A., et al., Early detection of PrPres in BSE-infected bovine PrP transgenic mice, *Arch. Virol.*, 148, 677–691, 2003.
118. Buschmann, A., Pfaff, E., Reifenberg, K., et al., Detection of cattle-derived BSE prions using transgenic mice overexpressing bovine PrP(C), *Arch. Virol.* (Suppl.) 75–86, 2000.
119. Vilotte, J.L., Soulier, S., Essalmani, R., et al., Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine PrP, *J. Virol.*, 75, 5977–5984, 2001.
120. Castilla, J., Gutierrez-Adan, A., Brun, A., et al., Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein, *J. Neurosci.*, 24, 5063–5069, 2004.
121. Browning, S.R., Mason, G.L., Seward, T., et al., Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP, *J. Virol.*, 78, 13345–13350, 2004.
122. Fischer, M., Rulicke, T., Raeber, A., et al., Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie, *EMBO J.*, 15, 1255–1264, 1996.
123. Buschmann, A. and Groschup, M.H., Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle, *J. Infect. Dis.*, 192, 934–942, 2005.
124. Castilla, J., Brun, A., Diaz-San Segundo, F., et al., Vertical transmission of bovine spongiform encephalopathy prions evaluated in a transgenic mouse model, *J. Virol.*, 79, 8665–8668, 2005.
125. Saá, P., Castilla, J., and Soto, C., Pre-symptomatic detection of prions in blood, *Science*, 313, 92–94, 2006.

126. Eloit, M., Adjou, K., Coulpier, M., et al., BSE agent signatures in a goat, *Vet. Rec.*, 156, 523–524, 2005.
127. Heikenwalder, M., Zeller, N., Seeger, H., et al., Chronic lymphocytic inflammation specifies the organ tropism of prions, *Science*, 307, 1107–1110, 2005.
128. Linsell, L., Cousens, S.N., Smith, P.G., et al., A case-control study of sporadic Creutzfeldt-Jakob disease in the United Kingdom: Analysis of clustering, *Neurology*, 63, 2077–2083, 2004.
129. Montagna, P., Gambetti, P., Cortelli, P., et al., Familial and sporadic fatal insomnia, *Lancet Neurol.*, 2, 167–176, 2003.
130. Sundstrom, D.G. and Dreher, H.M., A deadly prion disease: Fatal familial insomnia, *J. Neurosci. Nurs.*, 35, 300–305, 2003.
131. Gambetti, P., Kong, Q., Zou, W., et al., Sporadic and familial CJD: Classification and characterisation, *Br. Med. Bull.*, 66, 213–239, 2003.
132. Salmona, M., Morbin, M., Massignan, T., et al., Structural properties of Gerstmann-Straussler-Scheinker disease amyloid protein, *J. Biol. Chem.*, 278, 48146–48153, 2003.
133. Brown, P., Transmission of spongiform encephalopathy through biological products, *Dev. Biol. Stand.*, 93, 73–78, 1998.
134. Klatzo, I., Gajdusek, D.C., and Zigas, V., Pathology of kuru, *Lab Invest*, 8, 799–847, 1959.
135. Elliott, H., Gubbins, S., Ryan, J., et al., Prevalence of scrapie in sheep in Great Britain estimated from abattoir surveys during 2002 and 2003, *Vet. Rec.*, 157, 418–419, 2005.
136. Bessen, R.A. and Marsh, R.F., Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent, *J. Virol.*, 66, 2096–2101, 1992.
137. Doherr, M.G., Bovine spongiform encephalopathy (BSE)—Infectious, contagious, zoonotic or production disease?, *Acta Vet. Scand. Suppl.*, 98, 33–42, 2003.
138. Pearson, G.R., Wyatt, J.M., Gruffydd-Jones, T.J., et al., Feline spongiform encephalopathy: Fibril and PrP studies, *Vet. Rec.*, 131, 307–310, 1992.

Apéndice 3

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1772 (2007) 681–691

Biochimica et Biophysica Acta

BBA

www.elsevier.com/locate/bbadis

Review

The prion strain phenomenon: Molecular basis and unprecedented features

Rodrigo Morales [a,b], Karim Abid [a], Claudio Soto [a,*]

[a] *Protein Misfolding Disorders Laboratory, George and Cynthia Mitchell Center for Neurodegenerative Diseases, Departments of Neurology, Neuroscience and Cell Biology and Biochemistry and Molecular Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0646, USA*

[b] *Facultad de Ciencias, Universidad de Chile, Santiago, Chile*

Received 5 September 2006; received in revised form 9 December 2006; accepted 11 December 2006

Available online 15 December 2006

Abstract

Prions are unconventional infectious agents responsible for transmissible spongiform encephalopathies. Compelling evidences indicate that prions are composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) that replicates in the absence of nucleic acids. One of the most challenging problems for the prion hypothesis is the existence of different strains of the infectious agent. Prion strains have been characterized in most of the species. Biochemical characteristics of PrP^{Sc} used to identify each strain include glycosylation profile, electrophoretic mobility, protease resistance, and sedimentation. *In vivo*, prion strains can be differentiated by the clinical signs, incubation period after inoculation and the lesion profiles in the brain of affected animals. Sources of prion strain diversity are the inherent conformational flexibility of the prion protein, the presence of PrP polymorphisms and inter-species transmissibility. The existence of the strain phenomenon is not only a scientific challenge, but it also represents a serious risk for public health. The dynamic nature and inter-relations between strains and the potential for the generation of a large number of new prion strains is the perfect recipe for the emergence of extremely dangerous new infectious agents.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Prions; Prion strains; Protein misfolding; Scrapie; Creutzfeldt-Jakob disease

1. Introduction

Transmissible Spongiform Encephalopathies (TSEs), also known as prion disorders, are infectious and fatal neurodegenerative diseases affecting humans and other mammals. In humans, TSEs include Creutzfeldt–Jakob disease (CJD), fatal familial insomnia (FFI), Gertsmann–Straussler–Scheinker Syndrome (GSS) and Kuru [1,2]. In other mammals, bovine spongiform encephalopathy (BSE) is found in cattle, scrapie in sheep and goats and chronic wasting disease (CWD) in elk and deer [1,2]. Although the clinical symptoms vary in distinct diseases, they usually include dementia and/or ataxia with progressive loss of brain function, irreversibly resulting in death [3]. The hallmark of prion diseases is the misfolding of the prion protein observed in the brain of affected individuals [1]. Misfolded proteins have the intrinsic tendency to form large aggregates and fibrillar structures, that may form amyloid deposits in a similar fashion as observed in Alzheimer's, Parkinson's diseases and many other protein misfolding disorders [4].

Although of rare occurrence, prion diseases have drawn considerable attention and led to severe economic and political consequences in Europe and in the United States. The two main reasons of this impact include the unique nature of the infectious agent and the appearance of a new human disease (vCJD) linked to consumption of cattle meat infected by BSE. At present, it is impossible to estimate accurately the number of upcoming cases of vCJD due to the very long incubation time of the disease in humans [5–7]. Prion research has been plagued with the discovery of new and heretic scientific findings that have confronted the most solid paradigms in modern biology. The current evidence suggest that an abnormal form of the prion protein (termed PrP^{Sc}) is the main, and possibly the only, constituent of prion infectious agent [1]. This so-called protein-only hypothesis [1,8] proposes that replication of PrP^{Sc} occurs at expenses of the normal host's version of the prion protein (termed PrP^{C}). PrP^{Sc} has different biochemical characteristics compared to PrP^{C}, for example its insolubility, resistance to denaturation, and its partial resistance to protease degradation

* Corresponding author.
E-mail address: clsoto@utmb.edu (C. Soto).

0925-4439/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2006.12.006

[9]. PrP^{Sc} treatment with proteases reveal the protease resistant core of the infectious agent (termed PrP^{27-30} according to its molecular weight) [10].

2. Molecular basis of prion strains

Among the unique features that have contributed to place the prion field in the spotlight, one of the most interesting is the prion strain phenomenon. It has been observed that animals affected by prion diseases may develop different pathologies and the clinical and biochemical outcomes could be maintained through several passages in rodents models of prion diseases. In analogy to other infectious agents, these variants have been termed strains. A classical definition of strain makes mention to a genetic variant or subtype of the infectious agent responsible for the disease, but this concept, valid in virology, cannot be extended to prions. In early days, the strain phenomenon was claimed as one of the strongest evidences against the protein-only hypothesis [11,12]. It was assumed that the different phenotypes found in animals were due to differences in the genetic information contained within the TSE causing agent. However, currently it is widely accepted that the main differences between prion strains arise from alternative conformations of PrP^{Sc} that can be stably and faithfully propagated [13,14].

The first evidence about the existence of prion strains was described in goats affected by scrapie by Pattison and Millson in 1961 [15]. In this report, goats infected by the same batch of infectious scrapie agent developed two different clinical phenotypes, termed by the authors "scratching" and "drowsy", according to disease's manifestation. The differences between these infectious agents were alleged to be the consequence of differences in the genetic background of the host. The current evidence supports this hypothesis. In some cases, clinical signs could be very useful to differentiate between prion strains [15–17]. Each prion strain has the capability to affect specific brain areas producing differences in clinical signs. In the case of scrapie in sheep and goats, after identification and isolation of the prion protein gene (*prnp*) several polymorphic differences were recognized when numerous sheep flocks were compared [18].

Prion strains can be classified by different parameters. Incubation periods, profile of histological damage and clinical signs are the main *in vivo* characteristics which can be used to differentiate between prion strains [16,19,20]. The most commonly used is incubation period which corresponds to the time elapsed between experimental inoculation of the infectious agent and clinical onset of the disease. Intra-species inoculation of prions is usually very reproducible [19]. Inoculation of different prion strains preparations usually results in different and reproducible incubation times [19,21]. Histological studies have also shown substantial differences when animals were inoculated with distinct strains. The differences are mainly on the distribution and characteristics of PrP^{Sc} deposition and the degree of vacuolation in specific brain regions [22–25]. In order to quantify this aspect, a well-standardized procedure for vacuolization scoring (lesion profile) in mice [25] has been described; six gray matter and three white matter brain areas are analyzed and scored according to the magnitude of the damage. Using this approach, prion strains having similar incubation times were differentiated, such as ME7 and 79A [25]. In a similar way, PrP^{Sc} accumulation profile has been useful to track the origin of the infectious material. For example, in the case of the transmission of BSE into humans, originating vCJD, similar neuropathological signatures were produce [26,27]. Finally, the clinical signs are also a characteristic that can be very useful to differentiate strains. For example, in human prion diseases, motor incoordination, dementia, ataxia, depression, and insomnia are just few from a much larger list of clinical symptoms that can appear with more or less intensity depending on the strain of the agent [3]. In other animals, such as the case of hamsters, the clinical features can be diametrically opposed. That is the case of the Drowsy (DY) and Hyper (HY) prion strains [16]. Unfortunately, clinical signs cannot always be applied to differentiate and classify prion strains. In mouse for example, several prion strains have the same rough signs, which include ataxia, rough coat, and hunch [28,29]. However, studies using more detailed tests have identified dissimilar behavioral deficits when different prion strains are administrated to mice [30]. Since different brain lesion patterns appear to be responsible for the variation in clinical signs, behavioral studies could give us more specific information about the type of brain damage produced by different prion isolates [30,31].

In addition to the *in vivo* differences, each prion strain has a particular group of biochemical characteristics in the infectious protein that could be specifically associated to them. Among them, the most important are the electrophoretical mobility after proteinase K (PK) digestion [32–34], glycosylation pattern [33–35], extent of PK resistance [32], sedimentation [32] and resistance to denaturation by chaotropic agents [32,36]. Recently, differences in the binding affinity for copper among strains have been described [37]. As illustrated in Fig. 1, the biochemical features of PrP^{Sc} in various forms of CJD are different. The western blot profile of different sources of human PrP^{Sc} shows diversity in terms of glycosylation pattern and electrophoretical mobility after PK digestion [32–34]. Fourier Transform Infrared Spectroscopy (FTIR) studies involving

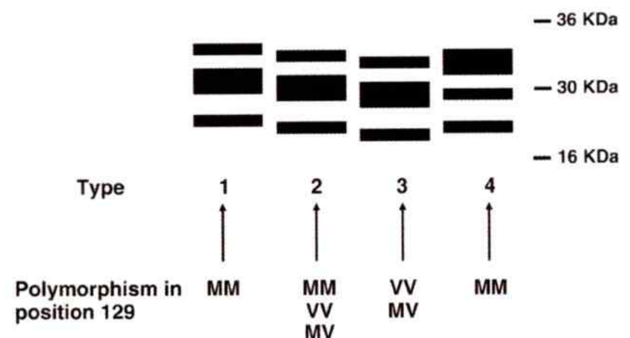


Fig. 1. PrP^{Sc} western blot profiles associated to different strains of human prions. Schematic representation of human PrP^{Sc} types after PK digestion. Particular polymorphic groups in position 129 are associated with specific PrP^{Sc} patterns. Types 1 and 2 are associated with sCJD, type 3 is mostly associated with iCJD and type 4 is found exclusively in vCJD.

different prion strains [36,38,39], conformation dependent immunoassays [36,40] and atomic force microscopy of synthetic prion protein polymers [41] confirm the hypothesis that differences between prion strains lies in the diversity of structures that PrP^{Sc} can acquire. However, the definitive proof for the structural nature of the differences between prion strains is still missing.

3. Species barrier and generation of new prion strains

The principal source of strain diversity arise from inter-species infection [23,42–45]. One of the characteristics of the agent responsible for prion diseases is its ability to infect some species and not others. This phenomenon is known as "species barrier" and is manifested as the prolongation in the incubation periods when prions from one species are used to infect a different one [46,47]. Differences in the sequence of prion protein could lead to different conformations, explaining both, species barrier and diversity of PrP^{Sc} conformations [41,48–50]. In some species, PrP^{C} conformation does not permit conversion by prions coming from other species. A clear example of this is found in rabbit, an animal that has been unable to be infected by various sources of prions. In these cases, it is considered that the species barrier is absolute.

Interspecies prion transmission from cattle to human is probably the most relevant problem in terms of public health [51–53]. It is widely accepted that consumption of BSE infected material is the cause of vCJD in humans [27,54]. Strikingly, vCJD presents many different features compared to the previously known human strains, arisen sporadically. Differences between vCJD and sCJD include the clinical manifestation of the disease, the profile of brain damage and the biochemical features of PrP^{Sc} [55]. The BSE epidemic in the United Kingdom demonstrated how dangerous prions could be. So far, BSE is the only non-human prion described to be transmissible to humans. Despite the fact that people have consumed for centuries sheep potentially affected by scrapie, no correlation has been found between patients suffering by CJD and sheep consumption. Scrapie transmissibility experiments using transgenic animal models expressing chimeric human/mouse PrP support this assumption [56].

BSE has not only been transmitted to humans. The extensive use of cow-derived material for feeding other animals led to the generation of new diseases in exotic felines such as tiger and cheetah, non human primates, and domestic cats [52,57–60]. The transmission of BSE into these different species could create many new prion strains, each one of them with particular biological and biochemical characteristics and thus a potentially new hazard for human health. Successful transmission of BSE in pigs has been described [61,62] and also in transgenic mice expressing pig PrP [63]. Porcine derivates are widely consumed and the hypothetic case of "mad pigs" could increase the events of zoonotic transmission of prions to humans. Fortunately, transmission of BSE to pigs is possible only in very drastic experimental conditions, not likely to be occurring naturally [62,63]. More frightening is perhaps the possibility that BSE has been passed into sheep and goats. Studies have already shown that this transmission is possible and actually relatively easy and worrisomely produces a disease clinically similar to scrapie [64]. The cattle origin of this new scrapie makes possible that the new strain may be transmissible to humans. Transmission experiments of BSE infected sheep brain homogenate into transgenic animal models expressing human PrP are currently ongoing in several laboratories. It is important to note that all materials generated by transmission of BSE in experimental and natural cases show similar biochemical behavior compared to the original inoculum [65], suggesting that these new generated infectious agents might be hazardous for humans. The origin of BSE is still a mystery. Abundant evidence supports the hypothesis that BSE was produced by cattle feeding with scrapie derivated material [66,67], indicating that bovine PrP^{Sc} might be a "conformational intermediary" between ovine PrP^{Sc} and human PrP^{C}.

There is currently no mean to predict which will be the conformation of a newly generated strain and how this new PrP^{Sc} conformation could affect other species. One interesting new prion disease is CWD, a disease affecting farm and wild species of cervids [68,69]. The origin of CWD and its potential to transmit to humans are currently unknown. This is worrisome, considering that CWD has became endemic in some parts of USA and the number of cases continues to increase [69]. It is presumed that a large number of hunters in the US have been in contact or consumed CWD-infected meat [70]. CWD transmissibility studies have been performed in many species in order to predict how this disease could be spread by consumption of CWD meat [71–73]. In these studies, a special attention has been done to scavenging animals [74], which are presumed to be exposed to high concentration of cervid prions, resulting in the putative generation of many new forms of TSEs. Fortunately negative results were obtained in experiments done in raccoons infected with CWD [74]. Transmission of CWD to humans cannot be ruled out at present and a similar infective episode to BSE involving CWD could result in catastrophic events, spreading the disease in a very dangerous way through the human population. No clinical evidence linking CWD exposed humans and CJD patients have been found [70], but experimental inoculation of CWD prions into squirrel monkeys propagated the disease [71]. Nevertheless, the species barrier between humans and cervids appears to be greater than with cattle, as judged by experiments with transgenic mice models [75]. Finally, it is important to be aware about CWD transmissibility to other species in which a "conformational intermediary" could be formed, facilitating human infection.

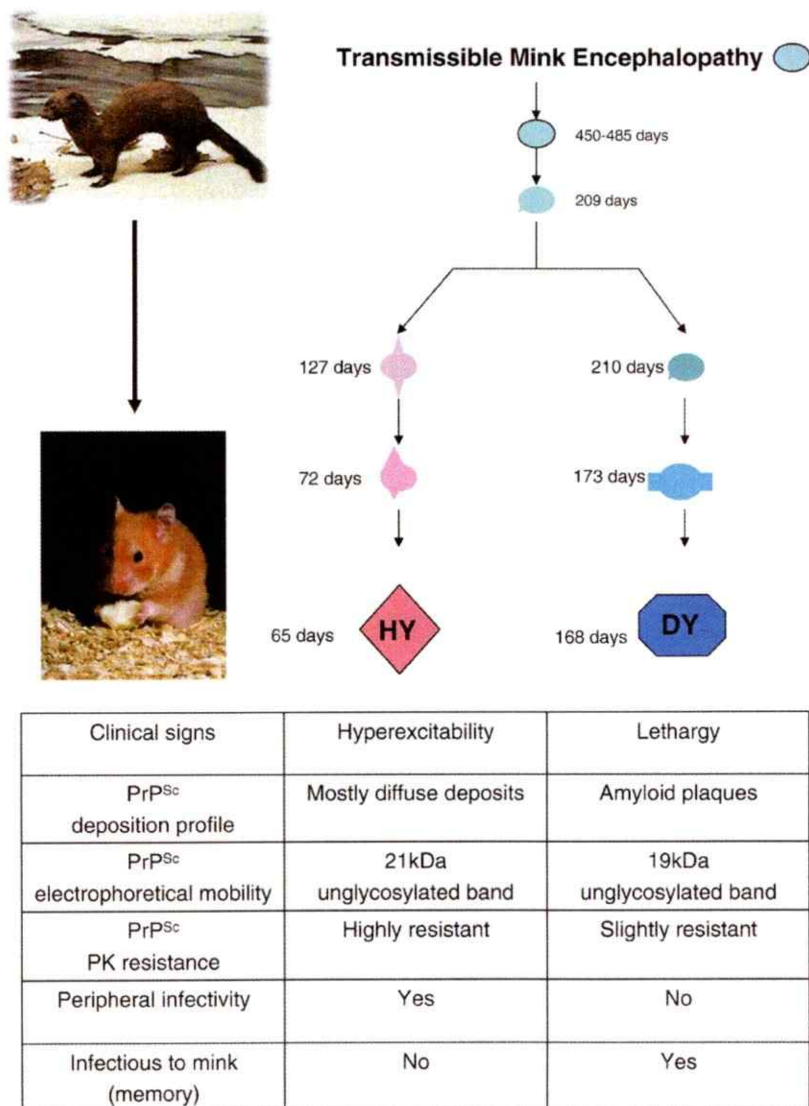
4. Use of experimental animals to study prion strains

Probably the best way to study the strain phenomenon is using experimental animals for the generation of diverse strains through inoculation with prion infectious material coming from different species. Among the experimental models, perhaps mouse is the most useful one, in which more than 20 phenotypically distinct strains have been isolated [19]. Many of these strains have their origin in the transmission of different

sources of scrapie from goat and sheep, BSE derived material from cattle [23,24,76] and human sources as sCJD and GSS [44,77,78]. Serial passage of infectious prions in one species, with constant biological background is necessary to stabilize and define a prion strain.

PrP^{Sc} obtained from mouse adapted scrapie prion strains such as RML, ME7, 139A and 79A show similar electrophoretical characteristics after PK digestion. PrP^{Sc} coming from these strains show an electrophoretical mobility of ~21 kDa for the unglycosylated band and a similar glycosylation pattern, with the monoglycosylated form as the most abundant [79–83]. Despite the lack of biochemical differences, these strains can be differentiated when inoculated in mice by measuring the incubation time or the profile of brain lesions [25,28,82,84]. Other mouse strains have been generated by inoculation of animals with BSE and sCJD prions, leading to strains termed 301C and Fukuoka, respectively [42,43,85]. The transmission of BSE into mice generated two different phenotypes: one presenting PK resistant isoform of PrP^{Sc}, and another lacking this characteristic [76]. This phenotype is maintained after two serial passages in mice, but finally only the PK resistance phenotype remains. The presence of a PK sensitive infectious material (termed $sPrP^{Sc}$) has been also described in some cases of human prion diseases [86,87].

The characteristics of mouse strains generated from scrapie or from BSE are quite different. For example, intracerebral inoculation of RML strain into mice present an onset of ~150 days post inoculation (dpi), while 301C preparations cause the disease at ~200 dpi [19,88]. Intraperitoneal inoculation of RML and 301C material in the same animals shows a larger difference in the incubation periods: 200 and 300 dpi, respectively [24,84]. There are also differences in the



Clinical signs	Hyperexcitability	Lethargy
PrPSc deposition profile	Mostly diffuse deposits	Amyloid plaques
PrPSc electrophoretical mobility	21kDa unglycosylated band	19kDa unglycosylated band
PrPSc PK resistance	Highly resistant	Slightly resistant
Peripheral infectivity	Yes	No
Infectious to mink (memory)	No	Yes

Fig. 2. Origin and properties of the HY and DY prion strains in hamsters. The Hyper (HY) and Drowsy (DY) scrapie strains were generated upon serial passage of transmissible mink encephalopathy (TME) infectious material in Syrian hamsters. The initial passage resulted in a very large incubation period that was upon successive passage stabilized in two different strains exhibiting strikingly different clinical, neuropathological, biochemical and infectious properties. The HY and DY hamster strains represent a prototype example of strain diversity without changes in amino acid sequence of the prion protein.

brain affected areas by both mouse adapted prions (Castilla J., Morales R., Saá P., and Soto C.; unpublished data). In addition, it is also possible to find biochemical differences between RML and 301C strains. As mentioned above, PK digestion pattern of RML shows an electrophoretical mobility of ~21 kDa for the unglycosylated band and is rich in the monoglycosylated isoform of prion protein. In contrast, 301C prion strain show a different electrophoretical pattern compared to RML. Unglycosylated isoform of BSE adapted mouse strain shows an electrophoretical mobility of ~19 kDa and its glycoform distribution favors the diglycosylated isoform [83].

How different PrP^{Sc} conformations could induce stable conformational changes in the same host protein is still unknown. Even more interesting is the isolation of different prion strains from the same host after inoculation of PrP^{Sc} from a single species. Probably the most representative experience is the isolation of DY and HY strains after inoculation of the agent associated to transmissible mink encephalopathy (TME) in Syrian hamsters (Fig. 2) [13,16]. This interspecies transmission of prions presented the expected behavior of the species barrier phenomenon: a long incubation period in the first passage, but shorter incubation periods after inoculation of serial passages from the resulting infectious material into Syrian hamsters. Incubation periods became stable in two different groups with different clinical signs: the first one with an incubation period of ~150 dpi presented lethargy, while a shorter incubation period strain (~60 dpi) presented hyperactivity. These strains were called Drowsy (DY) and Hyper (HY) respectively according to their clinical signs [16]. Histopathological analysis of animal groups infected with both TME hamster adapted agents show differences in the vacuolation distribution among different brain regions [16] and also in the PrP^{Sc} deposition areas [89] (Fig. 2).

As 301C and RML in mouse, DY and HY present differences in their electrophoretical mobility after PK treatment. The unglycosylated band of DY has a molecular weight of 19 kDa, while HY show the same band at 21 kDa [32,90]. This is the most direct evidence that suggested conformational differences between both PrP^{Sc} species. Supporting this assumption, structural differences using Fourier transform infrared spectroscopy (FTIR) between both Syrian hamster adapted TME strains were found [38]. Another biochemical difference found between DY and HY lies in their differential resistance to PK digestion, where DY is the most sensitive to digestion compared to HY [32] (Fig. 2). All these biological and biochemical characteristics make DY and HY one of the most intriguing examples of prion strain variation.

5. Polymorphisms and prion strains

Polymorphisms in the prion protein and their effects in the prion strain phenomenon were indirectly described a long time before the prion hypothesis was developed [15]. Differences in prion pathology were found and extensively described in sheep and mice [17–19,25]. The drowsy and scratchy phenotypes found in sheep were attributed to polymorphic differences in the host [17]. The identification of "scrapie incubation period gene" (*sinc*) and its polymorphic differences was a very big hit in the study of prion strains [91]. In mouse, two polymorphic animal groups were originally described: $sinc^{s7}$ and $sinc^{p7}$. Later, it was discovered that the *sinc* gene was indeed the gene encoding PrP and the polymorphisms resulted in differences in the prion protein at positions 108 and 189 [92]. The transmission of infectious agents from sheep, goats and cattle to both mice groups resulted in the emergence of a wide diversity of prion strains [19,28]. When incubation periods of mouse adapted prions were stabilized in each group, new generated infectious agent could be assayed in the other animal group. It was found that the presence of the polymorphism produced a prolongation in the incubation period in a similar way as observed in the species barrier phenomenon [19]. It was postulated that prion strains in mouse could be differentiated inoculating infectious material in both animals' types and identifying the short and long incubation period animal cluster [19]. Interestingly, when a mouse prion strain is inoculated in *sinc* heterozygous animals, either intermediate or longer incubation periods are observed [28]. Inter-polymorphic transmissions can lead to the generation of new prion strains [19], which implies new vacuolation, infectivity and/or dominance characteristics, among others. All this information suggests that polymorphisms in the prion protein are able to favor strain diversity. Table 1 shows mice and prion strains corresponding to each polymorphic group.

After the isolation of prion protein gene [93], it was described that *sinc* and *prnp* genes were congruent [94]. Analysis of long and short incubation period animal groups revealed the expected polymorphic differences in the prion protein gene [21]. These findings strongly supports the prion hypothesis, because as observed in the species barrier phenomenon, differences in the sequence of the prion protein affect extensively the transmission and strain characteristics of the infectious agent. According to a new nomenclature generated, $sinc^{s7}$ animals are re-baptized as $prnp^{a}$, while $sinc^{p7}$ as $prnp^{b}$ [21]. Recently a new group of mice have been identified and named $prnp^{c}$ (Table 1) [88].

PrP polymorphisms are not unique of mouse. Indeed, polymorphisms in the prion protein have been described in most of the species. In sheep, several inter-bred crosses have been performed in order to optimize the quality and productivity

Table 1
Polymorphisms associated to prion diversity in mouse

Mouse prnp genotype	Mouse strain	Prion strains	Associated polymorphisms
$prnp^{a}/sinc^{s7}$	C57	RML-ME7-	Leu-108
	RIII	139A-301C-	Thr-189
	Swiss	22C-79A-	
	NZW	87A-	
	SJL		
$prnp^{b}/sinc^{p7}$	VM	301V-22A-	Phe-108
	IM	87V-79V-	Val-189
$prnp^{c}$	Mai/Pas		Phe-108
	C57 MAI-Prnp		Thr-189

The table shows different mouse strain and prion strains isolated in each polymorphic group.

of these animals, producing a wide range of polymorphic variants for *prnp* [18]. However, only five alleles of the PrP gene are significantly present giving a total of 15 possible PrP genotypes, each likely to favor or disfavor the selection of different scrapie strains [18]. These five common polymorphic alleles are ARQ, ARR, AHQ, ARH and VRQ. Polymorphic changes are present principally in codons 136, 154 and 171, but in order to simplify the nomenclature they are designated by the amino acid present in each position. In a recent revision by Baylis and Goldman [18] it is documented that sheep carrying the VRQ/VRQ, ARH/VRQ and ARQ/VRQ alleles are most susceptible to develop scrapie, whereas the less vulnerable are animals having ARR/ARR, ARR/ARH and AHQ/ARH alleles. Therefore, it is generally agreed that the VRQ allele promotes susceptibility to scrapie, whereas ARR diminishes the manifestation of the disease. Interestingly, other alleles such as ARH alone appear to favor the development of the disease while in combination with other alleles appear to confer resistance. In the same study, a correlation was established between incubation periods and the type of polymorphism. A linear relationship between age of death and five polymorphic groups was observed. Many prion strains have been described for scrapie. Each strain is associated to a particular allelic group, and allelic groups are associated to a particular breed of animals [42,95]. However, as previously described, prion protein diversity could exist with the same sequence in the prion protein and sheep is not the exception. CH1641, a prion strain with clear biochemical differences compared to other scrapie strains was isolated from a natural case of scrapie in Cheviot sheep [96]. Recently a new scrapie strain designated Nor98 has been described [95], mostly in animals having AHQ/AHQ and AHQ/ARQ genotypes (a variation relatively resistant to scrapie). In this case, "classically susceptible" alleles seem to be resistant to this class of prions [18,97]. All this information arise questions about how natural cases of scrapie are developed.

In humans there is a polymorphism at codon 129, where an ATG or GTG results in either a methionine (Met) or a valine (Val) at that position. A large body of evidence indicates that this polymorphism alone or in conjunction with mutations in the prion gene modulates disease susceptibility and phenotypic expression of human TSE [2,34,98–104]. Both Met and Val homozygous are over-represented, while heterozygous cases are under-represented in sCJD [98,99,105]. About 40% of the normal population is Met-homozygous, however 78%, 50% and 100% of patients affected by the sporadic, iatrogenic and variant forms of CJD are Met-homozygous, respectively [105–109]. These data suggest that the presence of Met at position 129 confers a higher susceptibility for the protein to be converted into the pathogenic isoform. The polymorphism has also been shown to alter the neuropathological pattern of lesions in sporadic CJD, the glycoform profile of protease-resistant PrP^{Sc} and the duration and severity of the disease [34,102,103,110–113]. A study involving 300 patients showed that Met-homozygous develop a more aggressive phenotype characterized by a short duration of disease (4.5 months), while heterozygous and Val-homozygous have a much longer disease duration (14.3 and 16.9 months, respectively) [103]. Val-homozygous seems to cause damage preferentially in the deep gray matter, while Met-homozygous seems to target mainly cortical structures [103]. Codon 129 polymorphism also influences the phenotypic expression of mutations elsewhere in the prion gene [104,114–119]. For example, people with a mutation at codon 178 resulting in a change of aspartic acid to asparagine develop either familial CJD or FFI depending on whether the amino acid at codon 129 is Val or Met, respectively [104].

Despite the clear importance of PrP polymorphism at position 129 in the disease propensity and pathogenesis, the molecular mechanism of this effect is unknown. Experimental and computational modeling studies of the tridimensional structure of PrP have been unable to identify any significant difference between the two isoforms [120]. In addition, no difference was reported on the in vitro thermodynamic stability of recombinant PrP bearing either Met or Val at position 129 [120,121]. Structural studies show evidence for hydrogen bonding between Asp178 and Tyr128, which might provide a structural basis for the influence of the polymorphism on the disease phenotype that segregates with the mutation Asp178Asn [121]. In addition, it has been reported that a slightly different conformation of recombinant Met- or Val-containing PrP isoforms was induced upon copper binding [122]. Using short model peptides, we found that M at position 129 increases the propensity of this region to aggregate into β-sheet rich fibrillar structures [123]. These findings were interpreted to suggest that Met induces a higher local propensity to extend the short β-sheet present in the normal protein into a larger sheet, which results in an increase in the rate of PrP conversion to the pathological isoform [123].

6. Unique features of prion strains

The biological and infectious characteristics of prions are dramatically different to the conventional infectious agents. These differences are manifested in the prion strains phenomenon in unique and unprecedented features, such as for example strain adaptation and memory, the coexistence and competition of prion strains, among others. In this section, some of these interesting phenomena will be briefly described.

6.1. Adaptation of prion strains

Interspecies transmission of prions could result in the emergence of more than one variety of infectious material with different strain characteristics. That is the case of DY and HY prion strains generation [13,16]. When interspecies transmission of prions occurs, serial passages in the new host are needed in order to stabilize the characteristics of new generated infectious material. In the case of TME transmission in hamsters, at least four serial passages in the new species were required for stabilization (Fig. 2) [13]. The first passage was characterized by long incubation periods and a dominance of a 19 kDa fragment when newly obtained PrP^{Sc} was analyzed after PK digestion. In the three first passages, clinical symptoms were not characteristic of the hamster-adapted HY or DY TME

strains. This phenotype was attributed to the combination effects of both strains replicating simultaneously. Thereafter, each of the strains was stabilized in some of the animals and once they are adapted and stabilized, they can be serially propagated *in vivo* and the characteristics are maintained. It is accepted that both strains present differential conversion kinetics *in vitro*, with DY being the slowest and HY the fastest [124]. For this reason, in order to select efficiently this prion strain, limiting dilution experiments must be performed [13]. In that way, the most abundant and less convertible DY is favored against the less abundant but fastest HY strain.

6.2. Co-existence of prion strains

Related to the above, it has been shown that two or more prion strains can co-exist in natural cases of TSE. Co-existence of prion strains has been found in sporadic cases of CJD [113,125]. Analyses of several sCJD tissue showed that different biochemical profiles of PrP^{Sc} could be found in different brain areas from the same patient [113]. Co-existence of prion strains was mainly observed in patient heterozygous for codon 129 [113]. As many as 50% of these patients present different types of PrP^{Sc} in their brains, whereas 9% of MM patients were positive for co-existence of strains. On the other hand, more than one PrP^{Sc} type was not observed in VV patients [113].

The biochemical and structural properties of the protein seem to be the major cause of this differential distribution. This observation may explain why sCJD is so heterogeneous in terms of clinical manifestation [34,126,127]. In a recent publication by Bishop et al. [107], vCJD infected transgenic mice expressing human PrP^{C}, present changes in their PrP^{Sc} and vacuolation patterns in the brain according to their polymorphic classification for codon 129.

6.3. Competition of prion strains

In particular experimental conditions some prion strains can extend their specific incubation period when co-infected with another strain. Long incubation period prions increase the incubation period of "faster" prions. This phenomenon of "competition of prion strains" has been observed in mice and hamster. In mice, competition between 22A and 22C strains was reported in 1975 by Dickinson et al. [128]. In this study, RIII mice (homozygous for $sinc^{s7}$ allele) were used. 22A and 22C showed long and short incubation period (550 and 230 days), respectively. When 22C strain was intraperitoneally inoculated 100, 200 and 300 days after intraperitoneal administration of the 22A agent, all three experimental groups resulted in incubation periods and lesion patterns matching 22A prions, suggesting that 22C prions were degraded or excreted, in animals previously infected by 22A. Similar results were obtained by Kimberlin and Walker in 1985 [129] using a different strain of $sinc^{s7}$ mice. These authors treated mice using 22A and 22C prion strain. Before inoculation, 22A was treated with different chemical and physical agents in order to see if the "competitor" or "blocking" characteristics of 22A were maintained. From all treatments, 12 M urea was shown to almost abolish the blocking properties of 22A agent. This information suggests that infectious properties of long incubation period agent are strictly necessary in order to increase the incubation period of faster prions.

In hamster, similar observations were reported using DY and HY [130]. DY prion strain was inoculated 30 and 60 days prior intraperitoneal inoculation of HY at three different doses. When incubation periods of HY inoculated control group were compared with the animals inoculated at 60 days with DY, significant differences in the incubation periods were found, especially when HY prions were administrated in a higher dose [130]. On the other hand no differences were observed in the case of intranerve inoculation, revealing that competition phenomenon occurs only when peripheral inoculation is performed. These results are surprising considering the fact that DY was reported not to be infectious when intraperitoneally inoculated in hamsters [130]. These data suggest that replication of DY is occurring in peripheral tissues but is not able to reach the central nervous system.

In general, the principal variables that need to be observed for a successful competition are the route of infection, the interval between injections and the particular strains and doses of agent used. Prolongation of incubation periods in TSE are therapeutically beneficial and several strategies are under development to reach this aim, including antibodies, beta-sheet breakers, and other chemical agents [131–133]. The experimental evidence described above suggests that prions could be potentially useful for this purpose. For example, in order to prevent spread of prion disease in cattle or humans, prion strains with incubation periods longer than species' lifespan could be used to slowdown the replication of BSE or vCJD prions.

7. Concluding remarks

The existence of different strains of an infectious agent composed exclusively of a protein has been one of the most puzzling issues in the prion field. If is already difficult to understand how a protein can adopt two stable and different folded structures and that one of them can transform the other one into itself, it is unthinkable that the misfolded form can in turn adopt multiple conformations with distinct properties. Yet, compelling scientific evidence support the idea that PrP can adopt numerous folding patterns that can faithfully replicate and produce different diseases. The existence of the strain phenomenon is not only a scientific challenge, but it also represents a serious risk for public health. The dynamic nature and inter-relations between strains and the potential for the generation of many new prion strains depending on the polymorphisms and the crossing of species barrier is the perfect recipe for the emergence of extremely dangerous new infectious agents. Although, substantial progress has been made in understanding the prion strains phenomenon, there are many open questions that need urgent answers, including: what are the structural basis of prion strains? How are the phenomena of strain adaptation and memory enciphered in the conformation of the prion agent? To what species can a given prion strain be

transmissible? What other cellular factors control the origin and properties of prion strains?

Acknowledgement

This research was supported in part by NIH grant NS049173.

References

[1] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 13363–13383.

[2] J. Collinge, Prion diseases of humans and animals: their causes and molecular basis, Annu. Rev. Neurosci. 24 (2001) 519–550.

[3] H. Budka, A. Aguzzi, P. Brown, J.M. Brucher, O. Bugiani, F. Gullotta, M. Haltia, J.J. Hauw, J.W. Ironside, K. Jellinger, Neuropathological diagnostic criteria for Creutzfeldt–Jakob disease (CJD) and other human spongiform encephalopathies (prion diseases), Brain Pathol. 5 (1995) 459–466.

[4] C. Soto, Unfolding the role of protein misfolding in neurodegenerative diseases, Nat. Rev., Neurosci. 4 (2003) 49–60.

[5] C.H. Cohen, A.J. Valleron, When did bovine spongiform encephalopathy (BSE) start? Implications on the prediction of a new variant of Creutzfeldt–Jakob disease (nvCJD) epidemic, Int. J. Epidemiol. 28 (1999) 526–531.

[6] A.C. Ghani, N.M. Ferguson, C.A. Donnelly, T.J. Hagenaars, R.M. Anderson, Estimation of the number of people incubating variant CJD, Lancet 352 (1998) 1353–1354.

[7] R. Will, Variant Creutzfeldt–Jakob disease, Folia Neuropathol. 42 (2004) 77–83 (Suppl A).

[8] J.S. Griffith, Self-replication and scrapie, Nature 215 (1967) 1043–1044.

[9] F.E. Cohen, S.B. Prusiner, Pathologic conformations of prion proteins, Annu. Rev. Biochem. 67 (1998) 793–819.

[10] S.B. Prusiner, D.F. Groth, D.C. Bolton, S.B. Kent, L.E. Hood, Purification and structural studies of a major scrapie prion protein, Cell 38 (1984) 127–134.

[11] B. Chesebro, BSE and prions: uncertainties about the agent, Science 279 (1998) 42–43.

[12] C. Soto, J. Castilla, The controversial protein-only hypothesis of prion propagation, Nat. Med. 10 (2004) S63–S67.

[13] J.C. Bartz, R.A. Bessen, D. McKenzie, R.F. Marsh, J.M. Aiken, Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy, J. Virol. 74 (2000) 5542–5547.

[14] D. Peretz, M.R. Scott, D. Groth, R.A. Williamson, D.R. Burton, F.E. Cohen, S.B. Prusiner, Strain-specified relative conformational stability of the scrapie prion protein, Protein Sci. 10 (2001) 854–863.

[15] I.H. Pattison, G.C. Millson, Scrapie produced experimentally in goats with special reference to the clinical syndrome, J. Comp. Pathol. 71 (1961) 101–109.

[16] R.A. Bessen, R.F. Marsh, Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters, J. Gen. Virol. 73 (Pt 2) (1992) 329–334.

[17] A.G. Dickinson, Scrapie in sheep and goats, Front Biol. 44 (1976) 209–241.

[18] M. Baylis, W. Goldmann, The genetics of scrapie in sheep and goats, Curr. Mol. Med. 4 (2004) 385–396.

[19] M.E. Bruce, Scrapie strain variation and mutation, Br. Med. Bull. 49 (1993) 822–838.

[20] H. Fraser, Diversity in the neuropathology of scrapie-like diseases in animals, Br. Med. Bull. 49 (1993) 792–809.

[21] D. Westaway, P.A. Goodman, C.A. Mirenda, M.P. McKinley, G.A. Carlson, S.B. Prusiner, Distinct prion proteins in short and long scrapie incubation period mice, Cell 51 (1987) 651–662.

[22] M.E. Bruce, P.A. McBride, C.F. Farquhar, Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie, Neurosci. Lett. 102 (1989) 1–6.

[23] M.E. Bruce, A. Boyle, S. Cousens, I. McConnell, J. Foster, W. Goldmann, H. Fraser, Strain characterization of natural sheep scrapie and comparison with BSE, J. Gen. Virol. 83 (2002) 695–704.

[24] C.I. Lasmezas, J.P. Deslys, R. Demaimay, K.T. Adjou, J.J. Hauw, D. Dormont, Strain specific and common pathogenic events in murine models of scrapie and bovine spongiform encephalopathy, J. Gen. Virol. 77 (1996) 1601–1609.

[25] H. Fraser, A.G. Dickinson, Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation, J. Comp. Pathol. 83 (1973) 29–40.

[26] M.R. Scott, R. Will, J. Ironside, H.O. Nguyen, P. Tremblay, S.J. DeArmond, S.B. Prusiner, Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 15137–15142.

[27] M.E. Bruce, R.G. Will, J.W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, C.J. Bostock, Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent, Nature 389 (1997) 498–501.

[28] M.E. Bruce, I. McConnell, H. Fraser, A.G. Dickinson, The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis, J. Gen. Virol. 72 (1991) 595–603.

[29] A.G. Dickinson, V.M. Meikle, H. Fraser, Identification of a gene which controls the incubation period of some strains of scrapie agent in mice, J. Comp. Pathol. 78 (1968) 293–299.

[30] G. Dell'Omo, E. Vannoni, A.L. Vyssotski, M.A. Di Bari, R. Nonno, U. Agrimi, H.P. Lipp, Early behavioural changes in mice infected with BSE and scrapie: automated home cage monitoring reveals prion strain differences, Eur. J. Neurosci. 16 (2002) 735–742.

[31] C. Cunningham, R.M. Deacon, K. Chan, D. Boche, J.N. Rawlins, V.H. Perry, Neuropathologically distinct prion strains give rise to similar temporal profiles of behavioral deficits, Neurobiol. Dis. 18 (2005) 258–269.

[32] R.A. Bessen, R.F. Marsh, Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent, J. Virol. 66 (1992) 2096–2101.

[33] J. Collinge, K.C. Sidle, J. Meads, J. Ironside, A.F. Hill, Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD, Nature 383 (1996) 685–690.

[34] P. Parchi, R. Castellani, S. Capellari, B. Ghetti, K. Young, S.G. Chen, M. Farlow, D.W. Dickson, A.A. Sima, J.Q. Trojanowski, R.B. Petersen, P. Gambetti, Molecular basis of phenotypic variability in sporadic Creutzfeldt–Jakob disease, Ann. Neurol. 39 (1996) 767–778.

[35] A. Khalili-Shirazi, L. Summers, J. Linehan, G. Mallinson, D. Anstee, S. Hawke, G.S. Jackson, J. Collinge, PrP glycoforms are associated in a strain-specific ratio in native PrPSc, J. Gen. Virol. 86 (2005) 2635–2644.

[36] J. Safar, H. Wille, V. Itri, D. Groth, H. Serban, M. Torchia, F.E. Cohen, S. B. Prusiner, Eight prion strains have PrP(Sc) molecules with different conformations, Nat. Med. 4 (1998) 1157–1165.

[37] J.D. Wadsworth, A.F. Hill, S. Joiner, G.S. Jackson, A.R. Clarke, J. Collinge, Strain-specific prion-protein conformation determined by metal ions, Nat. Cell Biol. 1 (1999) 55–59.

[38] B. Caughey, G.J. Raymond, R.A. Bessen, Strain-dependent differences in beta-sheet conformations of abnormal prion protein, J. Biol. Chem. 273 (1998) 32230–32235.

[39] P. Aucouturier, R.J. Kascsak, B. Frangione, T. Wisniewski, Biochemical and conformational variability of human prion strains in sporadic Creutzfeldt–Jakob disease, Neurosci. Lett. 274 (1999) 33–36.

[40] A. Bellon, W. Seyfert-Brandt, W. Lang, H. Baron, A. Groner, M. Vey, Improved conformation-dependent immunoassay: suitability for human prion detection with enhanced sensitivity, J. Gen. Virol. 84 (2003) 1921–1925.

[41] E.M. Jones, W.K. Surewicz, Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids, Cell 121 (2005) 63–72.

[42] M. Bruce, A. Chree, I. McConnell, J. Foster, G. Pearson, H. Fraser, Transmission of bovine spongiform encephalopathy and scrapie to mice:

strain variation and the species barrier, Philos. Trans. R. Soc. Lond., B Biol. Sci. 343 (1994) 405–411.
[43] H. Fraser, M.E. Bruce, A. Chree, I. McConnell, G.A. Wells, Transmission of bovine spongiform encephalopathy and scrapie to mice, J. Gen. Virol. 73 (1992) 1891–1897.
[44] T. Muramoto, T. Kitamoto, J. Tateishi, I. Goto, Successful transmission of Creutzfeldt–Jakob disease from human to mouse verified by prion protein accumulation in mouse brains, Brain Res. 599 (1992) 309–316.
[45] A.F. Hill, J. Collinge, Prion strains and species barriers, Contrib. Microbiol. 11 (2004) 33–49.
[46] D. Peretz, R.A. Williamson, G. Legname, Y. Matsunaga, J. Vergara, D.R. Burton, S.J. DeArmond, S.B. Prusiner, M.R. Scott, A change in the conformation of prions accompanies the emergence of a new prion strain, Neuron 34 (2002) 921–932.
[47] R.A. Moore, I. Vorberg, S.A. Priola, Species barriers in prion diseases–brief review, Arch. Virol., Suppl. (2005) 187–202.
[48] D.L. Vanik, K.A. Surewicz, W.K. Surewicz, Molecular basis of barriers for interspecies transmissibility of mammalian prions, Mol. Cell 14 (2004) 139–145.
[49] S.B. Prusiner, M. Scott, D. Foster, K.M. Pan, D. Groth, C. Mirenda, M. Torchia, S.L. Yang, D. Serban, G.A. Carlson, Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication, Cell 63 (1990) 673–686.
[50] S.G. Chen, P. Gambetti, A journey through the species barrier, Neuron 34 (2002) 854–856.
[51] P.G. Smith, R. Bradley, Bovine spongiform encephalopathy (BSE) and its epidemiology, Br. Med. Bull. 66 (2003) 185–198.
[52] J.G. Collee, R. Bradley, BSE: a decade on–Part I, Lancet 349 (1997) 636–641.
[53] J.G. Collee, R. Bradley, BSE: a decade on–Part 2, Lancet 349 (1997) 715–721.
[54] A.F. Hill, M. Desbruslais, S. Joiner, K.C. Sidle, I. Gowland, J. Collinge, L.J. Doey, P. Lantos, The same prion strain causes vCJD and BSE. Nature 389 (1997) 448–50, 526.
[55] J. Collinge, Variant Creutzfeldt–Jakob disease, Lancet 354 (1999) 317–323.
[56] A. Gombojav, I. Shimauchi, M. Horiuchi, N. Ishiguro, M. Shinagawa, T. Kitamoto, I. Miyoshi, S. Mohri, M. Takata, Susceptibility of transgenic mice expressing chimeric sheep, bovine and human PrP genes to sheep scrapie, J. Vet. Med. Sci. 65 (2003) 341–347.
[57] J.K. Kirkwood, A.A. Cunningham, Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles, Vet. Rec. 135 (1994) 296–303.
[58] G.R. Pearson, J.M. Wyatt, T.J. Gruffydd-Jones, J. Hope, A. Chong, R.J. Higgins, A.C. Scott, G.A. Wells, Feline spongiform encephalopathy: fibril and PrP studies, Vet. Rec. 131 (1992) 307–310.
[59] D.M. Taylor, S.L. Woodgate, Bovine spongiform encephalopathy: the causal role of ruminant-derived protein in cattle diets, Rev. Sci. Tech. 16 (1997) 187–198.
[60] N. Bons, N. Mestre-Frances, P. Belli, F. Cathala, D.C. Gajdusek, P. Brown, Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 4046–4051.
[61] S.J. Ryder, S.A. Hawkins, M. Dawson, G.A. Wells, The neuropathology of experimental bovine spongiform encephalopathy in the pig, J. Comp. Pathol. 122 (2000) 131–143.
[62] G.A. Wells, S.A. Hawkins, A.R. Austin, S.J. Ryder, S.H. Done, R.B. Green, I. Dexter, M. Dawson, R.H. Kimberlin, Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs, J. Gen. Virol. 84 (2003) 1021–1031.
[63] J. Castilla, A. Gutierrez-Adan, A. Brun, D. Doyle, B. Pintado, M.A. Ramirez, F.J. Salguero, B. Parra, S.S. Diaz, J.M. Sanchez-Vizcaino, M. Rogers, J.M. Torres, Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein, J. Neurosci. 24 (2004) 5063–5069.
[64] J.D. Foster, J. Hope, H. Fraser, Transmission of bovine spongiform encephalopathy to sheep and goats, Vet. Rec. 133 (1993) 339–341.
[65] M.J. Stack, M.J. Chaplin, J. Clark, Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies, Acta Neuropathol. (Berl) 104 (2002) 279–286.
[66] J.W. Wilesmith, G.A. Wells, M.P. Cranwell, J.B. Ryan, Bovine spongiform encephalopathy: epidemiological studies, Vet. Rec. 123 (1988) 638–644.
[67] J.W. Wilesmith, J.B. Ryan, M.J. Atkinson, Bovine spongiform encephalopathy: epidemiological studies on the origin, Vet. Rec. 128 (1991) 199–203.
[68] C.J. Sigurdson, M.W. Miller, Other animal prion diseases, Br. Med. Bull. 66 (2003) 199–212.
[69] E.S. Williams, Chronic wasting disease, Vet. Pathol. 42 (2005) 530–549.
[70] E.D. Belay, P. Gambetti, L.B. Schonberger, P. Parchi, D.R. Lyon, S. Capellari, J.H. McQuiston, K. Bradley, G. Dowdle, J.M. Crutcher, C.R. Nichols, Creutzfeldt–Jakob disease in unusually young patients who consumed venison, Arch. Neurol. 58 (2001) 1673–1678.
[71] R.F. Marsh, A.E. Kincaid, R.A. Bessen, J.C. Bartz, Interspecies transmission of chronic wasting disease prions to squirrel monkeys (*Saimiri sciureus*), J. Virol. 79 (2005) 13794–13796.
[72] A.N. Hamir, R.C. Cutlip, J.M. Miller, E.S. Williams, M.J. Stack, M.W. Miller, K.I. O'Rourke, M.J. Chaplin, Preliminary findings on the experimental transmission of chronic wasting disease agent of mule deer to cattle, J. Vet. Diagn. Invest. 13 (2001) 91–96.
[73] A.N. Hamir, R.A. Kunkle, R.C. Cutlip, J.M. Miller, K.I. O'Rourke, E.S. Williams, M.W. Miller, M.J. Stack, M.J. Chaplin, J.A. Richt, Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route, J. Vet. Diagn. Invest. 17 (2005) 276–281.
[74] A.N. Hamir, J.M. Miller, R.C. Cutlip, M.J. Stack, M.J. Chaplin, A.L. Jenny, E.S. Williams, Experimental inoculation of scrapie and chronic wasting disease agents in raccoons (Procyon lotor), Vet. Rec. 153 (2003) 121–123.
[75] Q. Kong, S. Huang, W. Zou, D. Vanegas, M. Wang, D. Wu, J. Yuan, M. Zheng, H. Bai, H. Deng, K. Chen, A.L. Jenny, K. O'Rourke, E.D. Belay, L.B. Schonberger, R.B. Petersen, M.S. Sy, S.G. Chen, P. Gambetti, Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models, J. Neurosci. 25 (2005) 7944–7949.
[76] C.I. Lasmezas, J.P. Deslys, O. Robain, A. Jaegly, V. Beringue, J.M. Peyrin, J.G. Fournier, J.J. Hauw, J. Rossier, D. Dormont, Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein, Science 275 (1997) 402–405.
[77] E.E. Manuelidis, E.J. Gorgacz, L. Manuelidis, Transmission of Creutzfeldt–Jakob disease with scrapie-like syndromes to mice, Nature 271 (1978) 778–779.
[78] J. Tateishi, Y. Sato, H. Nagara, J.W. Boellaard, Experimental transmission of human subacute spongiform encephalopathy to small rodents. IV. Positive transmission from a typical case of Gerstmann–Straussler–Scheinker's disease, Acta Neuropathol. (Berl) 64 (1984) 85–88.
[79] R.J. Kascsak, R. Rubenstein, P.A. Merz, R.I. Carp, N.K. Robakis, H.M. Wisniewski, H. Diringer, Immunological comparison of scrapie-associated fibrils isolated from animals infected with four different scrapie strains, J. Virol. 59 (1986) 676–683.
[80] R. Rubenstein, P.A. Merz, R.J. Kascsak, C.L. Scalici, M.C. Papini, R.I. Carp, R.H. Kimberlin, Scrapie-infected spleens: analysis of infectivity, scrapie-associated fibrils, and protease-resistant proteins, J. Infect. Dis. 164 (1991) 29–35.
[81] R.J. Kascsak, R. Rubenstein, P.A. Merz, R.I. Carp, H.M. Wisniewski, H. Diringer, Biochemical differences among scrapie-associated fibrils support the biological diversity of scrapie agents, J. Gen. Virol. 66 (1985) 1715–1722.
[82] G. Legname, H.O. Nguyen, I.V. Baskakov, F.E. Cohen, S.J. DeArmond, S.B. Prusiner, Strain-specified characteristics of mouse synthetic prions, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 2168–2173.
[83] T. Baron, C. Crozet, A.G. Biacabe, S. Philippe, J. Verchere, A. Bencsik, J. Y. Madec, D. Calavas, J. Samarut, Molecular analysis of the protease-resistant prion protein in scrapie and bovine spongiform encephalopathy

transmitted to ovine transgenic and wild-type mice, J. Virol. 78 (2004) 6243–6251.

[84] A.M. Thackray, M.A. Klein, R. Bujdoso, Subclinical prion disease induced by oral inoculation, J. Virol. 77 (2003) 7991–7998.

[85] J. Tateishi, M. Ohta, M. Koga, Y. Sato, Y. Kuroiwa, Transmission of chronic spongiform encephalopathy with kuru plaques from humans to small rodents, Ann. Neurol. 5 (1979) 581–584.

[86] J. Collinge, F. Owen, M. Poulter, M. Leach, T.J. Crow, M.N. Rossor, J. Hardy, M.J. Mullan, I. Janota, P.L. Lantos, Prion dementia without characteristic pathology, Lancet 336 (1990) 7–9.

[87] R. Medori, P. Montagna, H.J. Tritschler, A. LeBlanc, P. Cortelli, P. Tinuper, E. Lugaresi, P. Gambetti, Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178, Neurology 42 (1992) 669–670.

[88] S.E. Lloyd, S.R. Thompson, J.A. Beck, J.M. Linehan, J.D. Wadsworth, S. Brandner, J. Collinge, E.M. Fisher, Identification and characterization of a novel mouse prion gene allele, Mamm. Genome 15 (2004) 383–389.

[89] R.A. Bessen, R.F. Marsh, Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy, J. Virol. 68 (1994) 7859–7868.

[90] R.A. Bessen, D.A. Kocisko, G.J. Raymond, S. Nandan, P.T. Lansbury, B. Caughey, Non-genetic propagation of strain-specific properties of scrapie prion protein, Nature 375 (1995) 698–700.

[91] A.G. Dickinson, V.M. Meikle, Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent, Mol. Gen. Genet. 112 (1971) 73–79.

[92] R.I. Carp, R.C. Moretz, M. Natelli, A.G. Dickinson, Genetic control of scrapie: incubation period and plaque formation in I mice, J. Gen. Virol. 68 (1987) 401–407.

[93] G.A. Carlson, D.T. Kingsbury, P.A. Goodman, S. Coleman, S.T. Marshall, S. DeArmond, D. Westaway, S.B. Prusiner, Linkage of prion protein and scrapie incubation time genes, Cell 46 (1986) 503–511.

[94] N. Hunter, J.C. Dann, A.D. Bennett, R.A. Somerville, I. McConnell, J. Hope, Are Sinc and the PrP gene congruent? Evidence from PrP gene analysis in Sinc congenic mice, J. Gen. Virol. 73 (1992) 2751–2755.

[95] S.L. Benestad, P. Sarradin, B. Thu, J. Schonheit, M.A. Tranulis, B. Bratberg, Cases of scrapie with unusual features in Norway and designation of a new type, Nor98, Vet. Rec. 153 (2003) 202–208.

[96] J.D. Foster, D. Parnham, A. Chong, W. Goldmann, N. Hunter, Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats, Vet. Rec. 148 (2001) 165–171.

[97] M.A. Tranulis, A. Osland, B. Bratberg, M.J. Ulvund, Prion protein gene polymorphisms in sheep with natural scrapie and healthy controls in Norway, J. Gen. Virol. 80 (1999) 1073–1077.

[98] J. Collinge, M.S. Palmer, A.J. Dryden, Genetic predisposition to iatrogenic Creutzfeldt–Jakob disease, Lancet 337 (1991) 1441–1442.

[99] M.S. Palmer, A.J. Dryden, J.T. Hughes, J. Collinge, Homozygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease, Nature 352 (1991) 340–342.

[100] H.S. Lee, P. Brown, L. Cervenakova, R.M. Garruto, M.P. Alpers, D.C. Gajdusek, L.G. Goldfarb, Increased susceptibility to Kuru of carriers of the PRNP 129 methionine/methionine genotype, J. Infect. Dis. 183 (2001) 192–196.

[101] S. Mead, M.P. Stumpf, J. Whitfield, J.A. Beck, M. Poulter, T. Campbell, J.B. Uphill, D. Goldstein, M. Alpers, E.M. Fisher, J. Collinge, Balancing selection at the prion protein gene consistent with prehistoric kurulike epidemics, Science 300 (2003) 640–643.

[102] J.J. Hauw, V. Sazdovitch, J.L. Laplanche, K. Peoc'h, N. Kopp, J. Kemeny, N. Privat, N. Delasnerie-Laupretre, J.P. Brandel, J.P. Deslys, D. Dormont, A. Alperovitch, Neuropathologic variants of sporadic Creutzfeldt–Jakob disease and codon 129 of PrP gene, Neurology 54 (2000) 1641–1646.

[103] P. Parchi, A. Giese, S. Capellari, P. Brown, W. Schulz-Schaeffer, O. Windl, I. Zerr, H. Budka, N. Kopp, P. Piccardo, S. Poser, A. Rojiani, N. Streichemberger, J. Julien, C. Vital, B. Ghetti, P. Gambetti, H. Kretzschmar, Classification of sporadic Creutzfeldt–Jakob disease based on molecular and phenotypic analysis of 300 subjects, Ann. Neurol. 46 (1999) 224–233.

[104] L.G. Goldfarb, R.B. Petersen, M. Tabaton, P. Brown, A.C. LeBlanc, P. Montagna, P. Cortelli, J. Julien, C. Vital, W.W. Pendelbury, Fatal familial insomnia and familial Creutzfeldt–Jakob disease: disease phenotype determined by a DNA polymorphism, Science 258 (1992) 806–808.

[105] A.F. Hill, S. Joiner, J.D. Wadsworth, K.C. Sidle, J.E. Bell, H. Budka, J.W. Ironside, J. Collinge, Molecular classification of sporadic Creutzfeldt–Jakob disease, Brain 126 (2003) 1333–1346.

[106] J.P. Brandel, M. Preece, P. Brown, E. Croes, J.L. Laplanche, Y. Agid, R. Will, A. Alperovitch, Distribution of codon 129 genotype in human growth hormone-treated CJD patients in France and the UK, Lancet 362 (2003) 128–130.

[107] M.T. Bishop, P. Hart, L. Aitchison, H.N. Baybutt, C. Plinston, V. Thomson, N.L. Tuzi, M.W. Head, J.W. Ironside, R.G. Will, J.C. Manson, Predicting susceptibility and incubation time of human-to-human transmission of vCJD, Lancet Neurol. 5 (2006) 393–398.

[108] A. Aguzzi, Prion diseases of humans and farm animals: epidemiology, genetics, and pathogenesis, J. Neurochem. 97 (2006) 1726–1739.

[109] J. Collinge, J. Beck, T. Campbell, K. Estibeiro, R.G. Will, Prion protein gene analysis in new variant cases of Creutzfeldt–Jakob disease, Lancet 348 (1996) 56.

[110] M. Glatzel, K. Stoeck, H. Seeger, T. Luhrs, A. Aguzzi, Human prion diseases: molecular and clinical aspects, Arch. Neurol. 62 (2005) 545–552.

[111] P. Gambetti, Q. Kong, W. Zou, P. Parchi, S.G. Chen, Sporadic and familial CJD: classification and characterisation, Br. Med. Bull. 66 (2003) 213–239.

[112] P. Parchi, W. Zou, W. Wang, P. Brown, S. Capellari, B. Ghetti, N. Kopp, W.J. Schulz-Schaeffer, H.A. Kretzschmar, M.W. Head, J.W. Ironside, P. Gambetti, S.G. Chen, Genetic influence on the structural variations of the abnormal prion protein, Proc. Natl. Acad. Sci. U. S. A 97 (2000) 10168–10172.

