FACULTAD DE CIENCIAS
UNIVERSIDAD DE CHILE

PARTICIPACION DEL HIERRO EN LA PATOGENESIS DE LA ENFERMEDAD DE ALZHEIMER

Memoria de título entregada a la Universidad de Chile
en cumplimiento parcial de los requisitos para optar
al título de Ingeniero en Biotecnología Molecular

Por

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Julio 2004
Santiago-Chile

Director de tesis: Dr. Ricardo B. Maccioni Baraona

ESCUELA DE PREGRADO – FACULTAD DE CIENCIAS – UNIVERSIDAD DE CHILE

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Santiago de Chile, 26 de Julio de 2004

A mis padres, mis hermanos, la Paula y el Simón

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LISTA DE ABREVIATURAS

aa	Aminoácido.
Alzh-A	Alzheimer avanzado.
Alzh-T	Alzheimer temprano.
APP	Proteína precursora de amiloide.
BSA	Albúmina de suero bovino.
DC-A	Demencia cuestionable avanzada.
DC-T	Demencia cuestionable temprana.
DNA	Ácido desoxirribonucleico.
EA	Enfermedad de Alzheimer.
EAA	Espectrometría de absorción atómica.
EDTA	Acido etildiaminotetracético.
EE	Error estándar
EROs	Especies reactivas del oxigeno.
H_2O_2	Peróxido de Hidrogeno.
HBS	Suero fetal de caballo.
KDa	Kilo Daltons.
LCR	Liquido Cefaloraquideo.
MAP	Proteína asociada a los microtúbulos.
N	Sujetos normales (en los gráficos).
NFT	"Neurofibrilary tangles".
NO	Oxido nítrico.
ONF	Ovillos neurofibrilares.

PBS	Buffer fosfato salino.
PS	Placas seniles.
Ser	Serina.
SOD	Superóxido dismutasa.
SP	"Senile Plaque".
Tf	Transferrina.
Thr	Treonina.
µl	micro Litro.
µM	micro Molar.
4-HNE	4-Hidroxinonenal.

RESUMEN

La enfermedad de Alzheimer (EA) es una patología multifactorial que, de acuerdo a recientes investigaciones, se genera como resultado de una serie de cambios moleculares y celulares que incluyen la formación de agregados patológicos del péptido β-amiloide, factores pro inflamatorios liberados por la glia activada y el estrés oxidativo. Todos estos cambios involucran una susceptibilidad genética, aunque el Alzheimer es una enfermedad de origen esporádico en donde sólo un 3-4% de los casos obedece a mutaciones en determinados genes como las presenilinas 1 y 2, la proteína APP y otros. Recientes estudios indican que existe clara evidencia que la carga celular oxidativa, y en general el estrés oxidativo constituye uno de los principales elementos de daño a la célula neuronal en la patogénesis de la EA. En este contexto, la acumulación de hierro en ciertas regiones del cerebro junto con aumentos en las concentraciones plasmáticas de este metal, han sido fuertemente involucrados en la generación de daño oxidativo en diversas patologías neurodegenerativas como la enfermedad Parkinson, la esclerosis lateral amiotrófica y la EA. En la EA, el hierro se ha encontrado asociado a los dos marcadores histopatológicos más importantes: las placas seniles (PS) formadas por agregados extraneuronales del péptido β-amiloide y los ovillos neurofibrilares (ONF), compuestos principalmente por agregados citoplasmáticos de proteína tau hiperfosforilada.

En la primera parte de esta tesis presentamos los resultados de los efectos del tratamiento de neuronas de hipocampo de rata con hierro. Demostramos que el hierro se acumula en neuronas de manera dependiente de la concentración del metal y el tiempo de exposición a este, provocando un aumento en marcadores de daño oxidativo.

Interesantemente, el tratamiento de estas células con hierro y peróxido de hidrogeno (H_2O_2) indujo una disminución en los niveles de fosforilación de la proteína tau en epítopos del tipo Alzheimer. Esto posiblemente debido a cambios en los equilibrios en la actividad de proteínas quinasas y fosfatasas. Estos cambios no se observaron en estudios realizados en sistemas de co-cultivos neurona/glia, lo cual sugiere que las células gliales juegan un rol crítico en la modulación de este proceso.

En la segunda parte de esta tesis, mostramos que en liquido cefaloraquídeo (LCR) de sujetos seniles con diferentes grados de demencia, evaluados en un estudio longitudinal llevado a cabo en nuestro laboratorio en colaboración con el Dpto. de Ciencias Neurológicas de la Facultad de Medicina de la Universidad de Chile , se observabó una estrecha co-relación entre los niveles de hierro redox activo y el grado de deterioro cognitivo en estos sujetos. Estos hallazgos pueden ser de gran relevancia, y apuntan hacia la participación de los cambios en la homeostasis del hierro en la patogénesis de la EA, como factor clave que guíe el proceso neurodegenerativo.

ABSTRACT

Alzheimer disease (AD) corresponds to a multifactorial brain disorder, since many factors are involved in its pathogenesis. In agreement with recent findings, AD is generated by several molecular and cellular alterations including the abnormal aggregation of β-amiloid peptide, pro-inflammatory signals by activated glial cells and oxidative stress. In this context, most of AD cases are sporadic, even though cellular changes involve genetic susceptibility. The familial AD accounts for only 3-4 % of the cases, and it is related with mutations in genes like Presenilins 1 or 2, APP and others.

Recent studies provide strong evidence suggesting that cellular oxidative load in general constitutes one of the main stresses in neuronal cells in AD. In this context, iron accumulation in some brain areas together with high levels of this metal in the plasma has been strongly related to the production of oxidative damage in several neurodegenerative disorders, such as Parkinson disease, amyotrophic lateral sclerosis and AD. In AD, iron has been found as a constituent of the two most important histopathological markers: senile plaques (SP) mainly formed by an extraneuronal aggregation of β-amiloide peptide, and the neurofibrillary tangles (NFT) composed by aggregation of hyperphosphorylated tau protein.

In the first part of this thesis, we analyze the effects of iron treatments on rat hippocampal neurons. We show that iron accumulation in neurons depends of the concentration of this metal in the culture media, and on the exposure time of neurons to iron. Iron induces an increase in markers of oxidative damage. Interestingly, the treatments of neurons with iron or hydrogen peroxide induce a decrease in the tau phosphorylation in

epitopes characteristics from Alzheimer's disease. This decrease could be due to alterations in the balance between phosphatase and kinase activities in the neuron. These alterations were not observed when neurons were co-cultured with glial cells. This fact suggests that glial cells could play a pivotal role in modulating this process.

In the second part of this thesis, we showed that in cerebrospinal fluid (CSF) samples, obtained from elderly subjects with different degrees of dementia, evaluated in the framework of a longitudinal study carried by our laboratory in collaboration with the Neurology Department of the Medical School. There is a high correlation between active redox iron in CSF and the degree of cognitive damage within the analyzed groups. These findings could be very important in order to evaluate potential biological markers for the disease. Indeed, those alterations in iron homeostasis could be a key factor in the neurodegenerative process leading to AD.