[113] G. Schoch, H. Seeger, J. Bogousslavsky, M. Tolnay, R.C. Janzer, A. Aguzzi, M. Glatzel, Analysis of prion strains by PrP(Sc) profiling in sporadic Creutzfeldt–Jakob disease, PLoS Med. 3 (2005) e14.

[114] G. Puoti, G. Rossi, G. Giaccone, T. Awan, P.M. Lievens, C.A. Defanti, F. Tagliavini, O. Bugiani, Polymorphism at codon 129 of PRNP affects the phenotypic expression of Creutzfeldt–Jakob disease linked to E200K mutation, Ann. Neurol. 48 (2000) 269–270.

[115] M. Bianca, S. Bianca, I. Vecchio, R. Raffaele, C. Ingegnosi, F. Nicoletti, Gerstmann–Straussler–Scheinker disease with P102L-V129 mutation: a case with psychiatric manifestations at onset, Ann. Genet. 46 (2003) 467–469.

[116] P. Montagna, P. Cortelli, P. Avoni, P. Tinuper, G. Plazzi, R. Gallassi, F. Portaluppi, J. Julien, C. Vital, M.B. Delisle, P. Gambetti, E. Lugaresi, Clinical features of fatal familial insomnia: phenotypic variability in relation to a polymorphism at codon 129 of the prion protein gene, Brain Pathol. 8 (1998) 515–520.

[117] R.B. Petersen, L.G. Goldfarb, M. Tabaton, P. Brown, L. Monari, P. Cortelli, P. Montagna, L. Autilio-Gambetti, D.C. Gajdusek, E. Lugaresi, A novel mechanism of phenotypic heterogeneity demonstrated by the effect of a polymorphism on a pathogenic mutation in the PRNP (prion protein gene), Mol. Neurobiol. 8 (1994) 99–103.

[118] P. Cortelli, P. Gambetti, P. Montagna, E. Lugaresi, Fatal familial insomnia: clinical features and molecular genetics, J. Sleep Res. 8 (Suppl. 1) (1999) 23–29.

[119] J.A. Hainfellner, P. Parchi, T. Kitamoto, C. Jarius, P. Gambetti, H. Budka, A novel phenotype in familial Creutzfeldt–Jakob disease: prion protein gene E200K mutation coupled with valine at codon 129 and type 2 protease-resistant prion protein, Ann. Neurol. 45 (1999) 812–816.

[120] S. Liemann, R. Glockshuber, Influence of amino acid substitutions related to inherited human prion diseases on the thermodynamic stability of the cellular prion protein, Biochemistry 38 (1999) 3258–3267.

[121] R. Zahn, A. Liu, T. Luhrs, R. Riek, C. von Schroetter, G.F. Lopez, M. Billeter, L. Calzolai, G. Wider, K. Wuthrich, NMR solution structure of the human prion protein, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 145–150.

[122] B.S. Wong, C. Clive, S.J. Haswell, R.A. Williamson, D.R. Burton, P. Gambetti, M.S. Sy, I.M. Jones, D.R. Brown, Copper has differential effect on prion protein with polymorphism of position 129, Biochem. Biophys. Res. Commun. 269 (2000) 726–731.

[123] C. Petchanikow, G.P. Saborio, L. Anderes, M.J. Frossard, M.I. Olmedo, C. Soto, Biochemical and structural studies of the prion protein polymorphism, FEBS Lett. 509 (2001) 451–456.

[124] E.R. Mulcahy, R.A. Bessen, Strain-specific kinetics of prion protein formation in vitro and in vivo, J. Biol. Chem. 279 (2004) 1643–1649.

[125] G. Puoti, G. Giaccone, G. Rossi, B. Canciani, O. Bugiani, F. Tagliavini, Sporadic Creutzfeldt–Jakob disease: co-occurrence of different types of PrP(Sc) in the same brain, Neurology 53 (1999) 2173–2176.

[126] M. Pocchiari, M. Puopolo, E.A. Croes, H. Budka, E. Gelpi, S. Collins, V. Lewis, T. Sutcliffe, A. Guilivi, N. Delasnerie-Laupretre, J.P. Brandel, A. Alperovitch, I. Zerr, S. Poser, H.A. Kretzschmar, A. Ladogana, I. Rietvald, E. Mitrova, P. Martinez-Martin, J. Pedro-Cuesta, M. Glatzel, A. Aguzzi, S. Cooper, J. Mackenzie, C.M. van Duijn, R.G. Will, Predictors of survival in sporadic Creutzfeldt–Jakob disease and other human transmissible spongiform encephalopathies, Brain 127 (2004) 2348–2359.

[127] P. Brown, P. Rodgers-Johnson, F. Cathala, C.J. Gibbs Jr., D.C. Gajdusek, Creutzfeldt–Jakob disease of long duration: clinicopathological characteristics, transmissibility, and differential diagnosis, Ann. Neurol. 16 (1984) 295–304.

[128] A.G. Dickinson, H. Fraser, G.W. Outram, Scrapie incubation time can exceed natural lifespan, Nature 256 (1975) 732–733.

[129] R.H. Kimberlin, C.A. Walker, Competition between strains of scrapie depends on the blocking agent being infectious, Intervirology 23 (1985) 74–81.

[130] J.C. Bartz, J.M. Aiken, R.A. Bessen, Delay in onset of prion disease for the HY strain of transmissible mink encephalopathy as a result of prior peripheral inoculation with the replication-deficient DY strain, J. Gen. Virol. 85 (2004) 265–273.

[131] C. Weissmann, A. Aguzzi, Approaches to therapy of prion diseases, Annu. Rev. Med. 56 (2005) 321–344.

[132] C. Soto, R.J. Kascsak, G.P. Saborio, P. Aucouturier, T. Wisniewski, F. Prelli, R. Kascsak, E. Mendez, D.A. Harris, J. Ironside, F. Tagliavini, R. I. Carp, B. Frangione, Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides, Lancet 355 (2000) 192–197.

[133] D. Peretz, R.A. Williamson, K. Kaneko, J. Vergara, E. Leclerc, G. Schmitt-Ulms, I.R. Mehlhorn, G. Legname, M.R. Wormald, P.M. Rudd, R.A. Dwek, D.R. Burton, S.B. Prusiner, Antibodies inhibit prion propagation and clear cell cultures of prion infectivity, Nature 412 (2001) 739–743.

Apéndice 4

Are amyloids infectious?

Rodrigo Morales[1,2], Baian Chen[1,3] and Claudio Soto[1]*

1 Protein Misfolding Disorders Laboratory, George and Cynthia Mitchell Center for

Neurodegenerative diseases, Department of Neurology, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas, 77555, USA.

2 Facultad de Ciencias, Universidad de Chile, Santiago, Chile

3 National Animal TSE Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China

* To whom correspondence should be addressed at clsoto@utmb.edu.

Abstract

Misfolding and aggregation of proteins is the main feature of a group of maladies which include most of neurodegenerative diseases (such as Alzheimer's, Parkinson's, Huntington's and prion diseases), as well as several systemic amyloidosis. Among them, prion disease is the only one known to be infectious. Current evidence shows that the infectious agent in prion diseases is the misfolded protein and that the molecular mechanisms responsible for transmissibility is very similar to the process of amyloid formation in all protein misfolding disorders. In this chapter we discuss the theoretical and experimental evidences suggesting the possible infectious nature of several protein misfolding disorders.

Introduction

DNA's linear code is translated into the three dimensional information of proteins. This information is dictated by a proper folding process which depends on both the primary sequence of the polypeptide chain and the folding machinery present within the cell. Abnormal folding of proteins can lead to many pathological processes including loss of function or gain of toxic activity, which usually result from the accumulation of misfolded and aggregated proteins in specific tissues [1-3]. Protein Misfolding Disorders (PMDs) are a group of pathological conditions which include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), diabetes type-2, systemic amyloidosis and prion diseases, among others [1-3]. A list of the diseases and proteins involved in PMDs is shown in table 1.

It has been suggested that tissue deposition of misfolded protein aggregates might be responsible for the activation of cell impairment and death, leading subsequently to clinical symptoms in affected individuals. The insidious clinical symptoms, the progressive nature of the illness and the lack of efficient therapeutic treatments for these diseases lead to severe problems for the quality of life of affected people and their families in both social and economic aspects. The expenses for the treatment and care of patients are very high and these costs progressively increase due to the severity of the disease. In addition, it is expected that the number of people that will be affected by these maladies will increase at a high rate during the coming years. Moreover, the lack of early diagnostic methods or

effective treatments paints a bleak scenario for the future. For these reasons it is urgent to move forward in trying to understand the mechanisms involved in the origin and development of these diseases. Exacerbating this state of affairs is a new hypothesis proposing that misfolded proteins could be infectious [4, 5]. In this chapter we will discuss experimental evidence suggesting that PMDs could have an infectious origin, in a similar manner that is occurring for the transmissibility of prion diseases.

Common features of protein misfolding disorders

In spite of the important differences in clinical manifestation, PMDs share some common features such as their appearance late in life, the progressive and chronic nature of the disease and the presence of deposits of misfolded protein aggregates [6]. These deposits are a typical disease signature and although in each disease the main protein component is different (Table 1), they have similar morphological, structural and staining characteristics. Amyloid is the name originally given to extracellular protein deposits found in AD and systemic amyloid disorders [7], but it is nowadays used to refer in general to disease-associated protein aggregates [6]. All protein aggregates share similar structural and biochemical characteristics. Structurally, misfolding of proteins increases the level of β-sheet structure, leading to the formation of amyloid polymers organized as cross-β structures [8]. As a consequence, protein aggregates are resistant to proteolysis, denaturation and general cellular clearance mechanisms.

The cellular factors and processes leading to the misfolded conformation have been partially identified. Among them, several mutations destabilizing the folded conformation and promoting its shift to the misfolded form have been identified in each protein [9]. These mutations usually result in dominant inheritance of the disease. Nevertheless, familial forms of the disease represent a small percentage of the total incidence of PMDs, and the majority of the cases have a sporadic origin. The discovery of mutations has led the development of transgenic animal models over-expressing mutated proteins, where aggregates accumulation and cellular impairments occur in a similar form as in the human disease [10].

In spite of the key role of misfolded proteins in the disease, the mechanisms leading to cellular damage and tissue dysfunction are still unclear. The toxicity of protein aggregates has been extensively documented *in vitro* and *in vivo* [3, 11, 12] However it is still not clear which type of aggregates are the most toxic species. Recent evidence supports the hypothesis that smaller and soluble oligomeric aggregates on pathway to form the large fibrillar deposits could be the molecules mostly responsible for the toxic effects observed in these maladies [12-14]. Fibers could be acting as a protective mechanism in order to trap these particles and encapsulate them in tissue.

Prion diseases: The bizarre infectious member of the PMD group

Prion diseases, or Transmissible Spongiform Encephalopathies (TSEs), are a group of rare, fatal neurodegenerative diseases, affecting humans and several spe-

cies of mammals [15]. The most prevalent form of human TSE is Creutzfeldt-Jakob disease (CJD) and the most common animal prion disease is scrapie affecting sheep. However, the most worrisome TSEs are the new diseases, variant CJD in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids. According to how they arise, prion diseases can be classified as sporadic, familial or infectious. Transmission of prion diseases was first reported in 1937 when sheep were inoculated with a vaccine prepared from formalin-treated sheep brain [16]. Since then, the search for the TSE infectious agent was (and still is) a matter of great effort from many investigators worldwide [17]. At this time, experimental evidence points out that the only component of the infectious agent in TSEs is the misfolded prion protein (referred as PrP^{Sc}) [17-19]. Highly purified preparations of PrP^{Sc} are infectious and the quantity of this protein correlates very well with infectivity [20]. Supporting this information, transgenic animals lacking the normal version of the prion protein (PrP^{C}) are completely resistant to infection, whereas animals over-expressing this protein are more susceptible [21, 22].

Prion diseases have been identified and transmitted to several animal species. The development of transgenic mice expressing PrP from different TSE sensitive mammals has helped to understand prion propagation processes among relevant species such as sheep, cattle, cervids, pigs and humans [23]. Using these models, several features of the pathology have been studied. One interesting example is related with the transmission of disease to animal models by inoculation with dif-

ferent tissues and body fluids. The presence of the infectious material in body fluids presents an alarming scenario.

The central concept in the prion hypothesis is that PrP^{Sc} is the only component of the infectious agent, which can "replicate" in the brain in the absence of nucleic acid by converting the natively-folded PrP^{C} into the misfolded form [18, 19]. Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^{C}, catalyzing its conversion to the pathogenic form of the protein. The precise molecular mechanism of $PrP^{C} \rightarrow PrP^{Sc}$ conversion is not well understood. However, the available data support the seeding/nucleation model in which infectious PrP^{Sc} is an oligomer that acts as a seed to bind PrP^{C} and catalyze its conversion into the misfolded form by incorporation into the growing polymer (Fig. 1) [24, 25]. In this model, in the absence of seeds the spontaneous PrP conversion process will be thermodynamically unfavorable and kinetically very slow, which could account for the extremely low incidence of sporadic TSEs. However, the addition of enough seeds by infection leads to disease in 100% of the cases.

The recent generation of infectious prions *in vitro* has been an excellent support for the protein-only hypothesis of prion misfolding and replication. Recently, Legname *et al.* showed the generation of infectious prions in the test tube. In this study, they used a specially folded fragment (89-230) of the recombinant mouse prion protein [26]. The final folded product results in similar biochemical properties to PrP^{Sc} as assessed by resistance to proteases and insolubility. This preparation was inoculated in transgenic animals over expressing PrP and the animals de-

veloped a spongiform encephalopathy characterized by long incubation periods and the presence of proteinase-K resistant PrP. Although this report provides interesting data, several problems with their experimental design limits its scope as the final proof for the prion hypothesis. Using a different strategy, our group has developed an *in vitro* system to replicate prions. This technique, termed Protein Misfolding Cyclic Amplification (PMCA) is able to replicate *in vitro* the misfolded form of PrP^{Sc} at expenses of the normally produced PrP^{C} [27, 28]. This assay, based on the seeding/nucleation model, is able to generate large amounts of infectious material using a small amount of starting PrP^{Sc}. Experimentally, this process is performed by mixing minute amounts of infectious prions in brain homogenate from healthy animals. Using this technique we reported the successful amplification of hamster prions [27, 28]. By serial dilutions of the *in vitro* generated material in healthy brain homogenates we were able to dilute out the original inoculum obtaining only *in vitro* generated prions. Biochemical and structural analysis of the newly generated misfolded proteins showed similar characteristics to the original material. Importantly, *in vivo* challenge of this preparation to wild type hamsters showed that these prions were infectious producing a similar disease compared to the original prions [28].

Are other protein misfolding disorders infectious?

The misfolding and aggregation pathway, its mechanism and structural intermediates are very similar in all PMDs, including TSEs [4, 5]. The mechanism by which amyloid is formed is similar to the process of prion replication. The

seeding/nucleation model accounts for the generation of infectious PrPSc and for the formation of amyloid fibrils. *In vitro* experiments have shown that most PMDs associated proteins form aggregates with kinetic features of nucleated polymerization [29, 30]. The seeding-nucleation model provides a rationale and plausible explanation for the infectious nature of prions and suggests that protein misfolding processes as those associated with several human diseases have the inherent ability to be transmissible [4, 5]. Infectivity lies on the capacity of preformed stable misfolded oligomeric proteins to act as a seed to catalyze the misfolding and aggregation process (Fig. 1) [4, 5, 31]. The acceleration of protein aggregation by addition of seeds has been convincingly reported *in vitro* for several proteins implicated in diverse PMDs [6, 32, 33]. Extrapolating the *in vitro* results to the *in vivo* situation, the correct administration of pre-aggregated, stable misfolded structures should substantially accelerate the misfolding, aggregation and tissue accumulation of the protein. Provided that protein misfolding and aggregation is the cause of the disease, this should lead to the acceleration of a pathogenic process that in the absence of the seed was set to occur much later in life or not at all during the life-span of the individual.

Considering the knowledge gained in TSEs, it is likely that infection by misfolded proteins will produce a very long incubation period between the times of infection and the manifestation of clinical disease. For this reason, a possible infectious origin would be very hard to identify. The recognition of TSEs as infectious was possible principally due to a couple of fortuitous events: the use of contaminated material to treat a specific flock of sheep in the 1930's [16] and the

discovery of Kuru transmission by cannibalistic rituals [34]. The rare prevalence of TSEs makes it easy to identify an isolated infectious event. However, in more frequent diseases, such as Alzheimer's, Parkinson's or diabetes, it would be difficult to associate disease with a possible infectious exposure that occurred decades before. Nevertheless, the mechanistic similarities between TSEs and other PMDs have prompted investigators to search for this possibility [4, 5, 35]. As a result of these studies, there are several pieces of evidence in favor and against the transmissibility of misfolding related disorders. Next, we will discuss some of these studies in three PMDs where the literature is most abundant.

Alzheimer's disease: The idea that AD can be infectious has been around for a long time. The prime approaches to prove this hypothesis consisted in the transmission of TSE by injecting brain homogenates from AD patients to non-human primates; however the results of these experiments were conflicting, with some of them positive [36] and some negative [37]. One possible reason for this is the low number of primates used for these studies. In addition, a possible "species barrier" effect, similar to what is observed in prions [38], could also be responsible for the conflicting results. Altogether, the limiting number of animals tested and the lack of knowledge about prion related transmission at the time the experiments were performed suggest that these studies should be repeated using optimal experimental conditions.

With the emergence of transgenic mice models expressing the human amyloid protein and developing some of the neuropathological, biochemical and clinical

characteristics of AD [39] the search for a possible infectious origin for the disease took a new breath. Recently, Kane *et al.* has shown that the inoculation of brain homogenates from AD patients to the Tg2576 transgenic model can accelerate the appearance of amyloid plaques [40, 41]. In these studies, unilateral inoculation of AD preparations accelerated the aggregation process 5 months post-inoculation, but only in the inoculated hemisphere [40]. After 12 months, experimental animals presented Aβ accumulation in both hemispheres but the Aβ load was clearly greater in the injected one [41]. A followed up study reported that the seeding activity of brain extracts was reduced or abolished by Aß immunodepletion, protein denaturation, or by Aß immunization [42]. Interestingly, the phenotype of the exogenously induced amyloidosis depended on both the characteristics of the host and the source of the agent. These findings indicate that AD brain preparations can accelerate the aggregation process. However, since spontaneous accumulation of Aβ occurs in these animals at a later time it is not possible to state that AD misfolded aggregates are infectious, but just accelerating a process that was set to occur because of the genetic manipulation of the mice.

Reactive systemic amyloidosis: Reactive systemic amyloidosis is a potential complication of any disorder that gives rise to a sustained acute-phase inflammatory response [43]. The list of chronic inflammatory, infective or neoplastic disorders that can underlie it is almost without limit. The prevalence of amyloid deposition in patients with chronic inflammatory diseases is 3.6–5.8%, being the most common rheumatoid arthritis [44]. The protein deposited in this disease is called

amyloid-A (AA) and is derived from cleavage fragments of the circulating acute-phase reactant serum amyloid-A protein (SAA) [45]. SAA is an apolipoprotein of high-density lipoprotein which is synthesized by hepatocytes. The molecular weight is 11.4-12.5 kDa in different species under the transcriptional regulation of cytokines. SAA is usually at a low concentration in the plasma (~20mg/L) but its concentration can increase up to a 1000-fold under inflammatory conditions [46]. Although AA amyloidosis can develop rapidly, the median latency between presentation with a chronic inflammatory disorder and clinically significant amyloidosis is almost two decades. AA aggregates can be found in tissues such as liver, spleen and kidneys [47]. It has been found that experimental induction of inflammation followed by challenge with tissue homogenates containing AA seeds can produce a clear shortening in the lag phase of protein accumulation compared with non AA seed treated animals [48]. It has been established that the active principles of these preparations, referred to as Amyloid Enhancing Factor (AEF), are indeed the misfolded units of AA [49]. As in prion diseases, the oral challenge of AEF in minute amounts results in a decrease in the AA deposition time suggesting AEF is very resistant to degradation and elimination. Interestingly, AEFs propagating properties are also completely abolished after treatment with denaturing agents [50]. While AEF shares many properties with prions, its properties, however, as an "enhancer" and not transmissible factor *per se* make it difficult to consider AA aggregates as a bona-fide infectious agent.

Mouse senile amyloidosis: The accumulation of apolipoprotein AII (apoAII) in some strains of old mice leads to senile amyloidosis [51]. Young animals do not accumulate apoAII fibrils, but progressively with age animals begin to accumulate misfolded apoAII aggregates in diverse organs during aging [52]. It has been reported that inoculation of preformed apoAII seeds lead to the aggregation of this protein in young mice [53]. In contrast, no effects were observed after the administration of denatured apoAII preparations [54]. The "infective" effects of these aggregates were also reported after oral challenge [55]. Surprisingly, the fact that apoAII aggregates are present in the feces of the affected animals suggests a possible mechanism for the transmission of the disease by this route [55]. In order to answer this question, untreated animals were housed in the same cage with apoAII affected mice. It was found that untreated mice generated amyloidosis at early stages, just by sharing the cage with old animals. The oral transmission via feces was suggested because p.o. administration of feces from old animals into young animals induced disease [55]. Moreover, transmission of apoAII amyloidosis exhibits a "strain phenomenon" analogous to the prion strains [56]. This data suggests that apoAII misfolded aggregates might be really a prion-type of infectious agent.

Acknowledgments

We thank Dr. Kristi Green for critical review of the manuscript. This manuscript was supported by a grant from the CART Foundation to Claudio Soto

Reference List

[1] Soto, C. (2001) Protein misfolding and disease; protein refolding and therapy. FEBS Lett. 498, 204-207.

[2] Chiti, F. and Dobson, C.M. (2006) Protein misfolding, functional amyloid, and human disease. Annu. Rev. Biochem. 75, 333-366.

[3] Carrell, R.W. (2005) Cell toxicity and conformational disease. Trends Cell Biol. 15, 574-580.

[4] Soto, C., Estrada, L. and Castilla, J. (2006) Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. Trends Biochem. Sci. 31, 150-155.

[5] Walker, L.C., Levine, H., III, Mattson, M.P. and Jucker, M. (2006) Inducible proteopathies. Trends Neurosci.

[6] Soto, C. (2003) Unfolding the role of protein misfolding in neurodegenerative diseases. Nature Reviews Neuroscience 4, 49-60.

[7] Glenner, G.G. (1980) Amyloid deposits and amyloidosis. The beta-fibrilloses (first of two parts). N. Engl. J. Med. 302, 1283-1292.

[8] Blake, C.C., Serpell, L.C., Sunde, M., Sandgren, O. and Lundgren, E. (1996) A molecular model of the amyloid fibril. Ciba Found. Symp. 199, 6-15.

[9] Hardy, J. and Gwinn-Hardy, K. (1998) Genetic classification of primary neurodegenerative disease. Science 282, 1075-1079.

[10] Rockenstein, E., Crews, L. and Masliah, E. (2007) Transgenic animal models of neurodegenerative diseases and their application to treatment development. Adv. Drug Deliv. Rev. 59, 1093-1102.

[11] Canevari, L., Abramov, A.Y. and Duchen, M.R. (2004) Toxicity of amyloid beta peptide: tales of calcium, mitochondria, and oxidative stress. Neurochem. Res. 29, 637-650.

[12] Haass, C. and Selkoe, D.J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nature Reviews Molecular Cell Biology 8, 101-112.

[13] Glabe, C.G. (2006) Common mechanisms of amyloid oligomer pathogenesis in degenerative disease. Neurobiol. Aging 27, 570-575.

[14] Caughey, B. and Lansbury, P.T. (2003) Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. Annu. Rev. Neurosci. 26, 267-298.

[15] Collinge, J. (2001) Prion diseases of humans and animals: their causes and molecular basis. Annu. Rev. Neurosci. 24, 519-550.

[16] Cullie, J. and Chelle, P.L. (1939) Experimental transmission of trembling to the goat. Comptes Rendus des Seances de l'Academie des Sciences 208, 1058-1160.

[17] Aguzzi, A. and Polymenidou, M. (2004) Mammalian prion biology: one century of evolving concepts. Cell 116, 313-327.

[18] Soto, C. and Castilla, J. (2004) The controversial protein-only hypothesis of prion propagation. Nat. Med. 10, S63-S67.

[19] Prusiner, S.B. (1998) Prions. Proc. Natl. Acad. Sci. U. S. A 95, 13363-13383.

[20] Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. Science 216, 136-144.

[21] Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. Cell 73, 1339-1347.

[22] Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. 15, 1255-1264.

[23] Groschup, M.H. and Buschmann, A. (2008) Rodent models for prion diseases. Vet. Res. 39, 32.

[24] Caughey, B. (2003) Prion protein conversions: insight into mechanisms, TSE transmission barriers and strains. Br. Med. Bull. 66, 109-120.

[25] Soto, C., Saborio, G.P. and Anderes, L. (2002) Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. Trends Neurosci. 25, 390-394.

[26] Legname, G., Baskakov, I.V., Nguyen, H.O., Riesner, D., Cohen, F.E., DeArmond, S.J. and Prusiner, S.B. (2004) Synthetic mammalian prions. Science 305, 673-676.

[27] Saborio, G.P., Permanne, B. and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411, 810-813.

[28] Castilla, J., Saá, P., Hetz, C. and Soto, C. (2005) In vitro generation of infectious scrapie prions. Cell 121, 195-206.

[29] Jarrett, J.T. and Lansbury, P.T., Jr. (1993) Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? Cell 73, 1055-1058.

[30] Walker, L.C., Bian, F., Callahan, M.J., Lipinski, W.J., Durham, R.A. and LeVine, H. (2002) Modeling Alzheimer's disease and other proteopathies in vivo: is seeding the key? Amino. Acids 23, 87-93.

[31] Gajdusek, D.C. (1994) Nucleation of amyloidogenesis in infectious and noninfectious amyloidoses of brain. Ann. N. Y. Acad. Sci. 724, 173-190.

[32] Harper, J.D. and Lansbury, P.T., Jr. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. Annu. Rev. Biochem. 66, 385-407.

[33] Krebs, M.R., Morozova-Roche, L.A., Daniel, K., Robinson, C.V. and Dobson, C.M. (2004) Observation of sequence specificity in the seeding of protein amyloid fibrils. Protein Sci. 13, 1933-1938.

[34] Gajdusek, D.C., Gibbs, C.J. and Alpers, M. (1966) Experimental transmission of a Kuru-like syndrome to chimpanzees. Nature 209, 794-796.

[35] Sigurdsson, E.M., Wisniewski, T. and Frangione, B. (2002) Infectivity of amyloid diseases. Trends Mol. Med. 8, 411-413.

[36] Baker, H.F., Ridley, R.M., Duchen, L.W., Crow, T.J. and Bruton, C.J. (1994) Induction of beta (A4)-amyloid in primates by injection of Alzheimer's disease brain homogenate. Comparison with transmission of spongiform encephalopathy. Mol. Neurobiol. 8, 25-39.

[37] Goudsmit, J., Morrow, C.H., Asher, D.M., Yanagihara, R.T., Masters, C.L., Gibbs, C.J., Jr. and Gajdusek, D.C. (1980) Evidence for and against the transmissibility of Alzheimer disease. Neurology 30, 945-950.

[38] Morales, R., Abid, K. and Soto, C. (2007) The prion strain phenomenon: molecular basis and unprecedented features. Biochim. Biophys. Acta 1772, 681-691.

[39] Duff, K. (2001) Transgenic mouse models of Alzheimer's disease: phenotype and mechanisms of pathogenesis. Biochem. Soc. Symp., 195-202.

[40] Kane, M.D., Lipinski, W.J., Callahan, M.J., Bian, F., Durham, R.A., Schwarz, R.D., Roher, A.E. and Walker, L.C. (2000) Evidence for seeding of beta -amyloid by intracerebral infusion of Alzheimer brain extracts in beta -amyloid precursor protein-transgenic mice. J. Neurosci. 20, 3606-3611.

[41] Walker, L.C., Callahan, M.J., Bian, F., Durham, R.A., Roher, A.E. and Lipinski, W.J. (2002) Exogenous induction of cerebral beta-amyloidosis in betaAPP-transgenic mice. Peptides 23, 1241-1247.

[42] Meyer-Luehmann, M., Coomaraswamy, J., Bolmont, T., Kaeser, S., Schaefer, C., Kilger, E., Neuenschwander, A., Abramowski, D., Frey, P., Jaton, A.L., Vigouret, J.M., Paganetti, P., Walsh, D.M., Mathews, P.M., Ghiso, J., Staufenbiel, M., Walker, L.C. and Jucker, M. (2006) Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. Science 313, 1781-1784.

[43] Sipe, J.D. (2000) Serum amyloid A: from fibril to function. Current status. Amyloid. 7, 10-12.

[44] Buxbaum, J.N. (2004) The systemic amyloidoses. Curr. Opin. Rheumatol. 16, 67-75.

[45] Anders, R.F., Natvig, J.B., Michaelsen, T.E. and Husby, G. (1975) Isolation and characterization of amyloid-related serum protein SAA as a low molecular weight protein. Scand. J. Immunol. 4, 397-401.

[46] De Beer, F.C., Mallya, R.K., Fagan, E.A., Lanham, J.G., Hughes, G.R. and Pepys, M.B. (1982) Serum amyloid-A protein concentration in inflammatory diseases and its relationship to the incidence of reactive systemic amyloidosis. Lancet 2, 231-234.

[47] Sipe, J.D., Colten, H.R., Goldberger, G., Edge, M.D., Tack, B.F., Cohen, A.S. and Whitehead, A.S. (1985) Human serum amyloid A (SAA): biosynthesis and postsynthetic processing of preSAA and structural variants defined by complementary DNA. Biochemistry 24, 2931-2936.

[48] Kisilevsky, R. and Boudreau, L. (1983) Kinetics of amyloid deposition. I. The effects of amyloid-enhancing factor and splenectomy. Lab Invest 48, 53-59.

[49] Ganowiak, K., Hultman, P., Engstrom, U., Gustavsson, A. and Westermark, P. (1994) Fibrils from synthetic amyloid-related peptides enhance development of experimental AA-amyloidosis in mice. Biochem. Biophys. Res. Commun. 199, 306-312.

[50] Lundmark, K., Westermark, G.T., Nystrom, S., Murphy, C.L., Solomon, A. and Westermark, P. (2002) Transmissibility of systemic amyloidosis by a prion-like mechanism. Proc. Natl. Acad. Sci. U. S. A 99, 6979-6984.

[51] Higuchi, K., Naiki, H., Kitagawa, K., Kitado, H., Kogishi, K., Matsushita, T. and Takeda, T. (1995) Apolipoprotein A-II gene and development of amyloidosis and senescence in a congenic strain of mice carrying amyloidogenic ApoA-II. Lab Invest 72, 75-82.

[52] Higuchi, K., Naiki, H., Kitagawa, K., Hosokawa, M. and Takeda, T. (1991) Mouse senile amyloidosis. ASSAM amyloidosis in mice presents universally as a systemic age-associated amyloidosis. Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 60, 231-238.

[53] Xing, Y. and Higuchi, K. (2002) Amyloid fibril proteins. Mech. Ageing Dev. 123, 1625-1636.

[54] Higuchi, K., Kogishi, K., Wang, J., Chen, X., Chiba, T., Matsushita, T., Hoshii, Y., Kawano, H., Ishihara, T., Yokota, T. and Hosokawa, M. (1998) Fibrilization in mouse senile amyloidosis is fibril conformation-dependent. Lab Invest 78, 1535-1542.

[55] Xing, Y., Nakamura, A., Chiba, T., Kogishi, K., Matsushita, T., Li, F., Guo, Z., Hosokawa, M., Mori, M. and Higuchi, K. (2001) Transmission of mouse senile amyloidosis. Lab Invest 81, 493-499.

[56] Xing, Y., Nakamura, A., Korenaga, T., Guo, Z., Yao, J., Fu, X., Matsushita, T., Kogishi, K., Hosokawa, M., Kametani, F., Mori, M. and Higuchi, K. (2002) Induction of protein conformational change in mouse senile amyloidosis. J. Biol. Chem. 277, 33164-33169.

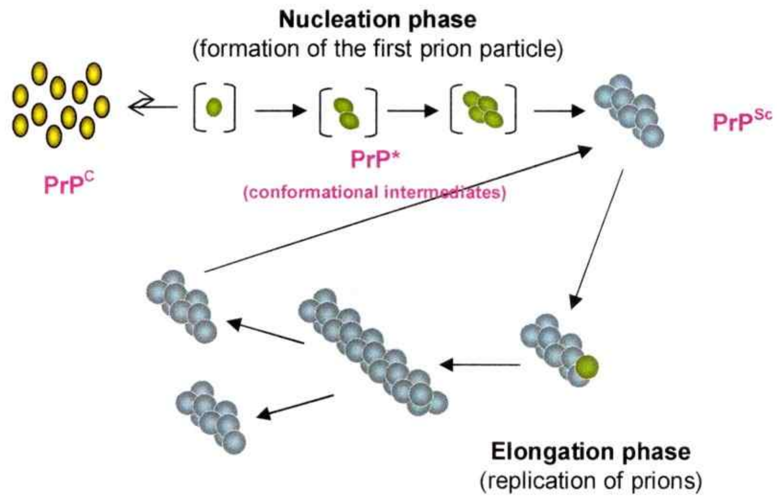
Figure legends.

Figure 1. Seeding/nucleation hypothesis of prion protein misfolding and propagation. Prion replication as well as amyloid formation follows a process in which the limiting step is the stabilization of misfolded structures through formation of a minimal stable oligomer. Misfolded monomers are transient, getting cleared by the normal biological clearance pathways or returning to the natively folded conformation. When several misfolded monomers bind, they can form a stable oligomer that act as a seed to induce and stabilize further misfolded monomers, which are integrated into the growing polymer. The infectious agent acts as such in virtue of its capacity to serve as a nucleus to catalyze the process of protein misfolding and aggregation that result in the disease. Therefore, all protein misfolding processes following a seeding/nucleation mechanism have the inherent possibility to be infectious.

Table 1. List of some Protein Misfolding Disorders, the protein implicated and the organ mostly affected by deposition of aggregates.

DISEASES	PROTEIN INVOLVED	AFFECTED ORGAN
Alzheimer's disease	Amyloid-β protein, Tau	Brain
Type II diabetes	Islet amyloid polypeptide	Pancreas
Parkinson's disease	α-synuclein	Brain
Primary amyloidosis (Implicated in Multiple myeloma, β-cell dyscracias)	Immunoglobulin light chain	Mostly kidney, liver, heart, nerves
Huntington's disease	Huntingtin	Brain
Secondary or reactive amyloidosis	Amyloid-A	Mostly spleen, liver and kidney
Spinocerebral ataxias	Ataxins	Brain
Transmissible spongiform encephalopathies	Prion protein	Brain
Hemodialysis-related amyloidosis	β2-microglobulin	Bones and joints
Amyotrophic lateral sclerosis	Superoxide dismutase	Brain
Familial dementia of British or Danish type	ABri or ADan polypeptides	Brain
Senile systemic amyloidosis, familial amyloid polyneuropathy	Transthyretin	Heart, Kidney, Lungs, peripheral nerves
Hereditary cerebral hemorrhage with amyloidosis Icelandic-type	Cystatin C	Brain
Familial amyloidosis, finnish-type	Gelsolin	Peripheral and Central nervous system
Familial amyloid polyneuropathy	Apolipoprotein A-I	Mostly in aorta
Senile Amyloidosis	Apolipoprotein A-II	Multiple organs
Hereditary systemic amyloidosis, familial visceral amyloidosis	Lysozyme	Liver, spleen, Gastro-intestinal tract
Serpin deficiency disorders (cirrhosis, angioedema)	Serpins	Liver, brain

Figure 1



Apéndice 5

Chapter 13

SIGNALING PATHWAYS CONTROLING PRION NEUROTOXICITY: ROLE OF ENDOPLASMIC RETICULUM STRESS-MEDIATED APOPTOSIS

Rodrigo Morales[1], Claudio Hetz[2] and Claudio Soto[1]

[1]*Department of Neurology, University of Texas Medical Branch, Galveston, TX USA*
[2]*Instituto de Ciencias Biomedicas, Universidad de Chile, Santiago, Chile*

13.1. Neurodegenerative diseases, protein misfolding and apoptosis

Cells can die by diverse mechanisms depending upon the stimulus triggering the death process. Among these mechanisms it is possible to include necrosis, apoptosis, autophagia, mitotic catastrophe, and others[1,2]. Apoptosis has been implicated in diseases affecting the nervous system such as neurodegenerative disorders and ischemia (review in 3). Programmed cell death[4] and its morphological manifestation "apoptosis"[5] is a conserved pathway that in its basic features appears to be operative in all metazoans. During embryonic developmental apoptosis is essential for successful organogenesis, and participates in the control of cellular populations (review in 6). Apoptosis also operates in adult organisms to maintain normal cellular homeostasis. This is an especially critical process in long-lived mammals, in which cells integrate multiple physiological as well as pathological signals.

Neurodegenerative diseases are some of the most debilitating disorders, affecting abstract thinking, skilled movements, emotional feelings, cognition, memory, and other abilities that distinguish human beings from other mammals. The analysis of the neuropathological characteristics of several neurodegenerative diseases has revealed common

features underlying the mechanism of the disease initiation and progression. Compelling evidence accumulated in the last few years suggest that the misfolding, aggregation and cerebral deposition of proteins play a central role on the pathogenesis of neurodegenerative diseases[7]. As we will discuss later on, the protein structural changes are associated with neuronal damage and the appearance of the disease-associated clinical symptoms. The most common neurodegenerative disorder is Alzheimer's disease (AD) where the extracellular accumulation of misfolded amyloid β peptide is one of the principal features that lead to apoptotic neuronal loss[7]. Other pathologies, like Parkinson Disease (PD), Huntington disease (HD) and Amyotrophic Lateral Sclerosis (ALS) are related to the accumulation of other misfolded proteins such as α-synuclein in PD, huntingtin in HD and superoxide dismutase (SOD)-1 in ALS[7]. Transmissible Spongiform Encephalopathies (TSEs) also known as prion disorders are the rarest, but perhaps the most famous neurodegenerative diseases. In TSEs the misfolded protein is not only associated to the disease, but is also the major or even the only component of the infectious agent[8].

13.2. TSE pathogenesis and misfolding of the prion protein

TSEs are a group of clinically diverse, but mechanistically similar neurological diseases affecting humans and animals. The group includes Creutzfeldt-Jakob (CJD), fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker (GSS) and kuru in humans as well as bovine spongiform encephalopathy (BSE), scrapie and chronic wasting disease (CWD) in animals[9]. The distinguishing pathological features of TSEs are the spongiform degeneration of the brain, accompanied by extensive neuronal loss, astrogliosis, and cerebral accumulation of a misfolded and protease-resistant form of the normal prion protein (PrP^{C}), termed PrP^{SC} (ref 10). No sequence or post-translational differences have been detected between the normal host cell surface PrP^{C} and the misfolded PrP^{SC}(ref 10). Based on structural studies, it has been proposed that during the pathogenesis of TSEs PrP^{C} undergoes a conformational transition from α helical to β sheet structure, resulting in the formation of PrP^{SC} (ref 11). This phenomenon leads to an "autocatalytic process", which replicates the abnormal protein structure and propagates new pathogenic prions in the brain of affected individuals.

TSEs can be initiated by different causes, including hereditary, sporadic or infectious[9]. It has been described that at least 10% of the CJD cases and all cases of GSS and FFI have an inherited origin. In these

cases mutations on the PrP gene stabilize the anomalous conformation of PrP, triggering the pathology[12]. Most of the CJD cases have been described as sporadic, where the cause that triggers the disease progression remains without a satisfactory explanation[9]. When brain homogenate of sick animals is injected into healthy animals the disease is transmitted in a dose-dependent manner[10]. Misfolded prion protein accumulates gradually in the brain at expense of the host PrP^C. In humans it has been described that treating patients with infected surgical supplies, cornea transplant or human growth hormone administration extracted from pineal gland of infected individuals, is possible to induce the pathology. An infectious origin for human prion diseases was also observed in the transmission of kuru by cannibalism in tribes from New Guinea and the recent transmission of BSE to human beings producing a new disease, named variant CJD (vCJD)[13,14].

A great deal of effort has been made to understand the remarkable biology of the prion replication process and the nature of the infectious agent[8]. The high β-sheet content of PrP^{SC} confers this protein distinct physicochemical properties from PrP^C, which are reflected in its insolubility in non-denaturating detergents, its partial resistance to proteolysis, and its ability to form fibrillar structures *in vitro*[10,12]. PrP^C is essential for the development of prion diseases since ablation of the gene which codifies PrP^C renders the mice resistant to prion infection[15]. Under certain conditions, the conversion of PrP^C into PrP^{SC} can be achieved in a cell-free replication assay, supporting the "protein-only hypothesis"[16,17].

Two alternative models have been proposed to explain the process of aggregation in TSE[18]. One of them is the "Nucleation-Polimerization" hypothesis, where PrP^{SC} is a multimeric particle, acting as a nucleus to induce and stabilize the misfolding of the monomeric protein by incorporating it into the oligomer. Another model termed "Template Assisted Conversion" postulates that PrP^{SC} serves as a template to direct the misfolding of a partially unfolded intermediate produced by interaction of PrPC with a yet unknown pathological chaperone named protein X.

13.3. Neuronal loss in TSE is mediated by apoptosis

A number of studies indicate that neuronal dysfunction in humans and animals affected with TSEs occurs through apoptosis[19–32]. The detection of cells with DNA degradation and the morphological characterization of the brain areas affected by prion infection have shown that neuronal apoptosis is observed mostly in terminally ill animals, and in those brain areas that show vacuolation[19]. Neuronal loss and apoptosis have also been described in experimental models for CJD in mice[21,33].

Interestingly, several groups have shown that in post-mortem samples of humans affected with FFI[22] and CJD[23–25], apoptotic cell death of neurons does not correlate well with the deposition of PrP (reviewed in 29). These findings suggest that the mechanism relating PrP^{SC} generation and neuronal loss is a complex phenomenon. It was proposed that the dissociation between neuronal damage and the amount of prion deposition only reflects variations in the selective neuronal vulnerability to PrP^{SC} toxicity[29].

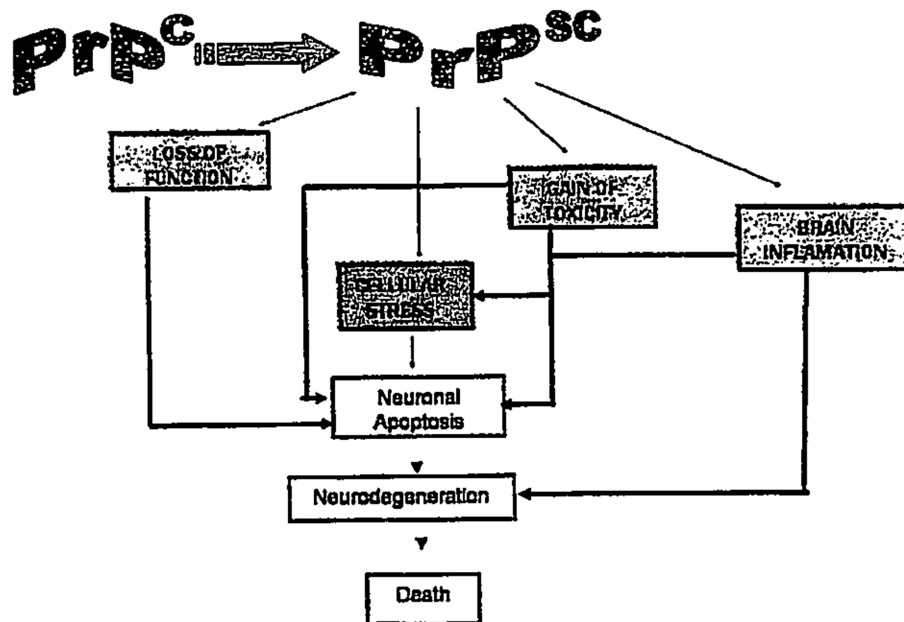
Different strategies have been developed to understand the relationship between PrP misfolding and neuronal dysfunction. A transgenic mice model of familial prion diseases has been developed by expressing the PrP homologue of a nine-octapeptide insertional mutation described in human patients affected with prion diseases[34]. This insertional mutation is genetically linked with a disease characterized by dementia and ataxia, and by the presence of PrP-containing amyloid plaques in the cerebellum and basal ganglia[35–37]. These transgenic mice showed accumulation of protease-resistant PrP^{SC} and apoptotic cell death of the cerebellar granule cells, in addition to progressive ataxia[38]. On the other hand, transgenic mice expressing PrP fragments die spontaneously by ataxia, showing an accumulation of protease resistant PrP within neuronal dendrites and cell bodies, apparently causing apoptosis[39,40]. Finally, in some inherited cases of prion diseases, the predominant form of mutant PrP detectable in the brain is ^{CTM}PrP, a transmembrane form of the prion protein. Transgenic mice expressing this particular PrP mutation also developed neurodegeneration[41]. In summary, neuronal cell death is a common feature observed in different forms of the disease, which can be reproduced when mutant PrP genes are expressed in transgenic mice or in mice experimentally infected with scrapie prions.

The general idea supported by many studies is that the structural conversion affecting PrP^{C} is a fundamental step in the neurodegeneration process, and at least two major hypotheses have been proposed to explain this causative relation: PrP^{SC} formation might be associated with the gain of a neurotoxic activity of this protein, or alternatively, the conversion of PrP^{C} into PrP^{SC} could cause neurodegeneration through the loss of the normal biological function of PrP^{C} (Figure13.1). We have recently reviewed the literature supporting each of these two hypotheses (review in 42, 43).

13.4. Pathways controlling neuronal apoptosis

Apoptosis can be induced by the ligation of plasma membrane death receptors, which constitute the "extrinsic" pathway, or by the perturbation of intracellular homeostasis, known as the "intrinsic" pathway[44–46].

Figure 13.1. Putative cellular pathways for neurodegeneration in TSEs. Conformational changes of PrPC induced by prion infections, mutations or unknown factors, lead to the production and accumulation of the misfolded PrPSC protein. The pathological protein may interact with different neuronal cell-surface receptors and with microglia and astrocytes, triggering signal transduction cascades which result in cellular stress and neuronal dysfunction. In addition, the conformational transition could lead to the lost of a beneficial activity of the natively folded protein.



The viability of a cell strictly depends on the functional and structural integration of a number of subcellular organelles like the nucleus, the mitochondria, the lysosomes and the endoplasmic reticulum[47]. Each organelle can sense stressful cellular conditions and initiate cellular responses either to adapt or to activate specific cell death signalling pathways, if a critical threshold of damage has been reached (reviewed in 48). In spite of the absence of large organelle ultra-structural changes in dying cells, it is now well established that most organelles manifest subtle biochemical alterations, such as permeabilization of membranes and changes on the concentration of messenger molecules, including calcium. In general the process of cell death has two phases: A terminal stage that is mediated by executer common molecules in which the different apoptotic signals converge; and an activator phase mediated by initiator molecules that are up-stream of executor molecules, and are associated with particular cell death stimuli. These complex processes involve cross talk between many signaling pathways and include

different molecular components that regulate the cell death response. In general, the apoptotic process can be subdivided in four different steps:

1. Initiation process: Under certain conditions, apoptosis can be triggered by cell death receptors binding to their corresponding ligands. This signal transduction event is known as the extrinsic pathway and is generally related with the triggering of Fas, TNF receptor or TRAIL[49]. Other extracellular signaling that disrupts ion-channel activity at the cell surface can play a central role in neuronal loss under pathological conditions. In addition, each organelle can sense and initiate cell death responses when the affected element can not restore the homeostasis under stress conditions. In general those events include mitochondrial stress, endoplasmic reticulum stress, DNA damage and others.
2. Amplification process: Independent on the initiator mechanism of cell death, common signaling pathways regulates the apoptotic process that leads to the execution of cell death. This involves phosphorylation steps, multiple proteolytic processes, calcium mobilization, activation of specific transcriptional factors and many others molecular events[48,50,51].
3. The cell death process which is an irreversible step and involves the self-degradation of macromolecules leading to DNA fragmentation, chromatin condensation and proteolytic degradation of many structural proteins and enzymes[1].
4. Finally a phagocytic event is involved in the clearance of the apoptotic bodies. Several cell types participates in this process, including dendritic cells, macrophages, and brain microglia, which recognize membrane receptors in the apoptotic cells and initiate their degradation.

The induction of apoptosis depends on the activation of cysteine proteases of the caspase family (review in 52). Caspases are produced as inactive zymogens, and after activation they cleave their substrates at aspartic acid residues contained within a tetrapeptide recognition motif. Currently, more than 14 caspases have been cloned and partially characterized in mammals, some of which are not involved in apoptosis but rather mediate inflammation and cytokine processing (such as caspase-1 and caspase-11)[53]. Activation of initiator caspases (such as pro-caspase-8, pro-caspase-9 and pro-caspase-12) leads to the proteolytic activation of downstream executor caspases (such as caspase-3), which cleave multiple substrates culminating with the morphological manifestation of apoptosis (review in 53).

The best studied apoptosis signaling pathways involve the activation of death receptors by their ligands and the mitochondrial stress

leading to the release of apoptogenic factors from this organelle. In neuronal death, most of the studies have been focused on the mitochondrial pathway. The central event in the regulation of apoptosis by this pathway is the release of mitochondrial proteins, such as cytochrome *c*, which triggers the activation of caspases through the formation of the "apoptosome" complex[54]. This protein complex is formed when cytosolic cytochrome c binds to the adaptor protein apaf-1, recruiting the inactive form of caspase-9 and triggering its self-proteolytic activation[55]. Cytocrome c release depends upon the opening of a mitochondrial pore termed "permeability transition pore" or PTP[56]. The opening of the PTP is highly regulated by the Bcl-2 family proteins, representing a critical intracellular checkpoint upstream of the caspase cascade (review in 57). The Bcl-2 family is comprised of pro- and anti-apoptotic members. Anti-apoptotic Bcl-2 family members include Bcl-2 and Bcl-X_L. Pro-apoptotic Bcl-2 members include for example Bax and Bak. Apoptotic signals trigger the conformational activation of Bax and Bak, inducing their oligomerization in the mitochondria and resulting in the opening of the PTP. Anti-apoptotic Bcl-2 proteins inhibit the activation of Bax and Bak. In general, the mitochondrial apoptotic pathway has been shown to be activated in neurons by growth factors deprivation, oxidative stress, DNA damage or by changes in the expression levels of Bcl-2 family proteins[59].

Although apoptosis probably participates in the development of all cell lineages, aberrations in genes encoding pro- or anti-apoptotic proteins have been implicated in the initiation of a variety of human diseases, such as cancer through a process of "cell inmortalization", while accelerated cell death is evident in immunodeficiency (review in 58, 59). In this sense, gain- and loss-of-function mice models for genes encoding proteins of the core apoptotic pathways indicate that the violation of cellular death homeostasis is a primary pathogenic event that results in disease (review in 60).

The analysis of signaling pathways involved in neuronal apoptosis in neurodegenerative diseases associated with the misfolding and accumulation of protein aggregates in the brain, has provided data for a novel apoptosis pathway implicating endoplasmic reticulum (ER) stress and the unfolding protein response process.

13.5. Caspase-12 and endoplasmic reticulum stress

Cellular alterations leading to a general accumulation of unfolded proteins in the ER, or modifying the homeostasis of calcium in this subcellular compartment results in a condition denominated ER stress. Some

of these triggering events include alterations on protein maturation, glucose depletion, expresion of mutant proteins, heat shock, and oxidative stress among others[61–65]. Experimentally the pharmacological targeting of the ER homeostasis with molecules such as brefeldin A (which block the trafficking between ER-Golgi), thapsigargin (an inhibitor of the ER calcium pump SERCA) and tunicamycin (an inhibitor of N-glycosylation) are able to induce ER stress and apoptosis.

It has been also described that under certain conditions, ER stress can be triggered by the accumulation of misfolded proteins outside of the reticular compartment[66]. The protein biosynthesis and degradation processes are tightly associated, determining that normal proteins synthesized in the ER and abnormally folded ones are recognized by the ER quality control[67,68]. During this process the abnormally folded proteins are subjected to ER-associated degradation (ERAD)-pathway, which includes the recognition of the protein by specific chaperones, deglycosylation, ubiquitination and translocation to the cytoplasm for degradation by the proteasome. The overload of the proteasome in the cytosol triggers a delay in the folding and degradation process inducing ER stress. Another known mechanism in which misfolded protein accumulation can involve reticular responses is the ER Overload Response (EOR), produced when the ER lumen is overloaded with proteins that are not transported to the Golgi apparatus, probably by a general saturation of the biosynthesis pathways[68].

The ER-stress response has mainly two phases: One anti-apoptotic phase mediated by a general decrease in protein synthesis and increases in the expression of different chaperones and folding enzymes of the glucose regulated family proteins (GRPs)[61,68]. This response is mediated by a signalling cascade known as the "unfolding protein response" or UPR. This process attenuates the toxicity of misfolded proteins in the ER by refolding the proteins or by promoting their degradation through the proteasome pathway[69]. The best characterized chaperon proteins involved in this process are the calcium-binding chaperones Grp78/Bip (involved in protein refolding processes) and Grp94 (involved in ER lumen calcium homeostasis). Experimentally, the overexpression of Grp78/Bip and Grp94 has been shown to protect cells against ischemia, and the pharmacological induction of ER stress. Conversely, the inhibition of the expression of these chaperones renders cells more susceptible to ER stress[65,70–76]. If the damage is too strong and the homeostasis cannot be restored, a second phase is initiated, which is mediated by several pro-apoptotic components triggering cellular death. This includes the activation of an ER-resident caspase, the induction of the pro-apoptotic transcriptional factor GADD153/CHOP, and the activation of several regulatory kinases (review in 47).

The induction of apoptosis by ER stress is dependent upon the activation of an ER-resident caspase, termed caspase-12[77]. Caspase-12 is ubiquitously expressed and is synthesized as an inactive pro-enzyme. Upon proteolytical processing, the active form of caspase-12 is generated consisting in a regulatory pro-domain and two catalytic (p20 and p10) subunits. The mechanism of caspases-12 activation is unclear but, unlike other caspases, caspase-12 is remarkably specific to insults that elicit ER stress[77]. Accordingly, caspase-12-null cells are resistant to apoptosis induced by ER stress, but not to other apoptotic stimuli related, for example, with normal cellular death during development or with the maintenance of the tissues homeostasis[77]. These findings may explain why caspase-12 knock out animals are viable.

It has been suggested that caspase-12 activation is linked to the ER stress pathway through the ER transmembrane kinase Ire1α and the adapter protein TRAF2.[68,78]. Ire1α is normally maintained in an inactive state through an association between its N-terminal lumenal domain and the chaperone Grp78/Bip. Under conditions of ER stress, Grp78/Bip dissociates to bind unfolded proteins and Ire1α undergoes homo-oligomerization, stimulating a trans-autophosphorylation within its serine/threonine kinase domains (review in 47). Upon activation, the cytosolic tail of Ire1α can recruit the adaptor protein TRAF-2[79]. TRAF-2 interacts with caspase-12 and induces its oligomerization and cleavage[78]. Moreover, Ire1α induces apoptosis when it is overexpressed, presumably due to caspase-12 activation[80]. Finally, overexpression of full-length caspase-12 induces its oligomerization and self-cleavage between the p20 and p10 subunits at position D318[81,82].

Alternatively, a second model for caspase-12 activation has been proposed. In mouse glial cells undergoing ER stress, caspase-12 was cleaved by calpain. *In vitro*, m-calpain cleaved caspase-12 at T132 and K158, which released the pro-domain from the catalytic subunits, increasing the enzymatic activity of this protease[83]. Thus, in this second model of activation, extensive intracellular calcium increases may trigger m-calpain activation with the subsequent proteolitic activation of caspase-12 at the ER membrane. Following ER stress, caspase-12, as a member of initiator caspasas group, may directly process downstream caspases in the cytosol or target, as yet unidentified substrates that influence the progression of apoptosis. Two groups have recently reported that caspase-12 directly cleaves caspase-9, leading to caspase-9-dependent activation of caspase-3[70,84]. This phenomenon does not require the expression of the adaptor protein apaf-1[70]. In addition, a direct activation of caspase-3 by caspase-12 through a protein complex formation has been described in other experimental systems[85,86,87].

13.6. ER stress involvement in TSEs neuronal apoptosis

In studies using cell lines which express a mutant form of PrP genetically linked with GSS (mutation PrPY145stop), retention of this protein in the ER and Golgi compartments was described[88]. The mutant PrPQ217R, which is also linked with GSS, was shown to be accumulated and aggregated in the ER[89,90]. This mutant form, as wild type PrP, is also subjected to ERAD, ubiquitinated and degraded by the proteasome system[90]. Moreover, it was described that after proteasome inhibition with different compounds, wild type misfolded PrP is accumulated in the cell leading to cytotoxicity[91–93]. This abnormal PrP^{C} molecule exhibits some of the biochemical properties of PrP^{SC}, such as insolubility in non-ionic detergents, increased aggregation and partial resistance to protease degradation[91,94,95]. Given these results, it was postulated that in sporadic forms of prion diseases, the conditions in which the proteasome ability to degrade PrP is compromised, like cellular stress and events associated with aging, the accumulation of unfolded PrP^{C} derived from the ER might promote neuronal degeneration.

Recently, it has been described that other PrP mutants involved in hereditary forms of the disease (14-octarepeats PrP and PrPD177N) are significantly delayed in their transit along the early part of the secretory pathway through the ER-Golgi[96]. This event opens the possibility that alteration of PrP maturation upon genetic mutations may be the triggering step in the toxicity of some abnormal PrP molecules. However, the mechanism involved in the neurotoxic effects of mutant PrP is not known. In summary, the overall of these data suggest that in sporadic and hereditary forms of TSEs, the ER is a key subcellular compartment where pathological PrP forms are generated and exert their lethal effects.

It has been shown that brain derived PrP^{SC} is cytotoxic *in vitro* in several experimental systems, however the toxicity mechanism was not investigated[97–100]. Recently, we have shown that treatment of mouse neuroblastoma cell cultures with nanoMolar concentrations of brain-derived PrP^{SC} purified from scrapie infected mice is able to induce ER stress, reflected in an increase expression of several ER chaperones and release of ER calcium. In addition, in dying cells, the induction of apoptosis by PrP^{SC} was associated with caspase-12 activation, confirming the participation of ER stress as a cytotoxic mechanism. Finally, the activation of the caspase-12 downstream target, caspase-3, was observed in these cells.

Experimentally, the targeting of anti-apoptotic protein Bcl-2 to the ER membrane was shown to decrease the susceptibility of neuroblastoma cells to PrP^{SC} toxicity. The protective effect was associated with the

inhibition of caspase-12 activation. In addition, expression of a dominant negative form of caspase-12 decreases the induction of apoptosis by PrP^{SC}.

Surprisingly, in post-mortem human samples of patients affected with sCJD or vCJD a high increase in the expression levels of the ER chaperones Grp58, Grp78/BiP and Grp94 was observed. Similar observations were described after a proteomic analysis of CJD brain samples, showing that Grp58, was the protein that showed the highest induction under disease conditions. However, no alteration in the expression levels of other chaperones such as Hsp70 was observed. In addition, a similar pattern to caspase-12 activation was observed in the same human samples reinforcing the hypothesis that ER stress is a central signaling pathway mediating neurodegeneration and neuronal loss. In agreement with these observations, the analysis of scrapie-infected mice revealed that the ER stress pathway correlates with the disease progression and brain damage. After the analysis of different brain samples (distinct brain regions and different times during the disease progression), it was shown that PrP^{SC} levels directly correlated with the rate of Grp58 upregulation (Hetz et al, manuscript submitted). Moreover, only in the brain regions that showed extensive neuronal loss, active caspase-12 fragments were detected. We are currently studying the exact contribution of ER chaperones to the cell death process and how their upregulation is related to the prion replication process. We have found that the upregulation of Grp58 levels occurs early in the disease progression, during the presymptomatic phase of the disease. *In vitro*, Grp58 was shown to be neuroprotectine against ER stress conditions and PrP^{SC} in vitro (Hetz et al, manuscript submitted). This observation is in agreement with previous findings showing that the expression of the chaperones Grp78 and Grp94 are protective against cell death caused by disturbances of ER homeostasis[65,70–76,101,102]. In addition, two close homologues of Grp58, PDI and EndoPDI, are induced during ischemia *in vivo*, and have a protective activity against cell death[73,101,102].

Interestingly, conditions that affect the formation of disulfide bridges induce PrP to adopt some PrP^{SC}–like properties, such as proteinase K-resistance and insolubility in non-denaturating detergents[103]. In addition, *in vitro* amplification of PrP^{SC} was shown to be dependent on the presence of free sulfhydryl groups[104] and reshuffling of cystein bridges from intra-molecular to inter-molecular forms has been proposed to play a role on PrP conversion and on the stabilization of the misfolded protein aggregates[105]. These findings may suggest that the protective activity of Grp58 against PrP^{SC} neurotoxicity may be mediated by a direct interaction between the two proteins, resulting in reduction of PrP^{SC} misfolding. We are currently investigating this possibility.

13.7. Endoplasmic reticulum stress in neurodegenerative diseases

Several other neurodegenerative diseases associated to the misfolding and cerebral accumulation of a particular protein have been shown to be related to ER stress, which has been proposed to mediate the neuronal cell death process observed in these diseases. This is the case of Alzheimer disease (AD), Parkinson's disease (PD) and Huntington disease (HD)[8,68]. These neurological diseases are characterized by accumulation of misfolded protein aggregates in the brain[7], which suggests that the triggering of ER stress could be a general mechanism related with the cellular toxicity of abnormally folded proteins. The main observations associated with the occurrence of ER stress in neurological diseases are the followings:

Alzheimer's disease—Caspase-12 activation has been reported in experimental models of AD and linked with neurotoxicity of amyloid fibrils. For instance, caspase-12 knockout neurons are less sensitive to Alzheimer's amyloid β cytotoxicity[77], and caspase-4 partially mediates the toxicity of this peptide in human neurons[87]. The injection of amyloid β in the hippocampus of aged rabbits induced the expression of GADD153/CHOP and its translocation into the nucleus, with the concomitant decrease of Bcl-2 expression[106]. In the same experimental system, the intracerebral injection of amyloid β triggered the activation of caspase-3 and caspase-12[107]. Additionally, in AD transgenic mice models, an increased susceptibility to ER stress induction was detected[108]. The observation described in these animals included an increased activation of caspase-12 and abnormal ER-calcium signaling after stimulation of apoptosis[109]. Also, increased expression of GADD153/CHOP in pharmacological paradigms of ER stress[110], and altered expression of Grp78/Bip[111] have been reported.

Huntington's disease and spinocerebral ataxias—The expansion of CAG trinucleotide encoding polyglutamine is the underlying cause of at least nine inherited human neurodegenerative disorders, including HD and spinocerebral ataxias. The expression of expanded polyglutamine repeats has been shown to induce ER stress in several cell lines, which is reflected as the activation of the Ire1α/TRAF-2 pathway[112], the induction of Grp78/Bip and the activation of caspase-12[113,114]. On the other hand, the expression of mutant ataxin-3, a polyglutamine repeat-containing protein involved in spinocerebellar ataxia[115], induces apoptosis and caspase-12 activation[114].

Parkinson's disease—In models of PD, oxidative stress and mitochondrial dysfunction are believed to be central players in the mechanism of neuronal dysfunction (review in 116, or in 117). However,

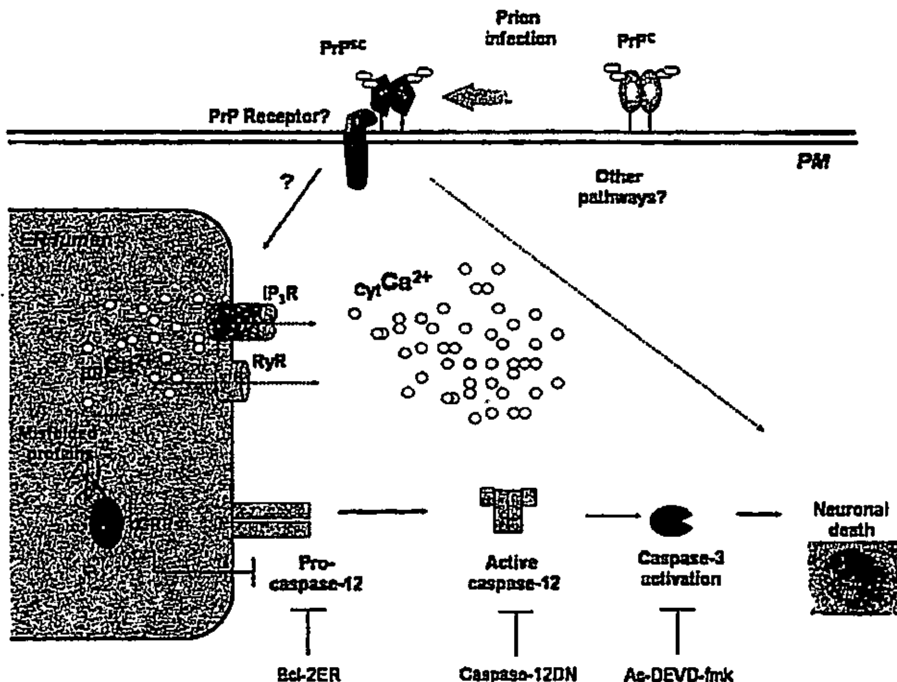
recent reports point out that the ER stress pathway is also involved in the disease process. Treatment of dopaminergic neurons with 6-hydroxydopamine, a parkinsonism-inducing neurotoxin, activates the UPR pathway reflected by the induction of several chaperones, such as Grp58, Grp78/Bip, GADD153/CHOP, as well as Ire1α activation[118]. Similar results have been described by another group, showing that toxic agents affecting the viability of dopaminergic neurons activate the ER-stress pathway, associated with a massive upregulation of chaperones (including calnexin, heat shock proteins and Grp chaperones), the expression of GADD153/CHOP, and the phosphorylation of Ire1α and other related ER kinases[119]. Hereditary parkinsonism has been genetically linked with mutations in the genes encoding the proteins parkin or α-synuclein[120]. ER stress-induced by mutant α-synuclein is decreased by the expression of wild type parkin.[114,117].

In addition, ER stress has been observed in other pathological conditions affecting the brain, such as ischemia, traumatic brain injury and retrovirus induced spongiform brain degeneration. The type of brain damage triggered by these retroviral infections closely resembles several pathological features of TSEs. At the molecular level, it has been described that the degeneration of the brain was associated with the induction of GADD153/CHOP, Grp58, Grp78/Bip, Grp94 and calreticulin[121]. Also, the activation of caspase-12 and caspase-3 was observed in this experimental system[122]. Finally, up-regulation of ER stress markers and activation of caspase-12 were described in animal models of neuronal loss by ischemia[123,124] and traumatic brain injury[125].

13.8. Concluding remarks

In the last few years it has become clear that ER-stress mediated apoptosis plays an important role in diverse diseases and in particular in neurodegenerative disorders associated with the misfolding and brain deposition of proteins. TSEs are a prototype of these diseases where the central role of the misfolded protein is widely accepted[7]. Data generated in our laboratory shows that an atypical form of ER stress features the pathogenesis of TSEs[126]. Figure 13.2 shows a schematic diagram of the cellular events occurring after treatment of neurons with PrP^{SC}. This signaling includes the release of calcium from the ER, the induction of the pro-apoptotic caspase-12 and the up-regulation of certain ER chaperones (Grp58, Grp78 and Grp94), but not others commonly detected under ER stress conditions (such as GAD153/CHOP, calreticulin, Hsp70). The initial molecular events associated with the apoptotic effect of PrP^{SC} remains unclear. We speculate that PrP^{SC} may interact with a

Figure 13.2. A working hypothesis for PrP^{SC} induced apoptosis in neuronal cells. Interaction of PrP^{SC} with an unknown receptor activates a signaling pathway which induces the release of calcium from the ER though the ryanodine receptors (RyR) and the IP3-receptors (IP3R). The alteration in calcium homeostasis promotes ER stress, which in turn triggers the accumulation of misfolded proteins in the ER, leading to a neuroprotective response associated with the induction of chaperones of the Grp family. Ultimately, ER stress leads to the activation of caspase-12, which in turn activates the executioner caspase-3, resulting in neuronal apoptosis. Experimentally, this process can be modulated *in vitro* by: expression of Bcl-2 in the ER membrane (Bcl-2ER), expressing a dominant negative form of caspase-12 (caspase-12DN), treating the cells with caspase-3 inhibitors (Ac-DEVD-fmk).



yet unknown receptor, triggering an abnormal release of calcium from the ER, activating the cell death program.

An alternative possibility that has been arising in recent years is that PrP^{SC} neurotoxic effect is mediated through PrP^{C} signalling. Mallucci and co-workers have found that the depletion of endogenous neuronal PrP^{C} in a post-natal knockout mice after prion infection, leads to a reversion of the early spongiform changes of the brain and prevents neuronal loss and progression to clinical disease[127]. This occurred despite the presence of extracellular PrP^{SC} deposition in the brain similar to the levels observed in terminally ill wild-type animals. In a similar study,

Brandner and colleagues showed that PrP-null brain tissue surrounding prion-infected *Prnp*$^{+/+}$ neurografts does not develop prion neuropathological changes[128]. In both experimental systems, PrP^{SC} seems unable to trigger neuronal death in the absence of PrP^{C}. These results suggest that PrP^{SC} requires the presence of PrP^{C} to be neurotoxic. This interpretation finds support in recent data from Solforosi et al., which shows that neurodegeneration could be directly triggered through a cross-linking of PrP^{C} by a monoclonal antibody[129]. The extrapolation of these findings is that PrP^{SC} could be the activator of a PrP^{C}–mediated signaling pathway[8]. This is in agreement with the observation that PrP knockout cells are resistant to the toxic activity of partially purified PrP^{SC} (review in 18). Hence, the possibility that normal PrP^{C} is the receptor for PrP^{SC} remains open for future research. An alternative model to explain PrP^{SC} cytotoxicity suggests that the misfolded protein might be transported to the ER and, by itself, induces ER stress. This mechanism has been described for bacterial toxins, like the cholera toxin, which binds to lipid rafts structures in the plasma membrane and is then internalised by endocytosis toward the ER[130]. PDI has been implicated in the regulation of the pathogenic effects of cholera toxin, since it recognizes the toxin in the ER and release it from the internalization-protein complex enabling its toxic effects[131]. This could be an interesting possibility to explore, since it has recently been shown that in neuroblastoma cells infected with scrapie, modification of intracellular trafficking between Golgi-ER or endosome-Golgi induces an accumulation of PrP^{SC} in the ER[132], opening the possibility that PrP^{SC} can reach the ER and be recognized by Grp58 as a stress factor.

The elucidation of the mechanism of neuronal apoptosis in TSE has clear implications for the development of TSE treatments directed to prevent neurodegeneration. Since caspases are central to both normal programmed cell death and injury-dependent apoptosis, inhibition of these proteases usually results in serious adverse effects. However, caspase-12 appears not to be essential for normal development or physiological cell death, but rather its activation seems confined to some specific pathological stress signals[50]. Indeed, caspase-12-deficient mice have no noticeable developmental or behavioral defects[77,133]. Therefore, inhibition of caspase-12 activation might provide a novel therapeutic strategy for TSEs and other neurodegenerative diseases initiated by protein misfolding. However, the participation of caspase-12 in human pathologies has been controversial, since the caspase-12 gene is not functional in humans[134]. Recent reports have shown that caspase-4 is the human caspase-12 homolog in terms of structure, function and subcellular localization[87]. Our data suggests that targeting caspase-4 or other components of the ER-stress mediated apoptosis pathway may lead to

therapeutic benefits for TSEs. Similarly, pharmacological treatment that induces the expression of Grp58 may have neuroprotective effects. An example of this class of drugs is valproate, which is used to treat brain-related alterations such as bipolar disorders. This compound is known to induce the expression of the chaperone Grp78/Bip without activating ER stress or any detectable ER-associated damage (review in 135). Also, recent reports suggest that under certain conditions, known endogenous ER-inducible chaperones can be expressed in the absence of any ER stress signal[61,136], suggesting that an strategy designed to generate drugs that induce Grps expression may be possible. Finally, the close relationship between Grp58 up-regulation and PrP^{SC} accumulation, which is even detected during the pre-symptomatic stages of the disease, may be exploited to generate a specific and early diagnosis for prion disorders. A biochemical and non-invasive diagnosis of these diseases is a high priority to minimize further spreading of the disease[7]. Therefore, the identification of a new putative surrogate marker for prion disease is of potentially high importance.

13.9. References

1. G. Majno and I. Joris, Apoptosis, Oncosis, and Necrosis—An Overview of Cell-Death. *Am. J. Pathol.* **146**, 3–15 (1995).

2. M. Castedo, J. L. Perfettini, T. Roumier, K. Andreau, R. Medema, and G. Kroemer, Cell death by mitotic catastrophe: a molecular definition. *Oncogene* **23**, 2825–2837 (2004).

3. J. Yuan, M. Lipinski, and A. Degterev, Diversity in the mechanisms of neuronal cell death. *Neuron* **40**, 401–413 (2003).

4. R. A. Lockshin and C. M. Williams, Programmed cell death I—Cytology of degeneration in the intersegmental muscles of the Pernyi Silknoth. *J. Insect Physiol* **11**, 123–133 (1965).

5. J. F. Kerr, A. H. Wyllie, and A. R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257 (1972).

6. D. L. Vaux and S. J. Korsmeyer, Cell death in development. *Cell* **96**, 245–254 (1999).

7. C. Soto, Unfolding the role of Protein Misfolding in Neurodegenerative Diseases. *Nature Rev. Neurosci.* **4**, 49–60 (2003).

8. J. Castilla, C. Hetz, and C. Soto, Molecular mechanisms of neurotoxicity of pathological prion protein. *Curr. Mol. Med.* **4**, 397–403 (2004).

9. J. Collinge, Prion diseases of humans and animals: their causes and molecular basis. *Annu. Rev. Neurosci.* **24**, 519–550 (2001).

10. S. B. Prusiner, Prions. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13363–13383 (1998).

11. K. M. Pan, M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R. J. Fletterick, F. E. Cohen and S. B. Prusiner, Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10962–10966 (1993).

12. F. E. Cohen and S. B Prusiner,. Pathologic conformations of prion proteins. *Annu. Rev. Biochem.* **67**, 793–819 (1998).

13. N. Streichenberger, D. Jordan, I. Verejan, C, Souchier, F. Philippeau, E. Gros, C. Mottolese, K. Ostrowsky, A. Perret-Liaudet, J. L. Laplanche, M. Hermier, J. P. Deslys, G. Chazot and N. Kopp, The first case of new variant Creutzfeldt-Jakob disease in France: clinical data and neuropathological findings. *Acta Neuropathol. (Berl)* **99**, 704–708 (2000).

14. G. Chazot, E. Broussolle, C. Lapras, T. Blattler, A. Aguzzi and N. Kopp, New variant of Creutzfeldt-Jakob disease in a 26-year-old French man. *Lancet* **347**, 1181 (1996).

15. H. Büeler, A. Aguzzi, A. Sailer, R. A. Greiner, P. Autenried , M. Aguet and C. Weissmann, Mice devoid of PrP are resistant to scrapie. *Cell* **73**, 1339–1347 (1993).

16. D. A. Kocisko, J. H. Come, S. A. Priola, B. Chesebro, G. J. Raymond, P. T. Lansbury and B. Caughey, Cell-free formation of protease-resistant prion protein. *Nature* **370**, 471–474 (1994).

17. G. P. Saborio, B. Permanne and C. Soto, Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411**, 810–813 (2001).

18. C. Hetz and C. Soto, Protein misfolding and disease: the case of prion disorders. *Cell Mol. Life Sci.* **60**, 133–143 (2003).

19. P. J. Lucassen, A. Williams, W. C. Chung and H. Fraser, Detection of apoptosis in murine scrapie. *Neurosci. Lett.* **198**, 185–188 (1995).

20. A. Giese, M. H. Groschup, B. Hess and H. A. Kretzschmar, Neuronal cell death in scrapie-infected mice is due to apoptosis. *Brain Pathol.* **5**, 213–221 (1995).

21. D. Jesionek-Kupnicka, R. Kordek, J. Buczynski and P. P. Liberski, Apoptosis in relation to neuronal loss in experimental Creutzfeldt-Jakob disease in mice. *Acta Neurobiol. Exp. (Wars.)* **61**, 13–19 (2001).

22. A. Dorandeu, L. Wingertsmann, F. Chretien, M. B. Delisle, C. Vital, P. Parchi, P. Montagna, E. Lugaresi, J. W. Ironside H. Budka, P. Gambetti and F. Gray, Neuronal apoptosis in fatal familial insomnia. *Brain Pathol.* **8**, 531–537 (1998).

23. I. Ferrer, Nuclear DNA fragmentation in Creutzfeldt-Jakob disease: does a mere positive in situ nuclear end-labeling indicate apoptosis? *Acta Neuropathol. (Berl)* **97**, 5–12 (1999).

24. F. Gray, H. Adle-Biassette, F. Chretien, T. Ereau, M. B. Delisle and C. Vital, [Neuronal apoptosis in human prion diseases]. *Bull. Acad. Natl. Med.* **183**, 305–320 (1999).

25. F. Gray, F. Chretien, H. Adle-Biassette, A. Dorandeu, T. Ereau, M. B. Delisle, N. Kopp, J. W. Ironside, C. Vital, Neuronal apoptosis in Creutzfeldt-Jakob disease. *J. Neuropathol. Exp. Neurol.* **58**, 321–328 (1999).

26. A. Williams, P. J. Lucassen, D. Ritchie and M. Bruce, PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Exp. Neurol.* **144**, 433–438 (1997).

27. D. W. Fairbairn, K. G. Carnahan, R. N. Thwaits, R. V. Grigsby, G. R. Holyoak and K. L.O'Neill, Detection of apoptosis induced DNA cleavage in scrapie-infected sheep brain. *FEMS Microbiol. Lett.* **115**, 341–346 (1994).

28. D. Theil, R. Fatzer, R. Meyer, M. Schobesberger, A. Zurbriggen and M. Vandevelde, Nuclear DNA fragmentation and immune reactivity in bovine spongiform encephalopathy. *J. Comp Pathol.* **121**, 357–367 (1999).

29. F. Chretien, A. Dorandeu, H. Adle-Biassette, T. Ereau, L. Wingertsmann, F. Brion, F. Gray, [A process of programmed cell death as a mechanisms of neuronal death in prion diseases]. *Clin. Exp. Pathol.* **47**, 181–191 (1999).

30. D. Jesionek-Kupnicka, J. Buczynski, R. Kordek, T. Sobow, I. Kloszewska, W. Papierz and P. P. Liberski, Programmed cell death (apoptosis) in Alzheimer's disease and Creutzfeldt-Jakob disease. *Folia Neuropathol.* **35**, 233–235 (1997).

31. T. Ookohchi, H. Ito, T. Serikawa and K. Sato, Detection of apoptosis in the brain of the zitter rat with genetic spongiform encephalopathy. *Biochem. Mol. Biol. Int.* **41**, 279–284 (1997).

32. S. Siso, B. Puig, R. Varea, E. Vidal, C. Acin, M. Prinz, F. Montrasio, J. Badiola, A. Aguzzi, M . Pumarola and I. Ferrer. Abnormal synaptic protein expression and cell death in murine scrapie. *Acta Neuropathol. (Berl)* **103**, 615–626 (2002).

33. D. Jesionek-Kupnicka, J. Buczynski, R. Kordek and P. P. Liberski, Neuronal loss and apoptosis in experimental Creutzfeldt-Jakob disease in mice. *Folia Neuropathol.* **37**, 283–286 (1999).

34. R. Chiesa, P. Piccardo, B. Ghetti and D. A. Harris, Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. *Neuron* **21**, 1339–1351 (1998).

35. S. Lehmann and D. A. Harris, A mutant prion protein displays an aberrant membrane association when expressed in cultured cells. *J. Biol. Chem.* **270**, 24589–24597 (1995).

36. S. Lehmann and D. A. Harris, Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5610–5614 (1996).

37. N. Daude, S. Lehmann and D. A. Harris, Identification of intermediate steps in the conversion of a mutant prion protein to a scrapie-like form in cultured cells. *J. Biol. Chem.* **272**, 11604–11612 (1997).

38. R. Chiesa, B. Drisaldi, E. Quaglio, A. Migheli, P. Piccardo, B. Ghetti and D. A. Harris, Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation. *Proc. Natl. Acad. Sci .U.S.A.* **97**, 5574–5579 (2000).

39. S. Supattapone, P. Bosque, T. Muramoto, H. Wille, C. Aagaard, D. Peretz, H. O. Nguyen, C. Heinrich, M. Torchia, J. Safar, F. E. Cohen, S. J. DeArmond, S. B. Prusiner and M. Scott, Prion protein of 106 residues creates an artificial transmission barrier for prion replication in transgenic mice. *Cell*, **96**, 869–878 (1999).

40. S. Supattapone, E. Bouzamondo, H. L. Ball, H. Wille, H. O. Nguyen, F. E. Cohen, S. J. DeArmond, S. B. Prusiner and M. Scott, A protease-resistant 61-residue prion peptide causes neurodegeneration in transgenic mice. *Mol. Cell Biol.* **21**, 2608–2616 (2001).

41. R. S. Hegde, J. A. Mastrianni, M. R. Scott, K. D. Defea, P. Tremblay, M. Torchia, S. J. DeArmond, S. B. Prusiner and V. R. Lingappa, A transmembrane form of the prion protein in neurodegenerative disease. *Science* **279**, 827–834 (1998).

42. C. Hetz, K. Maundrell and C. Soto, Is loss of function of the prion protein the cause of prion disorders? *Trends Mol. Med.* **9**, 237–243 (2003).

43. C. Hetz, S. Benavent, S. Bieler and C. Soto, (2003). Prion Protein Misfolding and Brain Degeneration. *Curr. Med. Chem.*3, 137–147.

44. A. Eastman, Survival Factors, Intracellular Signal-Transduction, and the Activation of Endonucleases in Apoptosis. *Seminars Cancer Biol.* **6**, 45–52 (1995).

45. A. M. Davies, Regulation of neuronal survival and death by extracellular signals during development. *EMBO J.* **22**, 2537–2545 (2003).

46. A. Ashkenazi and V. M. Dixit, Death receptors: Signaling and modulation. *Science* **281**, 1305–1308 (1998).

47. K. F. Ferri and G. Kroemer, Organelle-specific initiation of cell death pathways. *Nature Cell Biology* 3, E255–E263 (2001).

48. T. Dragovich, C. M. Rudin and C. B. Thompson, Signal transduction pathways that regulate cell survival and cell death., *Oncogene.* 1998 Dec 24;17(25): 3207–13.

49. L. E. French and J. Tschopp, Protein-based therapeutic approaches targeting death receptors. *Cell Death and Differentiation* **10**, 117–123 (2003).

50. H. Mehmet, Caspases find a new place to hide. *Nature* **403**, 29–30 (2000).

51. M. S. Forman, V. M. Lee and J. Q. Trojanowski, 'Unfolding' pathways in neurodegenerative disease. *Trends Neurosci.* **26**, 407–410 (2003).

52. A. Degterev, M. Boyce and J. Y. Yuan, A decade of caspases. *Oncogene* **22**, 8543–8567 (2003).

53. S. J. Kang, S. Wang, H. Hara, E. P. Peterson, S. Namura, S. Amin-Hanjani, Z. Huang, A. Srinivasan, K. J. Tomaselli, N. A. Thornberry, M. A. Moskowitz and J. Yuan, Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J. Cell Biol.* **149**, 613–622 (2000).

54. Y. Tsujimoto, Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?, *Genes Cells*. **3**, 697–707 (1998).

55. K. Kuida, Caspase-9. *Int. J. Biochem. Cell Biol.* **32**, 121–124 (2000).

56. J. G. Pastorino, M. Tafani, R. J. Rothman, A. Marcinkeviciute, J. B. Hoek, J. L. Farber and A. Marcineviciute, Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. *J. Biol. Chem.* **274**, 31734–31739 (1999).

57. S. J. Korsmeyer, (1999). Programmed cell death and the regulation of homeostasis. *Harvey Lect.* **95**, 21–41.

58. N. N. Danial, and S. J. Korsmeyer, Cell death: Critical control points. *Cell* **116**, 205–219 (2004).

59. J. T. Opferman and S. J. Korsmeyer, Apoptosis in the development and maintenance of the immune system. *Nature Immunol.* **4**, 410–415 (2003).

60. A. M. Ranger, B. A. Malynn, and S. J. Korsmeyer, Mouse models of cell death. *Nature Gen.* **28**, 113–118 (2001).

61. J. N. Gass, K. E. Gunn, R. Sriburi and J. W. Brewer, Stressed-out B cells? Plasma-cell differentiation and the unfolded protein response. *Trends Immunol.* **25**, 17–24 (2004).

62. R. J. Kaufman, Orchestrating the unfolded protein response in health and disease. *J. Clin. Invest.* **110**, 1389–1398 (2002).

63. D. T. Rutkowski and R. J. Kaufman, A trip to the ER: coping with stress. *Trends Cell Biol.* **14**, 20–28 (2004).

64. A. J. L. Macario, Heat-Shock Proteins and Molecular Chaperones—Implications for Pathogenesis, Diagnostics, and Therapeutics. *Int. J. Clin. Lab. Res.* **25**, 59–70 (1995).

65. H. Liu, R. C. Bowes 3rd, B. van de Water, C. Sillence, J. F. Nagelkerke and J. L. Stevens, Endoplasmic reticulum chaperones GRP78 and calreticulin prevent

oxidative stress, Ca2+ disturbances, and cell death in renal epithelial cells. *J. Biol. Chem.* 272, 21751–21759 (1997).

66. K. J. Travers, C. K. Patil, L. Wodicka, D. J. Lockhart, J. S. Weissman and P. Walter, Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249–258 (2000).

67. M. Molinari and A. Helenius, Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* 288, 331–333 (2000).

68. W. Paschen, Endoplasmic reticulum: a primary target in various acute disorders and degenerative diseases of the brain. *Cell Calcium* 34, 365–383 (2003).

69. D. T. Rutkowski and R. J. Kaufman, A trip to the ER: coping with stress. *Trends Cell Biol.* 14, 20–28 (2004).

70. R. V. Rao, S. Castro-Obregon, H. Frankowski, M. Schuler, V. Stoka, G. del Rio, D. E. Bredesen and H. M. Ellerby, Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. *FEBS Lett.* 514, 122–128 (2002).

71. R. K. Reddy, C. Mao, P. Baumeister, R. C. Austin, R. J. Kaufman, A. S. Lee, Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors—Role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.* 278, 20915–20924 (2003).

72. R. K. Reddy, J. Lu and A. S. Lee, The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca(2+)-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis. *J. Biol. Chem.* 274, 28476–28483 (1999).

73. D. C. Sullivan, L. Huminiecki, J. W. Moore, J. J. Boyle, R. Poulsom, D. Creamer, J. Barker and R. Bicknell, EndoPDI, a novel protein-disulfide isomerase-like protein that is preferentially expressed in endothelial cells acts as a stress survival factor. *J. Biol. Chem.* 278, 47079–47088 (2003).

74. Y. Bando, T. Katayama, K. Kasai, M. Taniguchi, M. Tamatani and M. Tohyama, GRP94 (94 kDa glucose-regulated protein) suppresses ischemic neuronal cell death against ischemia/reperfusion injury. *Eur. J. Neurosci.* 18, 829–840 (2003).

75. H. Miyake, I. Hara, S. Arakawa and S. Kamidono, Stress protein GRP78 prevents apoptosis induced by calcium ionophore, ionomycin, but not by glycosylation inhibitor, tunicamycin, in human prostate cancer cells. *J. Cell Biochem* 77, 396–408 (2000).

76. Z. Yu, H. Luo, W. Fu and M. P. Mattson, The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. *Exp. Neurol.* 155, 302–314 (1999).

77. T. Nakagawa, H. Zhu, N. Morishima, E. Li, J. Xu, B. A.Yankner and J. Yuan, Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98–103 (2000).

78. T. Yoneda, K. Imaizumi, K. Oono, D. Yui, F. Gomi, T. Katayama and M. Tohyama, Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J. Biol. Chem.* **276**, 13935–13940 (2001).

79. F. Urano, X. Wang, A. Bertolotti, Y. Zhang, P. Chung, H. P. Harding and D. Ron, Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664–666 (2000).

80. X. Z. Wang, H. P. Harding, Y. Zhang, E. M. Jolicoeur, M. Kuroda and D. Ron, (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *EMBO J.* **17**, 5708–5717.

81. R. V. Rao, A. Peel, A. Logvinova, G. del Rio, E. Hermel, T. Yokota, P. C. Goldsmith, L. M. Ellerby, H. M. Ellerby and D. E. Bredesen, Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J. Biol. Chem.* **276**, 33869–33874 (2001).

82. E. Fujita, Y. Kouroku, A. Jimbo, A. Isoai, K. Maruyama and T. Momoi, Caspase-12 processing and fragment translocation into nuclei of tunicamycin-treated cells. *Cell Death Diff.* **9**, 1108–1114 (2002).

83. T. Nakagawa and J. Yuan, Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol.* **150**, 887–894 (2000).

84. N. Morishima, K. Nakanishi, H. Takenouchi, T. Shibata and Y. Yasuhiko, An endoplasmic reticulum stress-specific caspase cascade in apoptosis—Cytochrome c-independent activation of caspase-9 by caspase-12. *J. Biol. Chem.* **277**, 34287–34294 (2002).

85. M. Kilic, R. Schafer, J. Hoppe and U. Kagerhuber, Formation of noncanonical high molecular weight caspase-3 and -6 complexes and activation of caspase-12 during serum starvation induced apoptosis in AKR-2B mouse fibroblasts. *Cell Death. Differ.* **9**, 125–137 (2002).

86. J. Hoppe, M. Kilic, V. Hoppe, A. Sachinidis, and U. Kagerhuber, Formation of caspase-3 complexes and fragmentation of caspase-12 during anisomycin-induced apoptosis in AKR-2B cells without aggregation of Apaf-1. *Eur. J. Cell Biol.* **81**, 567–576 (2002).

87. J. Hitomi, T. Katayama, Y. Eguchi, T. Kudo, M. Taniguchi, Y. Koyama, T. Manabe S.Yamagishi, Y. Bando, K. Imaizumi, Y. Tsujimoto and M.Tohyama, Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and A beta-induced cell death. *J. Cell Biol.* **165**, 347–356 (2004).

88. G. Zanusso, R. B. Petersen, T. Jin, Y. Jing, R. Kanoush, S. Ferrari, P. Gambetti, and N. Singh, Proteasomal degradation and N-terminal protease resistance of the codon 145 mutant prion protein. *J. Biol. Chem.* **274**, 23396–404 (1999).

89. N. Singh, G. Zanusso, S. G. Chen, H. Fujioka, S. Richardson, P. Gambetti, and R. B. Petersen, Prion protein aggregation reverted by low temperature in transfected cells carrying a prion protein gene mutation. *J. Biol. Chem.* **272**, 28461–70 (1997).

90. T. Jin, Y. Gu, G. Zanusso, M. Sy, A. Kumar, M. Cohen, P. Gambetti, and N. Singh, The chaperone protein BiP binds to a mutant prion protein and mediates its degradation by the proteasome. *J Biol Chem.* **275**, 38699–704 (2000).

91. J. Ma, R. Wollmann and S. Lindquist, Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science* **298**, 1781–1785 (2002).

92. J. Ma and S. Lindquist, Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. *Science* **298**, 1785–1788 (2002).

93. D. E. Dimcheff J. L. Portis, and B. Caughey, Prion proteins meet protein quality control. *Trends Cell Biol.* **13**, 337–340 (2003).

94. Y. Yedidia, L. Horonchik, S. Tzaban, A. Yanai and A. Taraboulos, Proteasomes and ubiquitin are involved in the turnover of the wild-type prion protein. *EMBO J.* **20**, 5383–5391 (2001).

95. J. Ma and S. Lindquist, Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14955–14960 (2001).

96. B. Drisaldi, R. S. Stewart, C. Adles, L. R. Stewart, E. Quaglio, E. Biasini, L. Fioriti, R. Chiesa and D. A. Harris, Mutant PrP is delayed in its exit from the endoplasmic reticulum, but neither wild-type nor mutant PrP undergoes retrotranslocation prior to proteasomal degradation. *J. Biol. Chem.* **278**, 21732–21743 (2003).

97. C. Bate, S. Reid and A. Williams, Killing of prion-damaged neurones by microglia. *Neuroreport* **12**, 2589–2594 (2001).

98. K. Post, D. R. Brown, M. Groschup, H. A. Kretzschmar and D. Riesner, Neurotoxicity but not infectivity of prion proteins can be induced reversibly *in vitro*. *Arch. Virol. Suppl* **16** 265–273 (2000).

99. C. Bate, M. Salmona, L. Diomede and A. Williams, Squalestatin cures prion-infected neurons and protects against prion neurotoxicity. *J. Biol. Chem.* **279**, 14983–14990 (2004).

100. W. E. Müller, H. Ushijima, H. C. Schroder, J. M. Forrest, W. F. Schatton, P. G. Rytik and M. Heffner-Lauc, Cytoprotective effect of NMDA receptor antagonists on prion protein (PrionSc)-induced toxicity in rat cortical cell cultures. *Eur. J. Pharmacol.* **246**, 261–267 (1993).

101. S. Tanaka, T. Uehara and Y. Nomura, Up-regulation of protein-disulfide isomerase in response to hypoxia/brain ischemia and its protective effect against apoptotic cell death. *J. Biol. Chem.* **275**, 10388–10393 (2000).

102. H. S. Ko, T. Uehara and Y. Nomura, Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. *J. Biol. Chem.* **277**, 35386–35392 (2002).

103. J. Ma and S. Lindquist, De novo generation of a PrPSc-like conformation in living cells. *Nat. Cell Biol.* **1**, 358–361 (1999).

104. R. Lucassen, K. Nishina and S. Supattapone, In vitro amplification of protease-resistant prion protein requires free sulfhydryl groups. *Biochemistry* **42**, 4127–4135 (2003).

105. Tompa, P., Tusnady, G. E., Friedrich, P., and Simon, I. The role of dimerization in prion replication. *Biophys. J.* **82**, 1711–1718 (2002).

106. O. Ghribi, M. M. Herman, D. A. DeWitt, M. S. Forbes and J. Savory, Abeta(1–42) and aluminum induce stress in the endoplasmic reticulum in rabbit hippocampus, involving nuclear translocation of gadd 153 and NF-kappaB. *Brain Res. Mol. Brain Res.* **96**, 30–38 (2001).

107. O. Ghribi, M. M. Herman, and J. Savory, Lithium inhibits Abeta-induced stress in endoplasmic reticulum of rabbit hippocampus but does not prevent oxidative damage and tau phosphorylation. *J. Neurosci. Res.* **71**, 853–862 (2003).

108. F. Terro, C. Czech, F. Esclaire, W. Elyaman, C. Yardin, M. C. Baclet, N. Touchet, G. Tremp, L. Pradier and J. Hugon, Neurons overexpressing mutant presenilin-1 are more sensitive to apoptosis induced by endoplasmic reticulum-Golgi stress. *J.Neurosci.Res.* **69**, 530–539 (2002).

109. S. L. Chan, C. Culmsee, N. Haughey, W. Klapper and M. P. Mattson, Presenilin-1 mutations sensitize neurons to DNA damage-induced death by a mechanism involving perturbed calcium homeostasis and activation of calpains and caspase-12. *Neurobiol. Dis.* **11**, 2–19 (2002).

110. O. Milhavet, J. L. Martindale, S. Camandola, S. L. Chan, D. S. Gary, A. Cheng, N. J. Holbrook, M. P. Mattson, Involvement of Gadd153 in the pathogenic action of presenilin-1 mutations. *J. Neurochem.* **83**, 673–681 (2002).

111. Y. Yasuda, T. Kudo, T. Katayama, K. Imaizumi, M. Yatera, M. Okochi, H. Yamamori, N. Matsumoto, T. Kida, A. Fukumori, M. Okumura, M. Tohyama and M.Takeda, FAD-linked presenilin-1 mutants impede translation regulation under ER stress. *Biochem. Biophys. Res. Commun.* **296**, 313–318 (2002).

112. H. Nishitoh, A. Matsuzawa, K. Tobiume, K. Saegusa, I. Takeda K, . Inoue, S. Hori, A. Kakizuka and H. Ichijo, ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes and Development* **16**, 1345–1355 (2002).

113. Y. Kouroku, E. Fujita, A. Jimbo, T. Kikuchi, T. Yamagata, M. Y. Momoi, E. Kominami, K. Kuida, K. Sakamaki, S. Yonehara and T. Momoi, Polyglutamine aggregates stimulate ER stress signals and caspase-12 activation. *Hum. Mol. Genet.* **11**, 1505–1515 (2002).

114. Tsai, Y. C., Fishman, P. S., Thakor, N. V., and Oyler, G. A. Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. *J. Biol. Chem.* **278**, 22044–22055 (2003).

115. H. L. Paulson, M. K. Perez, Y. Trottier, J. Q. Trojanowski, S. H. Subramony, S. S. Das, P. Vig, J. L. Mandel, K. H. Fischbeck and R. N. Pittman, Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron* **19**, 333–344 (1997).

116. T. M. Dawson and V. L. Dawson, Molecular pathways of neurodegeneration in Parkinson's disease. *Science* **302**, 819–822 (2003).

117. W. Dauer and S. Przedborski, Parkinson's disease: Mechanisms and models. *Neuron* **39**, 889–909 (2003).

118. W. A. Holtz and K. L. O'Malley, Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J. Biol. Chem.* **278**, 19367–19377 (2003).

119. E. J. Ryu, H. P. Harding, J. M. Angelastro, O. V. Vitolo, D. Ron and L. A. Greene, (2002). Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J. Neurosci.* **22**, 10690–10698.

120. R. E. Burke, Recent advances in research on Parkinson disease: Synuclein and parkin. *Neurologist* **10**, 75–81 (2004).

121. D. E. Dimcheff, S. Askovic, A. H. Baker, C. Johnson-Fowler and J. L. Portis, Endoplasmic reticulum stress is a determinant of retrovirus-induced spongiform neurodegeneration. *J.Virol.* **77**, 12617–12629 (2003).

122. H. T. Kim, K. Waters, G. Stoica, W. Qiang, N. Liu, V. L. Scofield and P. K. Wong, (2004). Activation of endoplasmic reticulum stress signaling pathway is associated with neuronal degeneration in MoMuLV-ts1-induced spongiform encephalomyelopathy. *Lab Invest.*

123. M. Shibata, H. Hattori, T. Sasaki, J. Gotoh J. Hamada and Y. Fukuuchi, Activation of caspase-12 by endoplasmic reticulum stress induced by transient middle cerebral artery occlusion in mice. *Neuroscience* **118**, 491–499 (2003).

124. G. Mouw, J. L. Zechel, J. Gamboa, W. D. Lust, W. R. Selman and R. A. Ratcheson, Activation of caspase-12, an endoplasmic reticulum resident caspase, after permanent focal ischemia in rat. *Neuroreport* **14**, 183–186 (2003).

125. S. F. Larner, R. L. Hayes, D. M. McKinsey, B. R. Pike, and K. K. W.Wang, Increased expression and processing of caspase-12 after traumatic brain injury in rats. *J.Neurochem.* **88**, 78–90 (2004).

126. C. Hetz, M. Russelakis-Carneiro, K. Maundrell, J. Castilla, and C. Soto, Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein. *EMBO J.* **22**, 5435–5445 (2003).

127. G. Mallucci, A. Dickinson, J. Linehan, P. C. Klohn, S. Brandner and J. Collinge, Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science.* **302**, 871–874 (2003).

128. S. Brandner, S. Isenmann, A. Raeber, M. Fischer, A. Sailer, Y. Kobayashi, S. Marino, C. Weissmann and A. Aguzzi, Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*, **379**, 339–343, (1996).

129. L. Solforosi, J. R. Criado, D. B. McGavern, S. Wirz, M. Sanchez-Alavez, S. Sugama, L. A. DeGiorgio, B. T. Volpe, E. Wiseman, G. Abalos, E. Masliah, D. Gilden, M. B. Oldstone, B. Conti and R. A. Williamson, Cross-linking cellular prion protein triggers neuronal apoptosis *in vivo*. *Science* **303**, 1514–1516 (2004).

130. K. Sandvig and B. van Deurs, Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. *FEBS Lett.* **529**, 49–53 (2002).

131. B. Tsai and T. A. Rapoport, Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J Cell Biol.* **159**, 207–216 (2002).

132. F. Beranger, A. Mange, B. Goud and S. Lehmann, Stimulation of PrP(C) retrograde transport toward the endoplasmic reticulum increases accumulation of PrP(Sc) in prion-infected cells. *J.Biol.Chem.* **277**, 38972–38977 (2002).

133. T. Nakagawa and J. Yuan, Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* **150**, 887–894 (2000).

134. H. Fischer, U. Koenig, L. Eckhart and E. Tschachler, Human caspase 12 has acquired deleterious mutations. *Biochem. Biophys. Res. Commun.* **293**, 722–726 (2002).

135. C. D. Bown, J. F. Wang, B. Chen and L. T. Young, Regulation of ER stress proteins by valproate: therapeutic implications. *Bipolar Disorders* **4**, 145–151 (2002).

136. M. Flores-Diaz, J. C. Higuita, I. Florin, T. Okada, P. Pollesello, T. Bergman, M. Thelestam, K. Mori and A. Alape-Giron, A cellular UDP-glucose deficiency causes overexpression of glucose/oxygen-regulated proteins independent of the endoplasmic reticulum stress elements. *J. Biol. Chem.* **279**, 21724–21731 (2004).

Apéndice 6

FEBS Letters 582 (2008) 3161–3166

Detection of infectious prions in urine

Dennisse Gonzalez-Romero, Marcelo A. Barria, Patricia Leon, Rodrigo Morales, Claudio Soto*

George and Cynthia Mitchell Center for Neurodegenerative diseases, Departments of Neurology, Neuroscience and Cell Biology and Biochemistry and Molecular Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0646, USA

Received 26 July 2008; accepted 4 August 2008

Available online 13 August 2008

Edited by Aleksander Benjak

Abstract **Prions are the infectious agents responsible for prion diseases, which appear to be composed exclusively by the misfolded prion protein (PrP^{Sc}). The mechanism of prion transmission is unknown. In this study, we attempted to detect prions in urine of experimentally infected animals. PrP^{Sc} was detected in ~80% of the animals studied, whereas no false positives were observed among the control animals. Semi-quantitative calculations suggest that PrP^{Sc} concentration in urine is around 10-fold lower than in blood. Interestingly, PrP^{Sc} present in urine maintains its infectious properties. Our data indicate that low quantities of infectious prions are excreted in the urine. These findings suggest that urine is a possible source of prion transmission.**
© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Prion; Transmissible spongiform encephalopathy; PMCA; Diagnosis; Scrapie

1. Introduction

Prions are the infectious agents responsible for a group of fatal neurodegenerative diseases, collectively called Transmissible Spongiform Encephalopathies (TSEs) that affect humans and several species of mammals [1–3]. Creutzfeldt–Jakob disease (CJD) is the most common TSE in humans, and scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids are the most prevalent prion diseases in animals [2]. Although prion diseases are relatively rare in humans, the recent appearance of variant CJD (vCJD), which is linked to consumption of BSE contaminated food, have raised concern about a possible epidemic outbreak in the human population [4]. BSE is still a significant health and economical problem, and other animal diseases have become a permanent source of concern. Scrapie, for example, is endemic in various countries and CWD is spreading dramatically fast among wild and captive cervids in North America. Finally, it is now clear that vCJD can be iatrogenically transmitted from human to human by blood transfusion raising fears of a second wave of infection [5,6].

Unlike conventional infectious micro-organisms, the TSE agent appears to be devoid of genetic material and instead composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) [3]. PrP^{Sc} has the intriguing ability to replicate in the body of infected individuals by propagating its misfolding to the normal prion protein (PrP^{C}) [3]. PrP^{Sc} is not only the main component of the infectious agent and the most likely triggering factor in brain damage, but it is also the only validated surrogate biomarker for the disease and its sensitive detection is critical for disease diagnosis and to prevent further spreading of TSEs [7]. Currently, there is no validated method to detect PrP^{Sc}. Hampering the efforts to develop a reliable biochemical diagnosis for TSEs is the fact that PrP^{Sc} quantity in peripheral tissues or biological fluids is extremely low and under the limit of detection of standard techniques [7].

With the aim of facilitating PrP^{Sc} biochemical detection, we have developed a novel technique that enables PrP^{Sc} amplification in the test tube. This method, termed protein misfolding cyclic amplification (PMCA), is based on converting large amounts of PrP^{C} triggered by undetectable quantities of PrP^{Sc} [8]. In a cyclic manner, conceptually analogous to polymerase chain-reaction (PCR), PrP^{Sc} is incubated with excess PrP^{C} to enlarge the PrP^{Sc} aggregates, which are then sonicated to generate multiple smaller units for the continued formation of new PrP^{Sc} [8]. The newly generated protein exhibits the same biochemical and structural properties as brain-derived PrP^{Sc} and strikingly it is infectious to wild-type animals, producing a disease with similar characteristics to the illness produced by brain-isolated prions [9]. PMCA is highly specific for detection of PrP^{Sc} and leads to several million folds increase on sensitivity as compared to standard Western blot assays [10,11]. The technology has been applied to replicate the misfolded protein from diverse species [12] and has enabled detection of prions in the blood of infected animals, both at the symptomatic and pre-symptomatic phases of the disease [10,13].

Although the highest concentration of PrP^{Sc} is present in the nervous system, its presence has been reported with a variable degree of success in peripheral tissues, such as lymphoid organs, peripheral nerves, skeletal muscle, kidney, mammary glands, olfactory mucosa and CSF (for reviews, see [7,14,15]). Blood and urine represent the ideal biological fluids for routine non-invasive diagnosis. Various reports of experimental and natural transmission have shown that blood carries infectivity (for reviews, see [6,15,16]) and as described above, we have been able to detect successfully PrP^{Sc} in animal blood. Although, experiments with urine have been for the most part negative, recent studies reported very small infectivity titers in urine of a tiny proportion of scrapie sick rodents [17,18]. However, PrP^{Sc} was not detected in these studies, presumably because the quantity secreted in the urine is below the limit of detection of the technology employed. The main goal of the current study is to attempt detection of PrP^{Sc} in urine of experimentally infected animals using the highly sensitive PMCA technology.

*Corresponding author.
E-mail address: clsoto@utmb.edu (C. Soto).

0014-5793/$34.00 © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2008.08.003

2. Materials and methods

2.1. Sample collection and preparation

Urine from healthy and sick animals was collected using metabolic cages. Sick and healthy animals were of similar age. Urine was processed as schematically described in Fig. 1A. Brain tissue from healthy hamsters was used in PMCA reactions as a substrate for amplification. To prepare brain homogenate, animals were first perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA prior to harvesting the tissue. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing NaCl 150 mM, 1.0% Triton X-100, 4 mM EDTA and the Complete Protease Inhibitor Cocktail from Roche, Switzerland). The samples were clarified by a brief, low-speed centrifugation (2000 rpm for 40 s) using an Eppendorf centrifuge (Hamburg, Germany), model 5414. Samples were stored frozen at −80 °C.

2.2. PMCA procedure

Urine samples after the processing described in Fig. 1A were resuspended into 10% healthy brain homogenate. Samples were loaded onto 0.2-ml PCR tubes and positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY). Each PMCA cycle consisted of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at potency of 7. Samples were incubated without shaking immersed in the water of the sonicator bath. The microplate horn was kept in an incubator set at 37 °C during the whole process. After a round of 96 cycles, an aliquot of the amplified material was diluted 10-folds into normal brain homogenate and a new round of 96 PMCA cycles was performed. This procedure was repeated several times as indicated in the text. The detailed protocol for PMCA, including reagents, methods and troubleshooting, has been published elsewhere [19,20].

2.3. PrP^{Sc} detection

Samples were incubated with 50 μg/ml of PK for 60 min at 45 °C with shaking. The digestion was stopped by adding electrophoresis sample buffer. Proteins were fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 3F4 antibody (Signet, Dedham, MA) diluted 1:5000 in PBS, 0.05% Tween-20. The immunoreactive bands were visualized by enhanced chemoluminescence assay (Amersham, Piscataway, NJ). Western blots signals were analyzed by densitometry, using a UVP Bioimaging system EC3 apparatus (Upland, CA).

2.4. In vivo infectivity studies

Syrian Golden hamsters were used as an experimental model of scrapie. Animals were 4–6-weeks old at the time of inoculation. For urine collection animals were injected i.p. with 100 μl of 10% brain homogenate. To assess infectivity, anesthesized animals were injected stereotaxically into the right hippocampus with 2 μl of the sample. The onset of clinical disease was measured by scoring the animals twice a week using the following scale: (1) normal animal; (2) mild behavioral abnormalities including hyperactivity and hypersensitivity to noise; (3) moderate behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability and aggressiveness; (4) severe behavioral abnormalities including all of the above plus jerks of the

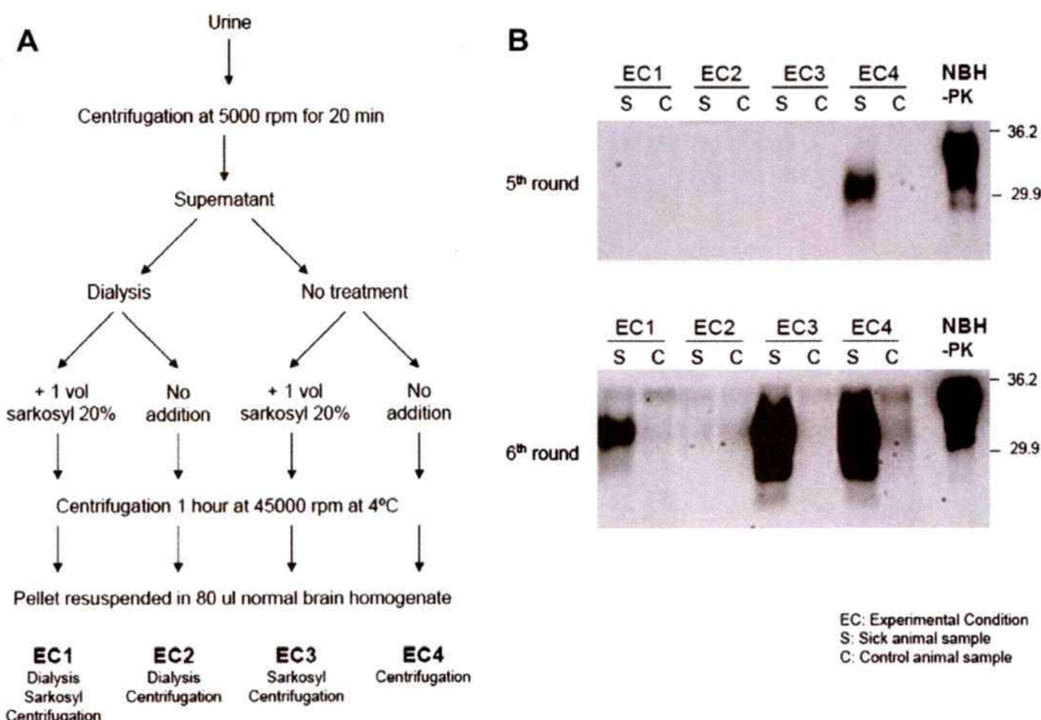


Fig. 1. Identification of the experimental conditions to process urine samples for PrP^{Sc} detection by PMCA. Urine from several hamsters at the symptomatic stage of the disease (and uninfected controls) produced by i.p. inoculation of 263K prions was collected with metabolic cages. Twelve milliliters of urine was pooled and processed as schematically illustrated in panel (A) First, urine was centrifuged at a low speed (5000 × g for 20 min) to remove debris. The supernatant was collected and divided into two groups: one was dialyzed overnight against PBS at 4 °C using a membrane with 30000 Da cutoff; the other sample was left untreated. Both samples were divided into two identical aliquots, one of which was supplemented with 1 volume of 20% sarkosyl and the other one was left untreated. After 30 min incubation at 4 °C, all the samples were centrifuged at 100000 × g for 1 h at 4 °C. Pellet of each sample was resuspended directly in 80 μl of 10% normal hamster brain homogenate and subjected to 96 cycles of PMCA (30 min incubation at 37 °C followed by a pulse of 30 s sonication, as described in Section 2). Then, 8 μl of this sample were diluted into 72 μl of normal brain homogenate and a new round of 96 PMCA cycles was performed. This process was repeated several times. After each round of PMCA, 20 μl of the sample was used for detection of PrP^{Sc} by Western blot after PK digestion (50 μg/ml for 1 h at 37 °C), using 3F4 antibody. Panel B shows the results of serial rounds 5 and 6 of PMCA. No signal was observed before the fifth round. S: samples from sick animals; C: samples from control animals. All samples were treated with PK before electrophoresis, except the normal brain homogenate (NBH) in which −PK is indicated.

head and body and spontaneous backrolls; and (5) terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during two consecutive weeks were considered sick and were sacrificed to avoid excessive pain using exposition to carbonic dioxide. Brains were extracted and one hemisphere was frozen for biochemical studies and the other was used for histological analysis. The scrapie infectious material used in these studies was titrated and 1 LD50 was obtained in a brain dilution of approximately 1×10^9.

2.5. Histopathological studies

Brain tissue was fixed in 10% formaldehyde solution, cut in sections and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin–eosin, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein, using our previously described protocols [9]. Immunoreactions were developed using the peroxidase–antiperoxidase method, following manufacturer's specifications. Antibody specificity was verified by absorption. Samples were visualized with a Zeiss microscope.

3. Results

In order to attempt PrP^{Sc} detection using PMCA, we collected urine from several hamsters at the symptomatic phase of the disease produced by intraperitoneal (i.p.) inoculation of 263K prions. First, we tested several experimental conditions to process urine samples for PrP^{Sc} detection by PMCA. Urine from either sick or control animals was collected using metabolic cages. Samples were pooled and processed as de-

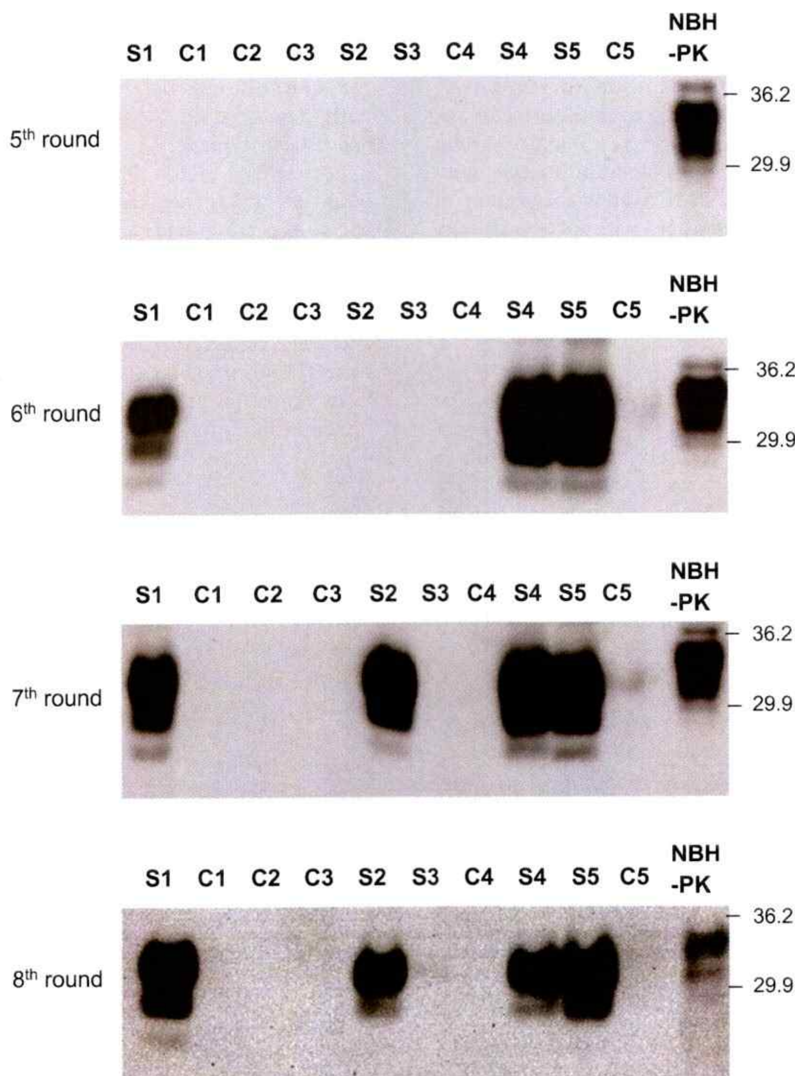


Fig. 2. PrP^{Sc} detection in urine of sick hamsters by PMCA. Three milliliters of urine from five clinically sick hamsters (infected i.p. with HY prions) and five control animals was collected using metabolic cages. The samples were processed as described in Fig. 1A. Briefly, urine was centrifuged at $5000 \times g$ for 20 min to remove debris. The supernatant was collected and subjected to a high speed centrifugation at $100\,000 \times g$ for 1 h at 4 °C to precipitate PrP^{Sc}. Pellet was resuspended directly in 80 μl of 10% normal hamster brain homogenate. Samples were subjected to serial rounds of 96 cycles of PMCA, as described [10,11]. Twenty microliters of the sample was used for detection of PrP^{Sc} by Western blot after PK digestion. The figure shows only the rounds 5, 6, 7 and 8, since the first four rounds of PMCA did not show signal in any of the samples. More than seven rounds of PMCA do not show any more positive signals. S: samples from sick animals; C: samples from control animals. All samples were treated with PK before electrophoresis, except the normal brain homogenate (NBH) in which –PK is indicated.

scribed in Fig. 1A. Four different conditions were tested including combinations between dialysis and sarkosyl precipitation. After processing, the samples were subjected to several rounds of 96 cycles of PMCA as described in Section 2. After each round of PMCA, 20 μl of the sample was used for detection of PrP^{Sc} by Western blot after PK digestion. As shown in Fig. 1B, after 6 serial rounds of PMCA, PrP^{Sc} was detectable in 3 of the 4 conditions tested. The conclusion of these results is that a simple high speed centrifugation, after removing large debris, is sufficient to obtain an adequate sample. Neither the dialysis nor the sarkosyl precipitation steps gave better results in terms of PMCA amplification.

To evaluate the presence of PrP^{Sc} in individual samples of urine, we collected urine from five scrapie sick animals i.p. infected by the hamster strain Hyper (HY) as well as control uninfected animals of similar age. As before, several serial rounds of PMCA were unsuccessful to detect PrP^{Sc} in any of the urine samples tested. However, after 6, 7 or 8 rounds of 96 PMCA cycles each, we were able to detect a PrP^{Sc} signal by Western blot in the urine of 3 or 4 of the 5 animals studied, respectively (Fig. 2). Conversely, PrP^{Sc} was not detected in the urine of any of the five control uninfected hamsters tested, indicating that the procedure is specific for detection of prion infected samples. These results indicate that PMCA enable detection of PrP^{Sc} in urine of scrapie sick hamsters with 80% sensitivity and 100% specificity. Larger number of samples would be needed to have a more accurate estimation of sensitivity and specificity. It should be noted that although we have been able recently to generate infectious PrP^{Sc} "de novo" (without the addition of brain PrP^{Sc}), this requires modification of some PMCA parameters (unpublished observations). Under standard PMCA conditions, as those used in this study, spontaneous generation of infectious material does not occur within the number of cycles used in this study.

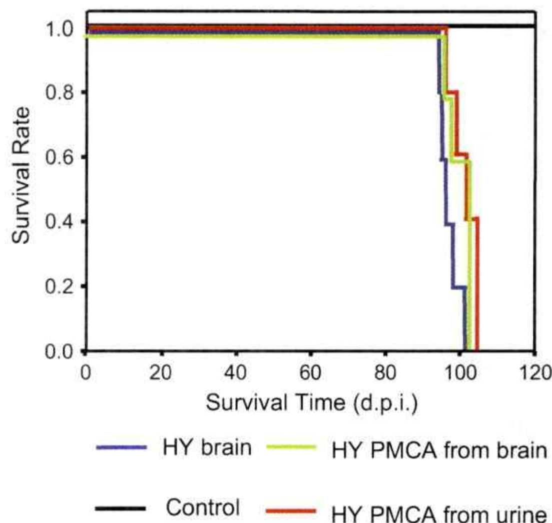


Fig. 3. Urinary PrP^{Sc} is infectious. To assess whether PrP^{Sc} amplified from urine maintain the infectious properties, we inoculated intracerebrally five wild-type hamsters with the sample S4 in Fig. 2 after seven serial rounds of PMCA. As controls, groups of hamsters were inoculated with equivalent quantities of brain-derived HY PrP^{Sc} and PMCA-generated PrP^{Sc} starting from HY brain. The later was generated as previously described [9]. Briefly a 10^4 dilution of HY brain was diluted into healthy hamster brain homogenate and subjected to 48 PMCA cycles. Thereafter, an aliquot of the amplified material was diluted 10-fold into healthy hamster brain homogenate and amplified again. This procedure was repeated to reach a 10^{20} dilution of brain inoculum in order to eliminate any brain derived PrP^{Sc}. A negative control group was included consisting of normal urine samples subjected to the same procedure of serial PMCA amplification. The onset of clinical signs was monitored as described in Section 2 and animals were considered sick when they reach clinical level 4, characterized by extensive behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, aggressiveness, jerks of the head and body and spontaneous backrolls. At this time, animals were sacrificed to avoid further pain and this is the number provided in the graph.

To assess whether PrP^{Sc} present in urine has the conformational properties required to produce infectivity, in vitro generated PrP^{Sc} by PMCA starting from urine was injected into wild-type hamsters. All inoculated animals developed clear signs of HY prion disease at an average of 101.6 days after inoculation (Fig. 3). The incubation period was not significantly different to that obtained with a similar quantity of PrP^{Sc} obtained from the brain of sick animals (97.8 days) or PMCA-generated starting with HY brain inoculum (100.1 days) (Fig. 3). Conversely, none of the animals inoculated with the equivalent material, but starting from urine of normal animals, developed disease. The disease exhibits the clinical characteristics typical of HY hamster scrapie, including hyperactivity, motor impairment, head wobbling, muscle weakness and aggressiveness. Histopathological and biochemical studies confirmed that the disease produced from urinary prions exhibited the typical characteristics of HY scrapie, including spongiform degeneration (Fig. 4A), PrP^{Sc} accumulation (Fig. 4B) and astroglyosis (Fig. 4C). The quantity and biochemical characteristics of PrP^{Sc} were also indistinguishable from HY prions (Fig. 4D). These data indicate that the infectious properties and strain characteristics of prions are maintained in the urinary excreted PrP^{Sc}.

4. Discussion

In this study we show that PrP^{Sc} with the capability to convert PrP^C into the misfolded form and produce disease is excreted in urine by most of scrapie sick hamsters. Detection of PrP^{Sc} in urine was possible only after extensive amplification by PMCA, suggesting that the quantity of prions in urine is very small. As reported previously, 7 serial rounds of PMCA enable detection of 1.3 ag of PrP^{Sc} in 20 μl of sample, which is equivalent to a 10^{-12} dilution from brain [11]. Considering that in this study we detected the majority of the samples after 6 serial rounds of PMCA, our estimation is that the quantity of PrP^{Sc} in urine is between 10^{10} and 10^{11}-fold lower than in brain. Since the PrP^{Sc} concentration in the brain of a sick hamster is around 50 μg/ml [11], we estimate that urine contains approximately 0.5–5 fg/ml of PrP^{Sc}, expressed as monomer concentration. Considering recent reports of the minimum size of the most infectious prion particle corresponds to a PrP^{Sc} oligomer containing around 20 molecules of PrP monomer [21], we estimate that there are around 500–5000 PrP^{Sc} oligomeric molecules in each ml of hamster urine. Comparison with the quantity of PrP^{Sc} present in blood of sick hamsters, which is around 10^9–10^{10} lower than in brain [10], we estimate that the concentration of PrP^{Sc} in urine is in average 10-fold lower than in blood.