I.- INTRODUCCION

La EA es la causa mas común de las demencia en personas mayores de 65 años (Maccioni y cols. 2001b). La EA constituye un problema psicosocial de gran relevancia además, debido a que es una de las enfermedades más costosas en las sociedades modernas y constituye un grave problema de salud pública por su impacto negativo en la economía de los países. Es por ello que se ha impulsado de manera notable el desarrollo de la investigación científica que permita explicar la etiopatogénesis de esta enfermedad. Uno de los mayores factores de riesgo lo constituye la edad de los pacientes, y en este contexto la esperanza de vida, aún en países en vías de desarrollo, ha incrementado de manera notable en las últimas dos décadas.

Además de la edad, numerosos factores de riesgo han sido descritos para explicar la etiología de la EA, incluyendo los efectos inflamatorios del peptido β-amiloide, sobreproducción de citoquinas secretados por la glia, NO, etc. Sin embargo ninguno de éstos por si solo es capaz de dar cuenta del proceso neurodegenerativo en su totalidad. En este contexto, es claro que de todos los factores de riesgo para EA, el envejecimiento es el factor que mejor se correlaciona con la incidencia de esta enfermedad, lo cual se observa en la Figura 1.

Figura 1. Incidencia de la EA respecto a la edad.

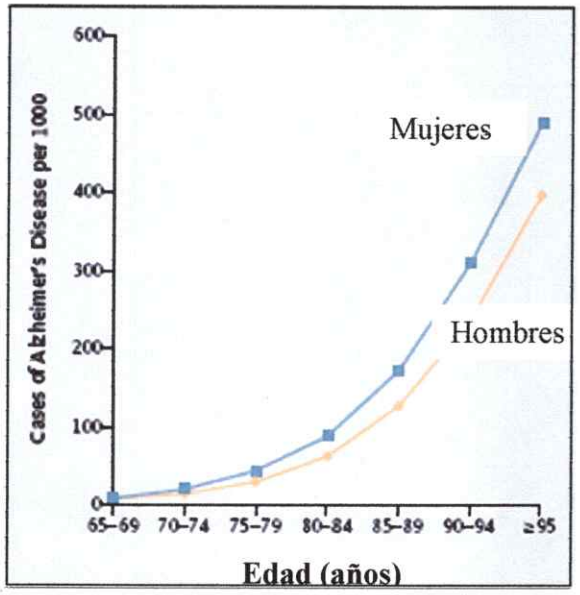

Fig. 1: En esta figura se muestra una correlación exponencial entre la EA y la edad para ambos sexos. Los valores se presentan como casos de enfermos de Alzheimer por cada 1000 personas. (Nussbaum y Ellis, 2003)

Numerosas teorías han sido desarrolladas para dar cuenta del porque ocurre el envejecimiento celular. La más aceptada hasta la fecha, es la teoría del envejecimiento por estrés oxidativo, que postula que en todas las células del cuerpo humano, se generan radicales libres, los cuales son capaces de oxidar diferentes componentes celulares como proteínas, lípidos y DNA. Esto genera daño oxidativo, el cual se acumula en el tiempo y puede inducir disfunción celular en los diferentes tejidos del cuerpo. El estrés oxidativo puede ser definido como una pérdida en el balance entre los sistemas de producción de especies reactivas de oxigeno (EROs) y la maquinaria celular antioxidante. Como resultado final de este estrés, se afecta la integridad celular. Este fenómeno es particularmente importante en neuronas ya que estas son células postmitoticas altamente diferenciadas. Debido a ello, tienen un tiempo de vida mucho mayor al que tienen la gran mayoría de las otras células del cuerpo, con lo cual las neuronas acumulan daño

oxidativo durante toda la vida. Esto las hace especialmente sensibles a factores que alteren el balance redox celular, por esto el estrés oxidativo ha sido fuertemente involucrado en la génesis de diversas patologías neurodegenerativas. Esto se ha evidenciado tanto en humanos como en modelos de animales, que muestran desórdenes cerebrales (Markesberry y cols 1997; Butterfield y cols. 2000) Durante los últimos años, un numero cada vez mayor de investigaciones se han centrado en estudiar la relación entre la presencia de metales de transición y los desórdenes neurodegenerativos (Lynch y cols. 2000; Qian y Shen 2001). Estos se han enfocado a analizar el rol del Fe, Cu, Mn y Zn en la producción de especies reactivas de oxigeno o nitrógeno y sugieren fuertemente que los metales de transición redox-activos son mediadores críticos en la generación de daño oxidativo en diversas patologías. Una contribución importante a este tema ha derivado de esta tesis, en cuya investigación logramos identificar al hierro como un agente que promueve procesos neurodegenerativos en neuronas, mediado por daño oxidativo (Egaña y cols. 2003; Zambrano y cols. 2004)

Numerosos trastornos neurodegenerativos involucran procesos de agregación de proteínas, tal como ocurre en las enfermedades de Huntington (Deckel 2001; Sayre y cols. 2001), esclerosis lateral amiotrófica (Estévez y cols. 1999), Creutzfeld-Jacob (Brown 2001), Parkinson (Goedert, 2001), demencia frontotemporal y la EA (Smith y cols. 2000; Maccioni y cols. 2001a, b). En esta última ha sido bien caracterizada la agregación extraneuronal del péptido β-amiloide que forma las PS la agregación a nivel citoplasmático de la proteína tau, que lleva a la formación de los ONF. Los orígenes de

estos procesos de agregación proteica no han sido esclarecidos hasta la fecha y su relación con el daño oxidativo es aun desconocida.

1.1 La proteína tau.

La proteína tau pertenece a la familia de las MAP´s o Proteínas Asociadas a los Microtúbulos. En humanos, ésta se encuentra casi exclusivamente en neuronas y se presenta en 6 isoformas, las que derivan de la expresión de un solo gen. Este gen se encuentra en el brazo largo del cromosoma 17 en la posición 21 (17q21) y contiene 13 exones, los cuales por un proceso de corte y empalme alternativo generan 6 isoformas, como se observa en la Figura 2.

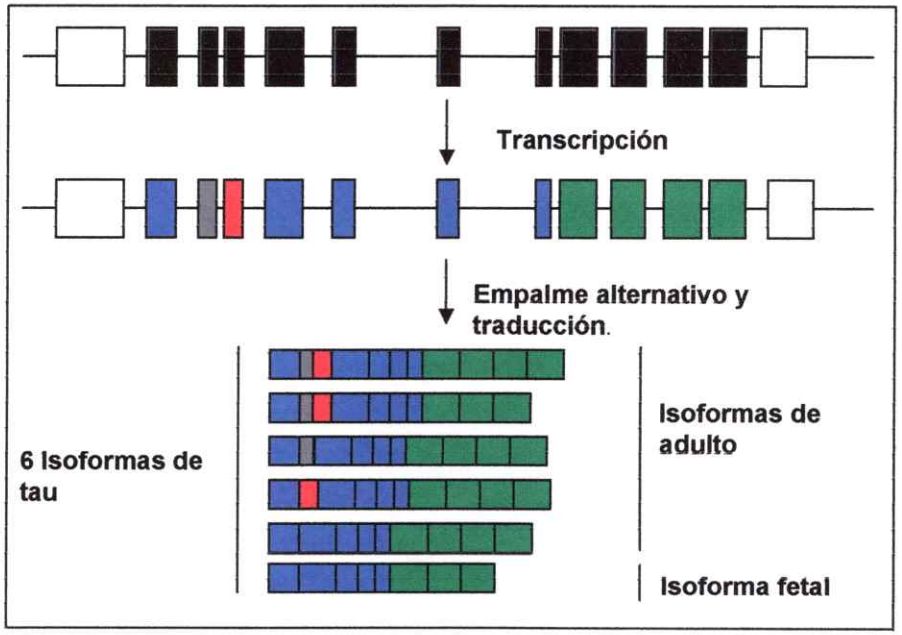

Fig. 2, En esta figura se muestra un esquema en donde se observa que mediante un proceso de corte y empalme alternativo se generan las 6 isoformas de tau. En azul se muestran los exones constitutivos; en gris o en rojo, los exones 2 y 3 que sufren corte y empalme y en verde (exón 10) los exones que codifican para los dominios con secuencias repetitivas de unión a microtúbulos (Maccioni y cols., 1989).