PrP^{Sc} in urine retains the infectious properties, since injection of the agent amplified from this fluid produced a disease indis-

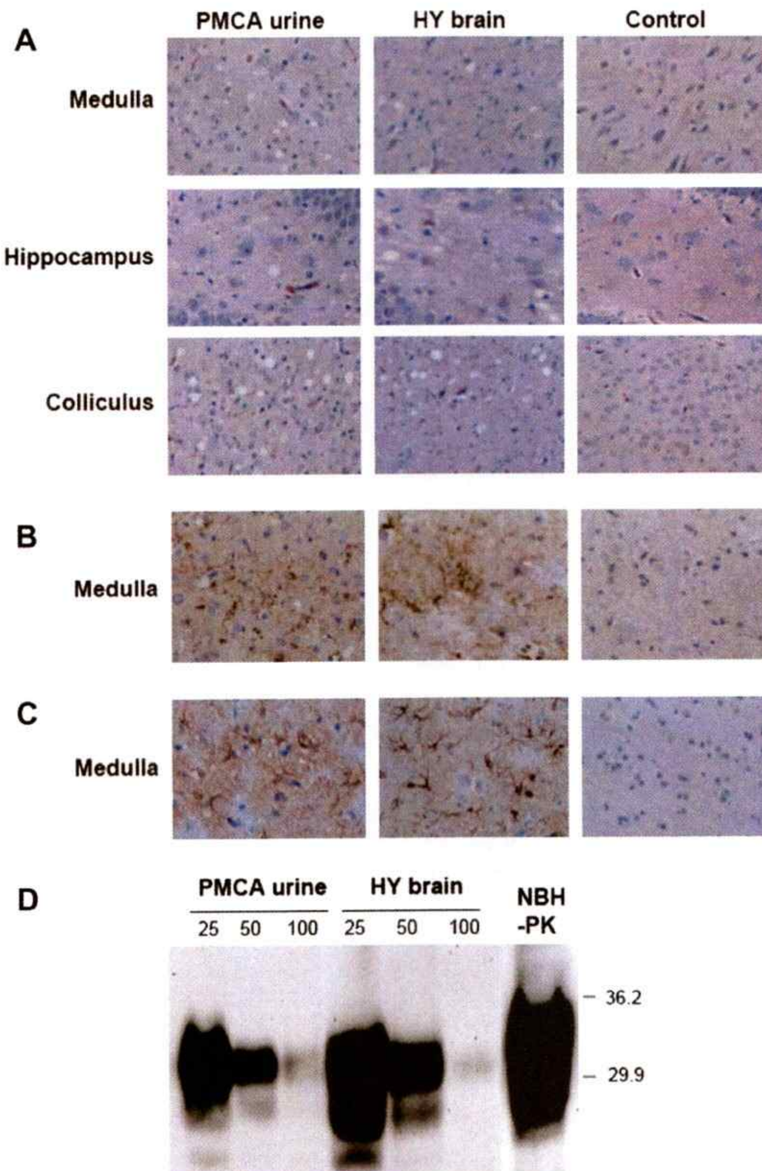


Fig. 4. Pathological and biochemical features of the disease produced by inoculation of PrP^{Sc} amplified from urine. As positive control we used animals inoculated with brain-derived HY prions and as negative control we analyzed brains of uninfected hamsters. (A) Spongiform degeneration was assessed by hematoxylin–eosin staining in diverse areas of the brain, including medulla, hippocampus and superior colliculus. (B) PrP^{Sc} accumulation was studied by histological staining with anti-PrP 3F4 antibody as described in methods. (C) Reactive astrocytes were visualized by immunological staining with antibodies against glial fibrillar acidic protein (GFAP). (D) PrP^{Sc} accumulation was evaluated by Western blot after PK digestion. Different quantities of brain homogenate (dilutions 1:25, 1:50 and 1:100 with respect to the brain) were loaded into the gel. All samples were treated with PK, except for the normal brain homogenate (NBH) were –PK is indicated. Results shown in panels A–D are representative of several animals analyzed.

tinguishable from the one induced by in vivo isolated material. Interestingly, animals inoculated with PrP^{Sc} amplified from the HY strain (both from brain and urine) showed a similar incubation time as those injected with the same quantity of PrP^{Sc} from sick brain. This is different to what we obtained previously with the 263K hamster prion strain in which the in vitro generated protein had consistently lower infectivity per unit of PrP^{Sc}, compared to the in vivo produced protein [9]. This is surprising considering that 263K and HY strains are fairly similar in most of the properties. So far, among the many prions from different species and strains we have amplified and shown to be infectious (data not shown), 263K is the only one that has a lower specific infectivity when amplified in vitro. Currently, we do not have an explanation for this phenomenon, but a recent publication from Kretschmar's group reported that immobilization of PMCA generated 263K PrP^{Sc} resulted in the same infectivity than brain derived PrP^{Sc} [22]. This result suggests that the in vitro produced protein was the same strain and the lower infectivity was due to a different size distribution which results in a more rapid biological clearance.

Our findings suggest that urine is a possible source of prion transmission. Since urine produced by animals potentially

infected with prions is permanently released and likely concentrated in environmental samples, such as soil and grass, this route may prove very relevant for spreading of TSEs in wild and captive animals such as cervids, sheep and cattle. It is known that PrP^{Sc} is highly resistant to degradation and infectivity can survive in the environment for a long time [23]. Recent studies have shown that PrP^{Sc} adsorbs efficiently into soil where it remains infectious and that both infectivity and PrP^{Sc} can stay intact in soil for long periods of time [24–26]. Contamination of soil with urinary prions may contribute to spreading prion disease among animals, which are known to ingest large amounts of soil, including cattle, sheep and cervids [24,26,27]. Worrisomely, the continuous excretion of urine and the extremely high resistance of prions may lead to a progressive accumulation of infectious material in the environment, with potentially catastrophic consequences in the future.

One of the top priorities in the prion field is to minimize further spreading of TSEs to humans or animals by limiting the exposure to contaminated material [7,14]. This is a difficult problem, because prion diseases have a long clinically-silent incubation period in which infected individuals may unknowingly transmit the disease. In addition, it is possible that many individuals may remain as sub-clinical carriers during their entire life, constituting a permanent source of prions [28]. Therefore, the development and validation of procedures to detect even the tiniest quantities of infectious material is of paramount importance [7,15]. Implementation of a large scale program to screen animals at risk of infection and diagnosis of the human population requires detection of prions in easily accessible samples, such as blood or urine. Our results showing that PrP^{Sc} can be detected in urine of a large proportion of infected animals provide a promising avenue for a sensitive and non-invasive biochemical diagnosis of prion diseases. Adaptation of PMCA for detection of prions in urine of naturally infected animals and humans may offer a great possibility for routine testing of prion infections.

Acknowledgements: This research was supported by NIH Grants NS049173, AI77774 and AG14359 to CS.

References

[1] Aguzzi, A. and Polymenidou, M. (2004) Mammalian prion biology: one century of evolving concepts. Cell 116, 313–327.
[2] Collinge, J. (2001) Prion diseases of humans and animals: their causes and molecular basis. Annu. Rev. Neurosci. 24, 519–550.
[3] Prusiner, S.B. (1998) Prions. Proc. Natl. Acad. Sci. USA 95, 13363–13383.
[4] Wadsworth, J.D. and Collinge, J. (2007) Update on human prion disease. Biochim. Biophys. Acta 1772, 598–609.
[5] Llewellyn, C.A., Hewitt, P., Knight, R.S., Amar, K., Cousens, S., Mackenzie, J. and Will, R.G. (2004) Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion. Lancet 363, 417–421.
[6] Ironside, J.W. (2006) Variant Creutzfeldt–Jakob disease: risk of transmission by blood transfusion and blood therapies. Haemophilia 12 (Suppl. 1), 8–15.
[7] Soto, C. (2004) Diagnosing prion diseases: needs, challenges and hopes. Nat. Rev. Microbiol. 2, 809–819.
[8] Saborio, G.P., Permanne, B. and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411, 810–813.
[9] Castilla, J., Saá, P., Hetz, C. and Soto, C. (2005) In vitro generation of infectious scrapie prions. Cell 121, 195–206.
[10] Castilla, J., Saa, P. and Soto, C. (2005) Detection of prions in blood. Nat. Med. 11, 982–985.
[11] Saa, P., Castilla, J. and Soto, C. (2006) Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. J. Biol. Chem. 281, 35245–35252.
[12] Soto, C., Anderes, L., Suardi, S., Cardone, F., Castilla, J., Frossard, M.J., Peano, S., Saá, P., Limido, L., Carbonatto, M., Ironside, J., Torres, J.M., Pocchiari, M. and Tagliavini, F. (2005) Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. FEBS Lett. 579, 638–642.
[13] Saa, P., Castilla, J. and Soto, C. (2006) Presymptomatic detection of prions in blood. Science 313, 92–94.
[14] Ingrosso, L., Vetrugno, V., Cardone, F. and Pocchiari, M. (2002) Molecular diagnostics of transmissible spongiform encephalopathies. Trends Mol. Med. 8, 273–280.
[15] Aguzzi, A. and Glatzel, M. (2006) Prion infections, blood and transfusions. Nat. Clin. Pract. Neurol. 2, 321–329.
[16] Brown, P., Cervenakova, L. and Diringer, H. (2001) Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt–Jakob disease. J. Lab. Clin. Med. 137, 5–13.
[17] Seeger, H., Heikenwalder, M., Zeller, N., Kranich, J., Schwarz, P., Gaspert, A., Seifert, B., Miele, G. and Aguzzi, A. (2005) Coincident scrapie infection and nephritis lead to urinary prion excretion. Science 310, 324–326.
[18] Kariv-Inbal, Z., Ben Hur, T., Grigoriadis, N.C., Engelstein, R. and Gabizon, R. (2006) Urine from scrapie-infected hamsters comprises low levels of prion infectivity. Neurodegener. Dis. 3, 123–128.
[19] Saa, P., Castilla, J. and Soto, C. (2005) Cyclic amplification of protein misfolding and aggregation. Meth. Mol. Biol. 299, 53–65.
[20] Castilla, J., Saa, P., Morales, R., Abid, K., Maundrell, K. and Soto, C. (2006) Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Meth. Enzymol. 412, 3–21.
[21] Silveira, J.R., Raymond, G.J., Hughson, A.G., Race, R.E., Sim, V.L., Hayes, S.F. and Caughey, B. (2005) The most infectious prion protein particles. Nature 437, 257–261.
[22] Weber, P., Giese, A., Piening, N., Mitteregger, G., Thomzig, A., Beekes, M. and Kretzschmar, H.A. (2007) Generation of genuine prion infectivity by serial PMCA. Vet. Microbiol. 123, 346–357.
[23] Brown, P. and Gajdusek, D.C. (1991) Survival of scrapie virus after 3 years' interment. Lancet 337, 269–270.
[24] Johnson, C.J., Phillips, K.E., Schramm, P.T., McKenzie, D., Aiken, J.M. and Pedersen, J.A. (2006) Prions adhere to soil minerals and remain infectious. PLoS Pathog. 2, e32.
[25] Seidel, B., Thomzig, A., Buschmann, A., Groschup, M.H., Peters, R., Beekes, M. and Terytze, K. (2007) Scrapie Agent (Strain 263K) can transmit disease via the oral route after persistence in soil over years. PLoS ONE. 2, e435.
[26] Johnson, C.J., Pedersen, J.A., Chappell, R.J., McKenzie, D. and Aiken, J.M. (2007) Oral transmissibility of prion disease is enhanced by binding to soil particles. PLoS. Pathog. 3, e93.
[27] Thornton, I. and Abrahams, P. (1983) Soil ingestion—a major pathway of heavy metals into livestock grazing contaminated land. Sci. Total Environ. 28, 287–294.
[28] Bishop, M.T., Hart, P., Aitchison, L., Baybutt, H.N., Plinston, C., Thomson, V., Tuzi, N.L., Head, M.W., Ironside, J.W., Will, R.G. and Manson, J.C. (2006) Predicting susceptibility and incubation time of human-to-human transmission of vCJD. Lancet Neurol. 5, 393–398.

Apéndice 7

[1] Protein Misfolding Cyclic Amplification for Diagnosis and Prion Propagation Studies

By JOAQUÍN CASTILLA, PAULA SAÁ, RODRIGO MORALES, KARIM ABID, KINSEY MAUNDRELL, and CLAUDIO SOTO

Abstract

Diverse human disorders are thought to arise from the misfolding and aggregation of an underlying protein. Among them, prion diseases are some of the most intriguing disorders that can be transmitted by an unprecedented infectious agent, termed prion, composed mainly (if not exclusively) of the misfolded prion protein. The hallmark event in the disease is the conversion of the native prion protein into the disease-associated misfolded protein. We have recently described a novel technology to mimic the prion conversion process *in vitro*. This procedure, named protein misfolding cyclic amplification (PMCA), conceptually analogous to DNA amplification by polymerase chain reaction (PCR), has important applications for research and diagnosis. In this chapter we describe the rational behind PMCA and some of the many potential applications of this novel technology. We also describe in detail the technical and methodological aspects of PMCA, as well as its application in automatic and serial modes that have been developed with a view to improving disease diagnosis.

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders of humans and animals usually characterized by the presence of PrP^{res}, an abnormal, protease-resistant isoform of a cellular protein called PrP^{C}. Historically, scrapie has been the most common TSE in animals, affecting sheep for more than 200 years (Collinge, 2001). TSEs have also been identified in mink and mule deer since the 1960s. The most recent and worrisome outbreak of an animal TSE disease is bovine spongiform encephalopathy (BSE) in cattle, which originated in Britain in the 1980s (Prusiner, 1997). BSE has important implications for human health, because the infectious agent can be transmitted to humans producing a new disease, termed variant Creutzfeldt-Jakob disease (vCJD) (Collinge, 1999; Will *et al.*, 1996). TSEs are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (Roos *et al.*, 1973). To date, no therapy or early diagnosis is available.

METHODS IN ENZYMOLOGY, VOL. 412
Copyright 2006, Elsevier Inc. All rights reserved.
0076-6879/06 $35.00
DOI: 10.1016/S0076-6879(06)12001-7

The pathogen responsible for TSEs, called "prion" (Prusiner, 1982), is composed mainly of a misfolded protein named PrP^{Sc}, which is a post-translationally modified version of the normal protein, PrP^{C} (Cohen and Prusiner, 1998). The conversion seems to involve a conformational change during which the α-helical content of the normal protein diminishes and the amount of β-sheet increases (Caughey *et al.*, 1991; Pan *et al.*, 1993). The structural changes are accompanied by alterations in the biochemical properties: PrP^{C} is soluble in nondenaturing detergents, PrP^{Sc} is insoluble; PrP^{C} is readily digested by proteases, whereas PrP^{Sc} is partially resistant, resulting in the formation of a N-terminally truncated fragment known as PrP^{res} (Baldwin *et al.*, 1995; Cohen and Prusiner, 1998).

At present, there is no accurate diagnosis for TSEs (Budka *et al.*, 1995; Weber *et al.*, 1997). In the case of sporadic CJD (sCJD) or vCJD, diagnosis is currently based almost entirely on clinical observation, because even though different molecules, such as protein S-100 or the 14-3-3 protein, have been proposed as markers of the disease, none of them are pathognomonic of the syndrome. For this reason, according to the operational diagnosis currently in use by the European Surveillance of CJD, definitive diagnosis can only be established by postmortem neuropathological examination and detection of PrP^{res} by immunohistochemistry, histoblot, or Western blot (Budka *et al.*, 1995; Weber *et al.*, 1997). Presymptomatic detection of sCJD or vCJD in living people is currently not possible.

To minimize the propagation of the bovine disease, several tests have been developed to diagnose BSE in postmortem brain tissue (Moynagh and Schimmer, 1999; Soto, 2004). However, in cattle, as in humans, there is no reliable way to identify affected animals early after infection (Schiermeier, 2001), because the problem of a diagnosis on the basis of PrP^{res} detection is that this form of the protein is abundant only in the brain at advanced stages of the disease.

Infectivity studies have been used to show that prions are also present in low amounts in peripheral tissues, such as lymphoid organs and blood (Aguzzi, 2000; Brown *et al.*, 2001; Collinge, 2001; Wadsworth *et al.*, 2001), and on the basis of these observations, different bioassays showing high sensitivity have been developed. In these methods, animals are injected with very low quantities of PrP^{Sc}, and the clinical signs indicating the presence of infectious material are monitored (Brown *et al.*, 2001). The biggest practical problem for using the infectivity assay in routine diagnosis, however, is that prion replication during the incubation phase progresses very slowly, and several months or even years may elapse before a detectable quantity of PrP^{Sc} has accumulated in the brain.

In vivo, prion replication is an extraordinary phenomenon that still remains not entirely understood. Although it is known that conversion of

PrP^C to PrP^{Sc} is an essential element in the etiology of the disease, the intrinsic mechanism by which this occurs, and whether other factors are involved, are crucial questions that remain to be answered.

The Birth of PMCA

To understand the mechanism of prion conversion, the nature of the infectious agent, and to attempt sensitive diagnosis, we have recently developed a technique referred to as protein misfolding cyclic amplification (PMCA) in which it is possible to simulate prion replication in the test tube in an accelerated mode (Saborio *et al.*, 2001). PMCA is a cyclic process leading to accelerated prion replication (Saborio *et al.*, 2001; Soto *et al.*, 2002). Each cycle is composed of two phases (Fig. 1). During the first phase, the sample, containing minute amounts of PrP^{Sc} and a large excess of PrP^C, is incubated to induce formation of PrP^{Sc} polymers. In the second phase, the sample is sonicated to break down the polymers, thus multiplying the number of growth sites for subsequent conversion. With each successive cycle, there is an exponential increase in the number of "seeds,"

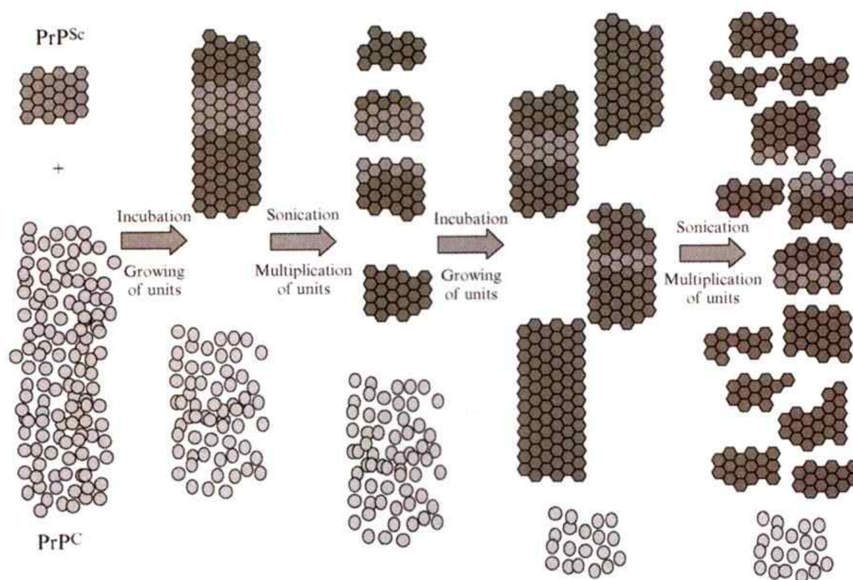


FIG. 1. Diagrammatic representation of principle behind PMCA. Cyclic amplification consists of subjecting a sample containing minute quantities of PrP^{Sc} and a large excess of PrP^C to cycles consisting of phases of growing of polymers and multiplication of converting units.

and thus the conversion process is dramatically accelerated (Fig. 1) (Saborio *et al.*, 2001). The cyclic nature of the system permits the use of as many cycles as is required to reach the amplification state needed for the detection of PrP^{Sc} in a given sample. Recently, we have shown that the *in vitro*–generated forms of PrP^{res} share similar biochemical and structural properties compared with PrP^{res} derived from sick brains (Castilla *et al.*, 2005a). Furthermore, inoculation of wild-type hamsters with *in vitro*–amplified PrP^{res} led to a scrapie-like disease identical to the illness produced using infectious material from diseased brains (Castilla *et al.*, 2005a). The technology has been automated, leading to a dramatic increase in efficiency of amplification and its application to detect PrP^{Sc} in blood of hamsters experimentally infected with scrapie (Castilla *et al.*, 2005b).

Applications of PMCA

Simulation of the process of prion conversion using PMCA represents a novel platform technology, which is likely to have a sustained impact in the field of prion biology. By using PMCA, we have been able to demonstrate definitively that the *in vitro*–generated PrP^{res} is fully infectious when injected into wild-type animals (Castilla *et al.*, 2005a). This provides the crucial demonstration that the *in vitro* conversion that occurs during PMCA closely mimics the events that take place over a protracted period *in vivo*, leading to disease and, ultimately, to death of the organism. The ability to simulate this process in accelerated mode, under controlled conditions *in vitro*, thus provides an opportunity to examine many aspects of prion biology that hitherto have been inaccessible to experimentation. Following is a brief description of the multiple areas in which PMCA may contribute.

The Molecular Mechanism of Species Barrier and Prion Strains Phenomena

As a consequence of the transmission of BSE to humans, a great concern has arisen regarding interspecies infectivity and tissues having a high enough quantity of prions to transmit the disease (Hill *et al.*, 2000; Wadsworth *et al.*, 2001). The molecular aspects that underlie the species barrier and the strain phenomena are still not understood (Bruce, 2003; Clarke *et al.*, 2001; Kascsak *et al.*, 1991). It has been shown that the sequence identity between infectious PrP^{Sc} and host prion protein plays a crucial role in determining species barrier (Telling *et al.*, 1996). It is clear that a few amino acid differences between both proteins can modify dramatically the incubation time and the course of the disease (Asante and

Collinge, 2001; DeArmond and Prusiner, 1996). So far, the investigation of the species barrier, prion strains, and the tissues carrying infectivity has been done mostly using the biological assay of infectivity (Clarke *et al.*, 2001; Wadsworth *et al.*, 2001). However, these studies are time consuming, because it is necessary to wait for several months or even years until the animals develop the clinical symptoms. In addition, the assessment of the species barrier for transmission of prions to humans is compromised by the use of animal models. PMCA can provide a complement to the *in vivo* studies of the species barrier and prion strains phenomenon by combining PrP^{Sc} and PrP^{C} from different sources in distinct quantities and evaluating quantitatively the efficiency of the conversion. In this sense, it has to be noted that the cell-free conversion system developed by Caughey and colleagues (Kocisko *et al.*, 1994) has been used successfully to compare and predict species barrier effects and the pertinent underlying mechanisms (Kocisko *et al.*, 1995; Raymond *et al.*, 1997).

Investigation of Factors Involved in the PrP^{C} to PrP^{Sc} Conversion

Another important issue in prion propagation is to know whether other factors have any role in the PrP^{C} to PrP^{Sc} conversion. We reported previously that the conversion procedure does not occur using highly purified prion proteins (PrP^{Sc} and PrP^{C}) under our experimental conditions (Saborio *et al.*, 1999). However, the activity is recovered when the bulk of cellular proteins is reincorporated into the sample (Saborio *et al.*, 1999). This finding provides direct evidence that other factors present in the brain are essential to catalyze prion propagation. In this direction, PMCA could also contribute to a better understanding of the mechanism of prion conversion and the identification of additional factors involved. Indeed, Supattapone and coworkers have used PMCA to show that metal cations, such as copper and zinc, and polyanions including diverse types of RNA molecules can modulate PrP conversion *in vitro* (Deleaut *et al.*, 2003, 2005; Nishina *et al.*, 2004).

Screening for Inhibitors of Prion Propagation

In the same manner that prion propagation can be used to discover novel drug targets for TSEs in culture cells, PMCA also shows a great advantage in these types of studies. Inhibitors and promoters could be tested quickly in different contexts using human and bovine prions, for which no prion-permissive culture cells have been generated. One of the best targets for TSE therapy is the inhibition and reversal of PrP^{C} to PrP^{res} conversion (Head and Ironside, 2000; Soto and Saborio, 2001). In drug development, it is crucial to have a relevant and robust *in vitro* assay to

screen compounds for activity before testing them in more time-consuming and expensive *in vivo* assays. PMCA represents a convenient biochemical tool to identify and evaluate the activity of drug candidates for TSE treatment, because it mimics *in vitro* the central pathogenic process of the disease. Also the simplicity of the method and the relatively rapid outcome are important features of these types of studies. Moreover, the fact that PMCA can be applied to prion conversion in different species provides the opportunity to validate the use in humans of drugs that have been evaluated in experimental animal models of the disease.

Diagnosis

One of the most valuable applications of PMCA is in TSE diagnosis. As stated previously, the biggest problem facing a biochemical test to detect PrP^{res} presymptomatically in tissues other than brain is the very low amount of PrP^{res} existing in them. Most of the efforts to develop a diagnostic system for prion diseases have been focused on the increase of sensitivity of the current detection methods. PMCA offers the opportunity to enhance existing methods by amplifying the amount of PrP^{res} in the sample. Combining the strategy of reproducing prions *in vitro* with any of the high-sensitive detection methods, the early diagnosis of TSE may be achieved. The aim would be not only to detect prions in the brain in early presymptomatic cases but also to generate a test to diagnose living animals and humans. For this purpose, a tissue other than brain is required and, to have an easier noninvasive method, detection of prions in body fluids such as urine or blood are the best options. A blood test for CJD can have many applications, including screening of blood banks, identification of populations at risk, reduction of iatrogenic transmission of CJD, and early diagnosis of the disease (Soto, 2004).

Extension to Other Protein Misfolding Diseases

Besides TSEs, several other diseases involve changes in the conformation of a natural protein to an altered structure with toxic properties capable of inducing tissue damage and organ dysfunction (Carrell and Lomas, 1997; Dobson, 2004; Kelly, 1998; Soto, 2001). This group of diseases called protein misfolding disorders includes several forms of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases, as well as a group of more than 15 distinct disorders involving amyloid deposition in diverse organs (Soto, 2001). In a similar way to PrP^{res} in TSE, the protein conformational changes associated with the pathogenesis of these diseases result in the formation of abnormal proteins rich in β-sheet structure, partially resistant to proteolysis and with a high tendency to aggregate (Soto,

2001). The process of misfolding and aggregation also follows a seeding-nucleation mechanism, and hence the principles of PMCA might be applied to amplify the abnormal folding of these proteins as well. Therefore, PMCA may have a broader application for research and diagnosis of diseases in which misfolding and aggregation of a protein are hallmark events.

Method and Technical Details

Protein misfolding cyclic amplification in its original mode was done by manual operation (Saborio *et al.*, 2001), but we have recently developed an automated mode (aPMCA), which has been developed to increase sensitivity, specificity, and throughput. The increased throughput of aPMCA has allowed us to evaluate the importance of numerous variables including temperature, pH, substrate concentration, type, and concentration of the detergents, power and length of sonication, and so on. In addition, the sensitivity has been increased further by the introduction of a new concept involving serial rounds of amplification. This procedure is named serial automated PMCA (saPMCA) and is similar to application of multiple rounds of PCR amplification to reach high sensitivity detection of DNA.

Buffer

Conversion Buffer. Composition of the conversion buffer (CB) has been established and optimized after exhaustive studies, and we have found that even small changes may dramatically affect the efficiency of the amplification process. Thus, we highly recommend using the following conversion buffer: PBS; NaCl, 0.15 *M*; Triton X-100, 1%; and complete protease inhibitor cocktail 1× (Roche, cat#: 1836145). A pH of between 7.0 and 7.3 is necessary to obtain the best results. Low concentrations of SDS may also be included in CB but are not usually necessary. When used, the SDS concentration should be optimized depending on the type of the PrP^{Sc} species to be amplified.

Equipment

Sonicator. In the original PMCA protocol, the proof of concept was established using a manual sonicator using a single microtip (Saborio *et al.*, 2001). However, with the increased need for high throughput and automation, we have implemented a programmable sonicator that uses a 96-well plate format (Misonix, USA, model S3000MP sonicator) and satisfies the principal requirements for PMCA even though the machine was originally designed for other purposes. Improvements to the equipment planned for the near future should lead to a full adaptation to the needs of PMCA.

Homogenizer. A principal component in the PMCA reaction is the PrP^C used as substrate. At this point we consider that a normal brain homogenate (NBH) is the best substrate for high-efficiency amplification. For brain homogenization, we recommend using the high-viscosity mixer Eurostar PWR BSC S1 (IKA, USA), whereas for manual homogenization, the Potter homogenizer is a perfectly satisfactory option.

Preparation of Samples for Amplification

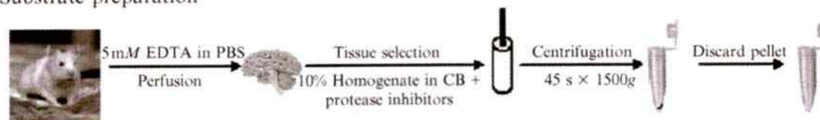
The correct preparation of the inoculum (PrP^{Sc} used as starting material) and substrate (material used as source of PrP^C) samples is critical to achieve a good efficiency of amplification (Fig. 2). To prepare the best samples for PMCA it is important to know several critical parameters concerning the material to be amplified, in particular, (1) the animal species; (2) the type of tissue in which PrP^{Sc} is to be detected; (3) an estimation of the amount of PrP^{Sc} in the sample to be amplified; (4) the storage conditions of the sample; and (5) possible inhibitors that may interfere with the amplification.

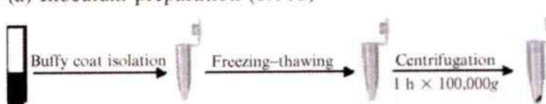
Whenever possible, it is preferable to use a substrate from the same species as the PrP^{Sc} to be amplified (see "Preparation of Substrate"). In this way, any potential problems caused by species barrier can be avoided. On the other hand, the use of substrates from different species can be useful, for example, in studies to understand the nature of the species barrier.

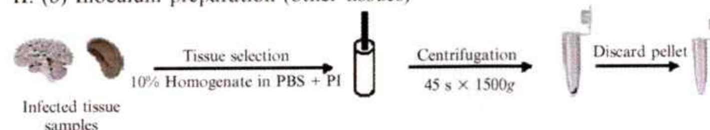
Another important parameter is the condition in which the sample to be amplified has been stored. Although PrP^{Sc} is resistant to high temperature (Castilla *et al.*, 2005a), treatment at >100° can promote the formation of large aggregates in the samples, which interfere with efficient amplification. The use of samples previously denatured using chaotropic agents or ionic detergents at high concentrations is also incompatible with PMCA and should be avoided. Although not many studies have been done using formalin-fixed samples, it is not recommended to use this type of sample for amplification.

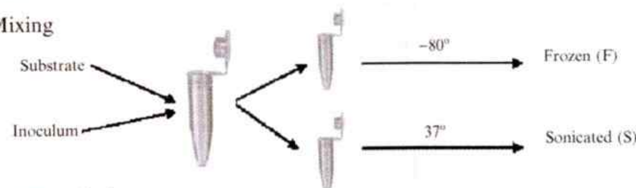
Finally, either the samples to be amplified or the substrates prepared for the amplification may contain potential inhibitory molecules such as plasminogen, cations, or other, as yet, unidentified blood compounds that have been found to interfere with PMCA (data not shown). Because it is extremely important to eliminate such molecules, some samples will require pretreatment before the amplification process (see "Pretreatment in Preparation of Samples from Peripheral Sources"). In addition, special precautions need to be taken when using blood, CSF, saliva, milk, urine, or feces (see "Preparation of Samples from Peripheral Sources").

Preparation of Prion-Infected Samples from CNS. Infectious brain material should be homogenized in conversion buffer (CB) at 10% (w/v)









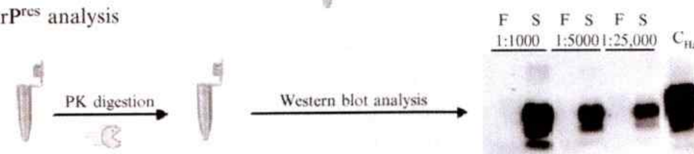


FIG. 2. PMCA method. The scheme shows a diagram of the different procedures involved in PMCA, including the preparation of the substrate (I), inoculum (II), the mixing and amplification process (III), and the PrP^{res} detection (IV).

at 4° using a high-viscosity mixer (see "Equipment"). The homogenate should be centrifuged at 1500*g* for 30 sec and the supernatant retained for further use. Because much of the PrP^{Sc} is present in the membrane fractions, centrifugation at a higher speed should be avoided to reduce the risk of losing infectious material in the pellet. Storage of the homogenate in aliquots at −80° is recommended.

Preparation of Prion-Infected Samples from Peripheral Sources. Although the most frequent samples for testing will be brain homogenates, other tissues could also be used as a source of infectious material. In most cases, the samples should be prepared in the same manner, with the exception of some special tissues that may require some form of pretreatment. We have

separated these latter samples into: (1) blood samples, (2) tissue samples containing high amount of blood, and (3) other fluids such as CSF, saliva, milk, urine, and feces.

BLOOD SAMPLES. Blood is probably the most interesting sample to use in prion diagnosis. However, it is also considered one of the most complicated tissues for these purposes. The extremely low amount of PrP^{Sc} present in blood and the presence of inhibitors of prion replication make it necessary to take special precautions. The blood should be collected using a syringe containing EDTA to avoid clotting and placed in tubes containing sodium citrate. For standard infectious blood material, it is necessary to use at least 1 ml of whole blood. One milliliter of PBS should be added to the total blood and buffy coat should be prepared by centrifugation on a Ficoll gradient using standard procedures. The isolated buffy coat fraction should be subjected to three consecutive freezing–thawing cycles to break the cells, and then centrifuged for 100,000*g* for 1 h at 4° to pellet PrP^{Sc}. For PMCA, the pellet should be resuspended directly in 100 μl of normal brain homogenate. The amount of PrP^{Sc} present in 1 ml of infectious blood is usually not sufficient for standard aPMCA, and in this case serial rounds of aPMCA are needed (Castilla *et al.*, 2005b) as described later (see "Serial Automatic PMCA Procedure").

TISSUE SAMPLES CONTAINING HIGH AMOUNT OF BLOOD. We have observed during development of PMCA that small amounts of plasma or serum can inhibit the PMCA reaction. In addition, the proteinase K treatment, which is performed after amplification to detect PrP^{Sc}, can be inhibited by protease inhibitors present in blood. For this reason, whenever possible, it is recommended that animals be perfused with PBS + 5 m*M* EDTA before dissection of the tissues. When this is not possible, freshly dissected tissues should be washed carefully in PBS + 5 m*M* EDTA before the preparation of the samples.

OTHER SOURCES OF PrP^{Sc} SUCH AS CSF, SALIVA, MILK, URINE, AND FECES. Although we have not yet attempted to use PMCA to amplify samples from these biological fluids, later we provide some advice based on our experience with similar samples. We would recommend diluting these samples with PBS + 5 m*M* EDTA followed by centrifugation at 100,000*g* for 1 h. This procedure requires exhaustive washing of the sample with CB and a further centrifugation at 100,000*g* for 1 h. The pellet, most probably invisible, should be resuspended directly in substrate to avoid more dilution. It is important to note that these special samples could contain enzymes, urea, and other molecules that may coprecipitate with the infectious material, thereby further complicating the amplification step.

Once prepared, samples for amplification should be divided into aliquots and frozen at −80°. It is known that PrP^{Sc} has a strong tendency to

aggregate, and this seems to increase with repeated freezing and thawing. Although higher levels of aggregation seem not to affect the ability of PrP^{Sc} to act as template for amplification (samples have been frozen and retested more than 20 times without any significant difference), the size and number of the large aggregates can lead to errors in sample dilution, which thus may produce variability in the level of amplification.

In vivo studies have shown that the PK-treated PrP^{Sc} is still infectious, even though its infectivity is diminished compared with that of nontreated PrP^{Sc}. The latter may be due to an increased propensity to aggregation. Samples digested with PK treatment can also be amplified by PMCA even though, as with *in vivo* infectivity, the level of amplification is slightly diminished. Despite this, the use of PK to remove proteinaceous contaminants might be a good option for certain samples.

Preparation of Substrate. We consider the preparation of the substrates to be the most critical step in achieving successful PMCA. In our hands, the best and most convenient substrate is normal brain homogenate from the same species as the prion sample to be amplified, although other substrates have also been used successfully (see "Other Substrates").

Normal Brain Homogenate. As mentioned previously, the presence of cations and certain blood components can seriously affect the amplification process. For this reason, we consider it highly beneficial to perfuse the animals with PBS + 5 m*M* EDTA before the brain extraction. After perfusion, a totally white brain can be obtained from the animal. We recommend the use of a CO_2 chamber for euthanizing, to avoid using anesthesia, which may also interfere with the subsequent amplification.

Whenever possible, we recommend preparing substrate from animals of the same species as the infectious sample to be amplified. In the case of larger animals such as cattle, sheep, goats, and so on in which perfusion before tissue dissection is not possible, we would recommend removing the entire brain as quickly as possible to reduce postmortem lysis, and then washing the fresh tissue immediately with cold PBS + 5 m*M* EDTA to remove as much blood as possible. In case obtaining brains from the same species is a problem (e.g., humans), we have successfully used transgenic mice brain overexpressing PrP^{C}.

We have not experimented extensively at this point to determine which part of the brain is the most suitable for PMCA studies; however, currently we would recommend using the entire encephalic area including brain stem. On the basis of *in vivo* experiments, we also recommend using animals as young as possible, although we avoid using fetal tissue.

After removal, the brain should be placed into conversion buffer at 4° and immediately homogenized at 10% (w/v) using a high-viscosity mixer (see "Equipment"). In our hands, the highest amplification is obtained using

7.5–10% of substrate, but it is also possible to use 2.5–5%, although with lower yield of amplification. After homogenization, large pieces of tissue and unbroken cells should be removed by a low-speed centrifugation. The low speed (1500*g* for 30 sec) is important to avoid losing or destabilizing membrane components that seem to be essential for conversion. The final substrate preparation should be turbid with visible membrane fragments still present. If the homogenate is transparent, the efficiency of conversion will not be good.

Homogenates, once prepared, should be stored at −80° and can be thawed and refrozen approximately 10 times without significant loss of efficiency. Storage at −20° is not recommended; however, for short-term use, homogenates can be kept at 4° for up to 7 days. Following the studies from Supattapone's group (Deleault *et al.*, 2003), it is recommended that the work be done in RNAse-free conditions.

Other Substrates. The normal brain homogenate is considered, at this moment, to be the most efficient substrate for amplification, particularly for TSE diagnosis purposes. However, the use of alternative substrates could be beneficial for other studies aimed, for example, at understanding the tissues capable to propagate prions or to localize other factors involved in PrP^C to PrP^{Sc} conversion.

CELLS. Protein extracts from whole cells can provide a good substrate for specific applications of PMCA. For these studies, transient or stably transfected knock out-PrP (PrP-KO) cells overexpressing different PrP^C transgenes can provide useful substrates for subsequent experimentation. Our experience at this point has been focused on PrP knock out N2a cells overexpressing hamster or mouse PrP as substrates. Cells should be resuspended in a small volume of PBS (0.5–1 ml) and centrifuged in a 1.5-ml Eppendorf tube for 5 min at 2000*g*. The supernatant must be completely removed, and the pellet containing cells should be resuspended in 50–100 μl of CB and the centrifugation step repeated. To enhance the level of amplification using cells as substrate, we have found that it is useful to supplement the PMCA reaction with a "PrP inert substrate" such as PrP-KO brain homogenate or normal brain homogenate from a species resistant to prion propagation such as rabbit. This material provides additional quantities of a yet unknown "conversion factor," which is highly expressed in brain.

LIPID RAFTS. The detergent-resistant membrane (DRM) or lipid–raft fraction, should also be considered as a good alternative substrate to the whole brain homogenate. PrP^C is attached to the outer cell membrane by a glycosyl phosphatidylinositol (GPI) anchor, and, like other GPI-anchored proteins, PrP^C is found in the cholesterol, glycosphingolipid, sphingomyelin-rich membrane subdomains known as lipid rafts (Vey *et al.*, 1996). This

membrane fraction seems to contain all elements required for prion conversion (unpublished data). Various methods have been described to isolate lipid rafts both from brain homogenates and from neuronal cell lines. We routinely use Optiprep (Axis-Shield) density gradients to isolate lipid raft from N2a neuroblastoma cells or from brain.

PURIFIED PrP^C. Currently, one of the most intriguing issues in the prion field is the identity of the factors required for PrP^C to PrP^{Sc} conversion. With this in mind, we have designed a purification technique to obtain PrP^C free of other components but suitable for conversion to PrP^{Sc}. This purified substrate can be used to study the effects of well-characterized biological fractions on the conversion process. Mixing PrP^C with a sample that is able to complement the conversion enables us to have a better understanding of the requirements of the conversion process (e.g., nucleic acids, lipids, proteins). The ability to purify PrP^C will allow us to mix it with PrP^{Sc} and screen for cellular components capable of reconstituting a conversion competent environment. This is currently being worked out and will be reported at a later time.

Automated PMCA Procedure

In the original PMCA procedure (Saborio *et al.*, 2001), sonication was performed manually using a single probe sonicator. More recently, an automated version of PMCA has been developed (Castilla *et al.*, 2005a) that shows improved efficiency and reproducibility. This procedure now referred to as automatic PMCA (aPMCA) uses an inverted 96-well sonicator that can be programmed for automatic operation (see "Equipment"). This technique has proved to be of great value for diagnosis and for other prion propagation studies. aPMCA overcomes one of the major drawbacks of manual PMCA, namely cross-contamination, because there is no direct contact between the sonicator probe and the sample. The following recommendations for standard use of this procedure should be observed:

1. Samples to be amplified are placed at different dilutions into 0.2-ml PCR tubes and mixed with 10% substrate (see "Preparation of Substrate"). The final volume should be between 60 and 100 μl. For each condition, three tubes are prepared. One is frozen immediately (frozen control), and the second is subjected to multiple cycles of incubation/sonication (PMCA samples) (Fig. 2).

2. Samples are incubated for 30–60 min at 37° in the reservoir of the automatic sonicator. The duration of the incubation phase needs to be optimized for each sample, because factors such as the prion strain and the amount of PrP^{Sc} in the sample will require different incubation periods. There are numerous parameters that can be modified (including time,

temperature, and agitation rate) to reach highest efficiency for a particular sample; however, in this chapter we will limit the description to our standard procedure. As further knowledge about prion replication *in vitro* accumulates, and as more sophisticated equipment becomes available, additional modifications to the technique will be implemented.

3. Samples are sonicated for a single pulse of 40 sec. The sonication is the most critical step in this technique, and variation in the level of sonication can generate huge differences in the results. Using the optimal level of sonication is crucial to break down and multiply the PrP^{Sc} polymers without affecting their capacity to act as "seed" for further PrP^{C} conversion. It is also important to note that the ultrasound strength needed to amplify PrP^{Sc} of distinct strains and from diverse species can be different (Soto *et al.*, 2005), and hence low or even no amplification at all may be obtained for new samples under conditions that work very well for others. These findings are probably related to the specific conformation/aggregation state of each strain of prion, which has been proposed to explain the differences in clinical, pathological, and biochemical features of distinct strains.

The sonication step is the most difficult to monitor adequately, and many factors can influence the final amplification observed. These factors, which we describe later, need to be taken into account to achieve maximal amplification.

a. *Power of sonication:* The power of sonication for the 263K hamster prion strain should be set to the maximum potency of this sonicator (level 8–10). For other species/strains, sonication power should be optimized experimentally and is in general lower than for 263K strain.
b. *Wavelength:* At present, we do not know how wavelength affects the effectiveness of sonication; however, we should be able to determine this once other equipment becomes available.
c. *Water in the sonication reservoir:* The reservoir has to be filled with 140 ml of water (see "Equipment"), which decreases at a rate of around 2.5 ml/h at 37°. Tubes should be incubated without touching the sonication plate.
d. *Tubes for sonication:* It is very important to use thin-walled 0.2-ml tubes to obtain the most effective penetration of ultrasound waves.
e. *Number of tubes:* The rack used in this sonicator is designed to hold 96 tubes. However, our experiments have shown that the effective power of the sonicator diminishes when the rack is completely full. This is probably because each tube attenuates to some extent the effect of the ultrasound waves. If all positions need to be used, we would recommend increasing the power of sonication. In our

standard procedure, only 60% of the rack is used, with tubes being placed at random positions across the plate.

4. The incubation/sonication cycle (steps 2–3) should be repeated as many times as needed to reach the desired level of amplification. For the standard reaction, we recommend approximately 24 h of cyclic amplification.

It is preferable to complete the entire amplification experiment without freezing–thawing the samples. If it is necessary to interrupt the amplification, samples should always be frozen at −80°. It is not necessary to use a quick freezing procedure.

Although the theoretical limit of amplification will be the amount of PrP^C substrate present in the tube, we regularly observe that the efficiency of amplification starts to decrease after approximately 150 cycles (75 h of incubation). This problem is most likely the result of a deleterious effect of prolonged incubation at 37°. Under these conditions, the PrP^C substrate or other brain-derived cofactors necessary to promote the conversion of PrP^C into PrP^{Sc} might be altered or consumed. In view of this, and to increase further the level of achievable amplification, we have extended the technique of aPMCA to include serial rounds, in which at each new round, the amplified samples are rediluted into fresh substrate. This new approach, termed serial automated PMCA (saPMCA), will be described in the following.

To maintain a good reproducibility of the aPMCA technique some points need to be carefully considered, especially when small amounts of PrP^{Sc} are used for amplification. In particular, the following situations should be avoided:

a. Low sample volumes (<50 μl).
b. Low water level (<100 ml) in the reservoir of the automatic sonicator.
c. Bubble formation in the sample that could prevent a good transmission of sonication waves.

5. After the last pulse of sonication is completed, the samples are ready for protease K digestion (see "Detection of Amplified Product"). If digestion is not performed immediately, the amplified samples should be stored at −80°.

Serial Automatic PMCA (saPMCA) Procedure

Serial automatic PMCA consists of successive rounds of aPMCA in which at each round the amplified sample is diluted into fresh substrate. This approach is highly recommended for experiments requiring elevated levels of amplification, especially when working with samples containing minute initial amounts of PrP^{Sc}, such as blood, CSF, or peripheral nonlymphoid tissues.

As mentioned previously, the efficiency of PMCA decreases after approximately 75 h of constant incubation at 37°. However, efficient conversion is restored when the amplified samples are diluted into fresh substrate. Therefore, after a first round of standard aPMCA, the amplified samples are diluted into NBH and amplified in a second round. The dilution factor depends on the purpose of the study and the original dilution of the PrP^{Sc}. For experiments in which the aim is to simply eliminate the original inoculum (e.g., for comparative studies, infectivity experiments), and where the initial amount of PrP^{Sc} is relatively high (4 log LD_{50}), a 100- or 1000-fold dilution can be performed at each stage of saPMCA. However, in studies where undetectable amounts or PrP^{Sc} are present, even after a first round of PMCA, a 10-fold dilution is enough to refresh the substrate. The rounds of saPMCA can be repeated as many times as is needed to reach the detection threshold of Western blotting. Samples remaining negative after eight rounds of saPMCA can be considered negative, because according to our experience, approximately six rounds of saPMCA can amplify the minimum amount of material required for amplification (approximately a few hundred molecules of PrP^{Sc} monomers) (unpublished data).

Because of the PCR-like nature of PMCA, special care should be taken during the manipulation of the samples when performing serial dilutions of the amplified material. Thus, after each round of aPMCA, the samples should be gently spun down to remove material present in the lid, which arises during sonication or because of condensation of the sample. Given the power of this procedure, inclusion of negative control samples (NBH without PrP^{Sc}) that are amplified and serially diluted in parallel with the experimental samples is highly recommended.

For safety conditions, filter tips should be used for liquid handling, and sonication should be performed in a closed container inside a BSL-2 hood to avoid the spread of infectious material.

Detection of Amplified Product

After amplification, the two samples (amplified and frozen) are digested with proteinase K (PK), and PrP^{res} is detected by immunological methods. Because distinct species/strains of prion show a different extent of resistance to proteolytic degradation, the optimal PK treatment condition should be determined beforehand. The critical issue is to make sure that no PrP^{C} remains undigested after PK treatment, because it is a common mistake to confuse incomplete digestion of PrP^{C} with false-positive PrP^{res} formation. When PrP is detected by Western blotting, it is easy to distinguish incomplete PrP^{C} digestion from bona-fide PrP^{res}, because the latter exhibit a switch on molecular weight because of the removal of the first ~90 amino acids. To ensure complete digestion, especially after extended incubations, a higher

concentration of PK may be required to digest increasingly larger aggregates. Addition of up to 0.05% SDS in the buffer used for the PK treatment may also help. Digestions using temperatures between 42° and 64° and with shaking at 350–450 rpm are also recommended. Our standard procedure, which can be taken as a basis for further optimization, is as follows.

1. Prion-containing samples (20 μl) are incubated with standard concentrations of PK (50 μg/ml for hamster, 25 μg/ml for mouse, 20 μg/ml for cattle, or 40–50 μg/ml for CJD) for 1 h at 45° with shaking at 350–450 rpm. Aliquots of PK (10 mg/ml) are stored frozen at −20°, and in the interests of reproducibility, any thawed, unused enzyme is discarded at the end of the experiment. Note: Blood contains protease inhibitors that can interfere with the PK digestion, and in those samples where the presence of blood is unavoidable, the PK concentration should be adjusted accordingly.

2. The PK digestion is stopped by addition of phenyl-methyl-sulfonyl-fluoride or SDS-PAGE loading buffer. Samples can be analyzed for the presence of PrP^{res} using any of the established immunological methods, such as Western blotting or ELISA (Soto, 2004).

Acknowledgments

This work is partially supported by NIH grants AG0224642 and NS049173. Dr. Soto is part of the European Community project TSELAB.

References

Aguzzi, A. (2000). Prion diseases, blood and the immune system: Concerns and reality. *Haematologica* **85,** 3–10.

Asante, E. A., and Collinge, J. (2001). Transgenic studies of the influence of the PrP structure on TSE diseases. *Adv. Protein Chem.* **57,** 273–311.

Baldwin, M. A., Cohen, F. E., and Prusiner, S. B. (1995). Prion protein isoforms, a convergence of biological and structural investigations. *J. Biol. Chem.* **270,** 19197–19200.

Brown, P., Cervenakova, L., and Diringer, H. (2001). Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jakob disease. *J. Lab. Clin. Invest.* **137,** 5–13.

Bruce, M. E. (2003). TSE strain variation. *Br. Med. Bull.* **66,** 99–108.

Budka, H., Aguzzi, A., Brown, P., Brucher, J. M., Bugiani, O., Gullotta, F., Haltia, M., Hauw, J. J., Ironside, J. W., Jellinger, K., Kretzschmar, H. A., Lantos, P. L., Masullo, C., Schlote, W., Tateishi, J., and Weller, R. O. (1995). Neuropathological diagnostic criteria for Creutzfeldt-Jakob disease (CJD) and other human spongiform encephalopathies (Prion diseases). *Brain Pathol.* **5,** 459–466.

Carrell, R. W., and Lomas, D. A. (1997). Conformational disease. *Lancet* **350,** 134–138.

Castilla, J., Saá, P., Hetz, C., and Soto, C. (2005a). *In vitro* generation of infectious scrapie prions. *Cell* **121,** 195–206.

Castilla, J., Saá, P., and Soto, C. (2005b). Detection of prions in blood. *Nat. Med.* **11,** 982–985.

Caughey, B. W., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, W. S. (1991). Secondary structure analysis of the scrapie-associated protein PrP 27–30 in water by infrared spectroscopy. *Biochemistry* **30,** 7672–7680.

Clarke, A. R., Jackson, G. S., and Collinge, J. (2001). The molecular biology of prion propagation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356,** 185–195.

Cohen, F. E., and Prusiner, S. B. (1998). Pathologic conformations of prion proteins. *Ann. Rev. Biochem.* **67,** 793–819.

Collinge, J. (1999). Variant Creutzfeldt-Jakob disease. *Lancet* **354,** 317–323.

Collinge, J. (2001). Prion diseases of humans and animals: Their causes and molecular basis. *Annu. Rev. Neurosci.* **24,** 519–550.

DeArmond, S. J., and Prusiner, S. B. (1996). Transgenetics and neuropathology of prion diseases. *Curr. Top. Microbiol. Immunol.* **207,** 125–146.

Deleault, N. R., Lucassen, R. W., and Supattapone, S. (2003). RNA molecules stimulate prion protein conversion. *Nature* **425,** 717–720.

Deleault, N. R., Lucassen, R. W., and Supattapone, S. (2005). PrPres amplification reconstituted with purified prion proteins and synthetic polyanions. *J. Biol. Chem.* **280,** 26873–26879.

Dobson, C. M. (2004). Protein chemistry. In the footsteps of alchemists. *Science* **304,** 1259–1262.

Head, M. W., and Ironside, J. W. (2000). Inhibition of prion protein conversion: A therapeutic tool? *Trends Microbiol.* **6,** 6–8.

Hill, A. F., Joiner, S., Linehan, J., Desbruslais, M., Lantos, P. L., and Collinge, J. (2000). Species-barrier-independent prion replication in apparently resistant species. *Proc. Natl. Acad. Sci. USA* **97,** 10248–10253.

Kascsak, R. J., Rubenstein, R., and Carp, R. I. (1991). Evidence for biological and structural diversity among scrapie strains. *Curr. Top. Microbiol. Immunol.* **172,** 139–152.

Kelly, J. W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* **8,** 101–106.

Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T., and Caughey, B. (1994). Cell-free formation of protease-resistant prion protein. *Nature* **370,** 471–474.

Kocisko, D. A., Priola, S. A., Raymond, G. J., Chesebro, B., Lansbury, P. T., Jr., and Caughey, B. (1995). Species specificity in the cell-free conversion of prion protein to protease-resistant forms: A model for the scrapie species barrier. *Proc. Natl. Acad. Sci. USA* **92,** 3923–3927.

Moynagh, J., and Schimmer, H. (1999). Test for BSE evaluated. *Nature* **400,** 105.

Nishina, K., Jenks, S., and Supattapone, S. (2004). Ionic strength and transition metals control PrPSc protease resistance and conversion-inducing activity. *J. Biol. Chem.* **43,** 2613–2621.

Pan, K. M., Baldwin, M., Njuyen, J., Gassett, M., Serban, A., Groth, D., Mehlhorn, I., and Prusiner, S. B. (1993). Conversion of alpha-helices into b-sheets features in the formation of scrapie prion proteins. *Proc. Natl. Acad. Sci. USA* **90,** 10962–10966.

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science* **216,** 136–144.

Prusiner, S. B. (1997). Prion diseases and the BSE crisis. *Science* **278,** 245–251.

Raymond, G. J., Hope, J., Kocisko, D. A., Priola, S. A., Raymond, L. D., Bossers, A., Ironside, J., Will, R. G., Chen, S. G., Petersen, R. B., Gambetti, P., Rubenstein, R., Smits, M. A., Lansbury, P. T., Jr., and Caughey, B. (1997). Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature* **388,** 285–288.

Roos, R., Gajdusek, D. C., and Gibbs, C. J., Jr. (1973). The clinical characteristics of transmissible Creutzfeldt-Jakob disease. *Brain* **96,** 1–20.

Saborio, G. P., Permanne, B., and Soto, C. (2001). Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411,** 810–813.

Saborio, G. P., Soto, C., Kascsak, R. J., Levy, E., Kascsak, R., Harris, D. A., and Frangione, B. (1999). Cell-lysate conversion of prion protein into its protease-resistant isoform suggests the participation of a cellular chaperone. *Biochem. Biophys. Res. Commun.* **258,** 470–475.

Schiermeier, Q. (2001). Testing times for BSE. *Nature* **409,** 658–659.

Soto, C. (2001). Protein misfolding and disease; protein refolding and therapy. *FEBS Lett.* **498,** 204–207.
Soto, C. (2004). Diagnosing prion diseases: Needs, challenges and hopes. *Nat. Rev. Microbiol.* **2,** 809–819.
Soto, C., and Saborio, G. P. (2001). Prions: Disease propagation and disease therapy by conformational transmission. *Trends Mol. Med.* **7,** 109–114.
Soto, C., Saborio, G. P., and Anderes, L. (2002). Cyclic amplification of protein misfolding: Application to prion-related disorders and beyond. *Trends Neurosci.* **25,** 390–394.
Soto, C., Anderes, L., Suardi, S., Cardone, F., Castilla, J., Frossard, M. J., Peano, S., Saá, P., Limido, L., Carbonatto, M., Ironside, J., Torres, J. M., Pocchiari, M., and Tagliavini, F. (2005). Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. *FEBS Lett.* **579,** 638–642.
Telling, G. C., Parchi, P., DeArmond, S. J., Cortelli, P., Montagna, P., Gabizon, R., Mastrianni, J., Lugaresi, E., Gambetti, P., and Prusiner, S. B. (1996). Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* **274,** 2079–2082.
Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., Anderson, R. G., Taraboulos, A., and Prusiner, S. B. (1996). Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. USA* **93,** 14945–14949.
Wadsworth, J. D., Joiner, S., Hill, A. F., Campbell, T. A., Desbruslais, M., Luthert, P. J., and Collinge, J. (2001). Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* **358,** 171–180.
Weber, T., Otto, M., Bodemer, M., and Zerr, I. (1997). Diagnosis of Creutzfeldt-Jakob disease and related human spongiform encephalopathies. *Biomed. Pharmacother.* **51,** 381–387.
Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P. G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* **347,** 921–925.

[2] Fractionation of Prion Protein Aggregates by Asymmetrical Flow Field-Flow Fractionation

By Jay R. Silveira, Andrew G. Hughson, and Byron Caughey

Abstract

Achieving the successful separation and analysis of amyloid and other large protein aggregates can be a difficult proposition. Field-flow fractionation (FFF) is a flow-based separation method like chromatography; however, FFF is capable of high-resolution separations in the absence of a stationary matrix. Thus, FFF is a relatively gentle technique and is well suited to the task of separating large macromolecules and macromolecular

METHODS IN ENZYMOLOGY, VOL. 412 0076-6879/06 $35.00
DOI: 10.1016/S0076-6879(06)12002-9

Apéndice 8

The EMBO Journal (2008) 27, 2557–2566 | © 2008 European Molecular Biology Organization | All Rights Reserved 0261-4189/08
www.embojournal.org

THE EMBO JOURNAL

Cell-free propagation of prion strains

Joaquín Castilla[1,5], Rodrigo Morales[1,2,5], Paula Saá[1,3], Marcelo Barria[1], Pierluigi Gambetti[4] and Claudio Soto[1,*]

[1]Department of Neurology, University of Texas Medical Branch, Galveston, TX, USA, [2]University of Chile, Santiago, Chile, [3]Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain and [4]Case Western Reserve University, Cleveland, OH, USA

Prions are the infectious agents responsible for prion diseases, which appear to be composed exclusively by the misfolded prion protein (PrP^{Sc}). Disease is transmitted by the autocatalytic propagation of PrP^{Sc} misfolding at the expense of the normal prion protein. The biggest challenge of the prion hypothesis has been to explain the molecular mechanism by which prions can exist as different strains, producing diseases with distinguishable characteristics. Here, we show that PrP^{Sc} generated *in vitro* by protein misfolding cyclic amplification from five different mouse prion strains maintains the strain-specific properties. Inoculation of wild-type mice with *in vitro*-generated PrP^{Sc} caused a disease with indistinguishable incubation times as well as neuropathological and biochemical characteristics as the parental strains. Biochemical features were also maintained upon replication of four human prion strains. These results provide additional support for the prion hypothesis and indicate that strain characteristics can be faithfully propagated in the absence of living cells, suggesting that strain variation is dependent on PrP^{Sc} properties.

The EMBO Journal (2008) 27, 2557–2566. doi:10.1038/emboj.2008.181; Published online 18 September 2008
Subject Categories: proteins
Keywords: infectious agent; prions; protein misfolding; strains; transmissible spongiform encephalopathies

Introduction

Prions are the unconventional infectious agents responsible for prion diseases, a group of fatal, neurodegenerative disorders, including Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease in deer, among others (Collinge, 2001). The infectious agent appears to be composed exclusively by a misfolded version of the prion protein (PrP^{Sc}) that replicates in the body in the absence of nucleic acids by inducing the misfolding of the cellular prion protein (PrP^C) (Prusiner, 1998). Although recent studies showing *in vitro* generation of infectious material by inducing or amplifying PrP misfolding have provided strong support for the prion hypothesis (Legname *et al*, 2004; Castilla *et al*, 2005; Deleault *et al*, 2007), it remains still highly controversial (Somerville, 2000; Chesebro, 2003; Soto and Castilla, 2004; Weissmann, 2004).

One of the main difficulties of the prion hypothesis has been to provide a molecular explanation for the prion strain phenomenon (Chesebro, 1998; Somerville, 2002; Aguzzi *et al*, 2007; Morales *et al*, 2007). Most TSEs are known to exhibit various 'strains' characterized by differences in incubation periods, clinical symptoms and biochemical and neuropathological features (Bruce, 2003; Aguzzi *et al*, 2007; Morales *et al*, 2007). In infectious diseases associated with conventional microbial agents (virus, bacteria and so on), different strains arise from mutations or polymorphisms in the genetic make-up of the agent. To reconcile the infectious agent composed exclusively of a protein with the strain phenomenon, it has been proposed that the strain characteristics are dependent on slightly different conformation or aggregation states of PrP^{Sc}, which can faithfully replicate at expenses of the host PrP^C (Telling *et al*, 1996; Prusiner, 1998; Morales *et al*, 2007). These different folding states of PrP^{Sc} may lead to selectively targeting distinct brain regions producing the diverse neuropathological alterations and clinical symptoms characteristic of prion strains. Support for this concept came from various studies showing that PrP^{Sc} from different strains have noticeable distinct biochemical properties and secondary structures (Caughey *et al*, 1998; Safar *et al*, 1998). However, up to now it is not known whether such differences are the cause or simply another manifestation of the prion strain phenomenon. Furthermore, the concept that a single protein can provide the conformational flexibility and the mechanism to propagate diverse strains is very intriguing and unprecedented. The definitive proof that the strain phenomenon is encoded in the PrP^{Sc} structure would be to reproduce in the test tube the folding of PrP^{Sc} associated with different strains and to show that the *in vitro*-generated infectious proteins maintain the *in vivo* strain characteristics. Important findings in this direction have been obtained for yeast prions, which are a group of 'infectious proteins' that behave as non-Mendelian genetic elements and transmit biological information in the absence of nucleic acid (Wickner *et al*, 1995). Compelling evidences have provided support for the prion nature of several yeast proteins, including Sup35p, Ure2p, Rnq1 (Uptain and Lindquist, 2002; Chien *et al*, 2004; Wickner *et al*, 2004). Recent studies showed that bacterially produced N-terminal fragments of Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (King and Diaz-Avalos, 2004; Tanaka *et al*, 2004; Brachmann *et al*, 2005). Remarkably, infection of yeasts with recombinant Sup35 folded in different conformations led to

*Corresponding author. Department of Neurology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, USA. Tel.: +1 409 747 0017; Fax: +1 409 747 0020;
E-mail: clsoto@utmb.edu
[5]These authors contributed equally to this work

Received: 24 December 2007; accepted: 13 August 2008; published *online: 18 September 2008*

the generation of distinct prion strains *in vivo* (Tanaka *et al*, 2004; Brachmann *et al*, 2005), indicating that structural differences in the infectious protein determine prion strain variation.

On the basis of theoretical considerations about the mechanisms of prion conversion, we have developed a strategy to reproduce PrP^{Sc} replication *in vitro* with a similar efficiency to the *in vivo* process, but with accelerated kinetics (Saborio *et al*, 2001). This system called PMCA (protein misfolding cyclic amplification) was designed to mimic PrP^{Sc} autocatalytic replication. In a cyclic manner, conceptually analogous to PCR cycling, a minute quantity of PrP^{Sc} (as little as one single particle) is incubated with excess PrP^{C} to enlarge the PrP^{Sc} aggregates that are then sonicated to generate multiple smaller units for the continued formation of new PrP^{Sc} (Saborio *et al*, 2001; Saá *et al*, 2006). PMCA confirms a central facet of the prion hypothesis, which is that prion replication is an autocatalytic process and that newly produced PrP^{Sc} can further propagate the protein misfolding (Soto *et al*, 2002). We have previously reported proof-of-concept experiments in which the technology was applied to replicate the misfolded protein from diverse species (Soto *et al*, 2005). The newly generated protein exhibits the same biochemical, biological and structural properties as brain-derived PrP^{Sc} and is infectious to wild-type animals, producing a disease with similar characteristics as the illness produced by brain-isolated prions (Castilla *et al*, 2005).

The main goal of this study was to assess whether the specific biological, biochemical and infectious properties of distinct prion strains can be maintained after *in vitro* replication. For this purpose, we propagated five different mouse and four distinct human prion strains *in vitro* by PMCA using as substrate PrP^{C} from the same normal mouse or human transgenic mouse brain extract, respectively. PrP^{Sc} associated with each strain was serially diluted and replicated *in vitro* to produce misfolded protein free from brain PrP^{Sc} inoculum. *In vitro*-generated PrP^{Sc} maintains the strain-specific biochemical properties and more importantly upon injection into wild-type mice produced a disease with indistinguishable characteristics as the parental strain. These results suggest that all strain-associated features can be maintained by cell-free formed PrP^{Sc}, suggesting that the prion strain phenomenon is enciphered on the characteristics of the misfolded prion protein.

Results

In vitro propagation of prion strains

To analyse whether *in vitro* replication of prion strains faithfully propagates the biochemical properties of PrP^{Sc}, we used five well-characterized strains from mouse and four from human. As many as 20 different prion strains have been identified in mouse after inoculation of animals with prions from diverse origins (Fraser and Dickinson, 1973; Kimberlin, 1976; Bruce, 2003). Four of the mouse strains used in this study (RML, 139A, ME7 and 79A) are originated from different scrapie sources and have been adapted into mouse by repetitive passages. These mouse-adapted scrapie strains have been shown to have some differences in brain lesion profiles, incubation times in diverse mouse backgrounds and susceptibility to PrP polymorphisms (Fraser and Dickinson, 1973; Bruce *et al*, 1991). On the other hand, 301C is a strain originated from cattle BSE, which has been serially passed in wild-type mice (Bruce *et al*, 2002). Mouse brain homogenates from animals experimentally infected with these different strains were diluted 10-fold into 10% normal mice brain homogenate. Samples were either immediately frozen or subjected to 96 PMCA cycles. The amplified samples were further diluted 10-fold and a new round of 96 PMCA cycles was carried out. This procedure of serial dilution/amplification was repeated many times to reach a 10^{-20} total dilution of the brain infectious material and these samples were used in the studies described below. In our estimation, this degree of dilution is at least 1 million-fold higher than what is necessary to remove the last molecule of PrP^{Sc} coming from the brain inoculum and at least a trillion (10^{12})-fold more than the last infectious dilution (Castilla *et al*, 2005). As determined by western blot after proteinase K (PK) digestion, a protease-resistant product was observed in all these amplifications, which remained constant despite the dilutions (data not shown). These results suggest that PrP^{Sc} is being produced *in vitro* and the newly generated PrP^{Sc} is capable of sustaining replication, as demonstrated before for hamster prions (Castilla *et al*, 2005). We conclude from these results that PMCA enables an infinite replication of PrP^{Sc} *in vitro* and the large dilution performed (10^{-20}) guarantees that the sample contains exclusively *in vitro*-produced misfolded protein. Assessment of the western blot profile after PK treatment for PMCA-generated PrP^{Sc} compared with *in vivo*-produced misfolded protein from each strain shows that both the proportion of glycoforms and the electrophoretic mobility were conserved after *in vitro* propagation (Figure 1). As shown in Figure 1A, the scrapie-derived mouse strains (RML, ME7, 139A and 79A) have a glycoform distribution dominated by the mono-glycosylated form, whereas in 301C PrP^{Sc} the most abundant isoform is the di-glycosylated species. Importantly, the glycoform ratio in PMCA-generated PrP^{Sc} was very similar to the brain-derived protein. The relative migration of the protein in gel electrophoresis was also strikingly similar, which is easily observed upon deglycosylation when comparing only the size of the non-glycosylated protein (Figure 1B). PrP^{Sc} from scrapie-adapted mouse strains migrate with an estimated molecular weight of 21 kDa, whereas the bovine-derived 301C protein exhibits a $\sim$19 kDa size. These findings indicate that the PK cleavage site in PrP^{Sc} generated *in vitro* by PMCA is similar to the site in which the protein obtained from the brain of sick animals is cleaved by this enzyme. This is important because differences in proteolytic cleavage likely reflect distinct foldings of the protein in diverse strains (Marsh and Bessen, 1994; Collinge *et al*, 1996; Chen *et al*, 2000; Gretzschel *et al*, 2005). It is noteworthy that the PrP^{C} substrate used for conversion was the same for all strains and thus the molecular characteristics of PrP^{Sc} in the inoculum are responsible for the propagation of the different biochemical properties of the protein. A control experiment in which samples of brain homogenate from 10 different healthy mice were serially diluted into itself and subjected to the same number of PMCA cycles as described above, but in the absence of PrP^{Sc} inoculum, did not show any protease-resistant PrP under the conditions used (data not shown). Although we have been able to generate infectious PrP^{Sc} '*de novo*' (without the addition of brain PrP^{Sc}) recently, this requires modification of some PMCA parameters (paper under review) and

 ©2008 European Molecular Biology Organization

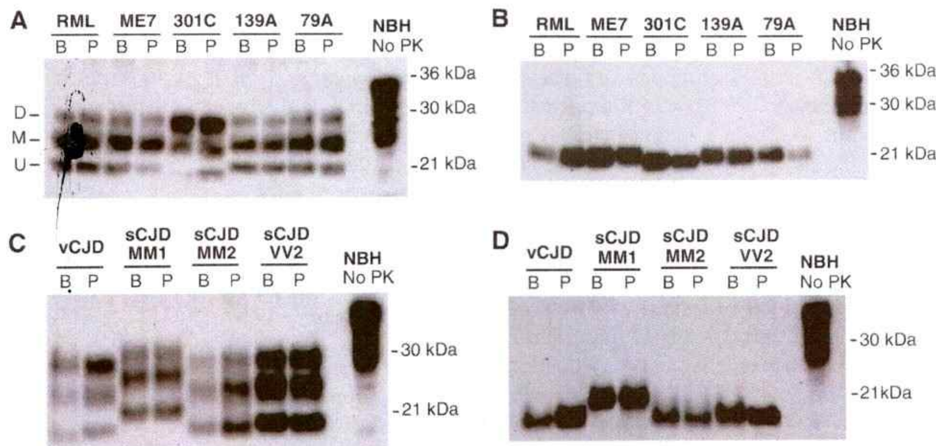


Figure 1 Biochemical properties of *in vitro*-generated PrP^{Sc} from different mouse and human strains. Brain homogenates from sick individuals (mice or humans) were diluted 10-fold into normal brain homogenate and subjected to 96 cycles of PMCA, as described in Materials and methods. The amplified material was diluted 10-fold into normal brain homogenate and amplified again. This procedure was repeated several times to reach a 10^{-20} dilution of the inoculum. (**A**) Aliquots containing similar amounts of brain-derived or PMCA-generated (after a 10^{-20} dilution) PrP^{Sc} from different mouse strains were subjected to proteinase K (PK) digestion (50 μg/ml for 60 min at 37°C) and loaded onto SDS–PAGE. Immunoreactive bands were observed using western blot. For clarity, the figure was composed from blots with different levels of exposure to enable a direct comparison of the position of the bands. (**B**) Aliquots of both proteins were subjected to deglycosylation by treatment with peptide N-glycosidase F for 2 h at 37°C. (**C**) Electrophoretical pattern of four different types of *in vitro*-generated or brain-derived human PrP^{Sc}. (**D**) The size of the PK-resistant PrP^{Sc} fragment in diverse human strains was assessed by western blot after deglycosylation. All samples were digested with PK before western blot, except in the normal brain homogenate (NBH), used as a control of PrP^C migration. B: brain-derived samples; P: PMCA-generated samples.

under standard PMCA conditions, as those used in this study, spontaneous generation of infectious material does not occur. These findings suggest that *de novo* formation of PrP^{Sc} can be experimentally distinguished from replication of pre-formed PrP^{Sc}, indicating that the biochemical, conformational or stability properties of the PrP structures involved in both processes are probably different.

To further assess whether the PrP^{Sc} features are maintained after *in vitro* replication by PMCA, we tested four different human strains with well-established differences in the biochemical properties of PrP^{Sc} (Gambetti *et al*, 2003). The samples came from patients affected by vCJD, and three distinct types of sCJD (types MM1, MM2 and VV2). PrP^{Sc} from vCJD and sCJD types MM1 and MM2 was amplified using as substrate transgenic mice brain expressing human PrP with MM genotype at position 129 and the sCJD type VV2 was amplified using transgenic mice expressing VV PrP^C. Little or no amplification was obtained when PrP^{Sc} and PrP^C have different polymorphism at position 129. Samples were subjected to the same scheme of serial PMCA and dilutions as described in the mouse experiment above to dilute out the PrP^{Sc} inocula used to trigger the reaction. Comparison of the glycoform proportion (Figure 1C) and the electrophoretic mobility after PK and deglycosylation (Figure 1D) between brain-derived and PMCA-generated PrP^{Sc} for each strains clearly show that the biochemical features were maintained upon *in vitro* propagation. Unseeded PMCA reactions of healthy human PrP transgenic mice brain homogenates did not show '*de novo*' PrP^{Sc} generation under the conditions tested (data not shown).

In vitro-generated PrP^{Sc} maintains strain-specific infectivity properties

To determine the infectious capability of *in vitro*-generated PrP^{Sc} and to assess the preservation of strain characteristics, we inoculated the five mouse strains into wild-type animals. Groups of mice were injected intracerebrally (i.c.) with a similar quantity of either brain-derived or PMCA-produced PK-resistant PrP^{Sc}. Appearance of clinical signs was monitored over time as described in Materials and methods. The first alterations observed were rough coat initially in the neck, which then extended to the lower back. This was followed by hunchback, urogenital lesions, cachexia and ataxia. Unfortunately, no substantial differences in clinical signs are evident among these strains other than the observation that 301C has a more severe urogenital damage, including abundant pus secretion. Nevertheless, the time of appearance of clinical alterations (Table I), and the type and severity of the signs were very similar in the animals inoculated with infectious material obtained from sick brain

Table I Infectivity of *in vitro*-generated prions

Prion strain	Brain	PMCA	PMCA—second passage	*P*-value*
RML	159 ± 7	164 ± 12	148 ± 1	0.382
ME7	156 ± 2	163 ± 6	164 ± 2	0.249
301C	189 ± 2	181 ± 2	183 ± 4	0.145
139A	162 ± 1	167 ± 6	169 ± 1	0.338
79A	161 ± 3	154 ± 4	154 ± 3	0.253
None	>500	>500	ND	—

Groups of five wild-type mice were injected i.c. with similar quantities of protease-resistant PrP^{Sc} either derived from the brain of sick animals (brain) or generated by PMCA *in vitro* (PMCA). Also shown is the second passage of the PMCA group. The values represent the average ± standard error of the time in which animals were killed with definitive signs of disease. The attack rate in all cases was 100%, except in the control experiment in which the normal brain homogenate was subjected to PMCA in the absence of any infectious material. ND: not done.
*The *P*-value was calculated by one-way ANOVA using the software GraphPad Instat, version 3.05.

©2008 European Molecular Biology Organization

or produced *in vitro* by PMCA. None of the animals inoculated with normal brain homogenate subjected or not to the same regimen of serial PMCA amplification showed disease signs up to 500 days post-inoculation (Table I). Interestingly, in none of these experiments we found a significant delay on the incubation time for the *in vitro*-generated prions compared with the *in vivo*-produced infectious agent, as we reported earlier in our experiments with hamster 263K scrapie prions (Castilla *et al*, 2005). These findings indicate that the infectivity associated with PMCA-generated PrP^{Sc} was the same as for misfolded protein obtained from the brain in all the mouse strains studied. A second passage of the *in vitro*-generated infectious material showed the same incubation periods, suggesting that the infectious agent was stable (Table I). Similar results were obtained when the material was inoculated intraperitoneally, albeit with longer incubation times (Data not shown). Statistical analysis of the incubation periods showed that the differences between the brain-derived material or PMCA-generated infectivity (first and second passages) were not significant for any of the strains studied (see *P*-values in Table I). Conversely, the incubation times for 301C were highly significantly different from all the other strains, as assessed by two-way ANOVA ($P<0.001$). The differences between the ovine-derived strains are nonsignificant, except for 139A versus 79A ($P=0.0031$).

Besides incubation time, one of the main differences among prion strains is the distribution and severity of damage in distinct regions of the brain (Fraser and Dickinson, 1973). To assess whether *in vitro*-generated infectious agent maintains the neuropathological signature of the parental strain, we evaluated the degree of vacuolation in various brain areas of infected animals, as described in Materials and methods. The profile of spongiform degeneration obtained for each strain using brain-derived or PMCA-generated material was statistically undistinguishable (Figures 2 and 3), indicating again that *in vitro* propagation of prions maintains the strains properties. As shown in Figure 3, statistical analyses of the brain lesion profile enable to differentiate three groups of strains: 301C, 79A and the other three ovine-derived strains (RML, 139A, ME7), which are not significantly different in their pattern of vacuolation. 301C-infected mice exhibit large extent of spongiosis in medulla and hippocampus, medium degree of damage in colliculus, hypothalamus and thalamus and very little vacuolation in cerebellum and cortex (Figures 2 and 3). On the other hand, the ovine strains RML, 139A and ME7 have a large extent of spongiform degeneration in cerebellum and only little damage in colliculus and hypothalamus. Finally, the 79A strain produces a more even pattern of vacuolation in the eight brain areas studied. These neuropathological differ-

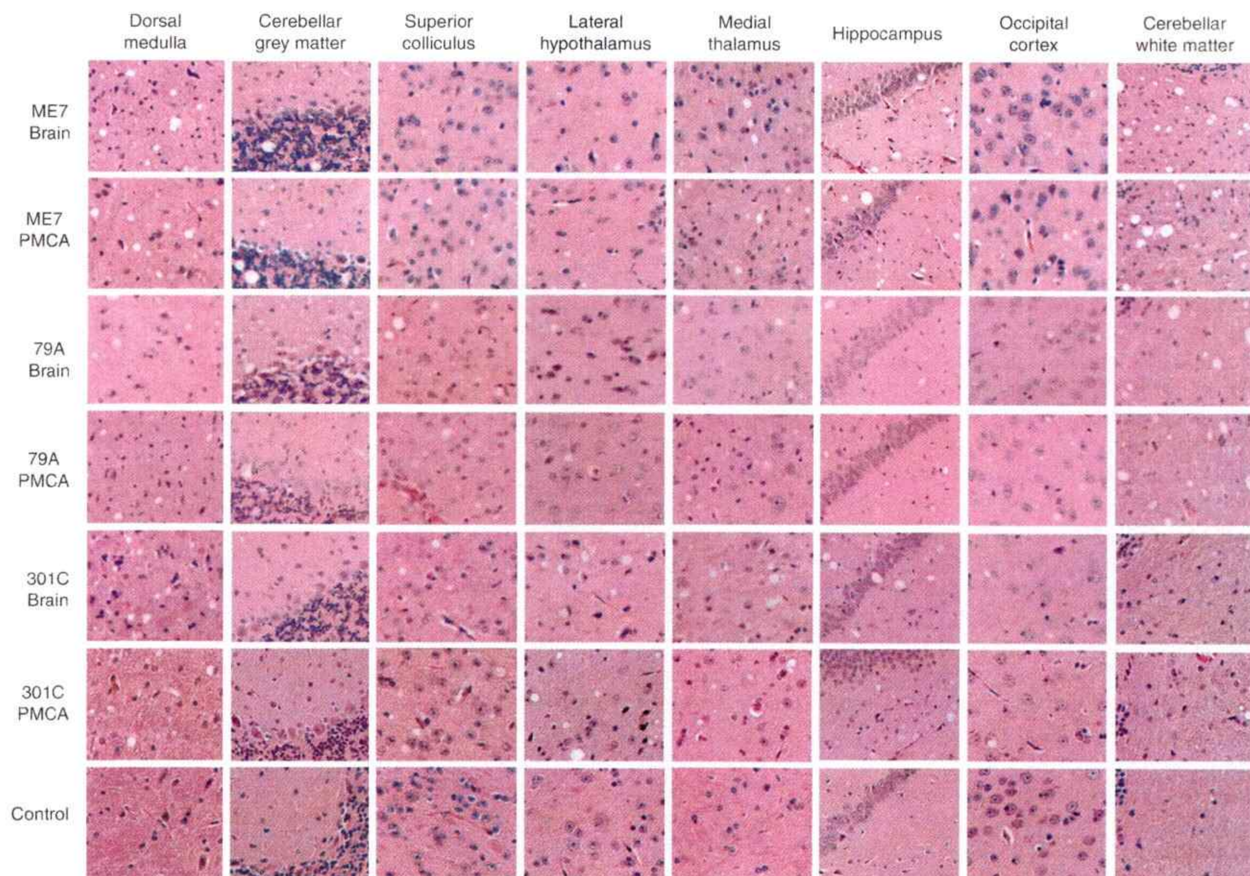
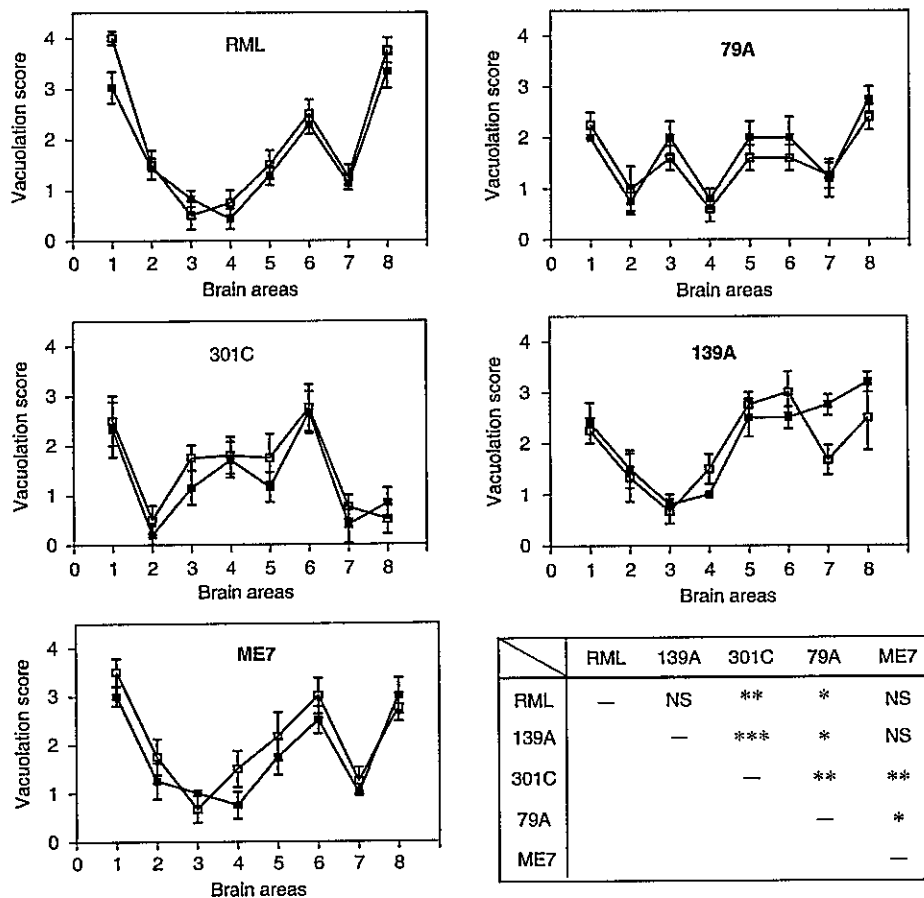


Figure 2 Histopathological profile of spongiform brain degeneration in mice inoculated with *in vivo*- and *in vitro*-produced prion strains. Pictures showing the vacuolation extent in eight brain regions in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions of three of the strains used in this study (ME7, 79A and 301C). As a control, we showed the brain staining of animals inoculated with brain homogenate subjected to the same serial PMCA procedure. These animals did not show clinical signs of the disease and were killed 500 days after inoculation. Samples were processed and stained by haematoxylin–eosin as described in Materials and methods and visualized at $\times 100$ magnification.

©2008 European Molecular Biology Organization



	RML	139A	301C	79A	ME7
RML	—	NS	**	*	NS
139A		—	***	*	NS
301C			—	**	**
79A				—	*
ME7					—

Figure 3 Profile of vacuolation in different brain areas of mice inoculated with different strains of *in vivo*- and *in vitro*-generated prions. Brains from mice clinically sick by inoculation with either brain-derived or PMCA-generated prions from the five strains used in this study were analysed histologically for spongiform degeneration after haematoxylin–eosin staining, as described in Materials and methods. Eight different brain areas were selected and the values represent the average ± s.e. of the extent of vacuolation from the five animals analysed. The brain areas studied were the following: (1) dorsal medulla; (2) cerebellar grey matter; (3) superior colliculus; (4) lateral hypothalamus; (5) medial thalamus; (6) hippocampus; (7) occipital cortex and (8) cerebellar white matter. Statistical analysis by two-way ANOVA for the results in each strain, using brain regions and prion origin (*in vivo*- or *in vitro*-produced) as the variables, indicated that the differences were not significant in any of the strains studied. Conversely, the differences in the vacuolation profile among distinct strains were statistically significant ($P<0.05$). The table on the bottom right side shows the statistical comparison among each individual strain. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

ences were also observed in the animals inoculated with PMCA-generated PrP^{Sc}. The neuropathological similarities produced by inoculation of *in vitro*- and *in vivo*-produced PrP^{Sc} were also evident by immunohistochemical staining with antibodies against PrP and GFAP (a marker for reactive astrocytes) (Figure 4).

Another difference among strains widely exploited to characterize and differentiate them is the biochemical properties of PrP^{Sc} accumulated in the brain. PrP^{Sc} associated with different strains can be distinguished biochemically by its glycosylation profile, PK cleavage, susceptibility to protease digestion, resistance to denaturation and secondary structure (Bessen and Marsh, 1992; Caughey *et al*, 1998; Safar *et al*, 1998; Hill *et al*, 2003). To assess some of these biochemical properties, we studied PrP^{Sc} from the brain of animals killed after showing signs of the disease produced by inoculation with different strains of infectious material generated *in vitro* by PMCA or obtained from the brain of sick mice. Comparison of the PrP^{Sc} western blot profile after PK digestion showed that both the glycoform distribution and the electrophoretic mobility are similar for each strain regardless of whether the infectious material was derived from brain or generated by PMCA (Figure 5A and B). As described before, PrP^{Sc} in the mouse strains originated from scrapie have a glycoform distribution dominated by the mono-glycosylated form, whereas in PrP^{Sc} from the 301C strain the most abundant isoform is the di-glycosylated form (Figure 5A). As the PrP^{C} used as substrate for conversion was the same for all strains, these results indicate that PrP^{Sc} from distinct strains can preferentially recruit and convert PrP^{C} with particular glycosylation types. Importantly, comparing the results shown in Figures 1A and 5A, it is possible to conclude that the glycoform distribution of PrP^{Sc} is maintained *in vitro* and *in vivo* and that the PMCA-generated PrP^{Sc} from each strain can further propagate these biochemical characteristics when inoculated into wild-type mice. Finally, we studied the susceptibility of the misfolded protein from different strains to PK degradation. Partial resistance to proteolysis is one of the typical features of PrP^{Sc} and data suggest that the misfolded proteins associated with diverse strains exhibit different susceptibility to PK digestion (Bessen and Marsh, 1992). To compare the protease resistance profile, similar quantities of PrP^{Sc} from the brain of animals inoculated with various strains of either brain-derived or PMCA-generated

©2008 European Molecular Biology Organization

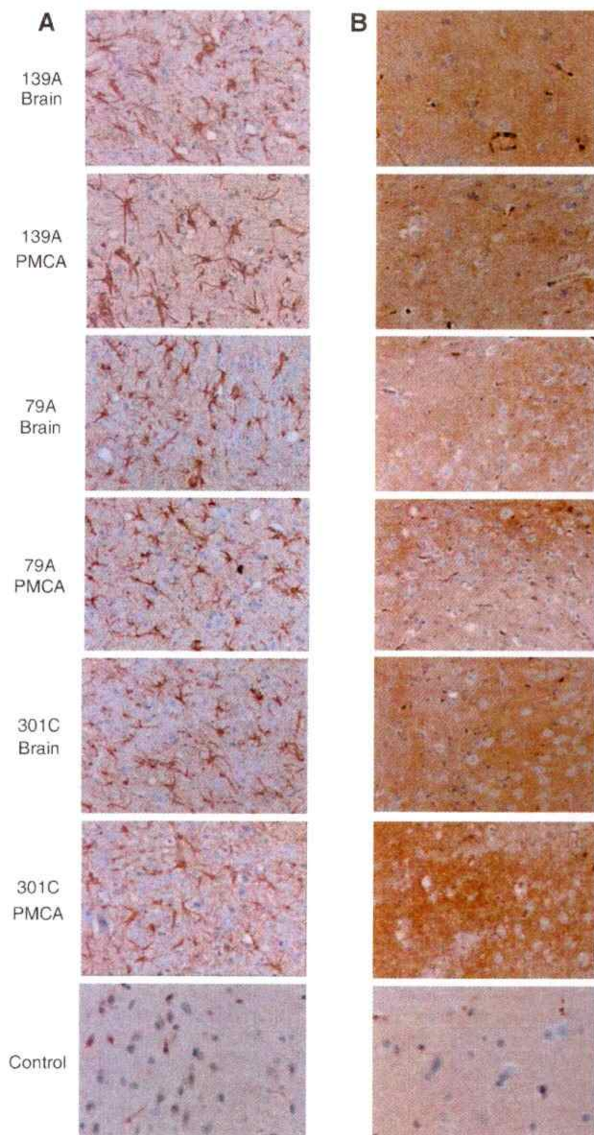


Figure 4 PrP^{Sc} accumulation and astroglyosis in animals inoculated with *in vitro-* or *in vivo*-generated prion strains. Pictures showing brain damage observed in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions of the three representative strains studied (139A, 79A and 301C). As a control, we showed the brain staining of animals inoculated with brain homogenate (without PrP^{Sc} seed) subjected to the same serial PMCA procedure. These animals did not show clinical signs of the disease and were killed 500 days after inoculation. (**A**) The profile of reactive astroglyosis in medulla as evaluated by histological staining with glial fibrillary acidic protein antibodies. (**B**) PrP accumulation in these animals as detected by staining the tissue with the 6H4 anti-PrP monoclonal antibody.

prions were treated for 60 min with 125, 250, 500, 750 and 1500 μg/ml of PK. The susceptibility of 301C PrP^{Sc} to PK degradation is dramatically different when compared with the ovine-derived strains. Figure 6 shows two of the ovine strains (139A and 79A), but similar results were obtained with the other strains used in this study (data not shown). Much larger concentrations of PK are required to degrade PrP^{Sc} from 301C than 139A or 79A strains as shown in the densitometric evaluation of the western blots from three independent experiments for each sample (graphs in

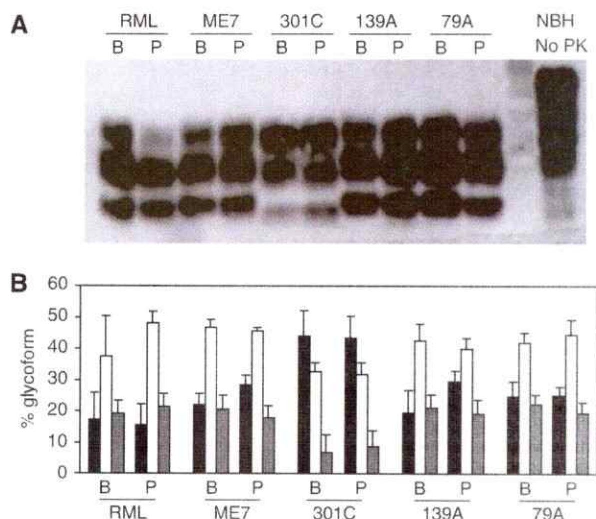


Figure 5 Proportion of distinct PrP^{Sc} glycoforms in animals inoculated with diverse strains of *in vivo-* and *in vitro*-generated prions. (**A**) Aliquots from the brain homogenate of clinically sick mice inoculated with either brain-derived (B for brain) or PMCA-generated (P for PMCA) PrP^{Sc} from different mouse strains were subjected to proteinase K (PK) digestion (50 μg/ml for 60 min at 37°C) and evaluated by western blot. All samples were digested with PK before western blot, except in the normal brain homogenate (NBH), used as a control of PrP^{C} migration. (**B**) Densitometric analysis of the western blots of three independent experiments as the one shown in (A) was done to calculate the proportion of each glycoform (di- in black bars, mono- in white bars or un-glycosylated in gray bars). The values correspond to the average ± s.e. of the three determinations. Statistical analysis by two-way ANOVA showed that none of the differences in glycoform distribution between animals inoculated with brain-derived or PMCA-generated prions was significant. Conversely, the differences in the glycoform distribution between the ovine- (RML, 139A, ME7 and 79A) and bovine-adapted (301C) strains were statistically significant ($P<0.01$). A full-colour version of this figure is available at *The EMBO Journal* Online.

Figure 6). Importantly, PrP^{Sc} from animals inoculated with *in vitro*-generated material exhibits the same characteristics of PK resistance.

Discussion

The prion hypothesis proposes that the infectious agent responsible for TSE transmission is a misfolded protein that has the unique capability to propagate the disease by transmitting its abnormal folding properties (Prusiner, 1998). Undoubtedly, the biggest challenge of the 'protein-only' hypothesis has been to provide a credible molecular explanation to the prion strain phenomenon (Chesebro, 1998; Somerville, 2002; Aguzzi *et al*, 2007; Morales *et al*, 2007). Prions as conventional infectious agents exist in different forms to produce diseases with distinct characteristics (Bruce, 2003; Aguzzi *et al*, 2007; Morales *et al*, 2007). In traditional infectious diseases, strains are determined by polymorphisms or mutations in the nucleic acid of the agent. However, prions do not appear to contain nucleic acids. Therefore, a key question in the field has been to understand how a single protein can encode the diversity of information needed to produce and propagate many strains. The proposal that strains are dependent on differences in the folding and aggregation stage of the prion protein that can be faithfully propagated in the animal to determine diverse

©2008 European Molecular Biology Organization

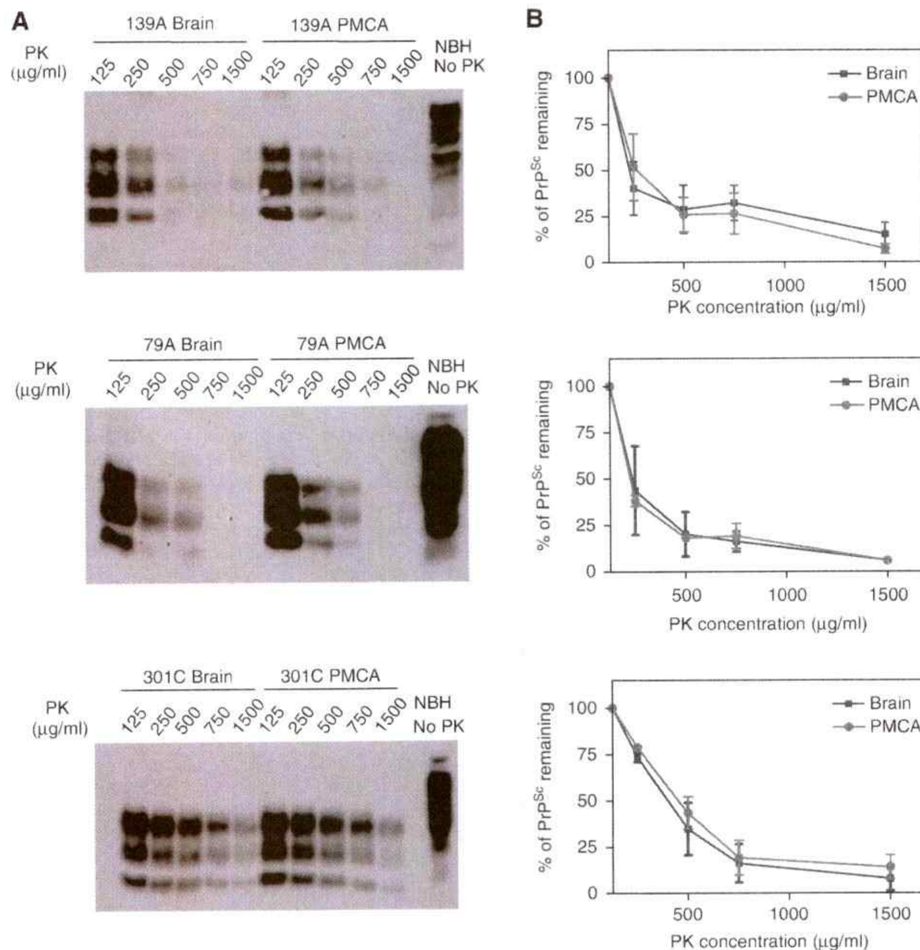


Figure 6 Extent of protease resistance of PrPSc associated with different mouse prion strains. (**A**) Western blot showing the extent of PK-resistant PrP^{Sc} observed in sick mice inoculated with brain-derived (brain) or PMCA-generated (PMCA) prions from three of the strains tested: 139A (upper panel), 79A (middle panel) and 301C (lower panel). For this study, aliquots of brain homogenate were incubated for 60 min at 37°C with the indicated concentrations of PK, and PrP^{Sc} signal remaining was detected by western blot analysis as described in Materials and methods. All samples were digested with PK before western blot, except for the normal brain homogenate (NBH), used as a control of PrP^{C} migration. (**B**) Densitometric analyses of the western blots of three independent experiments for each strain as those shown in (A). It is important to note that the values of the graphs shown in panels of (B) represent the average of three independent experiments, explaining why sometimes the numbers do not correspond exactly with the western blots. These data enable to determine the susceptibility of PrP^{Sc} to PK from the various sources and estimate the PK_{50} value, which corresponds to the PK concentration needed to degrade 50% of the protein. The PK_{50} values for the experiments shown in the figure are the following: RML brain = 224 µg/ml; RML PMCA = 248 µg/ml; 79A brain = 196 µg/ml; 79A PMCA = 192 µg/ml; 301C brain = 405 µg/ml; 301C PMCA = 438 µg/ml. Statistical analysis by two-way ANOVA showed that the differences between brain and PMCA infectious material were not significant for any of the strains studied.

disease phenotypes has been taken with scepticism (Farquhar *et al*, 1998). It is hard to reconcile the last 20 years of knowledge on protein folding with the idea that a single protein can adopt dozens of stable alternative structures that can imprint their unique folding into the natively folded normal prion protein. An alternative hypothesis, termed the 'unified theory', has been proposed to suggest that although PrP^{Sc} is the infectious agent, the strain diversity is dependent on the presence of an ancillary cofactor, likely a nucleic acid (Weissmann, 1991). Although many efforts to identify such nucleic acid cofactor have failed (Kellings *et al*, 1994; Safar *et al*, 2005), the consensus hypothesis is still considered a plausible explanation for the strain phenomenon. One of the strongest arguments against the idea that strains are enciphered exclusively in the PrP^{Sc} folding is that so far the strain-specific infectious properties have not been generated or propagated *in vitro* in a cell-free system.

In a previous study, we successfully generated and propagated infectious prions *in vitro* using the PMCA technology to autocatalytically replicate PrP^{Sc} in a cell-free system (Castilla *et al*, 2005). We showed that hamster PrP^{Sc} could be maintained indefinitely replicating even in the absence of any molecule of brain-derived PrP^{Sc}. Inoculation of wild-type hamsters with *in vitro*-produced PrP^{Sc} led to a scrapie disease similar to the illness produced by brain infectious material (Castilla *et al*, 2005). Subsequently, we showed that large amounts of infectious material can be generated from sub-infectious quantities of sick hamster brain, equivalent to a single particle of PrP^{Sc} oligomers (Saá *et al*, 2006). These studies provided a strong proof in favor of the prion hypothesis, but did not address the important issue of prion strains, as only one strain of hamster PrP^{Sc} was used. Also, the *in vitro*-generated infectious agent produced disease with a delay in the incubation period, and thus the possibility that

©2008 European Molecular Biology Organization

strain characteristics were not maintained could not be ruled out. A report from Kretzschmar's group confirmed our results and using nitrocellulose immobilization experiments, showed that the delay on the disease onset was likely not due to a strain switch, but possibly to a different size proportion of PrP^{Sc} oligomers that changed their clearance rate (Weber *et al*, 2007). Our current study shows that infectious prions can be generated *in vitro* by serial replication of PrP^{Sc} misfolding in a different species as the previous experiments. Interestingly, in contrast to our previous report, the current study shows that inoculation with *in vitro*-generated prions produced the same incubation time as *in vivo*-derived infectious material. These findings further support the concept that PrP^{Sc} is the only component needed for infectivity and that *in vitro*-produced prions are very similar to the agent generated in the brain of sick animals. Nevertheless, the most relevant result of the current study is the successful propagation of the PrP^{Sc} biochemical properties of five different mouse prion strains and four human strains. Furthermore, the *in vitro*-generated mouse prion strains were infectious to wild-type mice conserving the typical properties of each strain. PrP^{Sc} from four mouse-adapted scrapie strains (RML, 139A, ME7 and 79A) and one strain with cattle origin (301C) were propagated *in vitro* by 20 successive rounds of PMCA followed by dilutions to get rid of the initial inoculum progressively. After this procedure, the sick brain used as inoculum was diluted 10^{20}-folds, and therefore no molecules of PrP^{Sc} should be present unless they were generated *in vitro* during PMCA. Our data indicate that the strain-specific differences in biochemical features of PrP^{Sc} (electrophoretical mobility and proportion of various glycoforms) associated with these strains were maintained after *in vitro* propagation, despite the fact that all the strains were replicated using the same source of PrP^{C} substrate. More strikingly, when administered to wild-type mice, *in vitro*-generated PrP^{Sc} produced a disease in all cases with the same clinical features and incubation time as the parental strain. A detailed study of the neuropathological and biochemical characteristics of the brain of sick animals showed that *in vitro*-generated prions produced the same profile of damage as *in vivo*-derived infectious material. These results unequivocally demonstrate that strain characteristics were replicated *in vitro* by PMCA. Our findings provide additional support for the prion hypothesis and strongly argue that strain characteristics can be faithfully propagated in the absence of living cells, suggesting that strain variation is dependent on PrP^{Sc} biochemical changes.

Materials and methods

Biological samples

Five different mouse prion strains extensively adapted and characterized were used for these experiments. All strains were propagated several times in C57Bl6 wild-type mice. Four human prion strains, associated with vCJD or sCJD (types MM1, MM2 and VV2), were obtained from patients with clinically and neuropathologically confirmed prion disease. Human PrP^{Sc} was replicated using brains of transgenic mice overexpressing human PrP with MM or VV genotype at position 129. Brains from healthy animals were extracted after mice were perfused with phosphate-buffered saline (PBS) plus 5 mM EDTA. Brain homogenates (10%, w/v) were prepared in conversion buffer (PBS containing NaCl 150 mM, 1.0% Triton X-100, 4 mM EDTA and the complete^{TM} cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany). Brain from sick individuals were harvested and homogenized in PBS plus protease inhibitors. The samples were clarified by a brief, low-speed centrifugation (2000 r.p.m. for 45 s) using an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial replication of prions in vitro by PMCA

Aliquots of 10 μl of 10% brain homogenate from animals or humans infected with diverse strain were diluted into 90 μl of 10% normal brain homogenate and loaded onto 0.2-ml PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY) and programmed to perform cycles of 30-min incubation at 37°C followed by a 20-s pulse of sonication set at potency of 7. Samples were incubated, without shaking, immersed in the water of the sonicator bath. After a round of 96 cycles, a 10 μl aliquot of the amplified material was diluted into 90 μl of normal mouse brain homogenate and a new round of 96 PMCA cycles was performed. This procedure was repeated 19 times to reach a 10^{-20} final dilution of the initial sick brain homogenate. The detailed protocol for PMCA, including reagents, solutions and troubleshooting, has been published elsewhere (Castilla *et al*, 2004, 2006; Saá *et al*, 2004).

PK degradation assay

The standard procedure to digest PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 μg/ml) for 60 min with shaking at 37°C. The digestion was stopped by adding electrophoresis sample buffer and the protease-resistant PrP was revealed by western blotting, as indicated below. To study the profile of PK sensitivity for *in vitro*- and *in vivo*-generated PrP^{Sc}, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 125 to 1500 μg/ml.

Western blot

Proteins were fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted into nitrocellulose membrane and probed with 6H4 or 3F4 antibodies at a 1:5000 dilution for mouse and human samples, respectively. The immunoreactive bands were visualized by enhanced chemoluminescence assay (Amersham, Piscataway, NJ) using a UVP image analysis system. The immunoreactive bands were visualized by enhanced chemoluminescence assay (Amersham) using an UVP image analysis system. To assess the quantity of PrP^{Sc} in the western blot, densitometric analyses were done in triplicate.

Protein deglycosylation assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100 000 *g* for 1 h at 4°C, the supernatant was discarded and the pellet was resuspended in 100 μl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 13 μl of 50 mM sodium phosphate, pH 7.5 containing 1% Nonidet P-40 and 3 μl of peptide N-glycosidase F (New England Biolabs) were added. Samples were incubated overnight at 37°C with shaking and the reaction was stopped by adding electrophoresis buffer and samples analysed by western blot as indicated before.

PrP^{Sc} quantification

To inject the same quantity of PrP^{Sc} from each strain preparation, both *in vitro* generated and *in vivo* produced, the samples were compared by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artefacts due to saturation of the signal or to a too weak signal.

Infectivity studies

In vivo infectivity studies were done in C57Bl6 female mice purchased from Charles River. Animals were 4–6 weeks old at the time of inoculation. Anaesthetized animals were injected stereotaxically in the right hippocampus with 2 μl of the sample. The quantity of infectious material injected corresponds to the plateau portion of the incubation period (Supplementary Figure 1), therefore small differences in the amount of infectivity should not change incubation periods unless there are strain differences. To estimate the plateau of incubation period, we inoculated mice with different dilutions of infectious prions from distinct strains. The result indicates that dilutions lower than 150-fold of sick brain homo-

 ©2008 European Molecular Biology Organization

genate are at plateau of infectivity (Supplementary Figure 1). In our experiment, the amount of infectious material corresponds to between 15- and 30-fold dilution. The onset of clinical disease was measured by scoring the animals twice a week using the following scale: (1) normal animal; (2) roughcoat on limbs; (3) extensive roughcoat, hunckback and visible motor abnormalities; (4) urogenital lesions and (5) terminal stage of the disease in which the animal present cachexia and lies in the cage with little movement. Animals scoring level 5 were considered sick and were killed to avoid excessive pain using exposition to carbonic dioxide. Brains were extracted and analysed histologically. The right cerebral hemisphere was frozen and stored at −70°C for biochemical studies of PrP^{Sc} and the left hemisphere was used for histology analysis.

Histopathological studies

Brain tissue was fixed in 10% formaldehyde solution, embedded in paraffin and cut into sections. Serial sections (6 μm thick) from each block were stained with haematoxylin–eosin, or incubated with the 6H4 monoclonal antibody recognizing PrP or the glial fibrillary acidic protein, using our previously described protocols (Castilla *et al*, 2005). Immunoreactions were developed using the peroxidase–antiperoxidase method, following manufacturer's specifications. Antibody specificity was verified by absorption. Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by considering both number and size of vacuoles. Each analysed brain area was scored from 0 to 4 according to the extent of vacuolation in slides stained with haematoxylin–eosin and visualized at a ×40 magnification. Samples were analysed blindly by two different people and the scores represent the average of the two determinations. The brain areas studied are the following: (1) dorsal medulla; (2) cerebellar grey matter; (3) superior colliculus; (4) lateral hypothalamus; (5) medial thalamus; (6) hippocampus; (7) occipital cortex and (8) cerebellar white matter.

Statistical analysis

The quantitative differences in the vacuolation profile obtained by histopathological analysis were assessed by two-way ANOVA using brain areas and source of infectious material as the variables. Biochemical differences in PK susceptibility among strains were also analysed by two-way ANOVA using the PK concentration and source of infectious material as the variables. In both cases, the data were analysed using the GraphPad Instat, version 3.05 software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

This research was supported in part by NIH grants NS0549173 and AG014359 to CS.

References

Aguzzi A, Heikenwalder M, Polymenidou M (2007) Insights into prion strains and neurotoxicity. *Nat Rev Mol Cell Biol* **8:** 552–561

Bessen RA, Marsh RF (1992) Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *J Virol* **66:** 2096–2101

Brachmann A, Baxa U, Wickner RB (2005) Prion generation *in vitro*: amyloid of Ure2p is infectious. *EMBO J* **24:** 3082–3092

Bruce ME (2003) TSE strain variation. *Br Med Bull* **66:** 99–108

Bruce ME, Boyle A, Cousens S, McConnell I, Foster J, Goldmann W, Fraser H (2002) Strain characterization of natural sheep scrapie and comparison with BSE. *J Gen Virol* **83:** 695–704

Bruce ME, McConnell I, Fraser H, Dickinson AG (1991) The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *J Gen Virol* **72:** 595–603

Castilla J, Saá P, Hetz C, Soto C (2005) *In vitro* generation of infectious scrapie prions. *Cell* **121:** 195–206

Castilla J, Saá P, Morales R, Abid K, Maundrell K, Soto C (2006) Protein misfolding cyclic amplification for diagnosis and prion propagation studies. *Methods Enzymol* **412:** 3–21

Castilla J, Saá P, Soto C (2004) Cyclic amplification of prion protein misfolding. In *Methods and Tools in Bioscience and Medicine Techniques in Prion Research*, Lehmann S, Grassi J (eds), pp 198–213. Basel, Switzerland: Birkhauser Verlag

Caughey B, Raymond GJ, Bessen RA (1998) Strain-dependent differences in beta-sheet conformations of abnormal prion protein. *J Biol Chem* **273:** 32230–32235

Chen SG, Zou W, Parchi P, Gambetti P (2000) PrP(Sc) typing by N-terminal sequencing and mass spectrometry. *Arch Virol Suppl* 209–216

Chesebro B (1998) BSE and prions: uncertainties about the agent. *Science* **279:** 42–43

Chesebro B (2003) Introduction to the transmissible spongiform encephalopathies or prion diseases. *Br Med Bull* **66:** 1–20

Chien P, Weissman JS, DePace AH (2004) Emerging principles of conformation-based prion inheritance. *Annu Rev Biochem* **73:** 617–656

Collinge J (2001) Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci* **24:** 519–550

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383:** 685–690

Deleault NR, Harris BT, Rees JR, Supattapone S (2007) From the Cover: formation of native prions from minimal components *in vitro*. *Proc Natl Acad Sci USA* **104:** 9741–9746

Farquhar CF, Somerville RA, Bruce ME (1998) Straining the prion hypothesis. *Nature* **391:** 345–346

Fraser H, Dickinson AG (1973) Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J Comp Pathol* **83:** 29–40

Gambetti P, Kong Q, Zou W, Parchi P, Chen SG (2003) Sporadic and familial CJD: classification and characterisation. *Br Med Bull* **66:** 213–239

Gretzschel A, Buschmann A, Eiden M, Ziegler U, Luhken G, Erhardt G, Groschup MH (2005) Strain typing of German transmissible spongiform encephalopathies field cases in small ruminants by biochemical methods. *J Vet Med B Infect Dis Vet Public Health* **52:** 55–63

Hill AF, Joiner S, Wadsworth JD, Sidle KC, Bell JE, Budka H, Ironside JW, Collinge J (2003) Molecular classification of sporadic Creutzfeldt–Jakob disease. *Brain* **126:** 1333–1346

Kellings K, Prusiner SB, Riesner D (1994) Nucleic acids in prion preparations: unspecific background or essential component? *Philos Trans R Soc Lond B Biol Sci* **343:** 425–430

Kimberlin RH (1976) Experimental scrapie in the mouse: a review of an important model disease. *Sci Prog* **63:** 461–481

King CY, Diaz-Avalos R (2004) Protein-only transmission of three yeast prion strains. *Nature* **428:** 319–323

Legname G, Baskakov IV, Nguyen HO, Riesner D, Cohen FE, DeArmond SJ, Prusiner SB (2004) Synthetic mammalian prions. *Science* **305:** 673–676

Marsh RF, Bessen RA (1994) Physicochemical and biological characterizations of distinct strains of the transmissible mink encephalopathy agent. *Philos Trans R Soc Lond B Biol Sci* **343:** 413–414

Morales R, Abid K, Soto C (2007) The prion strain phenomenon: molecular basis and unprecedented features. *Biochim Biophys Acta* **1772:** 681–691

Prusiner SB (1998) Prions. *Proc Natl Acad Sci USA* **95:** 13363–13383

Saá P, Castilla J, Soto C (2004) Cyclic amplification of protein misfolding and aggregation. In *Amyloid Proteins: Methods and Protocols*, Sigurdsson EM (ed), pp 53–65. New York, NY: Humana Press

Saá P, Castilla J, Soto C (2006) Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. *J Biol Chem* **281:** 35245–35252

Saborio GP, Permanne B, Soto C (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411:** 810–813

Safar J, Wille H, Itri V, Groth D, Serban H, Torchia M, Cohen FE, Prusiner SB (1998) Eight prion strains have PrP(Sc) molecules with different conformations. *Nat Med* **4:** 1157–1165

©2008 European Molecular Biology Organization

Safar JG, Kellings K, Serban A, Groth D, Cleaver JE, Prusiner SB, Riesner D (2005) Search for a prion-specific nucleic Acid. *J Virol* **79:** 10796–10806

Somerville RA (2000) Is there more to TSE diseases than PrP? *Trends Microbiol* **8:** 157–158

Somerville RA (2002) TSE agent strains and PrP: reconciling structure and function. *Trends Biochem Sci* **27:** 606–612

Soto C, Anderes L, Suardi S, Cardone F, Castilla J, Frossard MJ, Peano S, Saá P, Limido L, Carbonatto M, Ironside J, Torres JM, Pocchiari M, Tagliavini F (2005) Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. *FEBS Lett* **579:** 638–642

Soto C, Castilla J (2004) The controversial protein-only hypothesis of prion propagation. *Nat Med* **10:** S63–S67

Soto C, Saborio GP, Anderes L (2002) Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. *Trends Neurosci* **25:** 390–394

Tanaka M, Chien P, Naber N, Cooke R, Weissman JS (2004) Conformational variations in an infectious protein determine prion strain differences. *Nature* **428:** 323–328

Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, Gabizon R, Mastrianni J, Lugaresi E, Gambetti P, Prusiner SB (1996) Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* **274:** 2079–2082

Uptain SM, Lindquist S (2002) Prions as protein-based genetic elements. *Annu Rev Microbiol* **56:** 703–741

Weber P, Giese A, Piening N, Mitteregger G, Thomzig A, Beekes M, Kretzschmar HA (2007) Generation of genuine prion infectivity by serial PMCA. *Vet Microbiol* **123:** 346–357

Weissmann C (1991) A 'unified theory' of prion propagation. *Nature* **352:** 679–683

Weissmann C (2004) The state of the prion. *Nat Rev Microbiol* **2:** 861–871

Wickner RB, Edskes HK, Ross ED, Pierce MM, Shewmaker F, Baxa U, Brachmann A (2004) Prions of yeast are genes made of protein: amyloids and enzymes. *Cold Spring Harb Symp Quant Biol* **69:** 1–8

Wickner RB, Masison DC, Edskes HK (1995) [PSI] and [URE3] as yeast prions. *Yeast* **11:** 1671–1685

©2008 European Molecular Biology Organization

Apéndice 9

Cell

Crossing the Species Barrier by PrP^{Sc} Replication In Vitro Generates Unique Infectious Prions

Joaquín Castilla,[1] Dennisse Gonzalez-Romero,[1,4] Paula Saá,[1,2,4] Rodrigo Morales,[1,3] Jorge De Castro,[1] and Claudio Soto[1,*]
[1]Department of Neurology, University of Texas Medical Branch, Galveston, TX 77555, USA
[2]Centro de Biología Molecular, Universidad Autónoma de Madrid, 28049 Madrid, Spain
[3]University of Chile, Santiago, Chile
[4]These authors contributed equally to this work
*Correspondence: clsoto@utmb.edu
DOI 10.1016/j.cell.2008.07.030

SUMMARY

Prions are unconventional infectious agents composed exclusively of misfolded prion protein (PrP^{Sc}), which transmits the disease by propagating its abnormal conformation to the cellular prion protein (PrP^{C}). A key characteristic of prions is their species barrier, by which prions from one species can only infect a limited number of other species. Here, we report the generation of infectious prions by interspecies transmission of PrP^{Sc} misfolding by in vitro PMCA amplification. Hamster PrP^{C} misfolded by mixing with mouse PrP^{Sc} generated unique prions that were infectious to wild-type hamsters, and similar results were obtained in the opposite direction. Successive rounds of PMCA amplification result in adaptation of the in vitro-produced prions, in a process reminiscent of strain stabilization observed upon serial passage in vivo. Our results indicate that PMCA is a valuable tool for the investigation of cross-species transmission and suggest that species barrier and strain generation are determined by the propagation of PrP misfolding.

INTRODUCTION

Prion diseases also known as transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative diseases affecting the brain of humans and several species of mammals (Collinge, 2001). Creutzfeldt-Jakob disease (CJD) is the most common TSE in humans, and scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in cervids are the most prevalent prion diseases in animals. Unlike conventional infectious microorganisms, the TSE agent appears to be devoid of genetic material and instead composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) (Prusiner, 1998). PrP^{Sc} has the unprecedented ability to replicate in the body by inducing the misfolding of the cellular form of the prion protein (PrP^{C}).

One of the characteristics of the agent responsible for prion diseases is its ability to infect some species and not others (Hill and Collinge, 2004; Moore et al., 2005). This phenomenon is known as species barrier. Even between close species, the species barrier is manifested as an incomplete attack rate and a prolongation of the time it takes for animals to develop the clinical disease when injected with another species' infectious material (Hill and Collinge, 2004). Primary interspecies transmission is usually not very efficient, and it takes a long time for the prion replication process to reach the point at which full-blown clinical disease appears. After sequential passages, the PrP^{Sc} in the new host adapts, resulting in a shortage of the incubation period and stabilization of the new strain (Hill and Collinge, 2004).

Compelling evidence indicates that the species barrier is largely controlled by the sequence of PrP (Moore et al., 2005). Unfortunately, we cannot predict the degree of a species barrier simply by comparing the prion proteins from two species. The barrier has to be measured by experimental studies in animals. These studies are long and costly, and in the case of the human species barrier, the studies have to be done with experimental models, the validity of which is not absolutely guarantied. Evaluation of the species barrier is of tremendous medical importance for risk assessment and to implement regulatory measures to avoid spreading of diseases (Moore et al., 2005). At this time, the epidemiological evidence suggests that among animal TSEs only cattle BSE has been transmitted to humans, generating a variant form of CJD (vCJD) (Will et al., 1996). It is unlikely that sheep scrapie is a concern for humans, because the disease has been described for centuries and no increased prevalence of human prion diseases has been found in scrapie-endemic areas (Caramelli et al., 2006; Hunter, 1998). However, the appearance of "atypical" strains of scrapie, as well as the known transmission of BSE to sheep, has generated new concerns of human infections with sheep-derived material (Buschmann and Groschup, 2005; Hunter, 2003). Similarly, the possibility that some of the newly identified animal prion diseases, such as CWD, could be transmitted to humans cannot be ruled out at the present time (Williams, 2005; Xie et al., 2005).

Recently, we reported the generation of infectious prions in vitro by amplification of PrP^{Sc} misfolding in the test tube (Castilla et al., 2005). For these experiments, we used a technology termed PMCA (protein misfolding cyclic amplification) that mimics in vitro some of the fundamental steps involved in PrP^{Sc} replication in vivo at an accelerated rate (Saborio et al., 2001). During PMCA, small quantities of PrP^{Sc} are mixed with excess of PrP^{C}, and through a cyclical process involving incubation and sonication, prion propagation occurs in an autocatalytic way. With this procedure, prions can replicate indefinitely in the test tube and, after successive rounds of dilutions followed by PMCA amplification, PrP^{Sc} used to begin the reaction can be eliminated, and only in vitro-generated misfolded protein remains in the sample (Castilla et al., 2005). Inoculation of PMCA-generated prions into wild-type animals resulted in a disease with the same clinical, neuropathological, and biochemical features as the disease produced by brain-derived infectious material (Castilla et al., 2005). The conclusion drawn from these findings is that all of the information required to propagate the infectious properties is enciphered in the structure of PrP^{Sc}. This is further supported by recent studies from Supattapone and coworkers in which infectious prions were generated in vitro by PMCA with purified PrP^{C} and PrP^{Sc} with the sole addition of synthetic polyanions (Deleault et al., 2007).

The goal of this study was to attempt crossing the species barrier in vitro to generate unique infectious prions in a cell-free system. For these studies, we used mice and hamsters, two experimental rodent systems widely employed in TSE studies and for which several prion strains are available (Bruce, 2003; Kimberlin and Walker, 1988). The PrP sequence shows nine differences between these two animal species (Figure 1A). Infectivity studies have shown that there is a large barrier for prion transmission between these species (Kimberlin et al., 1989; Kimberlin and Walker, 1988; Race et al., 2002). Our findings show that incubation of PrP^{C} from one of the species with PrP^{Sc} from the other resulted in new PrP^{Sc} that was infectious to wild-type animals. Interestingly, a detailed examination of the infectious, neuropathological, and biochemical features of the disease that was produced revealed characteristics that were different from other known prion strains. These results indicate that the prions generated in vitro by crossing of the mouse-hamster barrier represent new strains. Strikingly, studies of the infectious characteristics of these newly generated prions after different rounds of PMCA showed that the procedure not only enabled crossing of the species barrier but also resulted in stabilization of the new strain in vitro by successive rounds of amplification. Our findings show that prions can be propagated in vitro across the species barrier, leading to the generation and adaptation of unique prion strains.

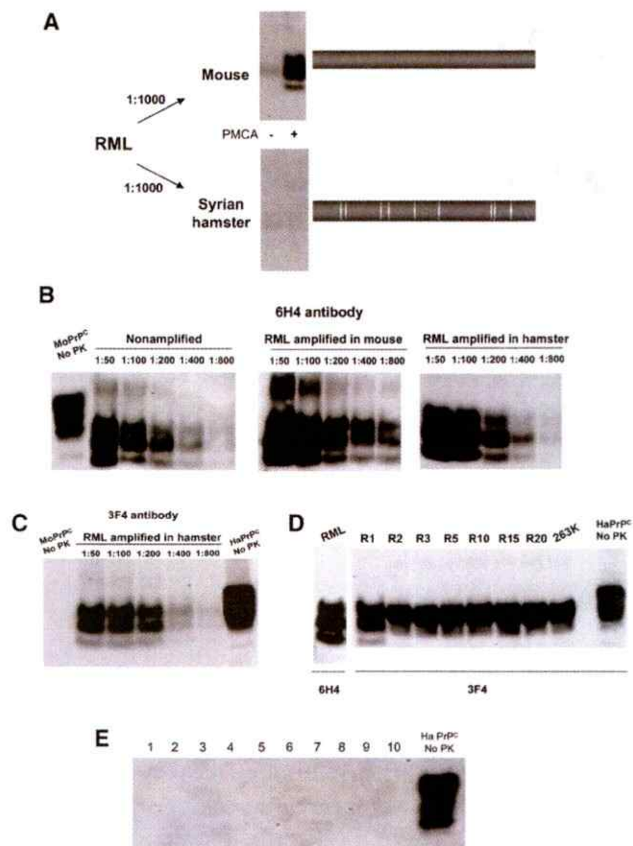


Figure 1. In Vitro Conversion of Hamster PrP^{C} Induced by Mouse RML PrP^{Sc}

(A) RML brain homogenate was diluted 1000-fold into either mouse or hamster normal brain homogenate and subjected to 96 PMCA cycles. The blot shows the results with and without PMCA in each species. At the right side, we show a scheme of PrP indicating the position in which there are amino acid differences between mice and hamsters.

(B) To attempt forcing conversion, we incubated larger quantities (dilutions 1:50 through 1:800) of RML PrP^{Sc} with mouse (central panel) or hamster (right panel) PrP^{C}. All samples (except for the control samples in the left panel labeled "nonamplified") were subjected to 96 PMCA cycles, and PrP^{Sc} signal was detected after PK digestion by western blot with the 6H4 antibody.

(C) The same samples as those in the right panel of (B) were developed with the 3F4 antibody.

(D) The newly generated RML-Ha PrP^{Sc} was serially passed in hamster brain homogenate by a series of 1:10 dilution followed by 48 PMCA cycles. "R" indicates the number of rounds of PMCA; i.e., R5 represent the samples after five serial rounds of PMCA.

(E) For the assessment of spontaneous generation of PrP^{Sc} by PMCA, samples from brain of ten different hamsters were subjected to the same process of serial PMCA as in (D). PrP^{Sc} formation was analyzed by western blot after PK treatment in each PMCA round. The figure shows the results obtained after 20 rounds of PMCA. In the experiments shown in this figure, all samples were treated with PK, except when indicated.