En el esquema de la Figura 2 se sintetiza la información sobre la estructura del gen de tau, y la expresión en las seis isoformas encontradas en cerebro humano. Producto de este proceso, las 6 isoformas que se generan van desde los 45 kDa hasta los 65 kDa, las cuales se expresan diferencialmente durante el desarrollo y pueden, además encontrarse distribuidas en diferentes subpoblaciones neuronales. (Kosik y cols. 1989). La región N-Terminal de la proteína tau es de longitud variable, dependiendo de si la isoforma presenta o no los exones 2 y/ó 3 (Figura 3). Esta región se conoce como dominio de proyección, debido a que una vez que la proteína tau interactúa con los microtúbulos se proyecta desde ellos y en estos dominios es capaz de interactuar con otros elementos del citoesqueleto como filamentos de actina o espectrina (Cross y cols., 1993; Farias y cols. 2002; Carlier y cols. 1984) o puede interactuar con la membrana plasmática. (Brandt y cols. 1995). Por otro lado en el dominio carboxilo terminal, se ubican los dominios de unión a microtúbulos los cuales se presentan en 3 o 4 copias y corresponden a una secuencia de 18 aminoácidos altamente conservados, los que se encuentran separados por regiones de alrededor de 13 aminoácidos (Maccioni y cols., 1989). Este dominio es el encargado del ensamblaje y estabilización de los microtúbulos (Mandelkow y cols. 1995) y además se ha demostrado su asociación con otras proteínas. En el siguiente cuadro (Fig. 3) se esquematiza la estructura general de la isoforma mayor de la proteína tau 42, de 441 aa.

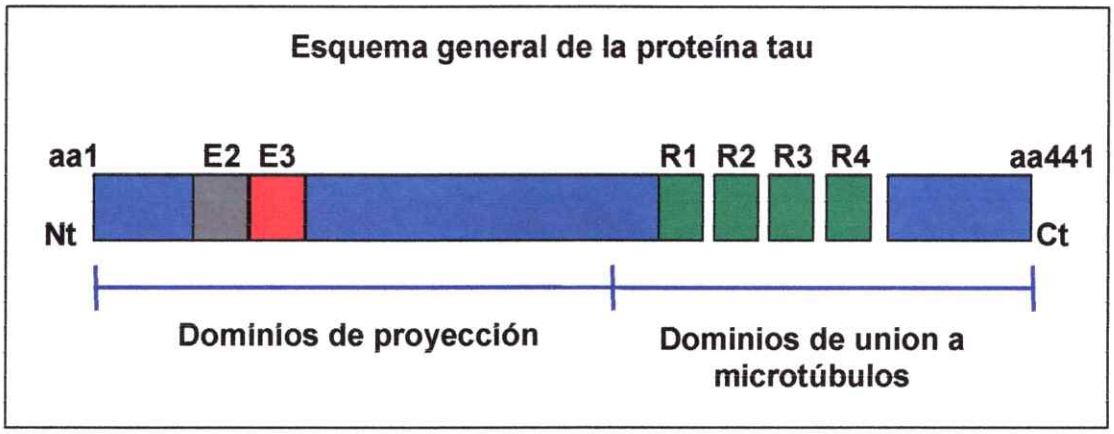

Fig. 3, En esta figura se esquematiza la isoforma mayor de la proteína tau, tau 42. Hacia el extremo amino terminal se muestra el dominio de proyección y hacia el carboxilo terminal el dominio de unión a microtúbulos.

A pesar de que la proteína tau es capaz de sufrir una serie de modificaciones postraduccionales, la fosforilación de juega un rol particularmente importante. La isoforma mayor de tau, tau 42 presenta 79 serinas o treoninas que actúan como potenciales sitios de fosforilacion. Debido a ello, la actividad combinada de diferentes proteínas quinasas y fosfatasa, que pueden actuar sobre las distintas isoformas de tau, pueden generar un alto número de estados estructurales en esta proteína conteniendo diferentes niveles de fosforilación en cada uno de estos residuos y en cada isoforma. Así el equilíbrio entre los finos mecanismos de fosforilación por proteínas quinasas, y de desfosforilación por proteínas fosfatasas, modulan finalmente un estado estructural de tau que en suma define su nivel de actividad. Es interesante hacer notar que una proteína como tau tan crucial para definir la polaridad de las neuronas y los procesos de transporte, generación de conos de crecimiento en el desarrollo axonal, etc., requiere ser modulada por mecanismos muy finos para que ésta cumpla de manera controlada su función. Así, cambios importantes en esta regulación pueden hacer cambiar su capacidad

para unirse a microtúbulos o a otros elementos del citoesqueleto, ó contribuir a generar condiciones patológicas como las observadas en la degeneración del tipo Alzheimer.

1.2 Proteína tau, cdk5 y enfermedad de Alzheimer

La enfermedad de Alzheimer es una de las enfermedades neurodegenerativas más comunes que afecta a las personas sobre los 65 años de edad y se manifiesta, en un comienzo por perdida en la memoria y luego afasia, agnosia, apraxia y alteraciones conductuales en general. Histopatológicamente se caracteriza por presentar formaciones de agregados proteicos tanto extra como intraneuronalmente, los que se conocen como PS y los ONF respectivamente. Las PS están compuestas principalmente de fibras de β-amiloide, que se originan por la agregación anómala de un péptido derivado de la proteolisis de la proteína precursora de amiloide (APP) la que se ubica en la membrana celular. Por otra parte, los ONFs están compuestos principalmente de proteína tau que se encuentra hiperfosforilada (Maccioni y cols., 2001b). Además, se ha sugerido que en el Alzheimer podrían ocurrir otros cambios sobre esta proteína, como son la ubiquitinación (Yang y Ksiezak-Reding. 1998), unión a proteoglicanos (Su y cols. 1992) y glicación (González y cols. 1998), los que también contribuirían a la formación de los ONF.

Se ha involucrado a las MARK quinasa, gsk3β, sistemas de MAP-quinasa y la proteína quinasa dependiente de ciclina cdk5 en la fosforilación de la proteína tau. Esta última es ubícua en el organismo y se ha detectado actividad solo en neuronas post-mitóticas, esto debido a que solo en ellas se expresan sus activadores p35 y p39 (Kesavapany y cols. 2003). La proteína cdk5 está directamente ligada, entre otros

fenómenos, a procesos de diferenciación y desarrollo neuronal (Homayouni y Curran. 2000) y en la migración neuronal (Ohshima y cols. 1996). Además se ha demostrado que es la proteína clave que mediaría la neurodegeneracion inducida por el péptido β amiloide. (Alvarez y cols. 1999, 2001)

En el caso de la fosforilación anómala de la proteína tau, el sistema cdk5/p35 hiperfosforila los residuos Ser^{202}, Thr^{205}, Ser^{235} y Ser^{404}, los que se ha demostrado que son independientes de la actividad de otras quinasas. (Madelkow y cols. 1995)

1.3 Hierro, estrés oxidativo y enfermedad de Alzheimer

Todos los organismos aeróbicos producen especies reactivas de oxigeno (EROs) como producto lateral de la cadena transportadora de electrones en la mitocondria. Sin embargo este daño oxidativo intracelular puede ser exacerbado por diversos factores, dentro de los que destacan, el aumento en la demanda celular de oxigeno y la presencia de metales redox activos. Estos metales son capaces de catalizar la producción de EROs por vía de la reacción de Fenton, la cual se esquematiza en el siguiente cuadro.

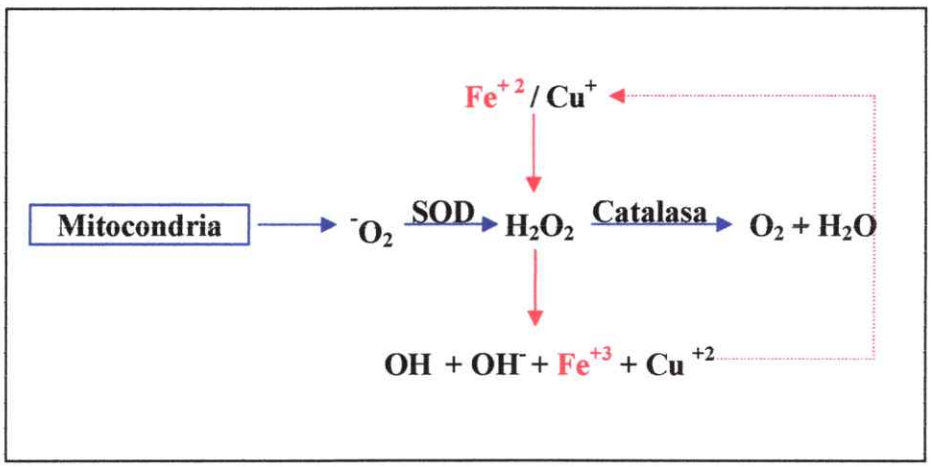

Fig. 4, En esta figura se muestra que el Fe y el Cu, por vía de la reacción de Fenton, pueden catalizar la producción del radical hidroxilo a partir de Peroxido de hidrogeno, generando daño oxidativo.

Como se puede ver en la Figura 4 tanto el Fe^{+2} como el Cu^{+} son capaces de reaccionar, cataliticamente, con el H_2O_2 para generar el radical hidroxilo ($OH^{\bullet}$), una de las especies mas reactivas que existe en la naturaleza, (Haupman y Cardenas, 1999; Symons y Gutteridge, 1998) oxidando a los distintos componentes celulares. Este radical ($OH^{\bullet}$) en ausencia de estos metales, no se genera en cantidades significativas debido a que la proteína Superóxido Dismutasa (SOD), cataliza la reacción de desdoblamiento del H_2O_2 en $H_2O + O_2$. De esta manera si aumentan las concentraciones de estos metales libres en el citoplasma aumenta también, la generación del radical hidroxilo (Symons y Gutteridge 1998).

El interés por el estudio de los metales, en particular el hierro y el cobre ha crecido exponencialmente durante los últimos años, debido, principalmente a que han sido fuertemente vinculados con patologías neurodegenerativas (Smith y cols. 1997). Estos metales son esenciales para una gran variedad de procesos celulares sin embargo, alteraciones en su homeostasis provocan graves consecuencias para la célula, incluidos fenómenos de neurotoxicidad (Kuperstein y Yavin, 2003; Liu y cols. 2003). Muchas de estas alteraciones en la homeostasis resultan en daño oxidativo debido a un incremento en la producción de especies reactivas de oxigeno (Sayre y cols. 2001).

Debido a que se ha descrito una acumulación de hierro cerebral en el tiempo, este proceso ha sido vinculado con envejecimiento (Zecca y cols. 2001) Además, en diversas patologías neurodegenerativas el hierro se ha encontrado acumulado en diferentes sitios. En la enfermedad de Parkinson el hierro se acumula en la sustancia nigra (Sofic y cols. 1988, 1991), en la enfermedad de Huntington en el estriado (Dexter y cols. 1991;

Bartzokis y cols. 1999) en patologías como Hallervorden-Spatz se acumula en el globus palídus (Hallíday. 1995) y en la EA se encuentra en los dos marcadores histopatológicos más importantes de la enfermedad: las PS y los ONF (Jellinger y cols 1990; Connor y cols. 1992). En general, no es claro si estas acumulaciones juegan un papel relevante en el desarrollo de la patología o son simplemente una consecuencia de esta, sin embargo por la naturaleza redox activa del hierro es probable que este metal juegue un rol importante en la generación de estrés oxidativo en estas regiones. En el caso particular de la EA, se ha observado que sujetos hemocromatóticos desarrollan EA mas tempranamente que sujetos normales (Sampietro y cols 2001) lo cual es una información importante que apoya los hallazgos sobre la contribución en la desregulación de la homeostasis del hierro en la patogénesis de la EA. Además, se ha descrito que en muestras de plasma de pacientes de EA se encuentran elevados los niveles de hierro (Ozcankaya y Delibas, 2002) y cobre (Squitti y cols 2002). Debido a la numerosa evidencia existente se ha sugerido que la acumulación de metales redox-activos podrían ser el elemento que contribuye con mayor importancia a la producción local de daño oxidativo mediado por EROs (Sayre y cols. 2001).

Los puntos planteados anteriormente nos llevan a formular las siguientes hipótesis de trabajo, las que constituyen la base para los diseños experimentales que se desarrollan mas abajo utilizando dos modelos de estudio: células cerebrales en cultivo por una parte y muestras de líquido cefaloraquídeo de pacientes, en el marco de un estudio longitudinal realizado con el Departamento de Ciencias Neurológicas de la Facultad de Medicina.

1.- "La acumulación citoplasmática de hierro en neuronas, provoca un aumento en la producción de especies reactivas de oxigeno. Este aumento induce un cambio en el balance redox neuronal que afecta a los estados de fosforilación de la proteína tau."

2.- "Muestras de liquido cefaloraquídeo de ancianos con distintos grados de demencia presentan alteraciones en el hierro circulante"

1.4 OBJETIVOS

Objetivo general

Estudiar alteraciones del hierro a nivel neuronal y sistémico que permitan explicar aspectos histopatológicos de la enfermedad de Alzheimer.

Objetivos específicos

1.- Realizar un estudio de la incorporación de hierro en cultivos primarios de células de hipocampo. Este objetivo, así como los otros objetivos en cultivos celulares, se desarrollaron en cultivos primarios de neuronas de hipocampo de embrión rata E18.5 y se analizaron de al menos 2 experimentos independientes.