RESULTS

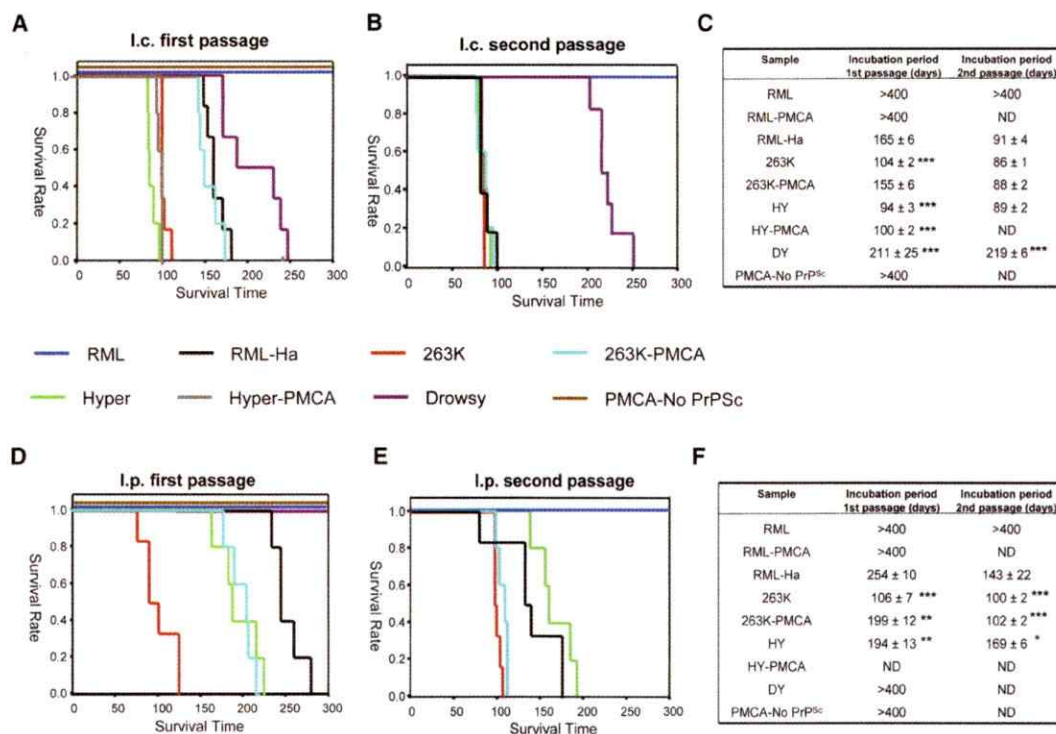
Crossing the Mouse-Hamster Species Barrier to Generate New Hamster Prions

To assess whether prions can be generated in vitro across the species barrier, we used hamsters and mice, two widely studied rodent experimental models of TSEs (Bruce, 2003; Kimberlin and Walker, 1988; Morales et al., 2007). A PMCA experiment done with our standard conditions for amplification of mouse RML prions showed no detectable formation of PrP^{Sc} when hamster PrP^{C} was used as a substrate (Figure 1A). Conversely, a robust PrP^{Sc} generation was observed with mouse PrP^{C} substrate. For this experiment, we mixed a 1000-fold dilution of RML PrP^{Sc}

into 10% brain homogenates of healthy hamsters and mice, respectively. We reasoned that if in vivo it takes longer for prions to replicate across species barriers, then in PMCA we should also encounter more difficulties to convert PrP^{C} when using PrP^{Sc} from a different species. To attempt forcing the in vitro conversion, we added a higher proportion of PrP^{Sc}-containing mouse brain homogenate into the hamster substrate. A range of dilutions from 50- to 800-fold were tested, but the problem with these experiments is that the large concentration of RML PrP^{Sc} used as inoculum makes it difficult to estimate convincingly whether new PrP^{Sc} generation was obtained (Figure 1B). Fortunately, the 3F4 monoclonal antibody can recognize hamster but not mouse PrP (Lund et al., 2007). Using this antibody for western blot, we could clearly observe that protease-resistant hamster PrP^{Sc} was being produced when the reaction was done with low dilutions (from 1:50 to 1:200) of mouse RML PrP^{Sc} (Figure 1C). When the amplification was attempted with 800-fold diluted PrP^{Sc}-containing mouse brain homogenate, only a very faint signal was observed, confirming the results obtained in Figure 1A and the idea that the combination of PrP^{C} and PrP^{Sc} from different species impairs PMCA efficiency.

Newly generated hamster PrP^{Sc} starting from RML prions was propagated many times in vitro by serial PMCA in order to remove by dilution the initial amount of mouse scrapie brain material added to begin prion replication (Figure 1D). As described before, using this procedure, we can completely remove all molecules of brain-derived PrP^{Sc} from the sample (Castilla et al., 2005). Hamster PrP^{Sc} of RML origin efficiently propagates in vitro at the expense of hamster PrP^{C}. Interestingly, in the first PMCA round, the glycoform distribution pattern of the in vitro-generated hamster PrP^{Sc} was comparable to the RML profile showing the three glycoform bands (Figure 1D). After further PMCA rounds, this pattern changed to become undistinguishable from PrP^{Sc} associated to the typical hamster strains, such as 263K (Figure 1D) or Hyper (HY), in which the diglycosylated band is highly predominant. This result suggests that the characteristics of the newly generated PrP^{Sc} are being adapted to the new species during successive PMCA cycling, reminiscent of the adaptation process occurring in vivo upon serial passage of the infectious material. After 20 serial rounds of PMCA, representing a dilution equivalent to 10^{-22} with respect to the brain (since the first round contains a 100-fold dilution of the material), our estimation is that no molecules of mouse brain PrP^{Sc} should be present in the sample. This in vitro-generated material was termed RML-Ha PrP^{Sc} to emphasize the RML origin of this new hamster misfolded prion protein. To make sure that newly formed PrP^{Sc} was indeed coming from conversion of hamster PrP^{C} induced by mouse PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in hamsters (Deleault et al., 2007), we did a large experiment to analyze in detail the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different hamsters were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure 1E).

Inoculation of wild-type hamsters with RML-Ha PrP^{Sc} (produced after a 10^{-22} dilution of RML scrapie brain homogenate) produced disease in 100% of the animals by both intracerebral (i.c.) and intraperitoneal (i.p.) routes (Figure 2). The disease exhibits the clinical characteristics typical of hamster scrapie, including hyperactivity, motor impairment, head wobbling, muscle weakness, and weight loss. The incubation time in the first passage was 165 $\pm$ 6 days by i.c. inoculation (Figures 2A and 2C). This is longer than the incubation time obtained with hamster scrapie strains, such as 263K and HY, in which a similar quantity of PrP^{Sc} produces disease at around 100 days by this route (Figures 2A and 2C). However, in agreement with our previously reported data (Castilla et al., 2005), when hamster 263K prions were replicated in vitro by PMCA, the newly generated PrP^{Sc} produced disease with a delay similar to that observed with the RML-Ha material (Figures 2A and 2C). The delay in our previous study was eliminated upon a second passage in vivo, in which the new infectious material was stabilized to acquire properties undistinguishable from in vivo-derived 263K (Figures 2B and 2C). Interestingly, in the HY hamster prion strain, PMCA-generated material did not show any statistically significant difference compared to in vivo-produced prions (Figures 2A and 2C). These results suggest that in vitro replication of prions by PMCA maintains the strain characteristics, at least in respect to the incubation periods. To assess the stability of RML-Ha and estimate the stabilized incubation period, we performed a second passage. As shown in Figure 2B, the incubation time of RML-Ha prions was decreased to around 90 days, which is very similar to that obtained with 263K and HY but different from the Drowsy (DY) strain. These results suggest that RML-Ha prions behave similarly to the 263K strain; both in vitro-generated prions show a delay in the first passage that gets corrected upon a second in vivo passage. This feature is not displayed by other hamster prion strains, such as HY, or other species of prions (see below for the results in mice), where PMCA-generated prions exhibited the same incubation period in the first passage as in vivo-produced infectious material. As expected, hamsters inoculated with RML prions did not develop disease during the time of the experiment (>400 days). Animals inoculated with hamster brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc} (control for the de novo generation of PrP^{Sc}) did not develop disease more than 400 days after inoculation (Figures 2A and 2C). Intraperitoneal inoculations of the infectious material showed a clear difference between the three prion strains used as reference, with 263K being the fastest and DY not producing disease by this route (Figure 2D). The incubation period produced by i.p. inoculation of RML-Ha prions was longer than that of the 263K and HY strains, with an average of 254 days in the first passage. This is also longer than 263K prions amplified in vitro by PMCA, which produced disease after 199 days postinoculation in the first passage (Figures 2D and 2F). A second in vivo passage again stabilized PMCA-generated 263K prions to produce disease at a time indistinguishable from that of brain-derived 263K infectious material. The second passage of RML-Ha prions showed that the stabilized incubation period for the i.p. route was on average around 140 days, which is significantly higher than 263K or 263K-PMCA material but shorter than HY prions (Figures 2E and 2F). The differences remained stable in a third passage (data not shown). These results indicate that in some aspects, RML-Ha prions are similar to the agent in the 263K strain but in other features are intermediate between 263K



C

Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	165 ± 6	91 ± 4
263K	104 ± 2 ***	86 ± 1
263K-PMCA	155 ± 6	88 ± 2
HY	94 ± 3 ***	89 ± 2
HY-PMCA	100 ± 2 ***	ND
DY	211 ± 25 ***	219 ± 6 ***
PMCA-No PrP^{Sc}	>400	ND

F

Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	254 ± 10	143 ± 22
263K	106 ± 7 ***	100 ± 2 ***
263K-PMCA	199 ± 12 **	102 ± 2 ***
HY	194 ± 13 **	169 ± 6 *
HY-PMCA	ND	ND
DY	>400	ND
PMCA-No PrP^{Sc}	>400	ND

Figure 2. Infectivity of Newly Generated RML-Ha PrP^{Sc} after Crossing the Species Barrier

RML-Ha PrP^{Sc} samples amplified by 20 serial PMCA rounds were inoculated i.c. or i.p. into six wild-type hamsters. For controls, we inoculated similar quantities of PrP^{Sc} from RML or three distinct hamster strains (263K, Hyper, and Drowsy). We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of 263K (263K-PMCA) or Hyper (HY-PMCA) PrP^{Sc} with healthy hamster brain homogenate and RML replicated at expenses of mouse PrP^{C} (RML-PMCA). The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal hamster brain homogenate (PMCA-No PrP^{Sc}). (A) and (D) show the survival curves obtained after i.c. and i.p. inoculation, respectively, of the in vitro-generated RML-Ha after 20 rounds of PMCA. (B) and (E) show the survival curves of the second passage (i.e., animals were inoculated with material obtained from the brain of sick animals in the experiments depicted in [A] and [D]) after i.c and i.p inoculation, respectively. (C) and (F) show the average incubation periods of the experiments done by i.c. and i.p. inoculation of various samples. The values correspond to the average ± standard error. The data was analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$). ND, not done.

and HY prions, providing a first indication that the material obtained by crossing of the mouse-hamster species barrier represents a unique hamster prion strain.

To further assess the characteristics of the disease produced by in vitro-generated RML-Ha prions, we studied in detail the neuropathological and biochemical features of the brain damage. Histopathological studies showed that animals inoculated with RML-Ha prions exhibit the typical brain lesions of scrapie, including spongiform degeneration, astrogliosis, and PrP^{Sc} deposition (Figures 3A–3C). Quantitative studies of the vacuolation profile in different brain areas showed that RML-Ha-infected hamsters showed the largest extent of spongiosis in medulla and cerebellum and less damage in hippocampus, cortex, and colliculum (Figure 3D). This pattern of brain damage was similar to that observed in 263K-inoculated animals and statistically different from that obtained in hamsters injected with HY and DY (Figure 3D). However, the extent of both astroglyosis (Figure 3B) and PrP^{Sc} accumulation (Figure 3C) in the medulla of RML-Ha-infected animals was lower than that in 263K-sick animals and similar to that observed in HY-injected hamsters (Figures 3B and 3C). These data suggest again that the RML-Ha prions are a unique strain with properties intermediate between the previously known 263K and HY hamster strains.

Comparative studies of the biochemical characteristics of PrP^{Sc} obtained from the brain of sick animals after inoculation with RML-Ha, 263K, HY, and DY were done by analysis of the electrophoretical pattern of the protein, its susceptibility to proteolytic degradation, and its resistance to denaturation. For comparison of the protease resistance profile, similar quantities of PrP^{Sc} from the new RML-Ha prions and PrP^{Sc} obtained from the brain of sick hamsters inoculated with the prion strains 263K, HY, and DY were treated for 60 min with various concentrations of proteinase K (PK) (Figure 4A). RML-Ha PrP^{Sc} was highly resistant to large PK concentrations. The misfolded protein associated to the newly generated strain was more resistant than HY or DY and similarly (but still significantly more) susceptible to PK digestion than 263K PrP^{Sc} (Figure 4A). The PK concentration in which 50% of the protein was degraded (PK50) was highest for PrP^{Sc} associated to RML-Ha, followed by 263K, HY, DY, and RML (Table S1 available online).

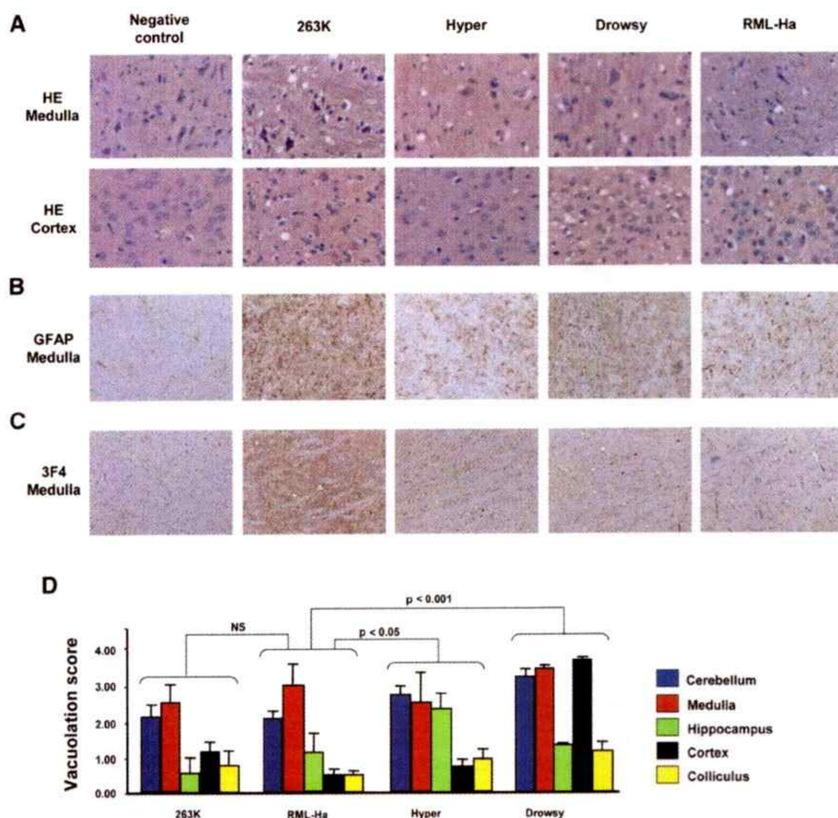


Figure 3. Histopathological Features of the Disease Induced by Inoculation of Hamsters with PMCA-Generated RML-Ha PrP^{Sc}

Brain from sick animals in which disease was produced by inoculation with the in vitro-generated RML-Ha PrP^{Sc} (first passage) or the known hamster strains 263K, Hyper, and Drowsy were analyzed by histological studies. As a control, we used the brain of a hamster inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin staining of medulla and occipital cortex sections and visualized by microscopy at a 40× magnification.

(B) Reactive astrogliosis was evaluated by histological staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated by staining of the tissue with the 3F4 anti-PrP monoclonal antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with the other hamster prion strains. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and RML-Ha, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

Another characteristic we studied was the electrophoretic mobility and glycosylation pattern of PrP^{Sc} associated to distinct strains. The predominant glycoform for the hamster strains (including the newly generated RML-Ha) is the diglycosylated band, whereas mouse RML PrP^{Sc} shows a more even distribution of the three bands with the main one being the monoglycosylated form. To assess the size of the protein after PK cleavage, we performed endoglycosidase treatment to remove the glycosylated chains (Figure 4B). Whereas PrP^{Sc} associated to the DY strain has a higher electrophoretical mobility, no significant differences were observed among the other proteins. Another biochemical property of misfolded PrP often used to differentiate prion strains is its resistance to chemical denaturation (Safar et al., 1998). Clear differences were observed in the guanidine concentrations required to denature PrP^{Sc} associated to different strains (Figure 4C). The concentration of the chaotropic agent needed to denature 50% of PrP^{Sc} RML-Ha was 1.11 M, substantially different from the 1.69, 1.56, and 1.72 M required for the proteins associated to HY, DY, and RML, respectively (Table S1).

Crossing the Hamster-Mouse Species Barrier to Generate and Stabilize New Mouse Prions

To study the barrier between these rodent species in the opposite direction, we mixed 263K hamster prions with mouse healthy brain homogenate. As before, when a standard PMCA assay was done by dilution of 263K brain homogenate 1000-fold into mouse healthy brain material, we did not see detectable generation of mouse PrP^{Sc} (data not shown). However, when a higher quantity of hamster PrP^{Sc} was added, we were able to generate new mouse PrP^{Sc} (termed 263K-Mo) that could be propagated by serial rounds of PMCA to reach a dilution of the hamster brain homogenate equivalent to 10^{-17} (Figure 5A). Since there are not available antibodies capable of recognizing mouse PrP but not hamster PrP, we could not compare the electrophoretical pattern of PrP^{Sc} generated in the first rounds of PMCA with the profile of PrP^{Sc} typically observed in mouse and hamster strains. However, the western blot pattern of 263K-Mo after 15 rounds of PMCA (when no more molecules of 263K PrP^{Sc} are present) is similar to the one observed for RML and other ovine-derived mouse strains, despite a slightly faster migration (Figure S1A) that will be investigated in more detail later. To assess whether newly generated PrP^{Sc} was indeed coming from conversion of mouse PrP^{C} induced by 263K hamster PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in mice, we did an experiment to analyze the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different mice were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure S1B).

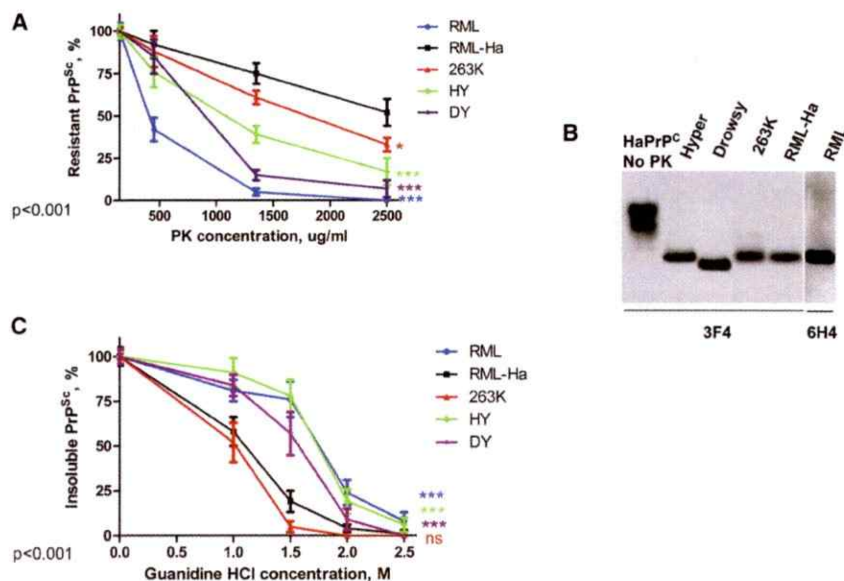


Figure 4. Biochemical Characterization of RML-Ha PrP^{Sc}

Samples from brains of animals inoculated with RML-Ha PrP^{Sc} (first passage in vivo) were used to study the PK resistance profile (A), the relative mobility after deglycosylation and PK treatment (B), and the susceptibility to guanidine denaturation (C). For controls, we used samples from RML or three distinct hamster strains (263K, Hyper, and Drowsy). The results in (A) and (C) correspond to the quantitative evaluation of western blots by densitometric analysis from three independent animals. The data represent the average ± standard error. The data were analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$).

To assess whether mouse PrP^{Sc} generated in vitro from hamster 263K is infectious to wild-type mice and to determine whether the infectious properties are being adapted upon serial PMCA passages, we inoculated several rounds of in vitro-generated material into mice (Figure 5A). Despite the fact that the same amount of PrP^{Sc} was inoculated (as determined by western blot), striking differences in the infectious properties were seen among in vitro-generated prions in distinct rounds of PMCA (Figure 5B). Only two of the six mice inoculated with material produced in the first round of PMCA showed disease symptoms, which appear at a very long time after inoculation (around 500 days) (Figures 5B and 5C). A complete attack rate was observed when animals were inoculated with material produced after three serial rounds of PMCA. However, the incubation period was long (around 310 days on average), and there was a large dispersion among animals (Figures 5B and 5C). The incubation period became stable, short (around 165 days), and there was little dispersion after the six serial rounds of PMCA. These findings indicate that upon successive rounds of PMCA, the newly generated prion, after crossing the species barrier, is becoming adapted and stabilized to the new host, a process very similar to what is seen after several passages in vivo. The large dispersion of incubation times observed in the third round of PMCA suggests that more than one strain has been generated upon crossing of the species barrier and that successive in vitro amplification leads to the selection and cloning of the most efficient of these strains. The incubation time for 263K-Mo after 15 rounds of PMCA (equivalent to a 10^{-17} dilution of the 263K inoculum) was around 165 days, similar to the one produced by scrapie-adapted mouse strains, such as RML, but different from that of the bovine strain 301C (Figure 5D). In vitro replication of the mouse strains RML and 301C at expense of mouse PrP^C produced PrP^{Sc} with identical properties as the brain-derived material, reflected as an indistinguishable incubation period (Figure 5D). As expected, mice inoculated with hamster 263K prions did not develop disease during the time of the experiment (>500 days). No disease was also observed in animals inoculated with mouse brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc}, which corresponds to the control experiment for the de novo generation of PrP^{Sc} (Figure 5D).

To analyze whether the newly generated 263K-Mo infectious material corresponded to a new strain of mouse prions, we studied the histopathological and biochemical features of the brain damage. Animals affected with the disease produced by inoculation of 263K-Mo showed extensive vacuolation in the medulla and hypocampus and moderate but clearly detectable damage in the cerebellum (Figures 6A and 6D). The pattern of spongiform degeneration does not correspond with any of the previously known mouse strains studied and indeed is statistically significantly different to the vacuolation profile produced by RML and 301C prions (Figure 6D). Differences were also detected in the extent of brain inflammation produced by 263K-Mo, since the degree of astroglyosis was less prominent than the one observed in animals inoculated with RML or 301C prions (Figure 6B). The profile of PrP^{Sc} accumulation consisted mostly of diffuse deposition and was not clearly different from the one observed in the other strains (Figure 6C). Then we studied the biochemical characteristics of PrP^{Sc} obtained from the brain of animals infected with 263K-Mo. Electrophoretical migration was assessed after PK digestion and endoglycosidase treatment to remove glycosylation chains. The PK-resistant core of PrP^{Sc} migrated slightly faster than RML but slightly slower than 301C, with an estimated molecular weight of 20 KDa (Figures 7A and 7B). These results indicate that the cleavage site after PK digestion is different from all of the currently known mouse strains. This is important because it is thought that differences in the PK cleavage site reflect disparities in the folding or aggregation of the protein (Chen et al., 2000; Collinge et al., 1996). To further search for biochemical differences, we subjected the protein to proteolytic degradation by using various concentrations of PK. 263K-Mo PrP^{Sc} was much more resistant to PK than to RML (Figure 7C), with a PK_{50} (the PK concentration needed to

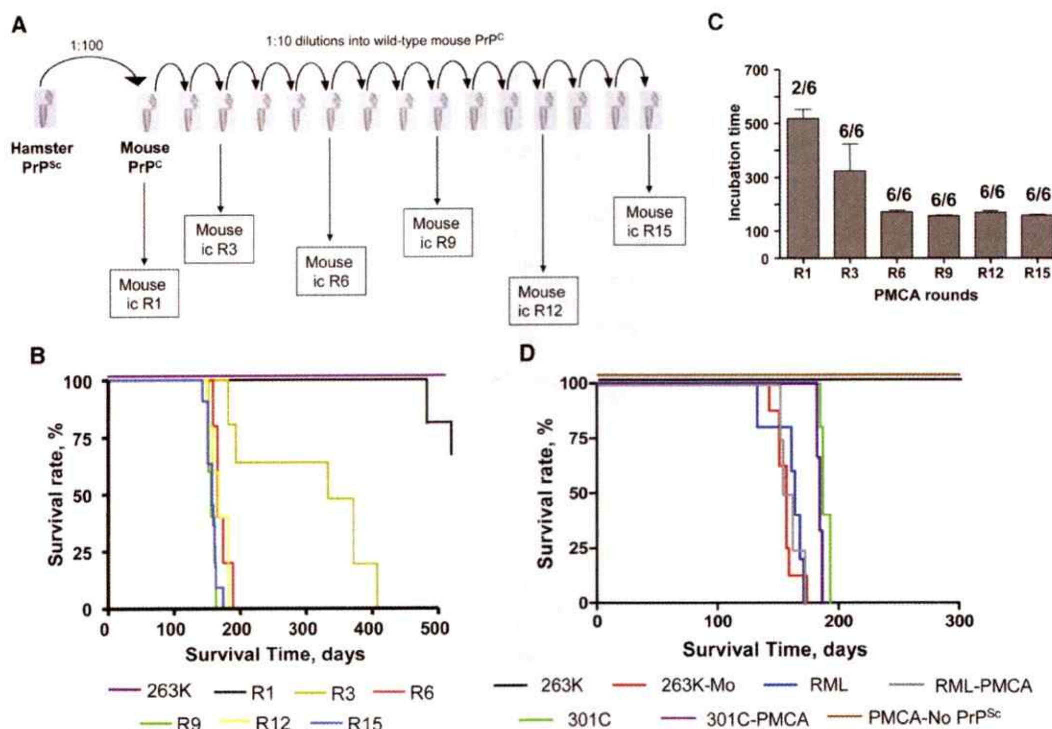


Figure 5. In Vitro Conversion of Mouse PrP^C Induced by Hamster 263K PrP^{Sc} Generates Infectious Prions

(A) Schematic representation of the dilutions done and the PMCA rounds used for our in vivo infectivity experiments.

(B) Survival curve observed after inoculation of six wild-type mice with the material generated after several rounds of PMCA. "R" indicates the number of rounds of PMCA. As a control, the animals were inoculated with 263K hamster prions.

(C) Average and standard error of the incubation times and attack rates observed after inoculation of wild-type mice with the material produced after different rounds of PMCA.

(D) Comparison of survival curves for the stabilized 263K-Mo infectious material (after 15 rounds of PMCA) with those obtained with RML and 301C, two mouse strains of different origin. We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of RML (RML-PMCA) or 301C (301C-PMCA) PrP^{Sc} with healthy mouse brain homogenate. The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal mouse brain homogenate (PMCA-No PrP^{Sc}), which correspond to the control for de novo generation of prions. For all of these experiments, the material was inoculated i.c. as described in the Experimental Procedures.

degrade half of the protein) of 1450 μg/ml (Figure 7D), much larger than the values obtained for RML (240 ug/ml) and 301C (430 ug/ml) (Table S2). Interestingly, the high resistance of PrP^{Sc} is typical of the hamster prions (Table S1), and indeed, 263K, the parental strain of the newly generated mouse prions, has a PK_{50} of around 1700 μg/ml.

DISCUSSION

The phenomenon of the species barrier, by which the agent coming from one species can infect only a limited number of other species, is a typical feature of prion diseases. The molecular basis of this process is not well-understood, but it is thought to be controlled by the structure and folding of the prion protein (Moore et al., 2005; Vanik et al., 2004). As with the related phenomenon of prion strains, it is difficult to imagine how an infectious agent lacking genetic material and composed by a single protein can encode the structural diversity and specificity required to control strains variability and species selectivity (Soto and Castilla, 2004).

In addition to the intriguing molecular mechanism behind the species barrier, understanding this phenomenon has profound implications for public health. Indeed, one of the scariest medical problems of the last decades has been the emergence of a new and fatal human prion disease (variant CJD) originated by cross-species transmission of BSE from cattle (Will et al., 1996). BSE has not only been transmitted to humans. The extensive use of cow-derived material for feeding other animals led to the generation of new diseases in exotic felines, nonhuman primates, and domestic cats (Doherr, 2003). Worrisomely, the transmission of BSE into these different species could create new prion strains with unique biological and biochemical characteristics and thus a potentially new hazard for human health. More frightening is perhaps the possibility that BSE has been passed into sheep and goats. Studies have already shown that this transmission is possible and actually relatively easy (Foster et al., 1993). The disease produced is clinically similar to scrapie, but since it comes from BSE it has the potential to be infectious to humans. Another concern is CWD, a disorder affecting farm and wild species of cervids (Sigurdson and Aguzzi, 2006; Williams, 2005). The

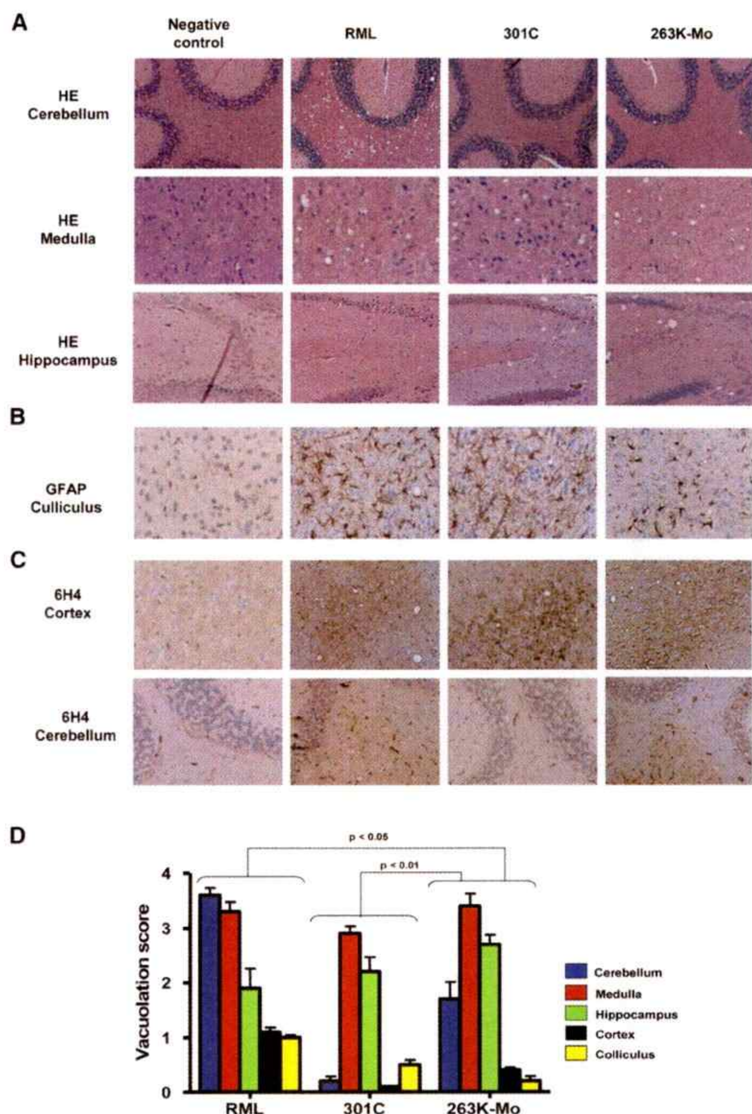


Figure 6. Histopathological Features of the Disease Induced by Inoculation of Mice with PMCA-Generated 263K-Mo PrP^{Sc}

Brains from sick mice in which disease was produced by inoculation with the newly generated 263K-Mo prions after 15 rounds of PMCA (first passage) or the known mouse strains RML and 301C were analyzed by histological studies. As a control, we used brain of a mouse inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin (HE) staining of three different brain areas (cerebellum, medulla, and hippocampus) and was visualized at a 40× magnification.

(B) Reactive astroglyosis was evaluated in the inferior culliculus by staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated in the occipital cortex and cerebellum by staining of the tissue with the 6H4 antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with RML and 301C. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and 263K-Mo, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

origin of CWD and its potential to transmit to humans are currently unknown. This is worrisome, considering that CWD has became endemic in some parts of the USA and that the number of cases continues to increase (Williams, 2005). CWD transmissibility studies have been performed in many species in order to predict how this disease could be spread by the consumption of CWD meat (Sigurdson and Aguzzi, 2006). Transmission of CWD to humans cannot be ruled out at present, and a similar infective episode to BSE involving CWD could result in catastrophic consequences.

The exciting scientific problem coupled with the relevant public-health issue prompted us to develop strategies to reproduce the species-barrier phenomenon in the test tube. We reported previously the generation of infectious prions in vitro by cyclic replication of the protein misfolding process featuring the pathogenesis of prion diseases (Castilla et al., 2005). These results were reproduced and extended by other groups to better dissect the elements required for prion replication (Deleault et al., 2007; Weber et al., 2007). The PMCA technology has been adapted to replicate prions from various species (Deleault et al., 2005; Jones et al., 2007; Kurt et al., 2007; Murayama et al., 2007; Sarafoff et al., 2005; Soto et al., 2005) and even to use bacterially produced recombinant PrP as substrate (Atarashi et al., 2007). The conclusion drawn from these studies together with the findings reported in this manuscript is that propagation of the PrP^{Sc} misfolding results in formation of infectious material, which maintains the strains and species-barrier properties of the original prions. Qualitatively similar conclusions have been obtained for yeast prions, which are a group of "infectious proteins" that behave as a non-Mendelian genetic element and transmit biological information in the absence of nucleic acid (Wickner et al., 1995). Recent studies showed that bacterially produced N-terminal fragments of the yeast prions Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (Brachmann et al., 2005; King and Diaz-Avalos, 2004; Tanaka et al., 2004). Infection of yeast with different conformers led to generation of distinct prion strains in vivo (Brachmann et al., 2005; Tanaka et al., 2004). Remarkably, yeast prions also show the species-barrier phenomenon, and recent data indicate that strain conformation is the critical determinant of cross-species prion transmission (Tanaka et al., 2005).

In the current study, we demonstrate the generation of new infectious prions across the species barrier. For this purpose, we

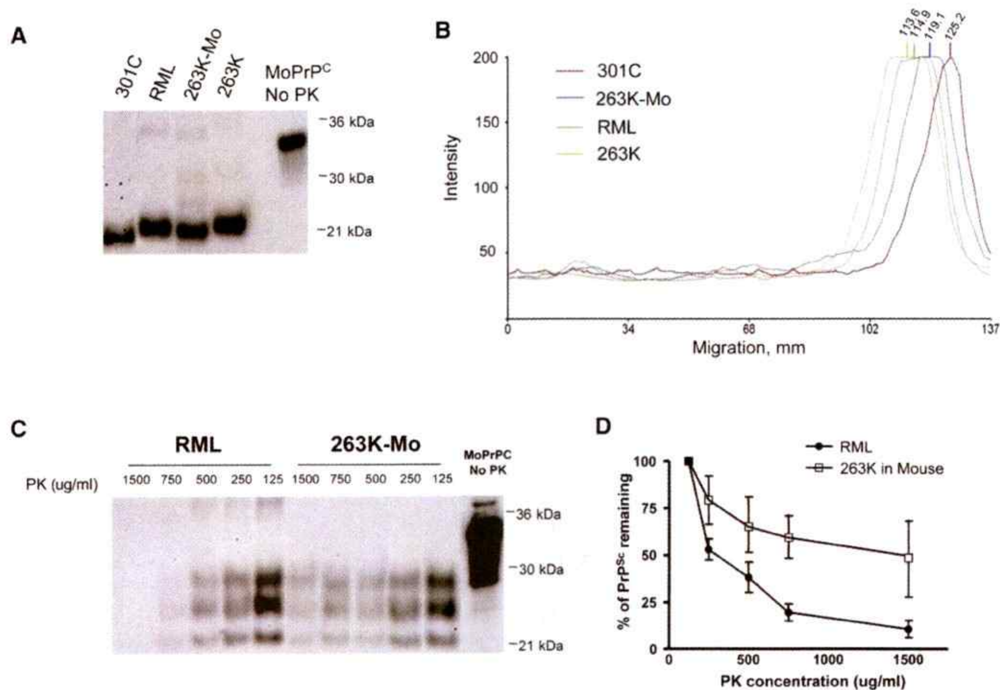


Figure 7. Biochemical Characteristics of 263K-Mo PrP^{Sc}

(A) Samples from brains of mice inoculated with 263K-Mo, RML, or 301C were used to study the electrophoretical migration after deglycosylation and PK treatment.

(B) For assessment of the electrophoretical differences among distinct strains, the blot in (A) was scanned and analyzed by software included in the UVP image analysis system to locate the exact position of the bands.

(C) The PK resistance profile of 263K-Mo PrP^{Sc} was studied and compared with RML.

(D) The results of the experiment shown in (B) were quantitated by densitometric analysis. The data in the figure represent the average ± standard error from three independent animals. The differences were statistically significant as evaluated by one-way ANOVA ($p < 0.01$).

mixed PrP^{Sc} from one species with PrP^{C} from a different animal species and subjected the mixture to serial rounds of PMCA to generate, propagate, and stabilize new prion strains. Hamster PrP^{Sc} generated from mouse RML prions was infectious to wild-type hamsters. Detailed analysis of the disease characteristics and comparison with the illness produced by several known hamster prion strains indicate that the in vitro-generated infectious material across the species barrier corresponds to a new prion strain in hamsters (termed RML-Ha). The main differences of the RML-Ha were on the incubation times after i.p. inoculation, the extremely high resistance to PK degradation, and the pattern of brain damage (Table S1). Similarly, PrP^{Sc} generated by conversion of mouse PrP^{C} with hamster PrP^{Sc} from the 263K strain was shown to be infectious to wild-type mice, with an incubation period comparable to that obtained after inoculation with some of the mouse-adapted scrapie strains, such as RML. Again, the disease produced by the new prions (termed 263K-Mo) was clearly distinguishable from the one produced by some of the currently known mouse prion strains. The major differences were seen in the electrophoretical migration, extremely high resistance to proteolytic degradation, and pattern of brain spongiform degeneration (Table S2). To rule out that newly generated PrP^{Sc} in these experiments was coming from "de novo" spontaneous conversion of PrP^{C} into PrP^{Sc} during PMCA, we used samples of healthy brain homogenate from ten different mice and hamsters that were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples. This material was inoculated into wild-type animals, and no disease was observed more than 400 days after inoculation. These results strongly indicate that the generation of PrP^{Sc} reported in the present study was due to interspecies prion conversion. Nevertheless, we would like to highlight that recently we have been able to generate in vitro PrP^{Sc} de novo without addition of PrP^{Sc} seed (data not shown). However, to reach this aim, the PMCA conditions need to be modified. The modifications include changes on the PMCA parameters (length of incubation and potency of sonication), preincubation, or pretreatment of the normal brain homogenate to induce/stabilize PrP misfolding prior to PMCA. These findings suggest that de novo formation of PrP^{Sc} can be experimentally distinguished from replication of preformed PrP^{Sc}, indicating that the biochemical, conformational, or stability properties of the PrP structures involved in both processes are probably different. Standard PMCA conditions, as those used in the current study, do not result in spontaneous PrP^{Sc} formation.

Interestingly, in our serial PMCA amplifications of RML PrP^{Sc} into hamster PrP^{C}, we observed a progressive change on the western blot profile of the newly generated RML-Ha PrP^{Sc}. Indeed, in the first round of PMCA, the glycoform distribution

pattern was reminiscent of RML and later switched to a profile typical of the hamster strains, characterized by the predomination of the diglycosylated form (Figure 1D). Our interpretation of this result was that consecutive rounds of PMCA may enable the new prion strain to adapt and stabilize. To further study this possibility in our experiments in which mouse prions were generated from 263K hamster prions, we inoculated the material generated after various rounds of PMCA. Strikingly, similar amounts of PrP^{Sc} generated after one and three rounds of PMCA produced disease with incomplete attack rates and/or very long incubation periods (Figures 5B and 5C). Incubation time stabilized after six rounds of serial PMCA, suggesting that at this point the new strain is fully adapted. These findings suggest that PMCA is not only able to reproduce the interspecies transmission of prions but is also able to mimic the strain adaptation process observed in vivo. In vivo adaptation and stabilization of prions generated after crossing the species barrier takes at least four consecutive passages, which requires several years of work (Race et al., 2001, 2002). Conversely, strain adaptation by PMCA takes only 2 or 3 weeks. Importantly, the kinetics of adaptation in vitro and in vivo, as well as the characteristics of the stabilized material, are very similar. Indeed, it has been reported that three serial passages of 263K in mice produce disease in all animals, with an incubation time of around 300 days (Race et al., 2002). This result is very similar to the data obtained with the material generated in vitro after three successive rounds on PMCA replication (Figures 5B and 5C). Moreover, less than three in vivo passages produced an incomplete attack rate, and more than three passages are needed to obtain a stable and low incubation period (Race et al., 2002), which is in the same range of our 263K-Mo infectious material. Finally, similar to our in vitro data, the in vivo cross-species transmission between hamsters and mice also led to the generation of unique prion strains (Race et al., 2001, 2002). Although we are tempted to speculate that each PMCA round has the same effect on strain adaptation as did each in vivo passage, more experiments with other species combinations are needed to reach this conclusion.

In summary, our results show that all elements controlling interspecies transmission of prions are contained in a cell-free system and that new prion strains can be generated, adapted, and stabilized upon crossing the species barrier in vitro by PMCA. These findings provide additional support for the prion hypothesis, suggesting that species-barrier transmission and strain generation are determined by the propagation of PrP misfolding. Furthermore, the data demonstrate that PMCA is a valuable tool for the investigation of the strength of the barrier between diverse species, its molecular determinants, and the expected features of the new infectious material produced. Finally, our findings suggest that the universe of possible prions is not restricted to those currently known but that likely many unique infectious foldings of the prion protein may be produced and that one of the sources for this is cross-species transmission.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Homogenates

Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM ethylenediaminetetraacetic acid (EDTA) before the tissue was harvested. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, and the complete cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany). The samples were clarified by a brief, low-speed centrifugation (1500 rpm for 30 s) with an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial Replication of Prions In Vitro by PMCA

Aliquots of 10% brain homogenate from clinically sick mice infected with RML or 301C and hamsters infected with 263K, HY, or DY prions were diluted into 10% hamster or mouse healthy brain homogenate. Samples were loaded onto 0.2 ml PCR tubes and positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY). Each PMCA cycle consisted of 30 min incubation at 37°C followed by a 20 s pulse of sonication set at potency of 7. Samples were incubated without being shaken immersed in the water of the sonicator bath. After a round of PMCA cycles, a 10 μl aliquot of the amplified material was diluted into 90 μl of more normal brain homogenate, and a new round of PMCA cycles was performed. This procedure was repeated several times to reach the final dilutions indicated in the text. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, has been published elsewhere (Castilla et al., 2006; Saa et al., 2005).

Proteinase K Degradation Assay

The standard procedure for digestion of PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 μg/ml) for 60 min at 37°C. The digestion was stopped by addition of electrophoresis sample buffer, and the protease-resistant PrP was revealed by western blotting. So that the profile of PK sensitivity for in vitro- and in vivo-generated PrP^{Sc} could be studied, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 0 to 2500 μg/ml. The PK_{50} values represent the concentration of PK needed to digest half of the protein, and these values are estimated on the basis of the densitometric analysis of three replicated western blots.

Guanidine Denaturation Assay

Samples were incubated with different concentrations of guanidine hydrochloride for 2 hr at room temperature with shaking. Thereafter, samples were incubated in the presence of 10% sarkosyl for 30 min at 4°C and centrifuged at 100,000 × g for 1 hr in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet of the centrifugation was resuspended in conversion buffer and treated with PK as described above. Equivalent aliquots of pellet were analyzed by western blot. The Gdn_{50} value corresponds to the concentration of guanidine hydrochloride required to denaturate 50% of the protein, and these values were estimated on the basis of the densitometric analysis of three replicated western blots.

Protein Deglycosylation Assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100,000 × g for 1 hr at 4°C, supernatant was discarded, and the pellet resuspended in 100 μl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 26 μl of 50 mM sodium phosphate (pH 7.5) containing 1% nonidet P-40 and 3 μl of peptide N-glycosidase F (New England Biolabs, Beverly, MA) were added. Samples were incubated for 2 hr at 37°C, and the reaction was stopped by the addition of electrophoresis buffer and samples were analyzed by western blot.

Western Blot

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 (for mouse samples) and 3F4 (for hamster samples) antibodies at a 1:5000 dilution. The immunoreactive bands were visualized by enhanced chemoluminesence assay (Amersham, Piscataway, NJ) with an UVp image analysis system. So that the quantity of PrP^{Sc} in the western blot would be assessed, densitometric analyses were done by triplicate.

PrP^{Sc} Quantification

To inject the same quantity of PrP^{Sc} from each preparation, we compared the samples by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artifacts due to saturation of the signal or to too weak of a signal.

Infectivity Studies

In vivo infectivity studies were done in C57Bl6 female mice or Golden Syrian female hamsters, purchased from Charles river. Animals were 4 to 6 weeks old at the time of inoculation. Anesthesized animals were injected stereotaxically into the right hippocampus with 2 or 4 μl of the mouse or hamster infectious material, respectively. For the i.p. infectivity studies, 100 μl of the sample were injected into the peritoneal cavity. The quantity of infectious material injected corresponds to the plateau portion of the incubation period; therefore, small differences in the amount of infectivity should not change incubation period unless there are strain differences. The onset of clinical disease was measured by scoring of the animals twice a week. For mice, the following scale was used: 1, normal animal; 2, roughcoat on limbs; 3, extensive roughcoat, hunckback, and visible motor abnormalities; 4, urogenital lesions; and 5, terminal stage of the disease in which the animal presented with cachexia and lies in the cage with little movement. For hamsters, the following scoring scale was used: 1, normal animal; 2, mild behavioral abnormalities including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness (or lethargy in case of the DY strain); 4, severe behavioral abnormalities including all of the above plus jerks of the head and body and spontaneous backrolls; and 5, terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during two consecutive weeks were considered sick and were sacrificed to avoid excessive pain via exposition to carbonic dioxide. Brains were extracted, the right cerebral hemisphere was frozen and stored at −70°C for biochemical examination of PrP^{Sc} with western blots, and the left hemisphere was used for histology analysis.

Histopathological Studies

Brain tissue was fixed in 10% formaldehyde solution, cut in sections, and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin-eosin, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein, via our previously described protocols (Castilla et al., 2005). Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by consideration of both number and size of spongiform degeneration in five different brain areas: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). Each analyzed brain area was scored from 0 to 4 according to the extent of vacuolation in slides stained with hematoxilin-eosin and visualized at a 40× magnification. Samples were analyzed blindly by two different persons, and the scores represent the average of the two determinations.

Statistical Analysis

The differences in incubation periods, histopathological profile of brain damage, and biochemical characteristics of PrP^{Sc} were analyzed by ANOVA, followed by the Dunnett Multiple Comparison post-test to estimate the significance of the differences between the newly generated strains and each of the other hamster and mouse prion strains studied. For these studies, the data were analyzed with the GraphPad Instat, version 3.05 software.

SUPPLEMENTAL DATA

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/5/757/DC1/.

ACKNOWLEDGMENTS

This research was supported in part by National Institutes of Health grants R01NS049173 and P01AI77774 to C.S. C.S. is a founder of Amprion, Inc., a company focused on the development of a diagnosis for prion diseases through the use of the PMCA technology.

Received: September 18, 2007
Revised: May 8, 2008
Accepted: July 21, 2008
Published: September 4, 2008

REFERENCES

Atarashi, R., Moore, R.A., Sim, V.L., Hughson, A.G., Dorward, D.W., Onwubiko, H.A., Priola, S.A., and Caughey, B. (2007). Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat. Methods *4*, 645–650.

Brachmann, A., Baxa, U., and Wickner, R.B. (2005). Prion generation in vitro: amyloid of Ure2p is infectious. EMBO J. *24*, 3082–3092.

Bruce, M.E. (2003). TSE strain variation. Br. Med. Bull. *66*, 99–108.

Buschmann, A., and Groschup, M.H. (2005). TSE eradication in small ruminants–quo vadis? Berl. Munch. Tierarztl. Wochenschr. *118*, 365–371.

Caramelli, M., Ru, G., Acutis, P., and Forloni, G. (2006). Prion diseases: Current understanding of epidemiology and pathogenesis, and therapeutic advances. CNS Drugs *20*, 15–28.

Castilla, J., Saá, P., Hetz, C., and Soto, C. (2005). In vitro generation of infectious scrapie prions. Cell *121*, 195–206.

Castilla, J., Saa, P., Morales, R., Abid, K., Maundrell, K., and Soto, C. (2006). Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Methods Enzymol. *412*, 3–21.

Chen, S.G., Zou, W., Parchi, P., and Gambetti, P. (2000). PrP(Sc) typing by N-terminal sequencing and mass spectrometry. Arch. Virol. Suppl. 209–216.

Collinge, J. (2001). Prion diseases of humans and animals: Their causes and molecular basis. Annu. Rev. Neurosci. *24*, 519–550.

Collinge, J., Sidle, K.C., Meads, J., Ironside, J., and Hill, A.F. (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature *383*, 685–690.

Deleault, N.R., Geoghegan, J.C., Nishina, K., Kascsak, R., Williamson, R.A., and Supattapone, S. (2005). Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. J. Biol. Chem. *280*, 26873–26879.

Deleault, N.R., Harris, B.T., Rees, J.R., and Supattapone, S. (2007). Formation of native prions from minimal components in vitro. Proc. Natl. Acad. Sci. USA *104*, 9741–9746.

Doherr, M.G. (2003). Bovine spongiform encephalopathy (BSE)–infectious, contagious, zoonotic or production disease? Acta Vet. Scand. Suppl. *98*, 33–42.

Foster, J.D., Hope, J., and Fraser, H. (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. Vet. Rec. *133*, 339–341.

Hill, A.F., and Collinge, J. (2004). Prion strains and species barriers. Contrib. Microbiol. *11*, 33–49.

Hunter, N. (2003). Scrapie and experimental BSE in sheep. Br. Med. Bull. *66*, 171–183.

Hunter, N. (1998). Scrapie. Mol. Biotechnol. *9*, 225–234.

Jones, M., Peden, A.H., Prowse, C.V., Groner, A., Manson, J.C., Turner, M.L., Ironside, J.W., MacGregor, I.R., and Head, M.W. (2007). In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrPSc. J. Pathol. *213*, 21–26.

Kimberlin, R.H., and Walker, C.A. (1988). Pathogenesis of experimental scrapie. Ciba Found. Symp. *135*, 37–62.

Kimberlin, R.H., Walker, C.A., and Fraser, H. (1989). The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J. Gen. Virol. *70*, 2017–2025.

King, C.Y., and Diaz-Avalos, R. (2004). Protein-only transmission of three yeast prion strains. Nature *428*, 319–323.

Kurt, T.D., Perrott, M.R., Wilusz, C.J., Wilusz, J., Supattapone, S., Telling, G.C., Zabel, M.D., and Hoover, E.A. (2007). Efficient in vitro amplification of chronic wasting disease PrPRES. J. Virol. *81*, 9605–9608.

Lund, C., Olsen, C.M., Tveit, H., and Tranulis, M.A. (2007). Characterization of the prion protein 3F4 epitope and its use as a molecular tag. J. Neurosci. Methods *165*, 183–190.

Moore, R.A., Vorberg, I., and Priola, S.A. (2005). Species barriers in prion diseases-brief review. Arch. Virol. Suppl. 187–202.

Morales, R., Abid, K., and Soto, C. (2007). The prion strain phenomenon: Molecular basis and unprecedented features. Biochim. Biophys. Acta *1772*, 681–691.

Murayama, Y., Yoshioka, M., Yokoyama, T., Iwamaru, Y., Imamura, M., Masujin, K., Yoshiba, S., and Mohri, S. (2007). Efficient in vitro amplification of a mouse-adapted scrapie prion protein. Neurosci. Lett. *413*, 270–273.

Prusiner, S.B. (1998). Prions. Proc. Natl. Acad. Sci. USA *95*, 13363–13383.

Race, R., Raines, A., Raymond, G.J., Caughey, B., and Chesebro, B. (2001). Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: Analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J. Virol. *75*, 10106–10112.

Race, R., Meade-White, K., Raines, A., Raymond, G.J., Caughey, B., and Chesebro, B. (2002). Subclinical scrapie infection in a resistant species: Persistence, replication, and adaptation of infectivity during four passages. J. Infect. Dis. *186*, S166–S170.

Saa, P., Castilla, J., and Soto, C. (2005). Cyclic amplification of protein misfolding and aggregation. Methods Mol. Biol. *299*, 53–65.

Saborio, G.P., Permanne, B., and Soto, C. (2001). Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature *411*, 810–813.

Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F.E., and Prusiner, S.B. (1998). Eight prion strains have PrP(Sc) molecules with different conformations. Nat. Med. *4*, 1157–1165.

Sarafoff, N.I., Bieschke, J., Giese, A., Weber, P., Bertsch, U., and Kretzschmar, H.A. (2005). Automated PrPres amplification using indirect sonication. J. Biochem. Biophys. Methods *63*, 213–221.

Sigurdson, C.J., and Aguzzi, A. (2006). Chronic wasting disease. Biochim. Biophys. Acta *1772*, 610–618.

Soto, C., and Castilla, J. (2004). The controversial protein-only hypothesis of prion propagation. Nat. Med. *10*, S63–S67.

Soto, C., Anderes, L., Suardi, S., Cardone, F., Castilla, J., Frossard, M.J., Peano, S., Saá, P., Limido, L., Carbonatto, M., et al. (2005). Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. FEBS Lett. *579*, 638–642.

Tanaka, M., Chien, P., Naber, N., Cooke, R., and Weissman, J.S. (2004). Conformational variations in an infectious protein determine prion strain differences. Nature *428*, 323–328.

Tanaka, M., Chien, P., Yonekura, K., and Weissman, J.S. (2005). Mechanism of cross-species prion transmission: An infectious conformation compatible with two highly divergent yeast prion proteins. Cell *121*, 49–62.

Vanik, D.L., Surewicz, K.A., and Surewicz, W.K. (2004). Molecular basis of barriers for interspecies transmissibility of mammalian prions. Mol. Cell *14*, 139–145.

Weber, P., Giese, A., Piening, N., Mitteregger, G., Thomzig, A., Beekes, M., and Kretzschmar, H.A. (2007). Generation of genuine prion infectivity by serial PMCA. Vet. Microbiol. *123*, 346–357.

Wickner, R.B., Masison, D.C., and Edskes, H.K. (1995). [PSI] and [URE3] as yeast prions. Yeast *11*, 1671–1685.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P.G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. Lancet *347*, 921–925.

Williams, E.S. (2005). Chronic wasting disease. Vet. Pathol. *42*, 530–549.

Xie, Z., O'Rourke, K.I., Dong, Z., Jenny, A.L., Langenberg, J.A., Belay, E.D., Schonberger, L.B., Petersen, R.B., Zou, W., Kong, Q., et al. (2005). Chronic wasting disease of elk and deer and Creutzfeldt-Jakob disease: Comparative analysis of the scrapie prion protein. J. Biol. Chem. *281*, 4199–4206.

Apéndice 10

Molecular Cross-talk between Prions and Amyloids in animal models of Alzheimer's and Prion diseases

Rodrigo Morales[1,2,3], Lisbell Estrada[2,3], Rodrigo Diaz-Espinoza[1,2], Joaquin Castilla[2] and Claudio Soto[1,2]*

[1]Protein Misfolding Disorders Laboratory, Department of Neurology, University of Texas Medical School at Houston, Texas.
[2]Protein Misfolding Disorders Laboratory, George and Cynthia Mitchell Center for Neurodegenerative diseases, Department of Neurology, University of Texas Medical Branch, Galveston, Texas.
[3]Facultad de Ciencias, Universidad de Chile, Santiago, Chile

*To whom correspondence should be addressed at Claudio.Soto@uth.tmc.edu.

Abstract

Protein Misfolding Disorders (PMDs) include several diseases such as Alzheimer's (AD), Parkinson's and prion disorders, among others. The central event in these diseases is the accumulation of a misfolded form of a naturally expressed protein. Despite the diversity of clinical symptoms associated to different PMDs, many similarities in their mechanism suggest that distinct pathologies may cross-talk at the molecular level to enhance each other. The main goal of this study was to analyze the interaction on the protein misfolding processes implicated in Alzheimer's and prion diseases. For this purpose we inoculated prions in an AD transgenic mouse model that develop typical amyloid plaques and followed the progression of pathological changes over time. Our findings show a dramatic acceleration and exacerbation of both pathologies. The onset of clinical symptoms of prion disease appeared significantly faster with a concomitant increase on the level of misfolded prion protein in the brain. A striking increase in amyloid plaque deposition was observed in prion infected mice compared with their non-inoculated counterparts. These results suggest a profound interaction between Alzheimer's and prion pathologies, indicating that one protein misfolding process may be an important risk factor for the development of a second one. Our findings may have important implications to understand the origin and progression of PMDs.

Introduction

Misfolding and aggregation of proteins have been described as the central pathogenic event for a group of diseases termed Protein Misfolding Disorders (PMDs) which include pathological conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease, diabetes type-2, systemic amyloidosis and Transmissible Spongiform Encephalopaties (TSEs), among others (*1, 2*). Compelling evidence indicates that misfolding and aggregation of an otherwise normal protein is the triggering event in the pathogenesis, leading to cell dysfunction and death, and consequentially to clinical symptoms in affected individuals (*1, 2*). The main structural changes include an increase of the β-sheet structure and a rearrangement of protein interactions resulting in the formation of fibrillar amyloid-like polymers (*3*). As a result, protein aggregates become resistant to proteolysis, denaturation and cellular clearance mechanisms.

In spite of the important differences in clinical manifestation, PMDs share some common features such as their appearance late in life, the progressive and chronic nature of the disease and the presence of deposits of misfolded protein aggregates (*4*). The misfolding and aggregation mechanisms and its structural intermediates are very similar in all PMDs (*4, 5*). Although for each disease the aggregates are composed by a different protein, they share similar morphological, structural and tinctorial characteristics. Moreover, the mechanism of aggregation in all cases seems to follow a seeding-nucleation process of polymerization (*4*). The critical step in this model is the generation of a misfolded seed that act as a nucleus to catalyze further aggregation, leading to the formation of oligomeric and fibrillar species and, in some cases, amyloid plaques (*4, 5*).

TSEs or prion disorders are the only PMD described as transmissible so far. In spite of their low incidence, TSEs have produce big health and economic problems due to the Bovine Spongiform Encephalopathy (BSE) epidemics and the emergence of the new variant form of Creutzfeldt Jakob disease (vCJD) in humans as a consequence (*6*). Compelling evidence indicates that the infectious agent in TSEs is composed exclusively by the misfolded and aggregated prion protein (termed PrP^{Sc}), which transmit the disease by inducing the auto-catalytic transformation of the normal host prion protein (termed PrPC) (*7, 8*). PrP^{Sc} accumulates in the brain leading to neurodegeneration and disease.

AD is the most common type of dementia in the elderly population and represents one of the largest problems for public health (*9*). This disease is pathologically characterized by the extracellular accumulation of the Aβ peptide and intracellular accumulation of hyperphosphorilated tau protein, both leading to synaptic dysfunction and cell death (*10, 11*). Extensive biochemical, neuropathological and genetic evidences suggest that the cerebral accumulation of misfolded Aβ peptide is the central event in the pathogenesis (*12*). AD is not considered transmissible, but recent work suggest that, under certain experimental conditions, exogenous administration of AD brain homogenates containing aggregated Aβ accelerates disease features in transgenic mice models (*13, 14*).

The co-existence of various misfolded aggregates has been described *in vivo* for several proteins such as α-synuclein, tau, prion protein and Aβ, among others (*15-19*). Specifically, the co-existence of PrP^{Sc} and Aβ in patients with clinical manifestation of both AD and CJD has been previously reported (*20-22*). PrP has been identified in senile plaques of AD patients (*23*) and Aβ aggregates have been found within PrP deposits in patients affected with CJD or Gertsmann-Straussler-Scheinker syndrome (*24, 25*). In

spite the reported co-occurrence of AD and TSEs in patients, it is unclear whether both pathologies interact or are simply co-existing simultaneously. In the present study we aimed to evaluate the interaction between AD and TSEs using mice models for both diseases. For this purpose we analyzed the appearance of prion related clinical features in a transgenic mouse model of AD inoculated with murine prions. We also analyzed the extent of Aβ and PrP^{Sc} deposition, as well as associated cerebral damage in these animals. Our results show a strong interaction between the two pathologies that has substantial implications for disease pathogenesis.

Results

In order to asses the *in vivo* interaction between the pathological processes implicated in AD and TSEs, we inoculated RML prions intra-peritoneally (i.p.) into Tg2576 mice at different stages of AD progression. One group of animals was inoculated at 45 days old when Aβ accumulation is not yet detectable and a second group was inoculated at the age in which amyloid deposition begin in these animals (365 days old). Age-matched WT (non-transgenic littermates) mice were treated in the same way. Evaluation of the onset of prion disease showed that Tg2576 mice develop clinical symptoms significantly faster than WT littermates (Fig. 1). Interestingly, the acceleration of the disease depended on the stage of AD-like pathology, since transgenic mice inoculated at 365 days old showed a substantially shorter incubation period than animals inoculated at 45 days old (Fig. 1). These differences are not purely an effect of the animal age, because WT mice inoculated at 45 or 365 days old did not show any statistically significant difference in the onset of prion symptoms. To assess whether the accelerated disease produced in Tg2576 animals

kept the infectious characteristics, we inoculated WT mice with brain homogenate of sick animals from the group of Tg2576 injected at 365 days old. The results of this second passage showed an average incubation period of 202.4 days, characteristic of RML prions inoculated i.p. and similar to the incubation time observed in our first passage (Fig. 1). Additional biochemical (glycosylation profile and electrophoretical mobility of PrP^{Sc}) and pathological (spongiform degeneration and astroglyosis) studies further showed that the RML-Tg2576 properties were the same as expected for RML prions (supplementary figure 1). These results indicate that the strain characteristics of RML prions are likely maintained after passage in AD transgenic mice.

Histopathological analyses of brains from Tg2576 prion infected mice showed the co-existence of both TSE and AD pathologies. The brain exhibited extensive spongiform degeneration (Fig. 2A), reactive astrogliosis (Fig. 2B), Aβ deposition (Fig. 2C and 2D) and PrP^{Sc} accumulation (see Fig. 5). Conversely, Aβ deposits were not detected in the brains of WT mice inoculated with RML prions and no vacuolation or PrP^{Sc} accumulation was seen in old non-infected Tg2576 mice (Fig. 2).

The degree of spongiform degeneration in animals with the double pathology did not differ from those affected only by TSE (Fig 2A). The extent of vacuolation in diverse areas of the brain is widely used to characterize TSEs. Indeed, different prion strains often produce a distinct pattern of spongiform degeneration (*26*). Evaluation of the lesion profile in different brain areas of Tg2576 mice infected with RML showed a similar pattern in all inoculated groups (Fig. 3). Vacuolation profiles were not statistically significantly different between Tg2576 and WT prion infected mice, indicating that spongiform degeneration did not change due to the presence of Aβ deposits.

Conversely, it is clear that both brain inflammation (Fig. 2B) and Aβ deposition (Fig. 2C and 2D) were substantially higher on animals bearing the double pathology. The increase on brain inflammation may be an additive result, since both pathologies are associated with astrogliosis (*27*) (Fig. 2B). More remarkably is the dramatic increase on Aβ deposition observed in the Tg2576 inoculated mice compared with non-infected animals. Indeed, some of the Tg2576 mice (2 out of 8) inoculated at 45 days old and sacrificed when prion disease was evident (around 185 days later), showed Aβ amyloid deposits at an age (~230 days) when these animals never show amyloid lesions (Fig. 2C). These deposits were recognized with anti-Aβ specific antibodies in both hippocampus and cortex (Fig. 2C) and were stained by Thioflavin S (Supplementary Fig 2). Moreover, the size, number and maturity of Aβ plaques in the Tg2576 group inoculated at 365 days old was dramatically higher than in the age-matched control inoculated with PBS (Fig. 2C). To quantitatively analyze the extent of Aβ aggregation in RML infected Tg2576 mice, we determined both the percentage of brain area covered by thioflavin S positive Aβ aggregates and the number of plaques between 365 days old RML infected and age-matched non-infected animals. The results showed that Aβ plaque area as well as the number of plaques was significantly higher in Tg2576 mice infected with prions (Fig. 4). These data strongly supports an interaction between the prion and AD pathologies, leading to a dramatic increase on the misfolding, aggregation and cerebral accumulation of Aβ.