2.- Estudiar las posibles alteraciones morfológicas inducidas por tratamientos con hierro. utilizando técnicas de inmunocitoquímica con marcadores específicos del citoesqueleto.

3.- Analizar la posible inducción de daño oxidativo gatillado por hierro utilizando anticuerpos anti 4-hidroxinonenal como marcador de daño oxidativo en ensayos de *Western-blots.*

4.- Analizar la acción del hierro sobre la alteración en los estados de fosforilación de la proteína tau utilizando anticuerpos específicos para fosfoepitopos de tau. Como control se investigó también los efectos sobre la proteína tau de otro estímulo pro-oxidante (H_2O_2) en sistemas de cultivos primarios de hipocampo.

5.- Analizar los posibles efectos moduladores de la glía en respuesta a estímulos con hierro, evaluados en sistemas de co-cultivos neurona/glía.

6.- Estudiar, por espectroscopia de absorción atómica, posibles alteraciones en los niveles totales de hierro y en el hierro readox activo, en muestras de LCR de sujetos seniles con diferentes grados de deterioro cognitivo y pacientes con la EA.

II. MATERIALES Y METODOS

2.1 Cultivos primarios de hipocampo: Los cultivos de neuronas hipocampales fueron obtenidos a partir de embriones de rata E18.5 (Banker y Cowan, 1977). Después de sacrificar ratas preñadas en estado E18.5, se obtienen los embriones, se separa en tejido cerebral y los hipocampos son disectados, para luego ser incubados en 0.25% tripsina-EDTA durante 10 minutos a 37 °C. Posteriormente, el tejido es lavado con una solución de HBS y disgregado para ser sembrado sobre poli-L-lisina a una densidad de 5000 células cm^2 para inmunofluorescencia y 15000 células cm^2 para estudios de *Western-blot*. Los cultivos fueron mantenidos en medio MEM suplementado con 10% suero fetal de bovino y 5% CO2 durante 3 horas, el que fue reemplazado por medio Neurobasal (GIBCO) suplementado con N2 (GIBCO). Los cultivos neuronales se mantuvieron por 4-5 días. Para los cultivos mixtos las células fueron tratadas en las mismas condiciones que los cultivos de hipocampo. Sin embargo se incubaron durante toda la noche en MEM.10% suero, luego de lo cual el medio se reemplazo por medio Neurobasal suplementado con N2. Los cultivos mixtos se mantuvieron por 4-5 días en cultivo.

2.2 Tratamientos con hierro: El hierro se agregó a los cultivos como hierro-citrato (dilución 1:100) a diferentes concentraciones. Paralelamente como quelante de hierro se agregaba desferal (desferoxamine mesylate) en una concentración de 100 μM, con el

objeto de controlar cuantitativamente los niveles de hierro en el medio (Control negativo).

2.3 Tratamientos con H_2O_2: El H_2O_2, previamente diluido en PBS estéril, fue agregado a los cultivos celulares a una concentración final de 100 μM en medio libre de suero y el cultivo se mantuvo durante 2 horas en esas condiciones.

2.4 Inmunocitoquímica: Los cultivos celulares fueron fijados en 4% paraformaldeído, 4% sacarosa durante 15 minutos a 37°C. Después de fijadas, las células fueron permeabilizadas con 0.2% tritón X-100 a temperatura ambiente durante 5 min. Luego las muestras fueron lavadas 3 veces con PBS y bloqueadas en PBS-5% BSA durante 1 hora a temperatura ambiente. Para la inmunodetección, el primer anticuerpo fue incubado en una cámara húmeda durante toda la noche, luego de lavar 3 veces con PBS, se agregó el segundo anticuerpo que fue posteriormente incubado por 1 hora a temperatura ambiente. Finalmente las muestras fueron lavadas y montadas con *Prolong mounting media* (Molecular Probes), y analizadas por microscopía confocal. Las imágenes fueron analizadas en un microscopio Zeiss modelo META.

2.5 *Western-blot.* Después de los tratamientos, los cultivos fueron homogenizados en buffer RIPA con inhibidores de proteasas, y la concentración de proteínas fue determinada por el método de Bradford. Cantidades iguales de proteínas fueron cargadas en geles denaturantes de poliacrilamida 12 %. Posteriormente fueron transferidas a una membrana de nitrocelulosa, y ésta bloqueada en PBS con 5% leche libre de grasa.

Posteriormente las membranas fueron incubadas con un anticuerpo primario toda la noche a 4°C o 2 horas a temperatura ambiente. Después de tres lavados con PBS-Tween (0.05%), las membranas fueron incubadas con un anticuerpo secundario asociado a peroxidasa. Finalmente la detección se realizó utilizando un sistema de luminiscencia (ECL, Amersham Pharmacia), y las muestras fueron analizadas en placas autorradiográficas. En este trabajo utilizamos los siguientes anticuerpos primarios para la inmunodetección.

Para estudiar los estados de fosforilación de la proteína tau utilizamos: (i) el anticuerpo AT8 que reconoce los epítopos fosforilados Ser ^{202} y Thr ^{205} en la proteína tau; (ii) Tau-1 que reconoce los mismos epítopos que AT8 pero desfosforilados, y (iii) Tau-5 que reconoce epítopos, en la proteína tau, independientes de su estado de fosforilación. Tau-1 y Tau-5 fueron generosamente donados por el Dr. Lester I. Binder, Northwestern University. Para normalizar las cargas en los estudios de inmunodetección, se utilizó un anticuerpo anti β-actina (Sigma) y como marcador de estrés oxidativo se utilizó un anticuerpo que reconoce lisinas modificadas por aductos de 4-hidroxinonenal (Calbiochem). Todos estos anticuerpos fueron utilizados de acuerdo con las instrucciones de los fabricantes

2.6 Ensayo de incorporación de hierro: Luego de 4 días de cultivo las células fueron mantenidas en medios suplementados con hierro radioactivo Fe^{55}, variando tanto el tiempo como la concentración a la cual estos fueron expuestos. Luego de 3 lavados se midió la incorporación de marca radioactiva en un contador de centelleo y se

extrapolaron las cpm en una curva de calibración para la obtención de su equivalencia en concentración de hierro, de esta manera los datos se graficaron como pmoles de Fe/mg de proteína.

2.7 Obtención de las muestras de líquido cefaloraquídeo. Nuestro laboratorio de Biología Celular, Molecular y Neurociencias ha participado desde hace cinco años en un estudio longitudinal realizado en colaboración con el Departamento de Ciencias Neurológicas de la Universidad de Chile, con el objeto de evaluar diferentes marcadores biológicos en el líquido cefaloraquídeo de cinco grupos de sujetos: (i) Sujetos normales; Total Box =0 (N) (ii) Pacientes con Desordenes Cognitivos Tempranos (DC-T); Total Box = 0.5-1 (iii) Desordenes Cognitivos Avanzados (DC-A); Total Box = 1.5-3 (iv) EA temprana (Alzh-T); Total Box = 4-6.5 y (v) Pacientes con EA avanzada (Alzh-A); Total Box = 7-10. En este estudio participaron los Drs. Renato Verdugo y Manuel Lavados del Servicio de Neurología, Hospital El Salvador y Departamento de Ciencias Neurológicas y el Dr. Ricardo B. Maccioni del Laboratorio de Biología Celular, Molecular y Neurociencias donde se llevó a cabo la tesis. Como parte de estos estudios, y en el contexto de esta memoria de título, se tomaron alícuotas de las muestras de LCR y se sometieron a diversos análisis, en un estudio que se realizó en colaboración con el Dr. Tulio Nuñez, Laboratorio de Biología del Envejecimiento, Instituto Milenio CBB, Facultad de Ciencias, Universidad de Chile; el Dr. Miguel Arredondo, Laboratorio de Microminerales del INTA, Universidad de Chile y el Grupo del Dr. Ioav Cavantchik, Instituto de Ciencias Biológicas de la Universidad Hebrea de Jerusalem en Palestina.

2.8 Medición de Fe total en muestras de LCR de sujetos seniles: Las muestras fueron analizadas por espectroscopia de absorción atómica (EAA). Para ello 100µl de LCR fueron mezclados con 100µl de ácido nítrico 5% y se incubaron a 60°C durante 12 horas. Una vez enfriado el medio de digestión, las muestras fueron centrifugadas a 12.000g por 2 minutos. Luego el sobrenadante se llevó a 1ml con ácido nítrico 0.2%. Finalmente el contenido total de Fe fue determinado por EAA en un equipo SIMAA 6100 (Perkin Elmer, Shelton CT). Como estándares de referencia para validar las mediciones de Fe se utilizaron MR-CCHEN-002 (*venus antiqua*) y DOLt-2 (*Dogfish liver*).

2.9 Mediciones de Fe-redox activo: Fe redox activo en LCR fue determinado según Esposito y cols. (2002), en las mismas muestras del estudio longitudinal en pacientes descrito más arriba. Brevemente, cuadruplicados de 20µl de LCR fueron incubados en una placa de 96 pocillos (Maxisorp 96, Nunc, Rotskilde, Denmark). En 2 pocillos se incubaron con 180µl de buffer salino HEPES, libre de Fe, con 40µM de ascorbato y 50µM del fluoroforo DHR (dihydrorhodamina 123, sal dihiroclorada, Biotium, Hayward, CA, USA). A otros 2 pocillos se les agregó la misma solución pero conteniendo 50µM del quelante de Fe Deferiprona (L1, Apotex, Toronto, Ont., Canada), y luego se analizó la cinética de fluorescencia a 37°C durante 40 minutos, con un ajuste de 485/538 nm excitación/emisión en un fluorímetro BMG Galaxy Fluostar (BMG Lab Instruments, Germany). Las mediciones se tomaron cada 2 minutos, y se compararon las diferencias en las pendientes de las curvas de excitación/emisión de las muestras de LCR

con y sin el quelante de Fe entre los 15 y 40 minutos. De esta manera, se analizó el componente de Fe sensible a quelantes el cual corresponde al Fe-redox activo de la muestra. Para la obtención de los valores en concentraciones μM se utilizaron curvas de calibración hechas con suero suplementado con Fe:NTA, 1:7 (mol:mol) hasta obtener concentraciones entre 40-100 μM, con las cuales se hicieron diluciones seriadas hasta obtener una curva apropiada.

2.10 Análisis estadístico: Los valores obtenidos para los promedios fueron comparados y se utilizo el test de Dunnett para analizar las significancias estadistas de estas diferencias. Las diferencias se consideraron significativas si $P < 0.05$.

III. RESULTADOS

3.1 ESTUDIOS DE LA ACCION DEL HIERRO EN CULTIVOS DE CELULAS HIPOCAMPALES. EFECTOS SOBRE LAS ALTERACIONES EN LA FOSFORILACION DE LA PROTEINA TAU ASOCIADAS A NEURODEGENERACION.

De acuerdo con nuestra hipótesis de trabajo, el hierro se acumularía en las neuronas lo que en el tiempo provocaría un aumento en los niveles de estrés oxidativo, por los mecanismos antes descritos. Para esto fue necesario estudiar como se comportaban nuestros cultivos en relación con este punto. Para ello hicimos un ensayo de incorporación de Fe^{55} y analizamos como variaba su incorporación, en relación la concentración de Fe total en el medio y a la duración del estimulo. Los resultados se muestran en la Figura 5.

Figura 5. - La incorporación de Fe ^{55} en cultivos primarios de hipocampo depende de su concentración en el medio y de la duración del estímulo.

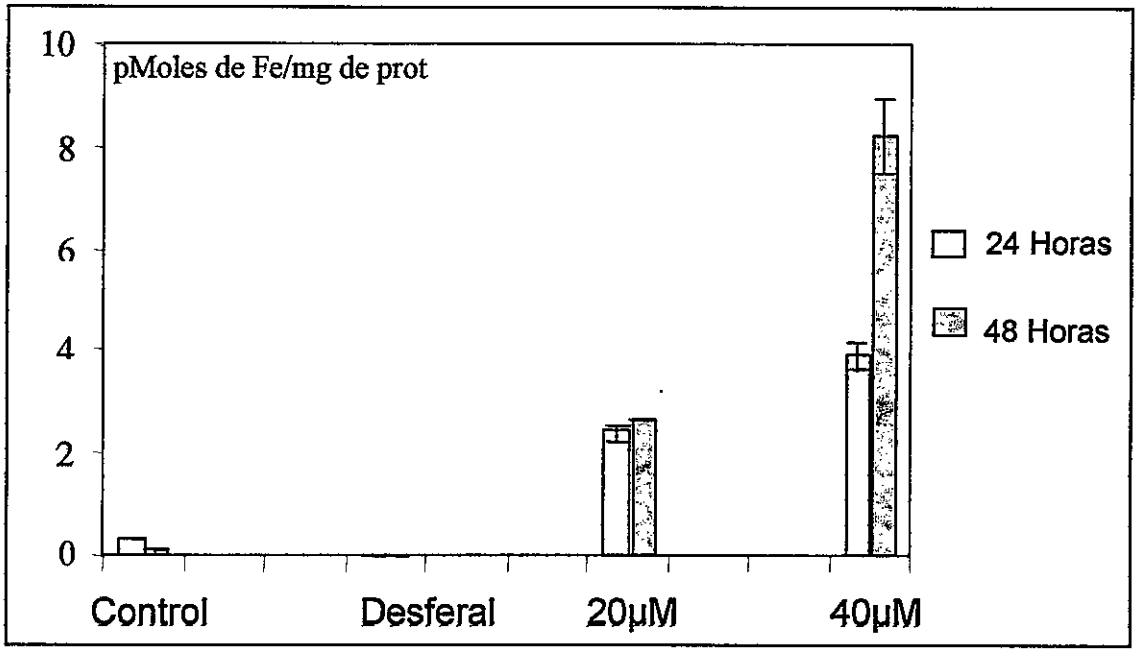

Figura 5. Concentraciones de 20 µM ó 40 µM de Fe ^{55}, como se indica en la Figura, ó de 100 µM de Desferal fueron agregadas en los medios de cultivo de neuronas primarias de hipocampo y se incubaron durante 24 ó 48 horas. Luego se realizaron extractos celulares y se graficó que existe una correlación positiva entre la concentración de Fe^{55} en el medio, la duración del estímulo y los niveles citoplasmáticos de este metal.