To evaluate whether PrP^{Sc} accumulation was also increased in the presence of AD pathology, we measured the quantity of PrP^{Sc} in the brain by Western blot analysis (Fig. 5). Infected Tg2576 mice were sacrificed when clinical signs of scrapie were confirmed,

in average 186 and 165 days post-inoculation (d.p.i.) in animals injected at 45 and 365 days old, respectively. As shown in figure 5A, the quantity of PrP^{Sc} in the brain was high and similar in these two groups (one representative animal is shown in each group). No PrP^{Sc} was detected in AD transgenic mice non-infected with RML prions. As comparison we analyzed by Western blot the levels of PrP^{Sc} in 365 days old RML inoculated WT mice at different time points after i.p. inoculation (Fig. 5B and supplementary table 1). In animals sacrificed at 140, 153 and 161 d.p.i. no PrP^{Sc} was detected even in highly concentrated samples. In contrast, at these times most of the Tg2576 mice inoculated at 365 days old showed clear clinical signs and strong deposition of PrP^{Sc} in their brains (Fig 1 and Fig 5A). At 169 d.p.i. only one out of three WT mice showed a very faint PrP^{Sc} signal (in absence of clinical signs) (Fig. 5B). The quantity of PrP^{Sc} in the brain of infected WT mice became higher and similar to the one in Tg2576 groups after around 225 post-inoculation (Fig. 5B). These results indicate that PrP^{Sc} formation and accumulation in the brain is accelerated in mice simultaneously affected by AD brain pathology.

One putative explanation for the acceleration of AD and TSE pathologies in animals affected by both diseases could be a direct interaction between Aβ and PrP misfolded proteins leading to speeding up the process of misfolding and aggregation. This is possible since both proteins aggregate by a seeding-nucleation mechanism and it has been described that seeds composed of one protein can cross-seed a second misfolding process (*28, 29*). To test this hypothesis, we evaluated the seeding capability of purified RML PrP^{Sc} in the aggregation of synthetic Aβ (Fig. 6A). The addition of various small quantities of prion seeds produced a clear and dramatic acceleration of Aβ aggregation,

measured as a shortening of the lag phase for polymerization. The acceleration of Aβ aggregation was directly proportional to the quantity of PrP^{Sc} seeds added to the sample (Fig. 6A). This data shows a direct interaction between infectious PrP^{Sc} and Aβ *in vitro*. These results may explain in part the large increase of Aβ deposition observed in animals inoculated with murine prions. In order to evaluate whether Aβ aggregates alter PrP misfolding, we studied the aggregation of recombinant prion protein (recPrP) in the presence of different quantities of Aβ fibrils. The formation of misfolded recPrP was studied by the standard proteinase K degradation assay followed by western blot. In the absence of Aβ fibrils recPrP does not acquire PK resistance under the conditions tested (Fig. 6B). However in the presence of various quantities of Aβ fibrils, a prominent PK-resistant band was observed with an apparent molecular weight of around 16KDa. The switch on the molecular weight is indicative of *bona-fide* PrP conversion and is similar to the size expected for the unglycosylated PK resistant PrP^{Sc} core. We are currently testing whether misfolded recPrP produced upon incubation with Aβ fibrils is infectious to animals. Interestingly, the extent of PK-resistant recPrP was directly proportional to the quantity of Aβ fibrils added to the reaction (Fig. 6B). This data provides further evidence for a functional interaction between Aβ and PrP resulting in mutual acceleration of protein misfolding and aggregation by cross-seeding.

Discussion

PMDs include more than 25 clinically diverse human disorders (*1, 2*). Despite the obvious differences among these diseases, the molecular mechanism triggering the pathology appears to be the same: the misfolding, aggregation and tissue accumulation of

a protein (*1, 2*). Although the protein involved in each disease is different, the structure of the misfolded aggregates as well as the mechanism and intermediates in the process are similar (*4, 5*). Indeed, a typical signature of most PMDs is the accumulation of amyloid-like fibrils, folded in a β-cross conformation (*30*). To reach this stage, a series of misfolding and protein-protein interaction events occur to form oligomers and protofibrils in a process of seeded/nucleated polymerization (*4*). The cellular consequences of the accumulation of misfolded aggregates in distinct diseases are also similar and include extensive tissue inflammation, cellular stress and activation of the unfolded-protein response (UPR) (*31*). The permanent accumulation of aggregates as well as the failure of the clearance system to eliminate them, leads to chronic endoplasmic reticulum stress, saturation of the proteasome system and activation of apoptotic pathways, resulting in cellular dysfunction and death (*32, 33*).

The mechanistic and pathological similarities among these diseases suggest that protein misfolding processes occurring simultaneously may synergistically interact among each other leading to an acceleration of the disease. Indeed, it has been reported extensively the co-existence of two PMDs in a single patient, including cases of AD, PD, TSEs, ALS, diabetes type 2, systemic amyloidosis, etc (*15-19, 34, 35*). Moreover, some PMDs involve the presence of more than one type of misfolded aggregated protein; the archetype case being AD, where intracellular neurofibrillary tangles (NFT) composed of hyperphorylated Tau are present simultaneously with extracellular Aβ amyloid plaques (*11*). Although it is possible that NFT and senile plaques are formed independently, a recent study showed that Aβ and tau form soluble complexes that may promote their self-aggregation into the insoluble forms observed in AD (*36*).

Our findings suggest that AD and TSE pathologies synergistically interact to accelerate the onset of both diseases. Indeed, scrapie clinical signs appear much faster after infection of animals that are simultaneously accumulating Aβ aggregates. The rate of acceleration depended on the stage of AD and correlated with a rapid accumulation of PrP^{Sc}. On the other hand, AD transgenic mice infected with prions developed a strikingly higher load of cerebral amyloid plaques that appeared much faster than in non-infected mice. There are at least three possible explanations for the synergistic effects: 1) The clearance mechanisms already impaired by one misfolded protein may be further diminished by a second misfolding event, leading to the faster and higher accumulation of both type of aggregates and subsequent brain damage. 2) Nerve cells stressed and injured by exposure to one toxic misfolded aggregated protein are more easily damaged by an additional toxic aggregate, leading to an accelerated and greater rate of cellular dysfunction and death. 3) A direct interaction between misfolded proteins may result in acceleration of protein misfolding and aggregation through a cross-seeding mechanism, leading to higher and faster accumulation of toxic aggregates. The complexity of the signaling events in the brain and the dynamic occurrence of all these processes simultaneously makes difficult to distinguish among these hypotheses and increase the likelihood that more than one of these possibilities is contributing to explain the results. Our *in vitro* data showing that purified PrP^{Sc} can substantially accelerate the misfolding and aggregation of synthetic Aβ *in vitro* and that preformed Aβ fibrils can induce the formation of misfolded protease-resistance PrP^{Sc}-like provide support for the possibility of a direct interaction between the proteins leading to cross-seeding and increased pathogenesis. Several studies have demonstrated the cross-seeding of misfolded

aggregates both *in vitro* and *in vivo* (*29, 37*). These data added to the now well-accepted idea that seeding is the general mechanism by which these proteins aggregate and the basis for disease propagation in TSEs (*4*), determine that cross-seeding between diverse misfolded proteins is a feasible mechanism.

Regardless of which is the molecular mechanism explaining the acceleration and exacerbation of AD and TSEs pathologies in animals affected by both diseases, our results suggest that one PMD is a significant risk factor for the emergence of a second disease. Whether this conclusion can be extrapolated to the diseases in humans will require additional epidemiological studies. In this sense, it is important to highlight that it has already been shown that the two most prevalent PMDs (AD and diabetes type 2) are a risk factor for each other (*38, 39*). In the case of AD and TSEs, it would be important to study whether the rare, but infectious prion diseases may contribute to increase the risk for AD in patients infected with prions. Since human TSEs have a very long incubation period, which can span several decades (*40*), it would be possible that these patients develop AD or other PMDs before showing the symptoms of prion disease, contributing to explain the low incidence of TSE. Therefore, our findings may have important implications to understand the origin and mechanism of progression of these devastating brain diseases.

Figure Legends

Figure 1: Alzheimer's pathology accelerates prion disease in mice models. A: To assess the effect of AD neuropathology in the onset of prion disease we inoculated i.p. Tg2576 mice with RML prions at 365 (orange) and 45 (green) days old. As control, age matched WT mice (non-transgenic littermates) were inoculated with the same stock and quantity of the infectious agent (black). WT animals infected at 45 or 365 days old were very similar and thus are presented together in the graph. Clinical signs were assessed as described in Methods. When animals were definitively diagnosed with prion disease they were sacrificed to avoid further pain. The data show that both groups of Tg2576 inoculated mice are more susceptible to prion infectivity compared to age matched WT controls. In addition, we performed a second infectivity passage in WT mice by inoculating infectious material from the brain of a sick Tg2576 animal injected at 365 days old (blue line). **B**: Average of incubation periods of the different groups showed in panel A, including the statistical comparison between each experimental group with the WT control mice, done using the student-t test.

Figure 2: Brain histopathological studies. Representative animals from different groups were studied histopathologically for spongiform brain degeneration after hematoxilin-eosin staining (**A**), reactive astroglyosis by GFAP staining (**B**) and Aβ deposition by immunohistochemistry using the 4G8 anti-Aβ antibody (**C**) and staining with the amyloid specific die thioflavin S (**D**). The pictures in panel A and B correspond to the medulla and panel D to the cortex.

Figure 3. Profile of vacuolation in different brain areas of Tg2576 and WT mice inoculated with RML prions. Brains from RML clinically sick mice were analyzed histologically for spongiform degeneration after hematoxilin-eosin staining. Nine different brain areas were selected for quantitative analysis of vacuolation, as described in Methods. The values represent the average ± standard error of the extent of vacuolation from 5 animals analyzed in each group. Statistical analysis by two-ways ANOVA (using animal group and brain areas as the variables) indicated that the differences were not significant in any of the groups studied. The brain areas analyzed were: 1) cerebellar white matter; 2) cerebellar grey matter; 3) medulla; 4) superior culliculus; 5) hypocamppus; 6) hypothalamus; 7) thalamus; 8) occipital cortex; 9) M1 cortex.

Figure 4: Burden of Aβ deposits in Tg2576 mice inoculated or non-inoculated with RML prions. Quantitative analysis of Thioflavin S reactive-plaques in several animals of the group of Tg2576 mice inoculated with RML prions or PBS at 365 days old, was studied by image analysis using the Metamorph software. **A**: The area of the brain covered by thioflavin S positive plaques was measured in cerebral cortex and expressed as a percentage. **B**: The number of Thioflavin S reactive plaques was counted in the cortex. Each bar represents the average ± standard error of 5 animals studied. Statistical analysis was done by student-t test ** $p<0.01$. As shown in figure 2, WT animals inoculated or not with RML prions did not show any Thioflavin S signal.

Figure 5: PrP^{Sc} levels in Tg2576 or WT mice inoculated with RML prions. A: Brain homogenates from clinically sick Tg2576 mice inoculated with prions at 45 or 365 days old or as a control Tg2576 mice non-inoculated with prions (right panel) were PK digested and Western blotted in order to analyze PrP^{Sc} burden. The result shown corresponds to an animal representative of all mice in the group. The animal of the 45 days group shown in the figure was sacrificed at 190 days post-inoculation (dpi) and the animal of the 365 days at 159 dpi. **B**: For comparison, we measured the PrP^{Sc} levels in the brain of WT mice challenged with RML prions and sacrificed at 140, 169, 193 and 225 dpi. Only the animal at 225 dpi was exhibiting signs of prion disease. Numbers at the top of each gel represent brain dilution. Brain dilutions were performed from a 10% brain homogenates and various dilutions are shown to facilitate the comparisons.

Figure 6: Cross-seeding of PrP and Aβ misfolding and aggregation *in vitro*. A: The effect of purified PrP^{Sc} on Aβ aggregation was measured overtime by sedimentation followed by sensitive ELISA. Seed-free soluble Aβ1-42 (0.01 mg/ml) was incubated with different concentrations of purified PrP^{Sc} seeds or PBS (control). The concentration of PrP^{Sc} is expressed as a percentage of oligomers per Aβ monomer and was calculated assuming that a PrP^{Sc} oligomer has an average 7,700 KDa. The latter was based on data coming from flow field fractionation of PrP^{Sc} and corresponds to the fraction with the highest concentration of PrP^{Sc} (*41*). Samples were incubated at 25°C with shaking for the indicated times. Thereafter soluble and aggregated Aβ were separated by centrifugation and the quantity of peptide in the supernatant was measured by ELISA. Experiment was done by triplicate and results represent the average and standard error. Analysis by two-

ways ANOVA (using condition and times as the variables) show that the kinetic of aggregation in the experiments containing PrP^{Sc} is highly significantly different from the control (P<0.0001). **B**: The effect of Aβ aggregates on PrP misfolding was studied by incubating 10 ug of recombinant mouse PrP in the presence of increasing concentrations of preformed Aβ fibrils. Fibrils were prepared as indicated in Methods and aliquots corresponding to 0.14% (1.2 ug of total Aβ), 0.28% (2.4 ug of total Aβ), 0.56% (4.8 ug of total Aβ) and 1.1% (9.6 ug of total Aβ) were added to monomeric recPrP (lanes 1, 2, 3, 4 and 5, respectively). The concentration of Aβ fibrils is expressed as a molar percentage per recPrP monomer and was calculated assuming that the average molecular weight of Aβ fibrils is 2,000 KDa, as estimated by a combination of size-exclusion chromatography, atomic force microscopy and electron microscopy (*42*). The mixture was incubated for 30 hrs at 37°C in an Eppendorf® Thermomixer with cycles of 1 minute agitation at 1500 rpm and 1 minute incubation. To assess PrP misfolding, samples were incubated at 37°C with 7 ug/mL of PK and PrPres signal analyzed by western blot. Lane 6 contains the molecular weight standard; lane 7 is the control with the same quantity of recPrP incubated in the absence of Aβ fibrils. Lane 8 corresponds to recPrP non-treated with PK to display the migration of the full length protein.

References and Notes

1. C. Soto, *FEBS Lett.* **498**, 204 (2001).
2. F. Chiti, C. M. Dobson, *Annu. Rev. Biochem.* **75**, 333 (2006).
3. R. Nelson, D. Eisenberg, *Adv. Protein Chem.* **73**, 235 (2006).
4. C. Soto, L. Estrada, J. Castilla, *Trends Biochem. Sci.* **31**, 150 (2006).
5. C. G. Glabe, *Neurobiol. Aging* **27**, 570 (2006).
6. R. Bradley, P. P. Liberski, *Folia Neuropathol.* **42 Suppl A**, 55 (2004).
7. S. B. Prusiner, *Cell* **116**, S109, 1 (2004).
8. C. Soto, J. Castilla, *Nat. Med.* **10**, S63 (2004).
9. Z. S. Khachaturian, *J. Alzheimers. Dis.* **9**, 409 (2006).
10. J. L. Cummings, *N. Engl. J. Med.* **351**, 56 (2004).
11. D. W. Dickson, *Clin. Geriatr. Med.* **17**, 209 (2001).
12. D. J. Selkoe, *Ann. N. Y. Acad. Sci.* **924**, 17 (2000).
13. M. D. Kane *et al.*, *J. Neurosci.* **20**, 3606 (2000).
14. M. Meyer-Luehmann *et al.*, *Science* **313**, 1781 (2006).
15. B. I. Giasson, V. M. Lee, J. Q. Trojanowski, *Neuromolecular. Med.* **4**, 49 (2003).
16. R. J. Moss, A. R. Mastri, L. J. Schut, *J. Am. Geriatr. Soc.* **36**, 237 (1988).
17. D. F. Brown *et al.*, *J. Neuropathol. Exp. Neurol.* **57**, 39 (1998).
18. A. H. Rajput, B. Rozdilsky, A. Rajput, *J. Geriatr. Psychiatry Neurol.* **6**, 170 (1993).
19. P. Brown *et al.*, *Neurology* **40**, 226 (1990).
20. K. Tsuchiya *et al.*, *Neuropathology.* **24**, 46 (2004).
21. S. Lin, J. Zhao, X. Jiang, M. Yao, X. Song, *Chin Med. J. (Engl.)* **111**, 756 (1998).
22. T. Muramoto, T. Kitamoto, H. Koga, J. Tateishi, *Acta Neuropathol. (Berl)* **84**, 686 (1992).

23. T. Voigtlander *et al.*, *Acta Neuropathol. (Berl)* **101**, 417 (2001).

24. J. A. Hainfellner *et al.*, *Acta Neuropathol. (Berl)* **96**, 116 (1998).

25. O. Bugiani *et al.*, *J. Neuropathol. Exp. Neurol.* **52**, 64 (1993).

26. R. Morales, K. Abid, C. Soto, *Biochimica et Biophysica Acta* **1772**, 681 (2007).

27. J. Diedrich *et al.*, *Microb. Pathog.* **2**, 435 (1987).

28. J. D. Harper, P. T. Lansbury, Jr., *Annu. Rev. Biochem.* **66**, 385 (1997).

29. B. O'Nuallain, A. D. Williams, P. Westermark, R. Wetzel, *J. Biol. Chem.* **279**, 17490 (2004).

30. M. Sunde *et al.*, *J. Mol. Biol.* **273**, 729 (1997).

31. D. T. Rutkowski, R. J. Kaufman, *Trends in Cell Biology* **14**, 20 (2004).

32. R. I. Morimoto, *Genes Dev.* **22**, 1427 (2008).

33. R. R. Kopito, *Trends Cell Biol.* **10**, 524 (2000).

34. A. Popescu, C. F. Lippa, V. M. Lee, J. Q. Trojanowski, *Arch. Neurol.* **61**, 1915 (2004).

35. J. Fernandez-Alonso, C. Rios-Camacho, A. Valenzuela-Castano, W. Hernanz-Mediano, *J. Clin. Pathol.* **47**, 560 (1994).

36. J. P. Guo, T. Arai, J. Miklossy, P. L. McGeer, *Proc. Natl. Acad. Sci. U. S. A* **103**, 1953 (2006).

37. K. Johan *et al.*, *Proc. Natl. Acad. Sci. U. S. A* **95**, 2558 (1998).

38. Z. Arvanitakis, R. S. Wilson, J. L. Bienias, D. A. Evans, D. A. Bennett, *Arch. Neurol.* **61**, 661 (2004).

39. J. Janson *et al.*, *Diabetes* **53**, 474 (2004).

40. J. Collinge *et al.*, *Lancet* **367**, 2068 (2006).

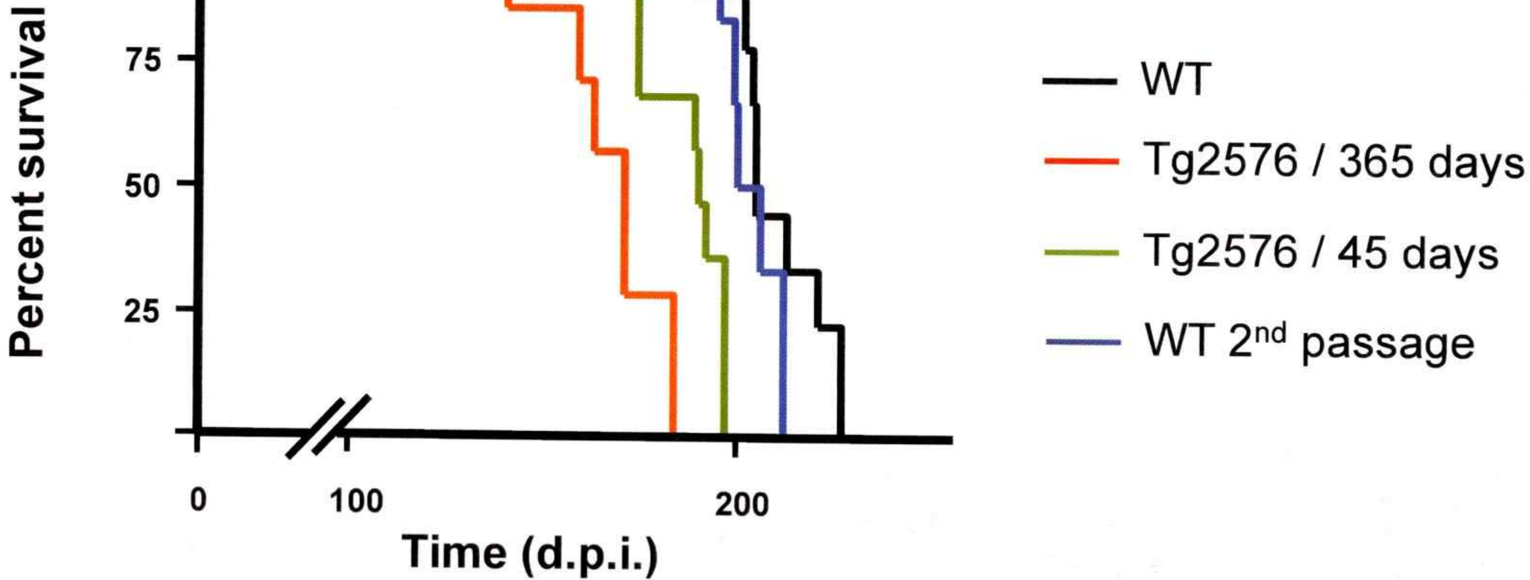
41. J. R. Silveira *et al.*, *Nature* **437**, 257 (2005).

42. C. S. Goldsbury *et al.*, *J. Struct. Biol.* **130**, 217 (2000).

43. These studies were supported in part by NIH grant AG028821, NS050349 and a Project from the Mitchell Foundation to CS.

Figure 1

A



B

Groups	Incubation period (Average ± st. error)	Number of animals	P
WT	208,4 ± 4,1	8	--
Tg2576/45 days	185,8 ± 3,7	8	0.0011
Tg2576/365 days	164,7 ± 6,0	6	<0.0001
2nd passage in WT	202,4 ± 3,0	5	ns

Figure 2

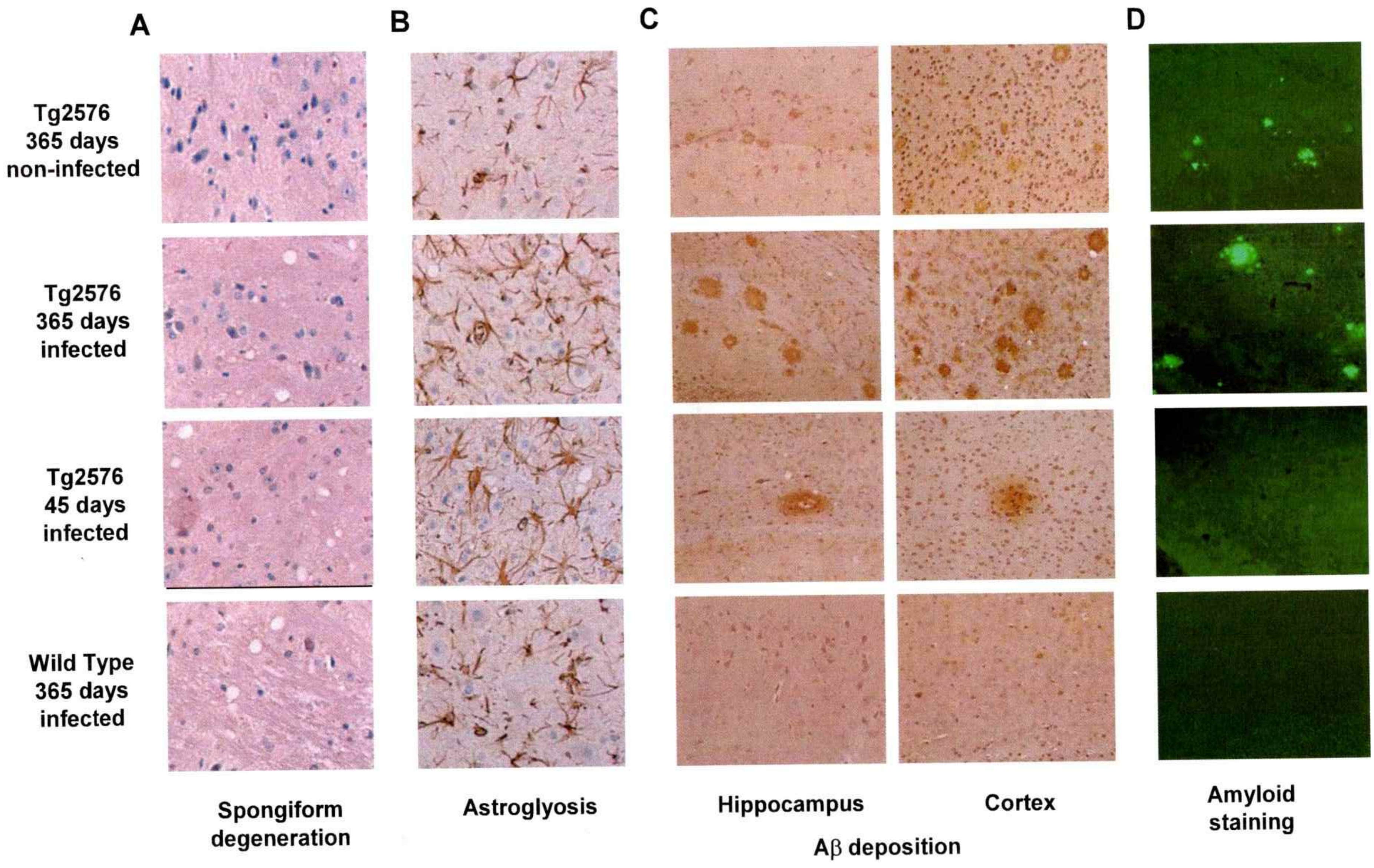


Figure 3

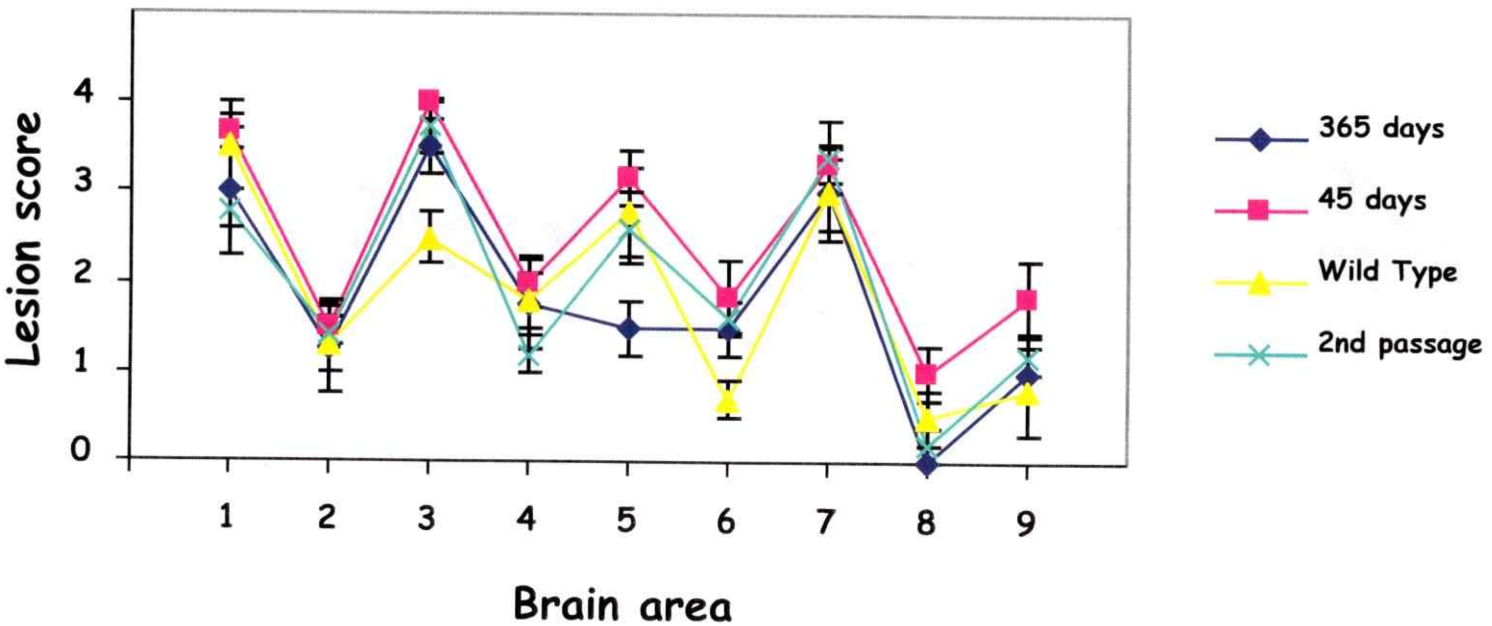


Figure 4

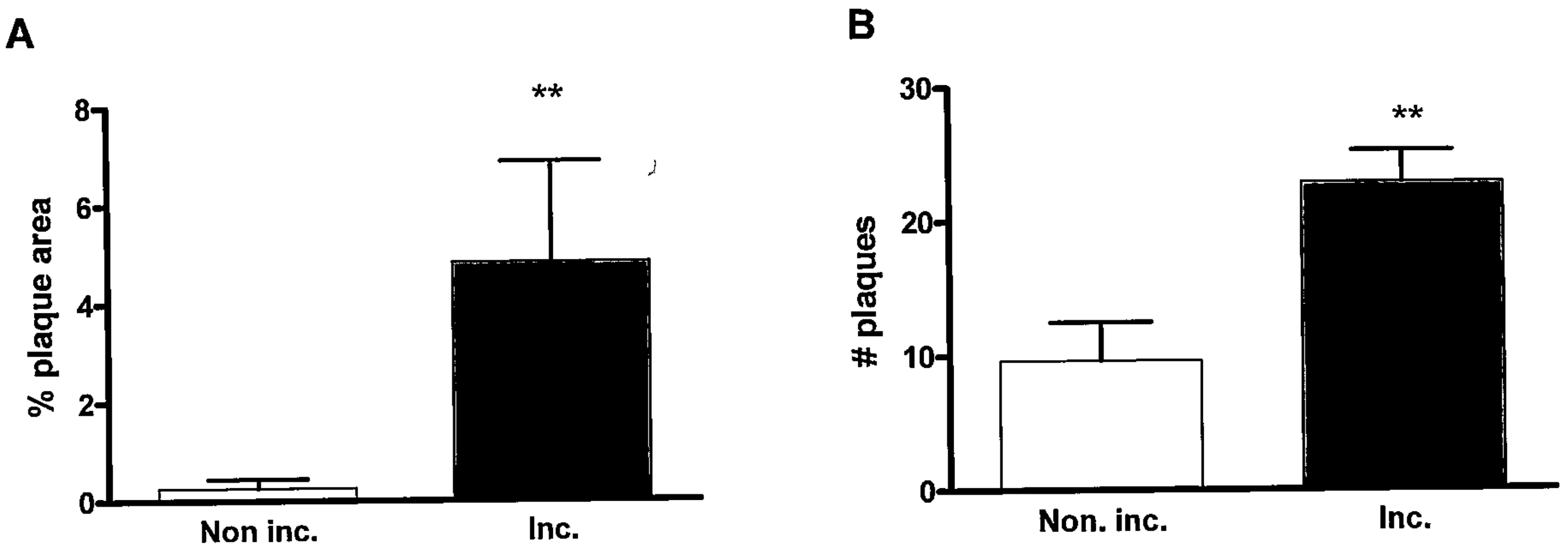


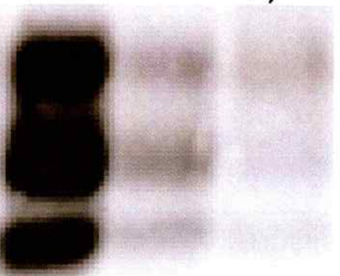
Figure 5

A

Brain dilution

Tg2576
45-days old

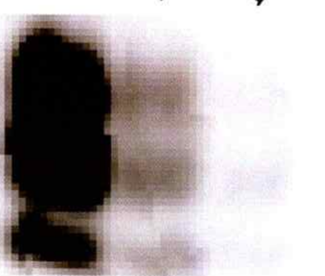
1/50 1/250 1/1250



190dpi

Tg2576
365-days old

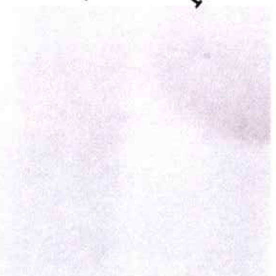
1/50 1/250 1/1250



159dpi

Tg2576
no PrPSc

1/50 1/250



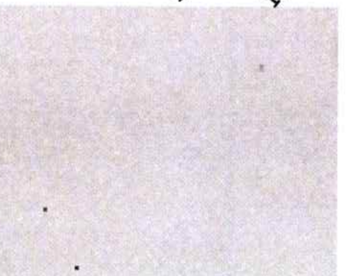
No clinical signs.
Sacrificed at 580 days old

B

Brain dilution

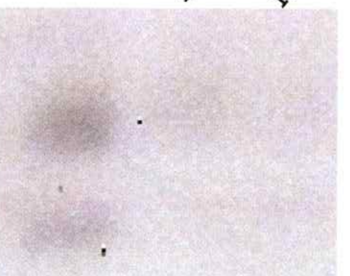
Wild Type
140-d.p.i.

1/50 1/250 1/1250



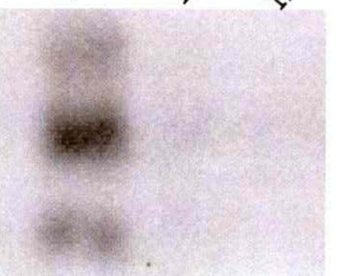
Wild Type
169-d.p.i.

1/50 1/250 1/1250



Wild Type
193-d.p.i.

1/50 1/250 1/1250

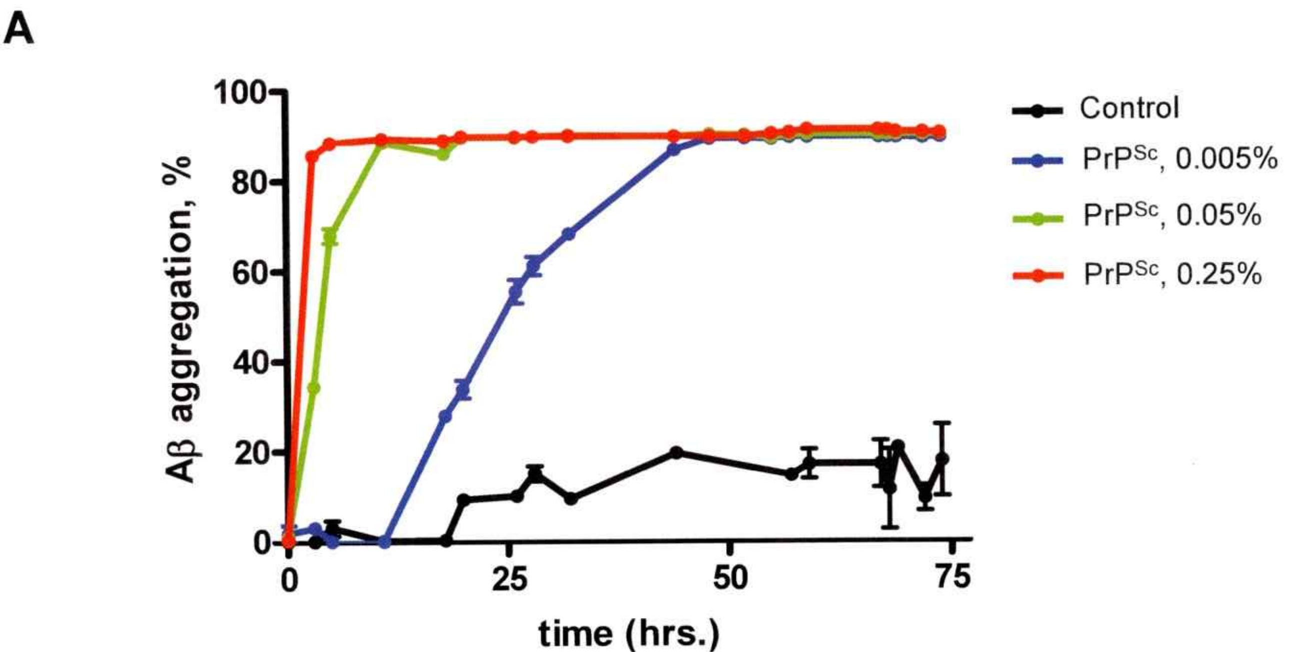


Wild Type
225-d.p.i.

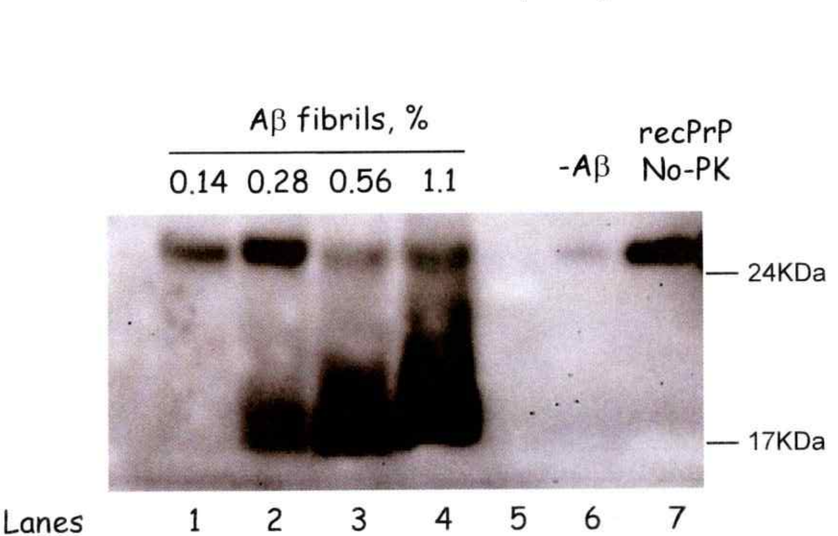
1/50 1/250 1/1250

Figure 6

A

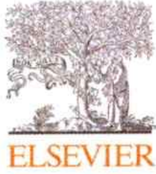


B



Apéndice 11

Biochemical and Biophysical Research Communications 377 (2008) 373–378



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reduction of prion infectivity in packed red blood cells

Rodrigo Morales [a,b], Kimberley A. Buytaert-Hoefen [c], Dennisse Gonzalez-Romero [a], Joaquin Castilla [a], Eric T. Hansen [c], Dennis Hlavinka [c], Raymond P. Goodrich [c], Claudio Soto [a,*]

[a] Department of Neurology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555, USA
[b] Facultad de Ciencias, Universidad de Chile, Santiago, Chile
[c] CaridianBCT Biotechnologies, L.L.C., Lakewood, CO, USA

ARTICLE INFO

Article history:
Received 25 September 2008
Available online 11 October 2008

Keywords:
Prions
Blood transfusion
Blood safety
Diagnosis
Prion diseases
Transmissible spongiform encephalopathy

ABSTRACT

The link between a new variant form of Creutzfeldt–Jakob disease (vCJD) and the consumption of prion contaminated cattle meat as well as recent findings showing that vCJD can be transmitted by blood transfusion have raised public health concerns. Currently, a reliable test to identify prions in blood samples is not available. The purpose of this study was to evaluate the possibility to remove scrapie prion protein (PrP^{Sc}) and infectivity from red blood cell (RBC) suspensions by a simple washing procedure using a cell separation and washing device. The extent of prion removal was assessed by Western blot, PMCA and infectivity bioassays. Our results revealed a substantial removal of infectious prions ($\geqslant$3 logs of infectivity) by all techniques used. These data suggest that a significant amount of infectivity present in RBC preparations can be removed by a simple washing procedure. This technology may lead to increased safety of blood products and reduce the risk of further propagation of prion diseases.

© 2008 Elsevier Inc. All rights reserved.

Transmissible spongiform encephalopaties (TSEs), also known as prion diseases, are a group of fatal neurodegenerative disorders affecting several mammalian species [1]. The main features of these diseases include the spongiform degeneration of the brain and the accumulation of an abnormal isoform of the prion protein, termed PrP^{Sc} [2]. PrP^{Sc} appear to be the main (or sole) component of the infectious agent [2,3]. Although prion diseases are rare in humans, the established link between the new variant form of Creutzfeldt–Jakob disease (vCJD) and the consumption of cattle meat contaminated by bovine spongiform encephalopathy (BSE) have raised concern about a possible outbreak of a large epidemic in the human population. Over the past few years, BSE has become a significant health and economic problem affecting many countries, principally in Europe. In addition to BSE epidemics, the incidence of other prionopaties in economically relevant species such as sheep, goats, and cervids presents an alarming scenario to the animal farming industry. Moreover, the possible spread of BSE in other species (i.e., sheep, goats, and porcine) has raised a concern about the emergence of multiple new sources of prions that may possibly affect human population [4].

The identification of PrP^{Sc} and infectivity in blood opens a new source of public health concern [5–9]. This alarming information urge the development of methodologies able to remove prions from organs and fluids designated for transplant or transfusion. Exacerbating this state of affairs is the lack of a reliable test to identify individuals incubating the disease during the long and silent period from the infection to the appearance of clinical symptoms [10]. Recent studies had demonstrated that vCJD can be iatrogenically transmitted from human to human by blood transfusion [11,12]. Transmission of prion disease through blood transfusion has also been described in sheep and experimental rodents [8,9]. Since blood used for transfusion was taken from animals and humans months or years before the onset of clinical disease, and because millions of people have been exposed to BSE infected material, there is a large concern that a portion of donated blood units might be contaminated with prions. The lack of routine methodologies approved to detect infectious prions in blood creates a need for prion elimination devices, which must be suitable for high throughput and effectiveness without affecting the quality of blood components.

The dynamic and distribution of PrP^{Sc} and infectivity in different blood fractions is unknown, but recent studies suggest that at pre-symptomatic stages of the disease PrP^{Sc} may be mostly attached to white blood cells (WBCs) originating from early peripheral prion replication in lymphoid tissues [6]. Later in the disease, a substantial amount of PrP^{Sc} is present in plasma and red blood cells (RBCs) where it seems to be mostly not cell associated and coming from brain leakage [6,13] (Marcelo Barria and CS, unpublished data). These findings suggest that there are two pools of infectious PrP^{Sc} in blood; one that is cell associated that can be mostly eliminated by efficient leukoreduction and a second pool freely circulating in plasma and contaminating RBC preparations. Indeed, it was reported that leucofiltration removed 42% of the

* Corresponding author. Fax: +1 409 7470020.
E-mail address: clsoto@utmb.edu (C. Soto).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.09.141

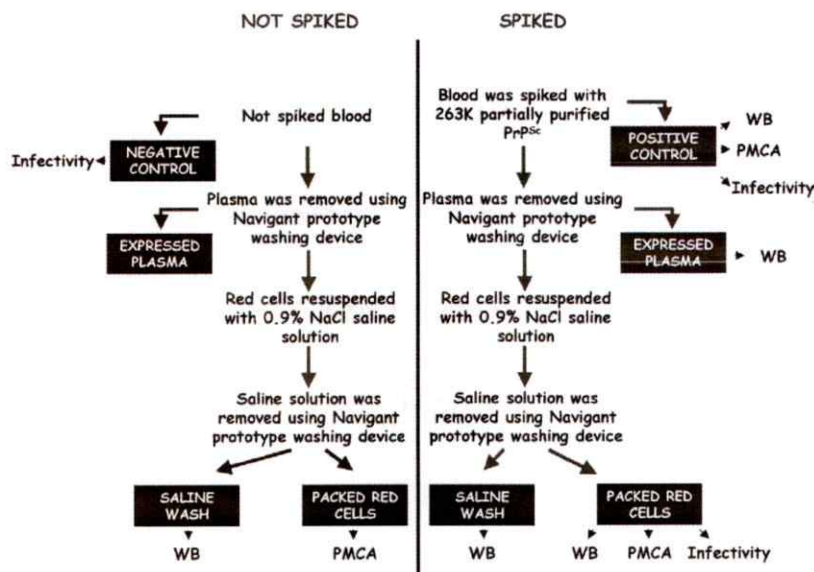


Fig. 1. Schematic representation of the RBC washing procedure. Different units of human whole blood were spiked with partially purified 263K PrP^{Sc} and samples were treated using the prototype device to separate and wash RBCs. From this purification protocol complete spiked blood (positive control), expressed plasma, saline wash, and pRBCs suspensions were obtained. PrP^{Sc} levels in each fraction were evaluated by Western blot, PMCA, and/or infectivity studies. Determinations were done by triplicate in independent experiments.

total TSE infectivity in endogenously infected blood [14]. Leucoreduction is efficient for the removal of WBC-associated TSE infectivity from blood; however, it is not, by itself, sufficient to remove all blood-borne TSE infectivity [14].

Every year, about 75 million units of blood are collected worldwide [15]. RBC transfusion is one of the treatments most extensively used in clinical practice. To reduce the risk for infection, RBCs can be filtered, washed, frozen, or irradiated for specific indications [15]. It has been suggested that PrP^{Sc} in RBCs can be depleted with specific adsorptive ligand resins. Indeed, a recent study reported that PrP-binding resins were able to reduce infectivity titer by 3 to more than 4 ID_{50} units [16]. We hypothesize that PrP^{Sc} in RBCs preparations can be removed by a simple washing of this fraction, since the infectious agent appears not to be tightly bound to RBCs. Various procedures to wash RBC preparations are currently being used to purify the cells before use for blood transfusion [15]. In order to assess PrP^{Sc} removal from the final pRBC fraction we used a hamster experimental model of prion diseases. We assessed the removal of prions by infectivity bioassays as well as by studying the levels of PrP^{Sc} using Western blot and protein misfolding cyclic amplification (PMCA) technology.

Materials and methods

Preparation of PrPSc samples. Symptomatic 263K infected hamsters were sacrificed by CO_2 inhalation and brains were collected. Ten percent brain homogenates (w/v) were prepared in phosphate-buffered saline (PBS) plus Complete™ cocktail of protease inhibitors (PI) (Boehringer Mannheim, Mannheim, Germany). The samples were clarified by a brief, low-speed centrifugation (1500 rpm for 30 s). Brain homogenates were mixed with 1 volume of 20% sarkosyl and the mixture was homogenized and sonicated using a Bandelin Sonoplus sonicator. This sample was centrifuged at 100,000g for 1 h at 4 °C. Supernatant was discarded and 2 volumes of PBS plus PI were added to pellets. Ultracentrifugation step was repeated. Supernatants were discarded and pellets were resuspended in 1 volume of PBS by pipetting and sonication. The sample was stored at −80 °C until use.

Treatment of spiked blood samples with the prototype cell separation and washing device. The cell separation and washing device was capable of processing two partial units of whole blood at one time. Three units of whole blood from Interstate Blood Bank (in Tennessee) were used in this study. Each unit was leukoreduced using a Pall™ Leukotrap Filter. Two hundred and thirty-five milliliters of each unit was transferred into a centrifugation bag. Five milliliters of the remaining whole blood from each unit was transferred into a 15 mL conical tube to serve as a negative control. Each of the three centrifugation bags was spiked with 5×10^6 LD_{50}/mL of PrP^{Sc}. Five milliliters of the mixture were removed from each bag and transferred to a 15 mL conical tube to serve as a positive control. Two units of prion spiked whole blood were loaded into the centrifuge (Fig. 1). Plasma was expressed by centrifugation of the whole blood for approximately 15 min at 3000 rpm bringing the pRBC hematocrit to ⩾95%. Each pRBC unit was washed with 120 mL of normal saline solution. Saline was expressed by centrifugation of the RBCs for approximately 15 min at 3000 rpm bringing the pRBC hematocrit to ⩾95%. Five milliliters of pRBCs final fraction were removed from each bag and transferred to a 15 mL conical tube to serve as a post-treatment sample (Fig. 1). The entire process can be performed in approximately 30 min.

Analysis of the quality of packed red blood cells. To assess RBC quality after washing, whole blood was washed as previously described without a PrP^{Sc} spike. After, the saline was expressed and discarded, and a full volume bag of AS-3 (GNI AS-3 Storage Solution, Larne, UK), approximately 107 mL, was added for storage at 4 °C. Stored RBCs were monitored on days 35 and 42 of storage to assess blood gases (pO_2, pCO_2), CBC (Becton–Dickinson Coulter AcT Diff, Fullerton, CA), and biochemistry; pH with Accumet research AR20 meter, lactate, glucose, Na+, K+, methemoglobin, plasma free hemoglobin, and hemolysis (calculated: % Hemolysis = [($hemoglobin_{free}$ mg/dL) × (1 − Hct)/($hemoglobin_{Total}$ g/dL)] × 100).

PrPSc concentration by sarkosyl precipitation. For detection of PrP^{Sc} in blood fractions, it was first necessary to concentrate diluted samples, which cannot be loaded directly into a gel or that interfere with electrophoresis and blotting. The concentration procedure consists in adding 1 volume of 20% sarkosyl, incubate the mixture for 10 min at room temperature and centrifuge it at 100,000g for

Table 2
Densitometric analysis of PrP^{Sc} removal by Western blot analysis. Percentage of remaining PrP^{Sc} in each fraction, obtained by densitometric analysis of three independent Western blots, as the one shown in Fig. 2. Values represent the mean ± standard deviation.

Fraction	% PrP^{sc} remaining
Spiked blood	100%
Expressed plasma	87% ± 7.6
Saline wash	5.8% ± 3.5
pRBC	0% ± 0

Table 3
Incubation time in animals infected with diverse blood preparations. Each group represents the data of three replicates that were combined to obtain the average ± standard error. The experiment was terminated at 350 days after inoculation. Attack rate represent the percentage of animals developing characteristic prion clinical signs.

Group	Attack rate (%)	Incubation time—days (mean ± SE)
Non-spiked blood	0/15 (0%)	–
Spiked whole blood	13/13 (100%)	99.7 ± 1.84
Spiked RBC washed	6/15 (40%)	135.8 ± 6.67

to perform cycles of 30 min incubation at 37 °C followed by a 20 s pulse of sonication set at 60% potency.

In vivo infectivity studies. Four to six-week-old female Syrian Golden hamsters were anesthesized and stereotaxically injected in the right hippocampus with 4 μL of the sample. The onset of clinical disease was measured by scoring the animals three times a week as described [3]. Brains were extracted and the right cerebral hemisphere was frozen and stored at −70 °C for biochemical examination of PrP^{Sc} using Western blot analysis and the left hemisphere was fixed in 10% formaldehyde solution, sectioned and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin–eosin, using standard protocols or incubated with an antibody recognizing the glial fibrillary acidic protein (GFAP) (DakoCytomation, Carpinteria, CA). Immunoreactions were developed using the peroxidase–antiperoxidase method, following the manufacturer's specifications.

Results

The experimental procedure consisted of spiking two groups of entire blood units with partially purified hamster PrP^{Sc} equivalent to a 5×10^6 LD_{50}. One group was washed by the prototype washing device and the other was left untreated (Fig. 1). The final RBC pellets were resuspended in PBS and aliquots used for Western blot, PMCA, and to infect wild-type hamsters. Washed, packed human RBCs produced by this procedure were able to be stored in standard additive solutions (AS-3) for at least 42 days while still meeting all *in vitro* blood bank standards for acceptable RBC quality (Table 1).

We first evaluated the amount of PrP^{Sc} present in different fractions by Western blot analysis. Blood components interfere with electrophoresis and blotting. To overcome this problem we implemented a procedure to clean and concentrate PrP^{Sc}. To make sure that this procedure does not result in any loss of PrP^{Sc}, we performed a control experiment in which the different blood fractions obtained during the washing procedure were first prepared and then spiked with the same quantity of PrP^{Sc}. Each of these fractions was subjected to the sarkosyl precipitation procedure and the pellet was analyzed by Western blot. As shown in Fig. 2A, all samples have similar quantity of PrP^{Sc}, which was equivalent to the amount added, indicating that the sarkosyl precipitation method recovered >95% of PrP^{Sc} and no differences were observed in the distinct blood fractions in which the protocol was tested. Using this procedure we were able to assess the presence and quantity of PrP^{Sc} in each of the steps of the washing procedure including the whole spiked blood, expressed plasma, saline wash, and pRBCs. The results indicate that the majority of PrP^{Sc} was eliminated in

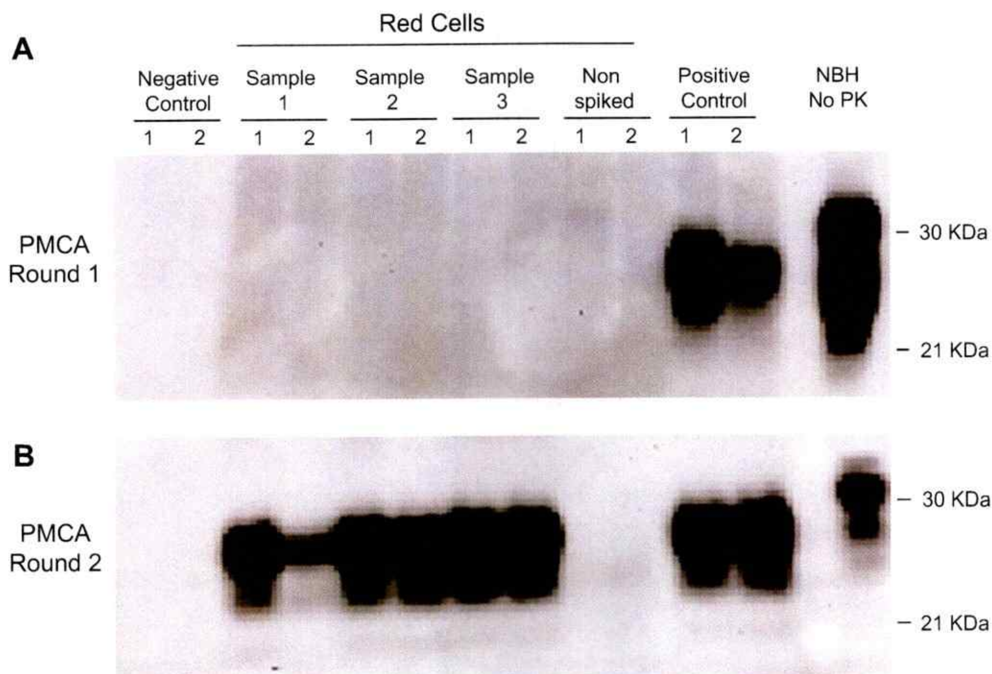


Fig. 3. Detection of PrP^{Sc} by PMCA in pRBCs samples obtained by the fractionation and washing procedure. RBC suspensions obtained from three independent experiments were subjected to two rounds of serial PMCA. Each PMCA reaction was performed in duplicate (numbers 1 and 2). For the negative control, we used normal Brain Homogenate (NBH) where no PrP^{Sc} is present and pRBCs obtained from non-spiked blood was used. For the positive control, whole blood spiked with 5×10^6 LD_{50} PrP^{Sc} was used. HaNBH represents control PrP^C without proteinase K (PK) digestion.

Table 1
Red blood cells quality after storage in AS-3. Biochemical parameters of packed red blood cells 35 and 42 days after treatment with the washing procedure. Values represent average ± standard deviation.

Storage	Lactate (mmol/L)	Glucose (mmol/L)	pH	pO_2 (mmHg)	pCO_2 (mmHg)	Plasma Hb (mg/dL)	% Hemolysis
Day 35 (mean ± SD)	143 ± 1.9	18.0 ± 5.1	6.3 ± 0.1	54.4 ± 12.8	51.9 ± 8.5	187.9 ± 96.1	0.4 ± 0.2
Day 42 (mean ± SD)	16.6 ± 2.4	16.2 ± 3.8	6.2 ± 0.1	58.1 ± 13.2	53.2 ± 8.3	244.1 ± 157.0	0.6 ± 0.3

	Na^+ (mmol/L)	K^+ (mmol/L)	WBC (10^6/mL)	Hb (g/dL)	% Hct	MCV (fL)	MCH (pg)	MCHC (g/dL)
Day 35 (mean ± SD)	106.2 ± 11.9	41.5 ± 3.4	0.1 ± 0.0	18.5 ± 0.9	57.1 ± 2.4	88.1 ± 7.8	28.7 ± 3.1	32.4 ± 1.0
Day 42 (mean ± SD)	101.2 ± 4.1	45.9 ± 3.6	0.1 ± 0.0	18.4 ± 0.9	57.1 ± 2.0	88.1 ± 7.1	28.3 ± 3.0	32.3 ± 1.1

1 h at 4 °C. Supernatants are discarded and pellets are resuspended into 2 volumes of 10% sarkosyl. Centrifugation process is repeated in order to assure the complete removal of blood components. Supernatants are discarded and pellets are resuspended in PBS by sonication. Following this protocol, PrP^{Sc} is recovered in the pellet fraction with a yield higher than 90%.

PrP^{Sc} detection. Samples are first digested with 12.5 μg/mL of proteinase K (PK) at 37 °C for 1 h and the reaction was stopped by adding NuPAGE LDS sample buffer. Proteins were then fractionated by electrophoresis using 4–12% sodium dodecyl sulphate–polyacrylamide gels (SDS–PAGE) under reducing conditions, electroblotted into Hybond-ECL nitrocellulose membrane and probed with the 3F4 antibody (Covance, Emeryville, CA) (dilution 1:5000). The immunoreactive bands were visualized by ECL Plus Western blotting detection system and quantified by densitometry using a UVP Bioimaging system EC3 apparatus (UVP, Upland, CA).

PMCA procedure. The detailed PMCA protocol, including troubleshooting, has been published elsewhere [17]. Briefly, samples were mixed with 10% normal brain homogenate prepared in conversion buffer and loaded onto 0.2-mL PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY) and programmed

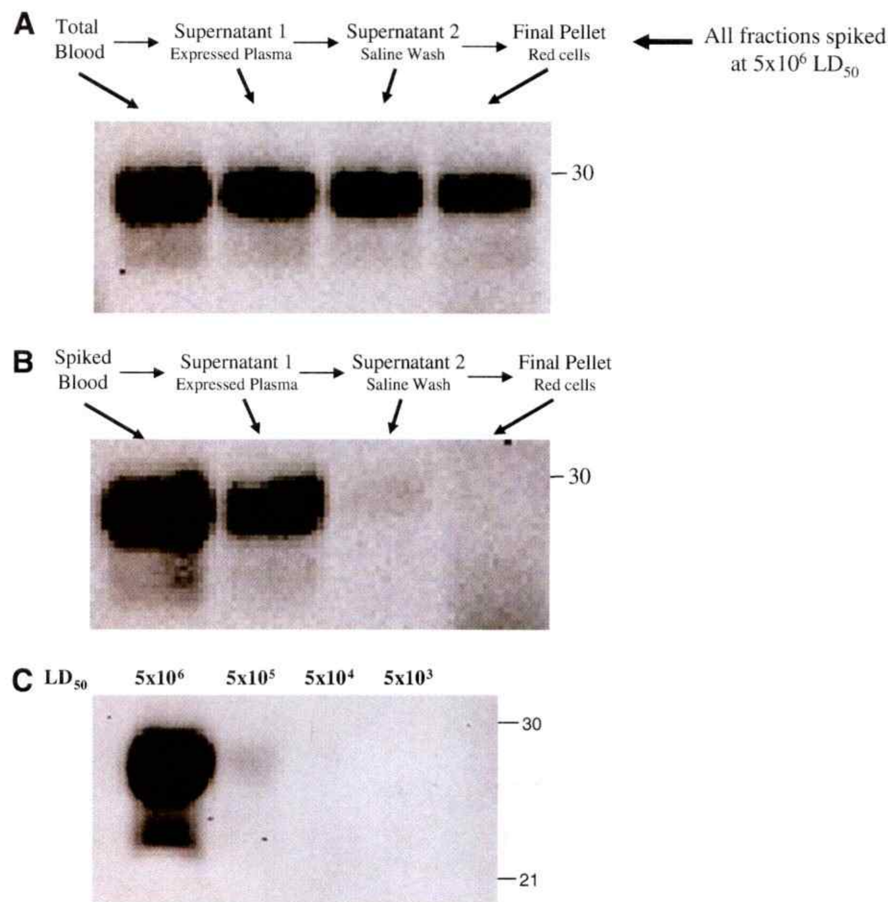


Fig. 2. Levels of PrP^{Sc} in different fractions of spiked blood obtained by the fractionation and washing procedure. (A) Control experiment to evaluate the yield of PrP^{Sc} recovery by the sarkosyl precipitation procedure. Blood samples were subjected to the protocol to separate and wash RBCs and all the fractions in the procedure were collected and spiked with PrP^{Sc} amount equivalent to 5×10^6 LD_{50}. Thereafter samples were precipitated with sarkosyl and equal amounts of the pellet were analyzed by Western blot. (B) Representative Western blot showing PrP^{Sc} levels in different fractions after treatment with the prototype cell separation and washing procedure. (C) Detection of PrP^{Sc} by Western blot at different dilutions. The first lane represents the quantity of PrP^{Sc} added to the blood samples, which contains the equivalent to 5×10^6 LD_{50}. This concentration was serially diluted by 10-fold to assess the limit of detection of Western blot assay.