Los datos de la Figura 5 muestran que para 24 y 48 horas existe una acumulación de Fe^{55} que es dependiente tanto de la concentración en el medio como de la duración del estímulo. Estos resultados nos sugieren que nuestro sistema aparece como un buen modelo de estudio, ya que nos permite simular un fenómeno biológico que ocurre en muchos años durante el envejecimiento, en un sistema en cultivo en pocos días. Pudiendo generar así, en pocos días, neuronas con elevados niveles de Fe en el citoplasma, y analizar los efectos biológicos de éste.

Luego de conocer como se incorporaba el hierro en nuestro sistema, analizamos si estos tratamientos afectaban a la morfología general de las neuronas en cultivo o a la organización integral del citoesqueleto. Para esto, las neuronas fueron tratadas como se describe en la sección de Materiales y Métodos y se evaluaron los cambios morfológicos así como la organización subcelular de elementos del citoesqueleto neuronal. Se utilizó para ello un anticuerpo primario anti-tubulina para la marcación de microtúbulos y faloidina asociada a rodamina para marcar los filamentos de actina.

Figura 6.- Los aspectos morfológicos de las neuronas tratadas con Fe no fueron afectados significativamente.

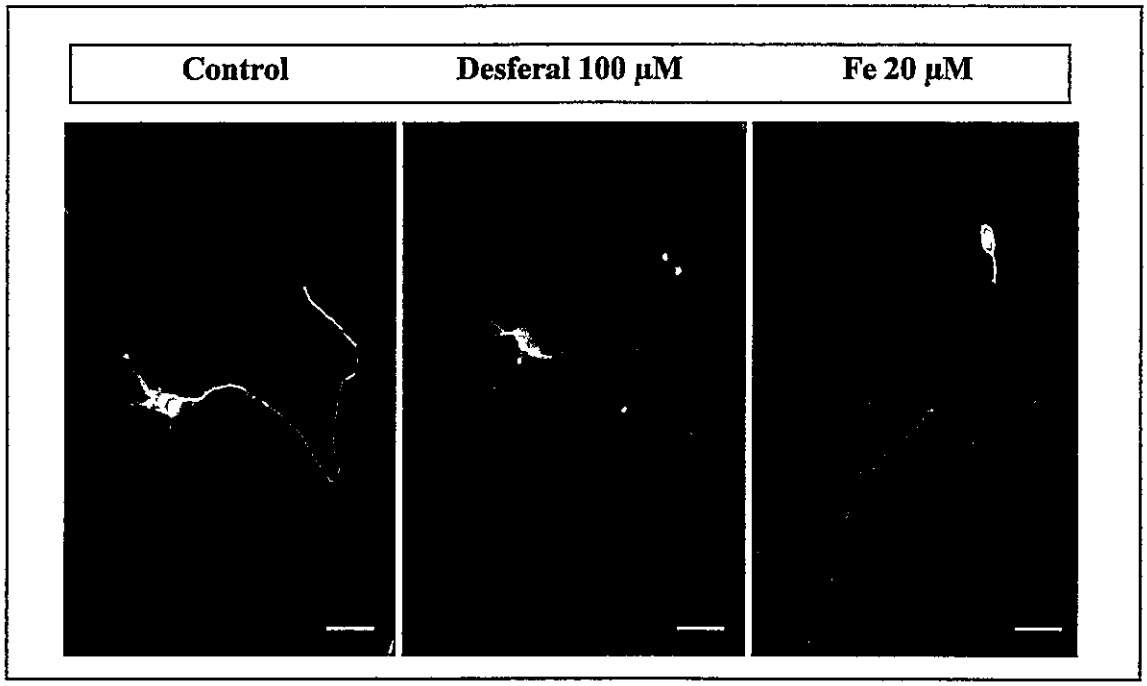

Figura 6. Cultivos primarios de hipocampo fueron tratados por 24 horas con 20 µM de Fe ó 100 µM de Desferal y fueron analizadas por técnicas de inmunocitoquímica para tinción de Tubulina (verde) y Actina (rojo). En la figura es posible observar que la morfología celular general no se ve afectada por estos niveles de Fe en el medio. Las barras corresponden a 15 µm.

En la Figura 6 se observa que tanto el citoesqueleto de actina (en rojo) como el de tubulina (en verde) no muestra variaciones importantes entre los diferentes grupos de tratamientos, lo cual sugiere que la citoestructura general de las neuronas no se ve afectada.

Un aspecto importante de nuestro estudio fue el confirmar trabajos de otros autores en el sentido de visualizar si los tratamientos con hierro inducían un significativo daño oxidativo. Para esto medimos los niveles de aductos de 4-HNE con proteínas neuronales, los cuales se generan como productos de peroxidación lipídica, los que modifican covalentemente a lisinas y argininas de éstas proteínas. El 4-HNE se utiliza comúnmente como marcador de estrés oxidativo ya que, permite medir daño directo sobre lípidos y proteínas.

Figura 7.-Tratamientos con Fe inducen daño oxidativo neuronal.

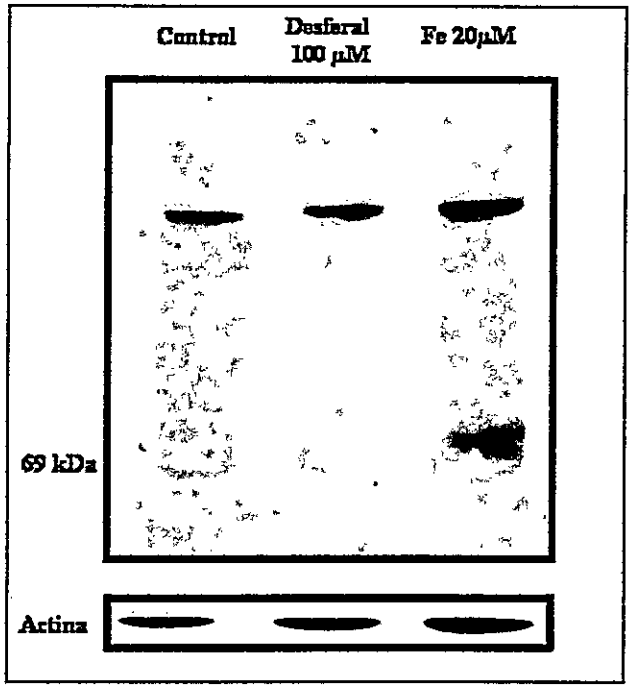

Figura 7. Fe induce estrés oxidativo, evaluado por cambios en los niveles de 4-HNE citosólicos. En esta figura se muestra un Western-blot de extractos proteicos de neuronas de hipocampo incubadas durante 24 horas en presencia de 20 μM de Fe o 100μM de Desferal en donde, 50 μg del homogeneizado total fueron cargados y la membrana fue revelada usando un anticuerpo policlonal anti aductos de 4-HNE en lisinas de proteínas. Interesantemente una banda de 69KDa aparece luego de los tratamientos con hierro.

La Figura 7 muestra un ensayo de *Western-blot* de extractos totales de células tratadas en las condiciones indicadas y revelado con un anticuerpo que reconoce la formación de aductos de 4-HNE en lisinas. En esta figura, para el caso de las células tratadas con Fe, se observa un aumento en la marca respecto de la condición control, situación que se ve reflejada en la aparición de una nueva banda de 69Kda. Este hecho sugiere que estos tratamientos generan daño oxidativo intracelular, el que afecta tanto a lípidos como a proteínas.