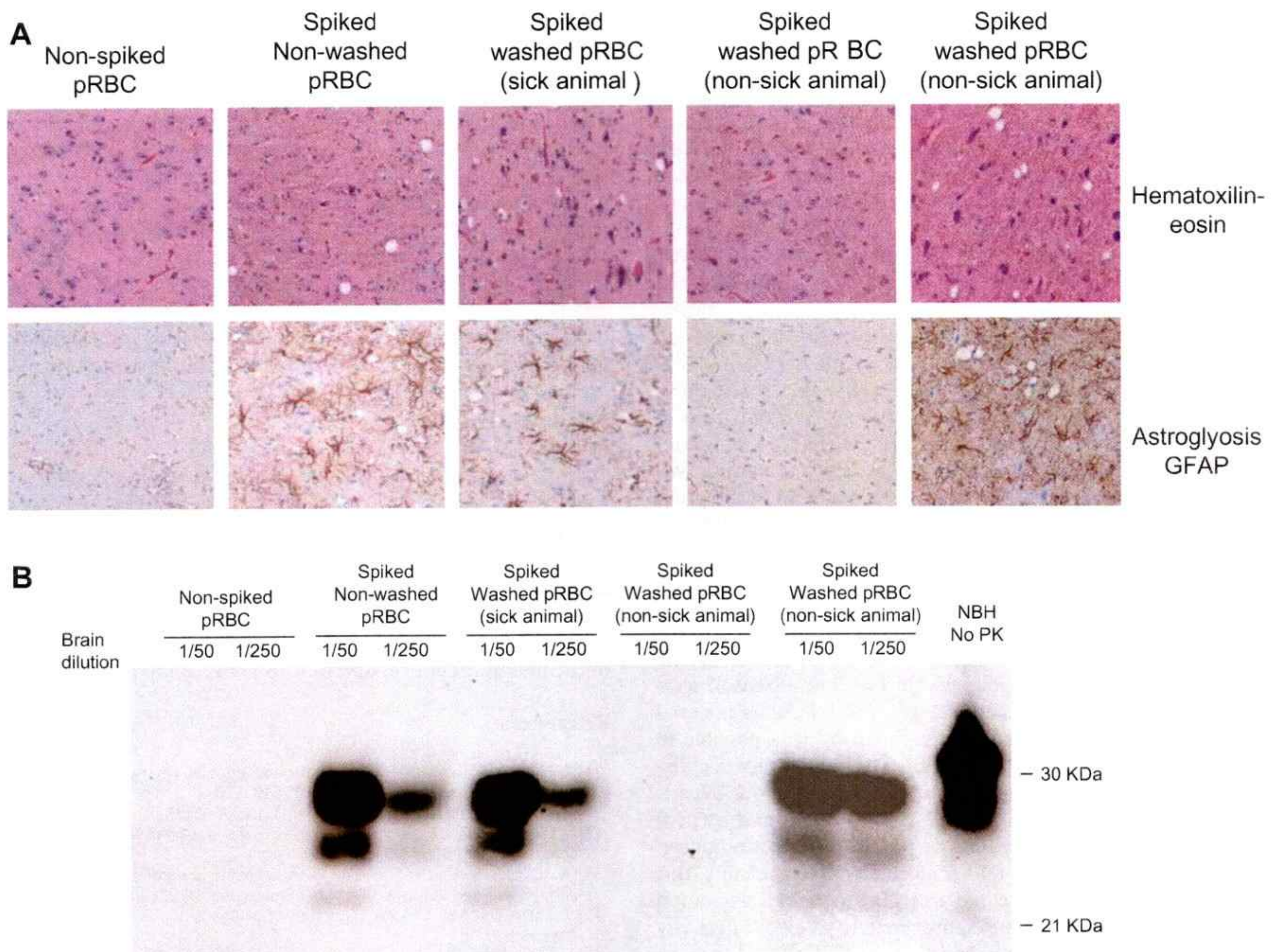


Fig. 4. Histological and biochemical characteristics of the brain of animals. Spongiform degeneration was assessed after staining with hematoxylin–eosin (A), astrogliosis was evaluated by immunohistochemistry with anti-GFAP antibodies (B) and Western blot showing the PrP27–30 (C). The figure includes representative pictures of animals inoculated with: non-spiked pRBCs (negative control), spiked blood not washed (positive control), and spiked washed pRBCs (three different animals from this group are shown to illustrate sick animals, non-clinically sick animals that developed brain pathology and non-sick animals that did not exhibited brain damage).

the plasma, whereas only a faint signal was found on the saline wash and no signal was observed in the RBC fraction (Fig. 2B). Densitometric evaluation of three replicates, indicate that 87.8% of PrP^{Sc} was present in plasma, whereas only 5.8% was detected in the saline washing (Table 2). The later amount is probably less reliable than the first, because of the weak signal observed. Assuming that both centrifugation and washing steps removed a similar amount of PrP^{Sc} (around 88%), our estimation from the Western blot studies would be that the RBC pellet contain around 1.5% of PrP^{Sc}. In other words the conclusion is that the prototype cell separation and washing device removes around 98.5% of PrP^{Sc} from the RBC preparation. This estimation is further supported by an experiment in which different dilutions of PrP^{Sc} were directly loaded into the gel to assess the limit of detection of the Western blot with the concentrations of PrP^{Sc} used in this study. As shown in Fig. 2C, a 100-fold dilution (or 1%) of a sample containing 5×10^6 LD_{50} of PrP^{Sc} is no longer detectable by Western blot.

In order to experimentally estimate the quantity of PrP^{Sc} present in the RBC fraction, we subjected the samples to serial PMCA [3,5,18]. A first round of 144 cycles did not show signal in any of the three pRBCs fractions subjected to the prototype cell separation and washing device (Fig. 3A). However, when a second round of PMCA was performed (Fig. 3B), we observed signal in all of the samples, indicating that PrP^{Sc} was indeed present in RBCs, albeit in very low quantity. As we previously reported [18], one round of 144 PMCA cycles enable to detect hamster PrP^{Sc} up to a brain dilution of 1×10^{-6}, which for this material corresponds to around 5×10^3 LD_{50}. Therefore, we conclude that pRBCs fraction contains less than 5×10^3 LD_{50} or <0.1% of PrP^{Sc} spiked into the blood. In other words, the PMCA results indicate that the blood processing by the prototype cell separation and washing device enable to remove >99.9% (~3 logs) of infectious prions.

Lastly, we assessed the reduction of infectivity from washed pRBC fractions by bioassay in experimental hamsters. Three groups of hamsters were injected intracerebrally with samples from three replicates of washed pRBCs. We also inoculated hamsters with blood spiked with 5×10^6 LD_{50} (three independent spiking experiments) and as negative controls we injected animals with non-spiked blood from three different donors (Fig. 1). We monitored the time that took for the animals in each group to exhibit typical clinical signs of scrapie. Brains from these animals were extracted for histological and biochemical analysis to confirm the disease. As expected, none of the negative control hamsters (animals inoculated with non-spiked blood) showed signs of scrapie up to 350 days after inoculation (Table 3). The disease onset in animals inoculated with an aliquot of whole blood spiked with 5×10^6 LD_{50}, the positive control was in average 100 days, which is well within the expected for this quantity of prions. The attack

rate (percentage of animals showing the disease) was 100%. The animals inoculated with pRBCs prepared by the washing procedure, showed a much longer incubation period (136 days in average) (Table 3). Strikingly of the 15 animals inoculated with washed spiked pRBCs only six developed clinical disease, representing an attack rate of 40%. These results indicate that the process of pRBCs preparation significantly removed infectious prions from the blood. Histopathological studies of the brain of all clinically sick animals showed the typical damage observed in scrapie animals, including spongiform degeneration (Fig. 4A) and astroglyosis (Fig. 4B). It is important to mention that from the nine animals inoculated with washed pRBCs that did not developed clinical signs of the disease, some (two of the nine) showed spongiform degeneration and astroglyosis when they were sacrificed at 350 days post-inoculation (Fig. 4A and B, lower panels). This result suggests that these correspond to subclinical animals. The other seven non-clinical animals did not show pathology at the time when they were sacrificed. The presence of PrP^{Sc} in the brain of infected animals was assessed by the detection of the PK resistant fragment of the prion protein (PrP27–30) by Western blot. As shown in Fig. 4C, PrP27–30 was detected in all hamsters inoculated with 5×10^6 LD_{50} spiked blood. Misfolded protein was also detected in clinically affected washed pRBC inoculated hamsters. As before two of the nine subclinical animals have PrP27–30 in their brains (Fig. 4C).

To estimate the extent of prion removal we compared the number of days in which hamsters developed clinical signs of scrapie after inoculation with known quantities of infectious material (Supplementary Figure 1). From this comparison it is possible to appreciate that administration of 5×10^6 LD_{50} should produce disease around 95 days, validating the results obtained in our experiment with 5×10^6 LD_{50} spiked blood. Extrapolation from this curve allows estimating that an incubation period of 135 days is produced when the equivalent to 3×10^3 LD_{50} is inoculated into hamsters. This result indicates that pRBCs prepared according to the cell separation and washing procedure, contains approximately 0.06% of infectivity spiked into the blood. Therefore, the *in vivo* results indicate that the cell separation and washing procedure removed 99.94% of infectious prions, which represents more than three orders of magnitude (logs of infectivity). This estimation only considers the incubation period and not the reduced attack rate, thus it is most likely that the rate of reduction of infectivity is even larger.

Discussion

Infectivity studies have shown that blood carries prions both in the symptomatic and pre-symptomatic stages of the disease in several animal species both in experimentally infected and naturally-produced disease [5–9]. Recently, four cases of vCJD have been associated with blood transfusion from asymptomatic donors who subsequently died from vCJD [11,19]. Because the incubation period of human prion diseases may be several decades, it is currently unknown how many people may be in an asymptomatic phase of vCJD infection. In addition, it is possible that some infected patients may never develop clinical symptoms but will remain asymptomatic carriers who can potentially transmit the disease to other individuals. All these considerations emphasize the importance of developing technologies capable to detect prions in blood and to remove the infectious protein from the blood supply.

In the current study, we analyzed whether a simple cell separation and washing procedure to prepare RBCs was sufficient to reduce PrP^{Sc} and infectivity from pRBCs used for transfusion. Similar washing procedures are currently being used to wash RBCs from other microbial agents and thus it would be very useful if simultaneously they can remove infectious prions. The results obtained on the biochemical estimation of PrP^{Sc} removal by Western blot and PMCA indicated that the washing procedure removed >98.5% and >99.9% of the infectious protein spiked into the blood. The *in vivo* results were even more encouraging, estimating that the procedure eliminated 99.94% of infectious material. Therefore, the overall conclusion of this study corroborated by the three different and independent techniques employed is that simple washing of blood fractions removed around or more than 3 logs of infectious material. This removal is highly significant and may well be enough to protect completely the blood supply, since the estimated quantities of prions in blood are close to the minimum required to transmit the disease. However, it remains to be shown that this procedure will perform similar removal with endogenously infected blood. A better understanding of this phenomenon will help to develop effective blood purification procedures that can efficiently reduce the risk of further spreading the disease through blood transfusion.

Acknowledgments

This work was supported by NIH Grant NS049173 and a grant from CaridianBCT Biotechnologies LLC to C.S. and by a project from the Department of Defense to CaridianBCT LLC.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.141.

References

[1] J. Collinge, Prion diseases of humans and animals: their causes and molecular basis, Annu. Rev. Neurosci. 24 (2001) 519–550.
[2] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. USA 95 (1998) 13363–13383.
[3] J. Castilla, P. Saá, C. Hetz, C. Soto, In vitro generation of infectious scrapie prions, Cell 121 (2005) 195–206.
[4] R. Morales, K. Abid, C. Soto, The prion strain phenomenon: molecular basis and unprecedented features, Biochim. Biophys. Acta 1772 (2007) 681–691.
[5] J. Castilla, P. Saa, C. Soto, Detection of prions in blood, Nat. Med. 11 (2005) 982–985.
[6] P. Saa, J. Castilla, C. Soto, Presymptomatic detection of prions in blood, Science 313 (2006) 92–94.
[7] P. Brown, Blood infectivity processing and screening tests in transmissible spongiform encephalopathy, Vox Sang. 89 (2005) 63–70.
[8] K. Holada, J.G. Vostal, P.W. Theisen, C. MacAuley, L. Gregori, R.G. Rohwer, Scrapie infectivity in hamster blood is not associated with platelets, J. Virol. 76 (2002) 4649–4650.
[9] F. Houston, J.D. Foster, A. Chong, N. Hunter, C.J. Bostock, Transmission of BSE by blood transfusion in sheep, Lancet 356 (2000) 999–1000.
[10] C. Soto, Diagnosing prion diseases: needs, challenges and hopes, Nat. Rev. Microbiol. 2 (2004) 809–819.
[11] C.A. Llewelyn, P.E. Hewitt, R.S. Knight, K. Amar, S. Cousens, J. Mackenzie, R.G. Will, Possible transmission of variant Creutzfeldt–Jakob disease by blood transfusion, Lancet 363 (2004) 417–421.
[12] A.H. Peden, M.W. Head, D.L. Ritchie, J.E. Bell, J.W. Ironside, Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient, Lancet 264 (2004) 527–529.
[13] Y. Murayama, M. Yoshioka, H. Okada, M. Takata, T. Yokoyama, S. Mohri, Urinary excretion and blood level of prions in scrapie-infected hamsters, J. Gen. Virol. 88 (2007) 2890–2898.
[14] L. Gregori, N. McCombie, D. Palmer, P. Birch, S.O. Sowemimo-Coker, A. Giulivi, R.G. Rohwer, Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood, Lancet 364 (2004) 529–531.
[15] H.G. Klein, D.R. Spahn, J.L. Carson, Red blood cell transfusion in clinical practice, Lancet 370 (2007) 415–426.
[16] L. Gregori, B.C. Lambert, P.V. Gurgel, L. Gheorghiu, P. Edwardson, J.T. Lathrop, C. MacAuley, R.G. Carbonell, S.J. Burton, D. Hammond, R.G. Rohwer, Reduction of transmissible spongiform encephalopathy infectivity from human red blood cells with prion protein affinity ligands, Transfusion 46 (2006) 1152–1161.
[17] J. Castilla, P. Saa, R. Morales, K. Abid, K. Maundrell, C. Soto, Protein misfolding cyclic amplification for diagnosis and prion propagation studies, Methods Enzymol. 412 (2006) 3–21.
[18] P. Saa, J. Castilla, C. Soto, Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification, J. Biol. Chem. 281 (2006) 35245–35252.
[19] J.W. Ironside, Variant Creutzfeldt–Jakob disease: risk of transmission by blood transfusion and blood therapies, Haemophilia 12 (Suppl. 1) (2006) 8–15.

Apéndice 12

Biochemistry **2006,** *45,* 15710–15717

Isolation and Characterization of a Proteinase K-Sensitive PrP^{Sc} Fraction[†]

Miguel A. Pastrana,[‡] Gustavo Sajnani,[‡] Bruce Onisko,[§] Joaquín Castilla,[‖] Rodrigo Morales,[‖] Claudio Soto,[‖] and Jesús R. Requena*,[‡]

Prion Research Unit, Department of Medicine, School of Medicine, University of Santiago de Compostela, Rue de S. Francisco s/n, Santiago de Compostela, Galiza, Spain 15782, Western Regional Research Center, United States Department of Agriculture, Room 3124, 800 Buchanan Street, Albany, California 94710, and Department of Neurology, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555

Received July 31, 2006; Revised Manuscript Received October 13, 2006

ABSTRACT: Recent studies have shown that a sizable fraction of PrP^{Sc} present in prion-infected tissues is, contrary to previous conceptions, sensitive to digestion by proteinase K (PK). This finding has important implications in the context of diagnosis of prion disease, as PK has been extensively used in attempts to distinguish between PrP^{Sc} and PrP^{C}. Even more importantly, PK-sensitive PrP^{Sc} (sPrP^{Sc}) might be essential to understand the process of conversion and aggregation of PrP^{C} leading to infectivity. We have isolated a fraction of sPrP^{Sc}. This material was obtained by differential centrifugation at an intermediate speed of Syrian hamster PrP^{Sc} obtained through a conventional procedure based on ultracentrifugation in the presence of detergents. PK-sensitive PrP^{Sc} is completely degraded under standard conditions (50 μg/mL of proteinase K at 37 °C for 1 h) and can also be digested with trypsin. Centrifugation in a sucrose gradient showed sPrP^{Sc} to correspond to the lower molecular weight fractions of the continuous range of oligomers that constitute PrP^{Sc}. PK-sensitive PrP^{Sc} has the ability to convert PrP^{C} into protease-resistant PrP^{Sc}, as assessed by the protein misfolding cyclic amplification assay (PMCA). Limited proteolysis of sPrP^{Sc} using trypsin allows for identification of regions that are particularly susceptible to digestion, i.e., are partially exposed and flexible; we have identified as such the regions around residues K110, R136, R151, K220, and R229. PK-sensitive PrP^{Sc} isolates should prove useful for structural studies to help understand fundamental issues of the molecular biology of PrP^{Sc} and in the quest to design tests to detect preclinical prion disease.

Since prions were defined as "proteinaceous infectious particles" and characterized as infectious agents composed almost exclusively of protein (*1*), substantial and diverse evidence has accumulated lending support to a stronger definition, according to which prions would be, in fact, infectious proteins (*2–5*). The prion protein, PrP,[1] is capable of adopting two basic conformations. Under the PrP^{C} form, it is a monomeric, glycosylated, α-helix-rich protein attached to cell membranes through a GPI anchor (*2*); in contrast, PrP^{Sc} appears as a β-sheet-rich, polymeric, insoluble molecule. PrP^{Sc} catalyzes the transformation of PrP^{C} into more PrP^{Sc} through a poorly characterized molecular mechanism (*2, 3, 6*). The structure of PrP^{Sc} is yet unknown, although several models have been proposed (*7–9*). These and future models need to conform to experimental constraints, such as those derived from electron micrographs of two-dimensional crystals of PrP^{Sc} (*7, 10*) or chemical cross-linking studies (*11*).

Traditionally, in the absence of definitive conformation-specific antibodies, and without structural data, PrP^{Sc} has been defined operationally by means of two physicochemical characteristics that distinguish it from PrP^{C}: its resistance to proteinase K and its insolubility in detergents (*2, 12*). Thus, treatment of a sample with 50 μg/mL PK for 1 h at 37 °C completely destroys PrP^{C}; in contrast, PrP^{Sc} is partially resistant to this treatment, resulting in cleavage of the amino terminal portion of this molecule, leaving a PK-resistant core termed PrP 27–30, 60–70 residues shorter than PrP^{Sc}. In the presence of detergent, PrP 27–30 forms characteristic rod-shaped structures readily visible by transmission electron microscopy (*13*). The amino terminal stretch of PrP^{C} spanning from the amino terminus to about position 121 is highly disordered, as surmised from NMR studies of recombinant PrP in solution, and it has been proposed that part of that stretch, up to the PK-cut position, is also disordered in PrP^{Sc}.

With regard to the second operational method to define PrP^{Sc}, insolubility in detergent, high-speed centrifugation under "standard conditions" (100000*g* for 1 h at 4 °C) allows pelleting PrP^{Sc}, leaving PrP^{C} in the supernatant (*12*). Resistance to PK and insolubility under "standard conditions", the two operational characteristics that define "*bona fide*" PrP^{Sc} (*12*), have been traditionally assumed to go hand in hand. Unexpectedly, more recent studies have revealed a more complex picture. Safar et al. first described the existence of a PK-sensitive fraction of PrP^{Sc} (*14*). These authors showed that denaturation with chaotropes enhances the immunoreactivity of PrP^{Sc} toward certain antibodies, a result of the uncovering of partially buried epitopes, which are equally available in native vs denatured PrP^{C} (*14*).

[†] This work was supported by research grants from the Spanish Ministry of Science and Technology (EET 2001-4861 to JRR) and the National Institutes of Health (NS049173 to CS).

* Corresponding author. Phone: +34-981-559904. Fax: +34-981-559904. E-mail: requenaj@usc.es.

[‡] University of Santiago de Compostela.

[§] United States Department of Agriculture.

[‖] University of Texas Medical Branch.

10.1021/bi0615442 CCC: $33.50 © 2006 American Chemical Society
Published on Web 12/06/2006

Intriguingly, pretreatment of samples with PK considerably weakens the denaturation-dependent immunoreactivity enhancement effect, indicating the existence of a subset of PrP^{Sc} molecules that are completely degraded by PK and that were termed, in accordance, PK-sensitive PrP^{Sc} ($sPrP^{Sc}$). Subsequent studies by Tzaban et al. showed that, when brain and cell homogenates from scrapie-infected animals and cultures were subjected to sucrose gradients, PrP distributed in a continuum of aggregation sizes. Although PrP^{Sc} is present in the more dense fractions, corresponding to larger polymers, and was PK-resistant, PrP^{Sc} recovered from intermediate fractions, corresponding to smaller oligomers, was not (*12*). In contrast, homogenates from control brains or cell cultures only contained PrP in the very light fractions, corresponding to monomeric or at the most, dimeric PrP (*12*). These results suggest that PrP^{Sc} is a heterogeneous collection of oligomers of different sizes and that resistance to PK is dependent, at least in part, on its quaternary structure.

More recent studies highlight the importance of $sPrP^{Sc}$; in a recent study, as much as 80% of PrP^{Sc} in the brains of individuals who had died as a consequence of Creutzfeldt–Jakob disease (CJD) was estimated to be $sPrP^{Sc}$ (*15*). Because many methods rely on the use of PK to distinguish between PrP^{Sc} and PrP^{C} (*2*), a considerable underestimation of the amount of PrP^{Sc} present in samples might be expected. This would be of particular importance in the quest to develop analytical methods to detect minute amounts of PrP^{Sc} in biological fluids such as blood, which could constitute the basis of a preclinical prion test. Beyond this practical consideration, $sPrP^{Sc}$ might hold important clues on the structure of PrP^{Sc} and the process that leads to its generation and propagation.

We reasoned that, starting from a sample of PrP^{Sc} isolated through standard procedures that rely on ultracentrifugation in the presence of detergent, it would be possible to isolate a lighter fraction of smaller PrP^{Sc} oligomers that would remain in the supernatant at lower centrifugal forces. On the basis of Tzaban's results, we also reasoned that such smaller oligomers of PrP^{Sc} would be more sensitive to the effect of PK. Thus, by operationally adjusting our experimental conditions, one would possibly be able to obtain a fraction of $sPrP^{Sc}$. We describe here the isolation of such a fraction and its further characterization and introduce its possible use for structural studies.

EXPERIMENTAL PROCEDURES

Isolation of PrP^{Sc}. PrP^{Sc} was isolated from brains of terminally ill Syrian hamsters infected with the 263K strain of scrapie, using a slightly modified version of the procedure of Diringer et al. (*16*). This procedure involves low-speed centrifugation of a 10% brain homogenate in 10% sarkosyl to remove cell debris and centrifugation of the supernatant at high speed (g_{av} 149008*g*) through a sucrose cushion. The pellet is resuspended in 0.1% of Z-3,14 detergent (*N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate, Boehringer Mannheim, Germany) and subjected to three additional cycles of pelleting at 149008*g* through sucrose cushions, followed by resuspension, first in this detergent, and later in deionized water at pH 8.5. Our modifications of the method consisted of inclusion of a cocktail of protease inhibitors (Complete, Roche Diagnostics, Penzberg, Germany) at a final concentration of 1X in all buffers used throughout the procedure up to the penultimate pellet, P145c, as defined in the mentioned study (*16*), which was resuspended, as described, in 20 mM Tris/HCl, pH 8.5 buffer (T8.5) not supplemented with the protease inhibitor cocktail. Also, treatment with PK of this fraction was omitted. The final pellet was resuspended in T8.5 containing 1% sarkosyl and no protease inhibitors at concentrations between 1 and 2 μg/μL by application of three to four 1 s pulses at an amplitude of 50% with a 4710 series probe ultrasonics homogenizer (Cole Parmer, Chicago, IL). The stock suspension thus prepared was frozen until further use; its purity was assessed by SDS-PAGE with Coomassie blue staining and MALDI and estimated to be approximately 80–85%. Several methods, all based on the same principle (pelleting in the presence of detergents) with minor variations in the number of centrifugation steps, centrifugation times, composition of buffers, etc., have been described to isolate PrP^{Sc} (*17,18*). Of note is that, when a control brain homogenate is subjected to the same procedure, no PrP is found in the pellets (Figure S1 in the Supporting Information and ref 17). Concentration of PrP^{Sc} was estimated by comparison of serial dilutions of this material with serial dilutions of a recombinant Syrian hamster, SHaPrP (90–231), standard, a generous gift of Giuseppe Legname, UCSF, on dot blots stained with Amido Black (*19*).

Isolation of $sPrP^{Sc}$. $sPrP^{Sc}$ was isolated from total PrP^{Sc} by ultracentrifugation at an intermediate speed. A 50–150 μL portion of the PrP^{Sc} stock suspension (see above) was homogenized by application of three to four sonication pulses of 1 s each, as described above, and spun in a TLX ultracentrifuge (Beckman, Fullerton, CA) using a TLA-120-1 rotor at 40 000 rpm (g_{av} 56806*g*) for 2 h at 20 °C. The supernatant, corresponding to $sPrP^{Sc}$ (see below), was collected, and the pellet was resuspended by brief sonication in a volume of T8.5 containing 1% sarkosyl equivalent to that of the supernatant. Fractions of supernatant and pellet were treated with 50 μg/mL PK at 37 °C for 1 h; the reaction was terminated with 2 M Pefabloc (Fluka, St. Louis, MO) and a fraction subjected to SDS-PAGE (*20*) and either stained with Coomassie blue or transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA) and analyzed by Western blotting using mAbs 3F4 (Dako, Glostrup, Denmark) or 6H4 (Prionics, Zurich, Switzerland) at 1:5000 and 1:2000 dilutions, respectively.

Conformation-Dependent Immunoreactivity. To measure the dependence of the immunoreactivity of $sPrP^{Sc}$ and $rPrP^{Sc}$ on protein denaturation, we used the dot blot procedure described by Serban et al. (*21*). Samples were diluted with deionized water to an approximate concentration of 12.5 ng/μL, and 2 μL of each sample was spotted on a dry nitrocellulose membrane. The dots were thoroughly air-dried. The membranes were then washed extensively with PBS

[1] Abbreviations: CB, conversion buffer (150 mM NaCl, 1% Triton X-100, 4 mM EDTA, complete 1X in PBS); CJD, Creutzfeldt–Jakob disease; GPI, glycosylinositol phospholipid; PBS, phosphate buffered saline; NBH, normal brain homogenate; PMCA, protein misfolding cyclic amplification; PK, proteinase K; PrP, prion protein; PrP^{C}, cellular prion protein isoform; PrP^{Sc}, scrapie prion protein isoform; $sPrP^{Sc}$, PK-sensitive PrP^{Sc}; $rPrP^{Sc}$, PK-resistant PrP^{Sc}; TIC, total ion current; TNS, 10 mM Tris, pH 7.5, 150 mM NaCl, 1% sarkosyl buffer; TSEs, transmisible spongiform encephalopathies; T8.5, 20mM Tris/HCl, pH 8.5 buffer; XIC, extracted ion chromatogram.

containing 0.3% Tween-20 and incubated for 10 min at room temperature in PBS with or without 4 M guanidinium hydrochloride; after thorough washing in PBS containing 0.3% Tween-20, the membrane was probed with mAb 3F4. Signal intensities were quantitated using the LabWorks 3.0 image analysis software (UVP, Cambridge, U.K.). Conformation dependence was calculated for each sample (N) as

$$Y(N) = I_{(N,0M)}/I_{(N,4M)}$$

where $I_{(N,0M)}/I_{(N,4M)}$ are, respectively, blot signal intensities of a given sample with and without 4 M guanidine hydrochloride treatment.

Velocity Sedimentation in Sucrose Gradients. Samples of $sPrP^{Sc}$ or $rPrP^{Sc}$, ~2.5 μg, were diluted in 1.2 mL of 10 mM Tris, pH 7.5, 150 mM NaCl, and 1% sarkosyl (TNS) and loaded on top of 10−60% sucrose step gradients (*12*). Gradients were formed in polyallomer (11 × 34 mm) tubes from 600 μL of each of the following sucrose concentrations: 10, 15, 20, 25, 30, and 60% in water. The gradients were spun for 1 h at 4 °C at 50 000 rpm (g_{av} 200620g) in a MLS-50 rotor in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA). Twelve 300 μL fractions, following the load volume, were collected starting from the top of the tube; fraction 12 included the pellet.

PMCA. Different fractions obtained from the sucrose gradient sedimentation experiment were diluted with a minimal amount of conversion buffer (CB), consisting of PBS containing 150 mM NaCl, 1% Triton X-100, 4 mM EDTA, and Complete 1X to obtain samples of a similar PrP concentration, as determined by Western blot. These samples were serially diluted in NBH (10% normal brain homogenate) prepared in CB from 1/20 to 1/320 (final volume 100 μL). From each final dilution, 18 μL aliquots were immediately withdrawn and frozen at −80 °C (control samples); the remaining 82 μL portions were subjected to PMCA amplification (*22*−*24*) in 0.2 mL PCR polypropylene tubes (Fisher Scientific, Hampton, NH). Tubes were inserted in an adaptor and placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY); the plate was then placed in the water bath of the sonicator and incubated without shaking. The sonicator was programmed to perform incubation cycles of 30 min at 37 °C followed by a 20 s pulse of sonication at 60−80% potency for 48 h. Amplified and control samples were incubated with 50 μg/mL of PK for 60 min at 37 °C; the digestion reaction was stopped by addition of Laemmli electrophoresis buffer and boiling. Samples were subjected to SDS-PAGE and Western blotting, as described above. In parallel experiments, $sPrP^{Sc}$ was treated with PK or buffer for 60 min at 37 °C and then diluted in CB as appropriate and subjected to PMCA.

Limited Proteolysis. $sPrP^{Sc}$ (~3.5 μg) was treated with trypsin (Promega, Madison, WI) in T8.5 (final volume 10 μL) at 37 °C for 1 h at the indicated enzyme/substrate ratios (figure legends). SHaPrP (90−231), kept at −70 in 6M guanidine hydrochloride, was freshly refolded in 50 mM phosphate buffer, pH 7.4, extensively dialyzed against the same buffer, and treated with trypsin in a similar way. Reactions were stopped with Complete (Roche) and reaction mixtures analyzed by Western blot. Alternatively, samples were denatured by addition of solid guanidine hydrochloride to a final concentration of 6 M, acidified with 10% trifluoroacetic anhydride, and adsorbed to C-18 ZipTips (Millipore). Peptides were eluted according to the manufacturer's instructions and dried by centrifugal evaporation (SpeedVac, Savant, Farmingdale, NY). For analysis by nanospray LC/MS/MS, samples were redissolved in 40 μL of 6 M guanidine hydrochloride.

Nanospray LC/MS/MS. NanoLC−ESI−MS−MS was done with an Applied Biosystems (ABI/MDS Sciex, Toronto, Canada) model QStar Pulsar equipped with a Proxeon Biosystems (Odense, Denmark) nanoelectrospray source. Redissolved trypsin digests (20 μL) were loaded automatically onto a C-18 trap cartridge, and after washing, the trapped peptides were chromatographed on a reversed-phase column (Vydac 238EV5.07515, 75 μm × 150 mm; Hesperia, CA) fitted at the effluent end with a coated spray tip (FS360-50-5-CE, New Objective Inc., Woburn, MA). An LC Packings nanoflow LC system (Dionex, Sunnyvale, CA) with an autosampler, column switching device, loading pump, and nanoflow solvent delivery system was used to elute the column. Elution solvents were A (0.5% acetic acid in water) and B (80% acetonitrile, 0.5% acetic acid). Samples were eluted at 250 nL/min with the following gradient profile: 8% B at 0 min to 80% B in a 15 min linear gradient (held at 80% B for 5 min then back to 8% B for 10 min). The QStar Pulsar was externally calibrated daily and operated above a resolution of 7000. The acquisition cycle time of 6 s consisted of a single 1 s MS "survey" scan followed by a 5 s MS/MS scan. Ions between m/z 400 and 1000 of charge states between +2 and +5 having intensities greater than 40 counts in the survey scan were selected for fragmentation. The dynamic exclusion window was set to always exclude previously fragmented masses. A collision energy optimized for charge state and m/z was automatically selected by the Analyst QS 1.1 software after adjusting parameters to obtain satisfactory fragmentation of the Glu fibrinogen peptide (+2) and ACTH (+3 and +4). Nitrogen was used for the collision gas, and the pressure in the collision cell ranged from 3×10^{-6} to 6×10^{-6} Torr. The externally calibrated TOF−MS survey scans were processed with the "LCMS Reconstruct" tool in the Analyst software. The output is a list of peptide molecular weights calculated by deconvolution of multiple charge states and then identification of the monoisotopic ^{12}C species.

RESULTS

Isolation of an $sPrP^{Sc}$ Fraction. Centrifugation of a PrP^{Sc} suspension at an intermediate centrifugal force, as described in the Experimental Procedures, generated pellets and supernatants with divergent resistance to PK. Supernatants, typically making up 35−55% of the total starting PrP^{Sc} material, were much less resistant than pellets. By fine-tuning the centrifugal force and length of centrifugation, we were able to obtain a supernatant PrP^{Sc} fraction that was completely hydrolyzed by the standard treatment with PK at 50 μg/mL for 1 h at 37 °C ($sPrP^{Sc}$), as judged by Western blot using antibody 3F4 and Coomassie staining (Figure 1). Under the same conditions, an equivalent amount of the pellet produced the characteristic PK-resistant core (PrP 27−30) with an increased electrophoretic mobility, a consequence of the trimming of 65−70 amino terminal residues. We thereafter term this pellet fraction "$rPrP^{Sc}$". Treatment of $sPrP^{Sc}$ with lower concentrations of PK showed that this

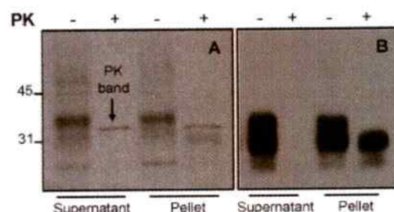


FIGURE 1: Isolation of a PK-sensitive PrP^{Sc} fraction: PrP^{Sc} was subjected to intermediate speed ultracentrifugation, as described in the Experimental Procedures, and supernatant and pellet fractions thus obtained subjected to PK treatment under standard conditions (50 μg/mL, 37 °C, 1 h) and analyzed by SDS-PAGE. A: Coomassie blue stain; B. Western Blot using mAb 3F4.

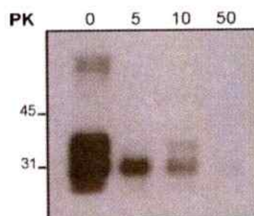


FIGURE 2: Partial PK resistance of $sPrP^{Sc}$. sPrPSc was treated with increasing concentrations of PK (μg/mL) at 37 °C for 1 h and analyzed by Western blot using mAb 3F4.

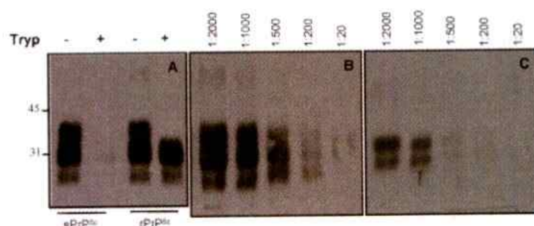


FIGURE 3: Susceptibility of $sPrP^{Sc}$ to trypsin digestion. Samples of $sPrP^{Sc}$ and $rPrP^{Sc}$ were treated with trypsin for 1 h at 37 °C and analyzed by Western blot. A: Equal amounts of $sPrP^{Sc}$ and $rPrP^{Sc}$ treated with a 1:60 enzyme/substrate ratio (mAb 3F4). B: Limited digestion of $sPrP^{Sc}$ with increasing trypsin/substrate ratios (mAb 3F4). C: Limited digestion of $sPrP^{Sc}$ (mAb 6H4).

material exhibits, as expected, some resistance to degradation by this enzyme (Figure 2).

We next studied susceptibility of $sPrP^{Sc}$ to trypsin. Treatment of $sPrP^{Sc}$ with trypsin at 37 °C for 1 h, at enzyme/substrate ratios in the range 1:20−1:60 (there was some variability with different substrate and trypsin batches) resulted in a virtually complete disappearance of the protein, as assessed by Western blot using antibody 3F4 (Figure 3A); under similar conditions, an equivalent amount of $rPrP^{Sc}$, treated with the same ratio of trypsin, yielded a band of approximately the same intensity as that of the untreated sample, albeit migrating slightly faster (Figure 3A). This apparent MW difference of 2000−3000 suggests clipping of $rPrP^{Sc}$ at arginines 37 and 48 in the putatively unstructured tail that, in contrast, is cleaved up to Gly90 by PK and agrees with previously published results relative to total PrP^{Sc} (*13*). Further experiments showed that decreasing trypsin/substrate ratios can be used to achieve limited proteolysis of $sPrP^{Sc}$ (Figure 3B). Western blots using mAb 6H4 (epitope 144−152) also showed a similar trypsin concentration-dependent signal disappearance (Figure 3C), suggestive of a generalized tryptic cleavage. When freshly refolded, recombinant SHaPrP-(90−231) was subjected to trypsin proteolysis under the same conditions, complete disappearance of the 3F4 signal was

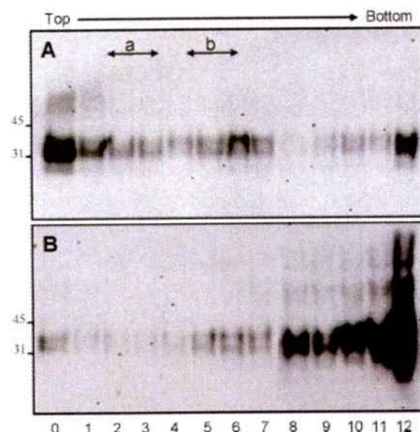


FIGURE 4: Size distribution of $sPrP^{Sc}$ (A) and $rPrP^{Sc}$ (B) in sucrose gradients. Equal amounts of $sPrP^{Sc}$ and $rPrP^{Sc}$ were loaded on the top of a preformed 10−60% step sucrose gradient; the gradient was spun for 1 h at 4 °C at g_{av} 200620*g*, fractions 0−12 collected from the top. Fractions were analyzed by Western blot using mAb 3F4. In parallel experiments under the same conditions, Apoferritin (443k) and Dextran Blue (2000k) emerged in the indicated fractions, a and b, respectively.

seen at an approximate enzyme/substrate ratio of 1:400, indicative of a much lower resistance to trypsin degradation.

Biochemical Characterization of $sPrP^{Sc}$. When subjected to sucrose gradient centrifugation, $sPrP^{Sc}$ exhibited a distribution throughout the upper fractions, particularly 0−7 with a considerable amount of material present in fractions 0 (load volume) and 1 (Figure 4). Little material tailed in the lower fractions (*8−11*); some material was also recovered in the pellet fraction. When $rPrP^{Sc}$ was subjected to a similar treatment, its distribution showed, as expected, a pattern complementary to that of $sPrP^{Sc}$ with very little material present in early fractions (0−7) and most of it present in late fractions (8−12) including the pellet (Figure 4). It is known that lipid micelles can interfere with the mobility of proteins in sucrose gradients; therefore, we chose a concentration of 1% sarkosyl in our samples, which is known to prevent micelle formation (*25*). Furthermore, we conducted control experiments in which we took individual sucrose gradient fractions, dialyzed them against TNS, and reloaded the sample to a freshly prepared gradient. As seen in Figure S2, Supporting Information, such samples migrated to the same position as in the first separation experiment, which confirms these fractions contain oligomers of a defined size.

The molecular weight markers apoferritin (MW 443k) and Dextran Blue (MW 2000k), loaded on a gradient run in parallel under identical conditions, emerged in fractions ~2 and ~5, respectively (Figure 4). Extrapolation of these results would lead to the conclusion that, if an approximate MW of 35k is assigned to each PrP subunit, and assuming that the aggregates are entirely composed of PrP (*26*), the majority of $sPrP^{Sc}$ aggregates would be composed of aggregates of up to 12−15 PrP subunits, and most $rPrP^{Sc}$ would contain over 60 PrP subunits.

Different fractions obtained from the sucrose gradient centrifugation experiment were assayed for their converting capacity using the PMCA assay (*22−24*). More specifically, we assayed fractions 0 and (1 + 2) from $sPrP^{Sc}$, corresponding to very light and heavier fractions of $sPrP^{Sc}$, and fraction 11 from $rPrP^{Sc}$. As seen in Figure 5, when equal amounts of

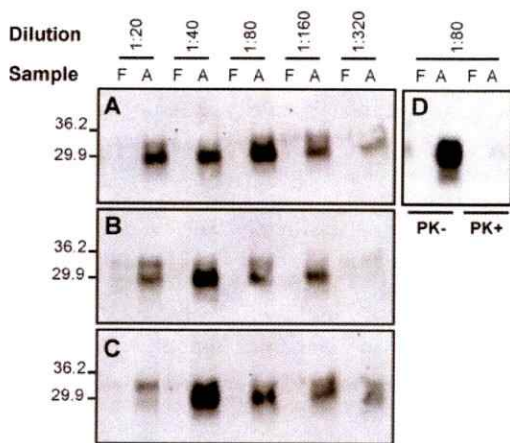


FIGURE 5: Transforming activity of sPrP^{Sc} as shown by PMCA. Equivalent amounts of PrP^{Sc} from different fractions (A, fraction 0 from sPrP^{Sc}; B, combined fractions 1 + 2 from sPrP^{Sc}; C, fraction 11 from rPrP^{Sc}) obtained from the sucrose gradient sedimentation experiment were diluted in NBH and split in two samples, one frozen (F) and the other subjected to amplification by PMCA (A). After treatment with 50 μg/mL PK, samples were analyzed by Western blot (mAb 3F4). D: Aliquots of a sPrP^{Sc} sample treated with or without PK at 37 °C for 1 h and subjected to PMCA.

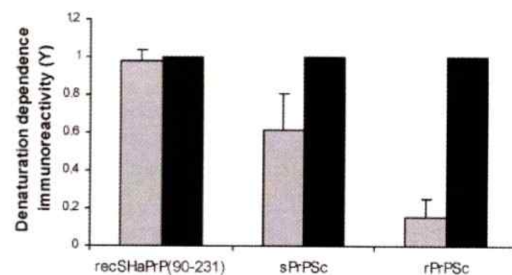


FIGURE 6: Denaturation dependent immunoreactivity of sPrP^{Sc}, rPrP^{Sc}, and recombinant SHaPrP (90−231). Samples were probed by dot blot (mAb 3F4) without (gray bars) or with (black bars) treatment with 4 M guanidinium hydrochloride. Y represents the ratio between signals obtained for a given sample without guanidinium hydrochloride treatment divided by signal obtained with 4 M guanidinium hycdrochloride; Y = 1 indicates a complete lack of conformation dependence. Data represent means and standard deviations (n = 4). Differences were statistically significant (student's t test) between recSHaPrP (90−231) and sPrP^{Sc} (p < 0.03), recSHaPrP (90−231) and rPrP^{Sc} (p < 0.0001), and sPrP^{Sc} and rPrP^{Sc} (p < 0.02).

PrP present in these fractions were subjected to PMCA, all three exhibited converting activity, surmised from the generation of PK-resistant PrP^{Sc} at the end of the PMCA cycles. In a control experiment, we compared the converting activity of sPrP^{Sc} with or without prior treatment with PK and found that, as expected, PK-induced destruction of sPrP^{Sc} abolished the converting activity (Figure 5D). It is of note that, in these experiments, the detection of PrP^{Sc} was done after PK treatment, which means that, in the case of sPrP^{Sc} present in fractions 0 and (1 + 2), the conversion process progresses to generation of PK-resistant PrP^{Sc}. It remains to be studied what the ratio of sPrP^{Sc}/rPrP^{Sc} produced by PMCA from the different fractions used is. Further dilutions of the samples, subjected to PMCA, were also conversion-competent. Given the high sensitivity of PMCA, it is not possible, from this experiment, to compare the relative efficacy of the different fractions tested, but it is clear that even the material obtained from fraction 0 is highly conversion-competent.

Further biochemical characterization of sPrP^{Sc} was carried out using a version of the dot blot format CDI introduced by Serban et al. (*21*). As expected, treatment of total PrP^{Sc} blotted on a nitrocellulose membrane with 4 M guanidine, prior to probing with monoclonal antibody 3F4 resulted in a considerable increase in intensity (data not shown), in agreement with published results (*21*); rPrP^{Sc} behaved also in a similar way, and for sPrP^{Sc}, guanidine treatment produced a significant yet lower enhancement of intensity (Figure 6).

Limited Proteolysis of sPrP^{Sc}. The fact that sPrP^{Sc} can be digested by trypsin (Figure 3) prompted us to explore the possibility that sPrP^{Sc} might be subjected to limited proteolysis experiments using this enzyme. This might allow for probing the susceptibility of individual lysine and arginine residues to cleavage, which in turn can provide some structural information. We digested sPrP^{Sc} using trypsin to substrate ratios ranging from 1:2 to 1:2000 and analyzed the reaction mixtures by nanoLC−MS/MS. In agreement with

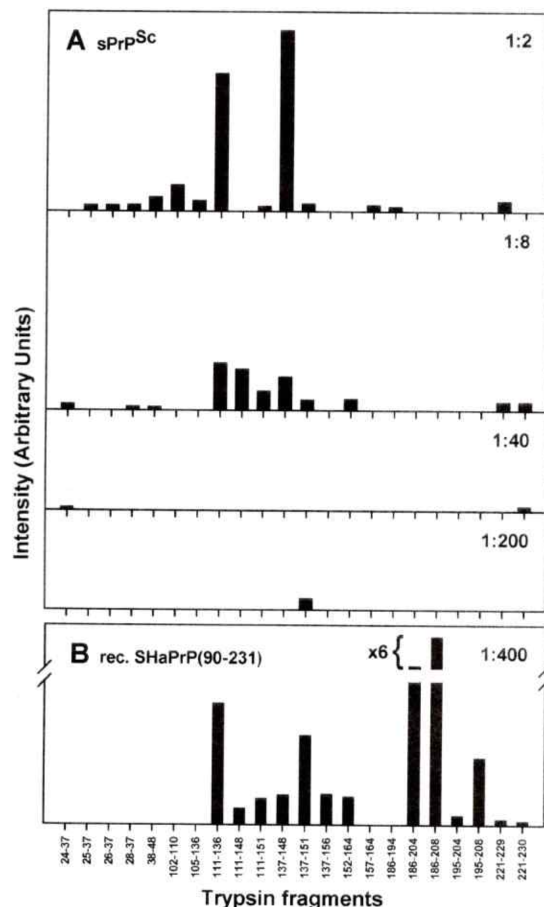


FIGURE 7: Tryptic fragments obtained by limited proteolysis of sPrP^{Sc}. Samples were treated with trypsin for 1 h at 37 °C at the indicated enzyme/substrate ratios. Tryptic fragments were identified by LC−MS, as described in the text. **A**: sPrP^{Sc}. **B**: recombinant SHaPrP (90−231). Bars represent relative peak areas in total intensity chromatograms (TIC) with all panels at the same scale, except as otherwise indicated. Data are representative of two independent experiments.

Western blot data (Figure 3), the lower enzyme/substrate ratios resulted in just a few detectable fragments (Figure 7). As expected, higher trypsin/substrate ratios resulted in detection of more fragments with overall higher intensities.

It is noteworthy that detection of a given fragment depends not only on cleavage of residues located at its boundaries but also on the intrinsic "detectability" of the resulting peptide; i.e., low MW peptides (<800) would not trigger MS–MS experiments, and others may suffer substantial losses during sample cleanup, etc. For PrP^{Sc}, glycosylation and the presence of the disulfide bond further limit the number of peptides that could be detected under our particular experimental conditions. With all this in mind, the most readily conspicuous fragments were 137–151, 24–37, and 221–230 (Figure 7 and Figures S3 and S4 in the Supporting Information). At higher concentrations of trypsin, besides these fragments, several others were detected, including 111–136, 137–148, 152–164, and 157–164. At an even higher trypsin concentration, intensity ratios of some fragments changed, indicating further fragmentation at previously intact internal residues. As an example, peptides 111–136 and 137–148, which do not have any intact internal K or R residues, increased steadily as the trypsin concentration increased, whereas fragments 111–148, 111–151, and 152–164 exhibited a bimodal behavior, with maximum relative intensities at intermediate trypsin concentrations.

DISCUSSION

There is a growing interest in PK–sensitive PrP^{Sc}, a previously unnoticed PrP^{Sc} fraction. There are several reasons for this: on one hand, recent studies suggest that, in many instances, $sPrP^{Sc}$ makes up a very sizable fraction of total PrP^{Sc} (*15*); on the other hand, $sPrP^{Sc}$ might offer explanations for some puzzling experimental results that have been difficult to accommodate within the current prion paradigm, such as reports of disease transmission in the absence of "PK-resistant PrP" (*27*), or the inherent difficulty to propagate some prions, such as those causing some genetic forms of disease (*28*). Finally, the existence of $sPrP^{Sc}$ has clear practical implications, as it limits the usefulness of analytical techniques relying on the use of PK to detect PrP^{Sc}. This should be carefully considered in the development of the urgently needed ultrasensitive tests capable of detecting PrP^{Sc} in preclinical disease-suffering subjects and animals. Furthermore, the possibility of missing infected specimens containing a high proportion of $sPrP^{Sc}$ becomes an issue with obvious health implications.

We report here a simple method to isolate a PK-sensitive fraction of PrP^{Sc}. It is evident that our definition of $sPrP^{Sc}$ is operational: we have chosen a specific combination of centrifugal force and centrifugation time that allows for isolation of a fraction of PrP^{Sc} that will, precisely, be completely degraded under the standard, yet arbitrary, conditions defined by treatment by 50 μg/mL PK at 37 °C for 1 h. However, it is also evident that modifying the parameters of centrifugation allows one to obtain an alternative fraction that would meet the definition of $sPrP^{Sc}$, were different operational PK-treatment parameters to be chosen. Our results show, in agreement with the work of Tzaban et al. (*12*), that PK resistance of PrP^{Sc} shows a strong dependence on the quaternary structure of PrP^{Sc}. Indeed, PrP^{Sc} isolated following a standard procedure based on ultracentrifugation in the presence of detergents is a collection of oligomers with a continuum of sizes (Figure 4). Our results confirm that larger PrP^{Sc} polymers exhibit higher PK resistance than smaller ones.

Resistance to digestion by PK is a function of accessibility to the enzyme, and larger oligomers offer the possibility of enhanced protection of certain PrP subunits. Because there is not a definitive model of PrP^{Sc}, it is difficult to envision how such enhanced protection is achieved. In contrast to PrP 27–30, obtained by proteolytic treatment of PrP^{Sc} in the presence of detergent, and organized in rods of measurable dimensions (*2, 13*), there are no available experimental data on the organization or shape of PrP^{Sc} oligomers. The most parsimonious view would be that PrP^{Sc} and PrP 27–30 share basic structural features, such as the way in which PrP subunits are stacked (*7*) and that clipping by PK of the very labile, random coil amino termini leads to further packing. Such a possibility is compatible with a mechanism of PrP^{Sc} growth and transmission based on nucleated polymerization. In this model, the β-sheet domains of a PrP subunit, whatever their specific organization might be (β-helices, simple parallel β-sheets, etc.; *7,8*), act as templates or scaffolds for the next PrP subunit (*6, 29*). The alternative possibility is that PrP^{Sc} and PrP 27–30 have unrelated quaternary structures and that PrP 27–30, with its proposed regular stacking, is formed totally *de novo* through a complete rearrangement of the subunits that constitute the PrP^{Sc} aggregate; in that case, PrP^{Sc} oligomers might even be formed by featureless aggregates. However, it is more difficult in that case to envision how one β-sheet rich PrP^{Sc} subunit might influence a PrP^{C} molecule to adopt a PrP^{Sc} conformation. In any case, it seems obvious that, whether well-structured stacks or random aggregates, the larger PrP^{Sc} oligomers become more protected from PK digestion.

A property of PrP^{Sc} that also shows, in our hands, a significant dependence on size is conformation-dependent immunoreactivity. With regard to this, $sPrP^{Sc}$ exhibited a distinct enhancement of immunoreactivity after guanidine hydrochloride treatment (Figure 6); however, such enhancement was significantly lower than that of $rPrP^{Sc}$, made up, as discussed, of larger polymers. This is in agreement with Tzaban et al. (*12*), who showed a decrease in conformational-dependent immunoreactivity in the lighter fractions of brain homogenates subjected to sucrose gradient fractionation.

Our results confirm that $sPrP^{Sc}$ is able to convert PrP^{C} into PrP^{Sc}. Fractions of PrP^{Sc} comprising oligomers of small relative size were as effective as larger polymers to convert PrP^{C} into PrP^{Sc} in the PMCA assay. This result strongly suggests that $sPrP^{Sc}$ is indeed *bona fide* PrP^{Sc} and not an off-route molecular species along the pathway that leads from PrP^{C} to a putative "full-fledged" PrP^{Sc} (*14*). Studies aimed at determining whether $sPrP^{Sc}$ is infectious and what the kinetics of its accumulation *in vivo* look like are in process. A recent study showed that the most infectious fraction of solubilized PrP 27–30 corresponds to relatively small oligomers comprising 14–28 PrP molecules; smaller and larger aggregates show reduced specific infectivity and, beyond certain given size thresholds, are not infectious at all (*30*). *In vitro* converting capacity also peaked at the same intermediate size. Even though extreme caution must be exercised when comparing results obtained with PrP^{Sc} and those obtained with MW markers, our data suggest that the material present in our loading fraction (fraction 0, Figure 4) corresponds to oligomers with very low molecular weights; a direct interpolation would suggest that they contain less than 12 PrP subunits. Thus, our data suggest that even

such low molecular weight oligomers are capable of initiating prion replication.

The susceptibility of sPrP^{Sc} to virtually complete degradation by trypsin opens the possibility of using limited proteolysis as a tool to probe its structure. This approach has been successfully used to obtain structural information on a variety of proteins that were not amenable to other techniques (*31*−*33*). Lysine and arginine residues that are accessible (solvent-exposed) and do not participate in strong ionic interactions are more easily cleaved by trypsin. The results described in Figure 7 constitute an example of the applicability of this strategy to PrP^{Sc}. Thus, comparison of yields of specific cleavage products obtained from sPrP^{Sc} and recombinant PrP after application of increasing amounts of trypsin allows one to conclude that residues R136, R151, K220, and R229 are particularly susceptible to cleavage in sPrP^{Sc}. Other residues, such as K110, R148, R156, R164, K185, and K194 are partially cleaved as well, but this requires larger amounts of trypsin. In contrast, evidence of cleavage of all these residues in recombinant SHaPrP (90−231) is seen even at the lowest concentration of trypsin tested (trypsin/substrate ratio of 1:400). A similar analysis of the rPrP^{Sc} showed an overall pattern that was very similar to that of sPrP^{Sc}, although with much lower yields of peptide fragments (data not shown). In a recent model of mouse PrP 27−30 based on a left-handed β-helical core (*7*), residue R150, equivalent to hamster R151, is located in a loop protruding from the central β-helical core, which would be in good agreement with accessibility of this residue; residues K219 and R228, equivalent to hamster K220 and R229, are located in the carboxy-terminal α-helix, again, allowing good accessibility, provided there is no hindrance from carbohydrates attached to PrP^{Sc} in the carboxy-terminal region. However, R135, equivalent to hamster R136, is placed in the middle of the β-helical assembly, a location that theoretically would not facilitate access of trypsin. It should be noted that an exhaustive analysis of cleavage patterns is complicated by the fact that in PrP^{Sc} several of the possible products are glycosylated, which renders them impossible to detect with the methods used. Additional and more extensive studies, including time course experiments, will provide structural information that, together with data obtained with other techniques, such as chemical cross-linking (*11*) and surface labeling studies, will allow testing of the structural models proposed thus far and the design of new models that can explain the physicochemical data obtained.

To summarize, we have developed an easy method to isolate a PK-sensitive fraction of PrP^{Sc}. A first survey of the biochemical properties of this material, composed by a light fraction of PrP^{Sc} oligomers, shows that it has prion-converting activity and that it is susceptible to digestion by specific proteolytic enzymes, such as trypsin. This material should prove useful to study fundamental aspects of the biochemistry of PrP^{Sc}.

ACKNOWLEDGMENT

We are grateful to Emilio Nogueira from the Department of Physiology, University of Santiago de Compostela, for outstanding technical advice. We also thank Alejandro Brun and José A. Rodríguez from the CISA, Madrid, for help with hamster inoculations.

SUPPORTING INFORMATION AVAILABLE

Figures showing the presence of PrP in supernatants and pellets of a healthy hamster brain homogenate subjected to the PrP^{Sc} isolation procedure, consistent location of PrP^{Sc} fractions in sucrose gradient sedimentation, examples of an extracted ion chromatogram (XIC), and an MS/MS spectrum corresponding to a tryptic peptide of sPrP^{Sc}. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Prusiner, S. B. (1982) Novel proteinaceous infectious particles cause scrapie, *Science 216*, 136−144.
2. Prusiner, S. B. (1998) Prions, *Proc. Natl. Acad. Sci. U.S.A. 95*, 13363−23383.
3. Soto, C., and Castilla, J. (2004) The controversial protein-only hypothesis of prion propagation, *Nat. Med. Suppl. 7*, S63−67.
4. Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., Cohen, F. E., DeArmond, S. J., and Prusiner, S. B. (2004) Synthetic mammalian prions, *Science 305*, 673−676.
5. Castilla, J., Saa, P., Hetz, C., and Soto, C. (2005) In vitro generation of infectious scrapie prions, *Cell 121*, 195−206.
6. Caughey, B. (2003) Prion protein conversions: insight into mechanisms, TSE transmission barriers and strains, *Br. Med. Bull. 66*, 109−120.
7. Govaerts, C., Wille, H., Prusiner, S. B., and Cohen, F. E. (2004) Evidence for assembly of prions with left-handed beta-helices into trimers, *Proc. Natl. Acad. Sci. U.S.A. 101*, 8342−8347.
8. DeMarco, M. L., and Daggett, V. (2004) From conversion to aggregation: protofibril formation of the prion protein, *Proc. Natl. Acad. Sci. U.S.A. 10*, 12293−12298.
9. Stork, M., Giese, A., Kretzschmar, H. A., and Tavan, P. (2005) Molecular dynamics simulations indicate a possible role of parallel beta-helices in seeded aggregation of poly-Gln, *Biophys. J. 88*, 2442−2451.
10. Wille, H., Michelitsch, M. D., Guénebaut, V., Supattapone, S., Serban, A., Cohen, F. E., Agard, D. A., and Prusiner, S. B. (2002) Structural studies of the scrapie prion protein by electron crystallography, *Proc. Natl. Acad. Sci. U.S.A. 99*, 3563−3568.
11. Onisko, B., Fernandez, E. G., Freire, M. L., Schwarz, A., Baier, M., Camiña, F., Garcia, J. R., Rodriguez-Segade Villamarin, S., and Requena, J. R. (2005) Probing PrP^{Sc} structure using chemical cross-linking and mass spectrometry: evidence of the proximity of Gly90 amino termini in the PrP 27-30 aggregate, *Biochemistry 44*, 10100−10109.
12. Tzaban, S., Friedlander, G., Schonberger, O., Horonchilk, L., Yedidia, Y., Shaked, G., Gabizon, R., and Taraboulos, A. (2002) Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes, *Biochemistry 41*, 12868−12875.
13. McKinley, M. P., Meyer, R. K., Kenaga, L., Rahbar, F., Cotter, R., Serban, A., and Prusiner, S. B. (1991) Scrapie prion rod formation in vitro requires both detergent extraction and limited proteolysis, *J. Virol. 65*, 1340−1351.
14. Safar, J., Wille, H., Itrri, V., Groth, D., Serban, H., Torchia, M., Cohen, F. E., and Prusiner, S. B. (1998) Eight prion strains have PrP^{Sc} molecules with different conformations, *Nat. Med. 4*, 1157−1165.
15. Safar, J. G., Geschwind, M. D., Deering, C., Didorenko, S., Sattavat, M., Sanchez, H., Serban, A., Vey, M., Baron, H., Giles, K., Miller, B. L., DeArmond, S. J., and Prusiner, S. B. (2005) Diagnosis of human prion disease, *Proc. Natl. Acad. Sci. U.S.A. 102*, 3501−3506.
16. Diringer, H., Beekes, M., Özel, M., Simon, D., Queck, I., Cardone, F., Pocchiari, M., and Ironside, J. W. (1997) Highly infectious purified preparations of disease-specific amyloid of transmissible spongiform encephalophathies are not devoid of nucleic acids of viral size, *Intervirology 40*, 238−246.
17. Bolton, D. C., Bendheim, P. E., Marmorenstein, A. D., and Potempska, A. (1987) Isolation and structural studies of the intact scrapie agent protein, *Arch. Biochem. Biophys. 258*, 579−590.

18. Caughey, B., Raymond, G. J., Priola, S. A., Kocisko, D. A., Race, R. E., Bessen, R. A., Lansbury, P. T., Jr., and Chesebro, B. (1999) Methods for studying prion protein (PrP) metabolism and the formation of protease-resistant PrP in cell culture and cell-free systems, *Mol. Biotechnol. 13*, 45–55.
19. Beekes, M., Baldauf, E., Cassens, S., Diringer, H., Keyes, P., Scott, A. C., Wells, G. A. H., Brown, P., Gibbs, C. J., Jr., and Gajdusek, D. C. (1995) Western blot mapping of disease-specific amyloid in various animal species and humans with transmissible spongiform encephalopathies using a high-yield purification method, *J. Gen. Virol.* 76, 2567–2576.
20. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680–685.
21. Serban, D., Taraboulos, A., DeArmond, S. J., and Prusiner, S. B. (1990) Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins, *Neurology 40*, 110–117.
22. Saborio, G. P., Permanne, B., and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding, *Nature 411*, 810–813.
23. Castilla, J., Saá, P., and Soto, C. (2004) Cyclic Amplification of Prion Protein Misfolding, in: *Techniques in Prion Research* (Lehman, S., and Grassi, J., Eds.), pp 198-213, Birkhouser, Basel.
24. Saá, P., Castilla, J., and Soto, C. (2004). Cyclic Amplification of Prion Protein Misfolding, in *Amyloid Proteins: Methods and Protocol.* (Sigurdsson, E. M., Ed.) pp 53–65, Humana Press, Totowa, NJ.
25. Riesner, D., Kellings, K., Post, K., Wille, H., Serban, A., Groth, D., Baldwin, M. A., and Prusine, r S. B. (1996). Disruption of prion rods generates 10-nm spherical particles having high α-helical content and lacking scrapie infectivity, *J. Virol. 70*, 1714–1722.
26. Chiesa, R., Piccardo, P., Quaglio, E., Drisaldi, B., Si-Hoe, S. L., Takao, M., Ghetti, B., and Harris, D. A. (2003) Molecular distinction between pathogenic and infectious properties of the prion protein, *J. Virol. 77*, 7611–7622.
27. Lasmezas, C. I., Deslys, J. P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J. M., Fournier, J. G., Hauw, J. J., Rossier, J., and Dormont, D. (1997) Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein, *Science 275*, 402–405.
28. Brown, P., Gibbs, C. J., Rodgers-Johnson, P., Asher, D. M., Sulima, M. P., Bacote, A., Goldfarb, L. G., and Gajdusek, D. C. (1994) Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease, *Ann. Neurol. 35*, 513–529.
29. Come, J. H., Fraser, P. E., and Lansbury, P. T., Jr. (1993) A kinetic model for amyloid formation in the prion diseases: Importance of seeding, *Proc. Natl. Acad. Sci. U.S.A. 90*, 5959–5963.
30. Silveira, J. R., Raymond, G. J., Hughson, A. G., Race, R. E., Sim, V. L., Hayes, S. F., and Caughey, B. (2005) The most infectious prion protein particles, *Nature 437*, 257–261.
31. Hubbard, S. J. (1998) The structural aspects of limited proteolysis of native proteins, *Biochim. Biophys. Acta 1382*, 191–206.
32. Kheterpal, I., Williams, A., Murphy, C., Bledsoe, B., and Wetzel, R. (2001) Structural features of the Aβ amyloid fibril elucidated by limited proteolysis, *Biochemistry 40*, 11757–11767.
33. Neurath, H. (1980) Limited Proteolysis, Protein Folding and Physiological Regulation, in *Protein Folding* (Jaenicke, R., Ed.) pp 501–523, Elsevier, Amsterdam, The Netherlands.

BI0615442