Los niveles totales de proteína tau permanecieron inalterados luego de tratamientos con hierro, lo que se observa en la Figura 8 al utilizar un anticuerpo que reconoce a tau en un epitopo que es independiente del grado de fosforilación (Tau-5). Sin embargo, cuando analizamos los niveles de tau hiperfosforilada en un epitopo característico de la enfermedad de Alzheimer, inesperadamente observamos una importante disminución en los niveles de marca para el anticuerpo AT-8. Ello sugiere que bajo estas condiciones, debido a los tratamientos con hierro, la proteína tau pierde esta hiperfosforilación. Consistentemente con esto, se observó un aumento en los niveles de proteína tau desfosforilada en el mismo epitopo lo que se evidencia con un aumento en la marca para el anticuerpo Tau-1, el cual reconoce el mismo epitopo que AT-8 pero en un estado desfosforilado.

Figura 8.- Fe induce alteraciones en el patrón de fosforilación de la proteína tau.

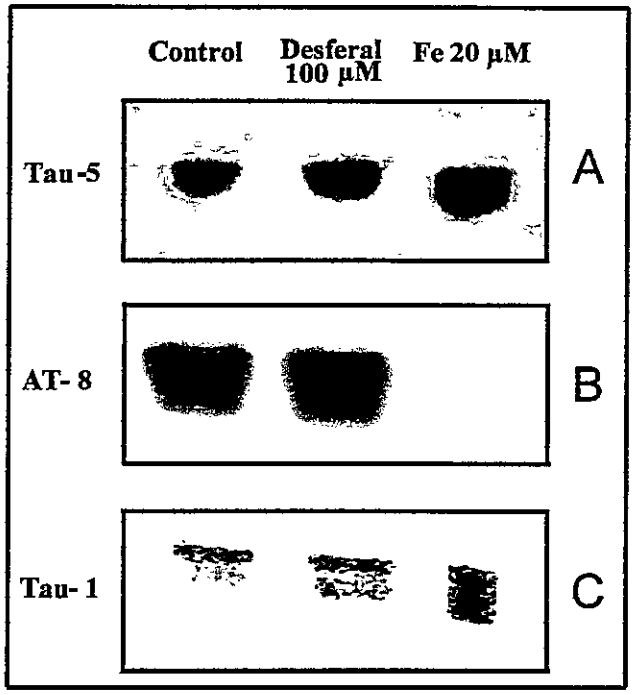

Figura 8. Fe induce alteración en el patrón de fosforilación de la proteína tau. 50µg de extractos proteicos de neuronas de hipocampo incubadas durante 24 horas en presencia de 20 µM de Fe o 100µM de Desferal. fueron analizados por Western-blot usando los anticuerpos Tau-5 que reconoce un epitopo conformacional independiente de fosforilación, AT-8 que reconoce un epitopo fosforilado en la proteína tau y el anticuerpo Tau-1 que reconoce el mismo epitopo pero desfosforilado. En esta figura se observa que en presencia de 20 µM de Fe, la marca para AT-8 disminuye (B), lo que se correlaciona con un aumento en la marca para Tau-1 (C), manteniéndose invariables los niveles totales de la proteína tau (A).

Para analizar si la capacidad del hierro de promover cambios sobre el estado de fosforilación de la proteína tau como se muestra en la Figura 8, se debe a un aumento en la producción de EROs por la vía de la reacción de Fenton, decidimos evaluar los efectos de otro tratamiento pro-oxidante que actúe sobre esta misma vía para inducir un aumento en la producción de EROs. De esta manera realizamos estímulos con H_2O_2 sobre nuestro sistema celular en cultivo. Los datos de la Figura 9 muestran que H_2O_2 inducen la misma modificación sobre los estados de fosforilación de la proteína tau, que los tratamientos con hierro. En esta Figura 9 se observa que la marca con el anticuerpo

AT-8 disminuye en presencia de H_2O_2 (panel B) lo cual es consistente con un aumento en la reactividad obtenida con el anticuerpo Tau-1 contra tau desfoforilada (Panel C). Además es posible observar en la Figura 9 que los niveles totales de la proteína tau no varían en respuesta al tratamiento lo que se detecta utilizando el anticuerpo Tau-5 (Panel A)

Figura 9.- El agente oxidante H_2O_2 induce las mismas alteraciones en el patrón de fosforilación de la proteína tau que los tratamientos con Fe.

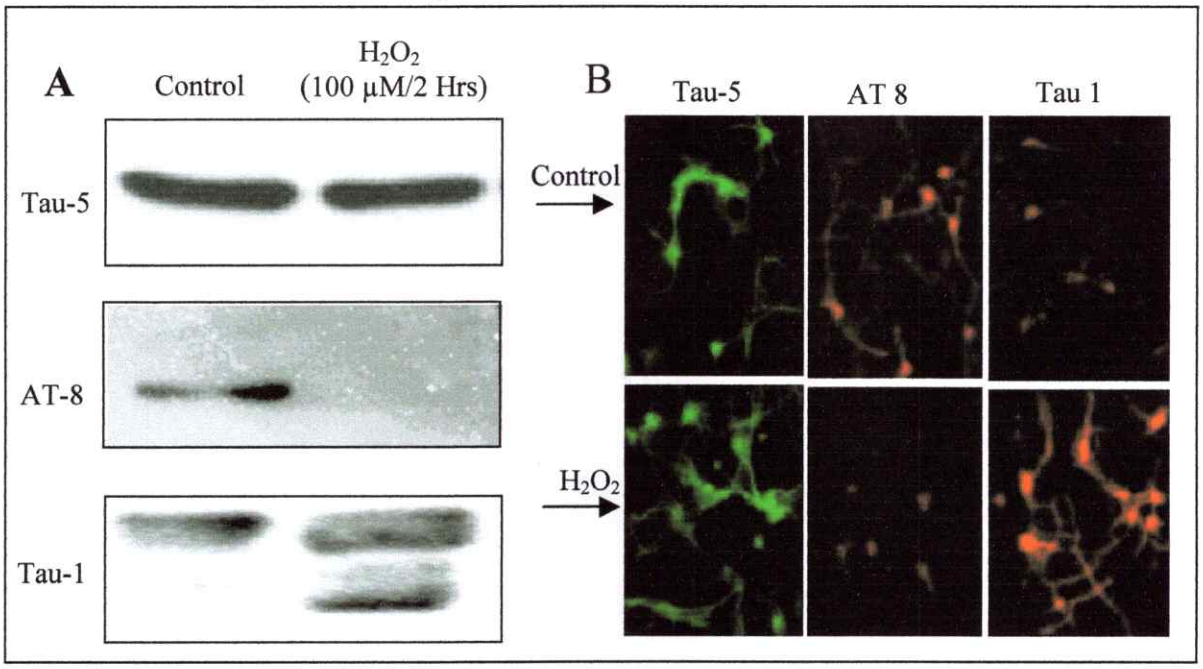

Figura 9. H_2O_2 induce alteración en el patrón de fosforilación de la proteína tau.
En esta figura se muestra un *western blot* (A) de extractos proteicos de neuronas de hipocampo incubadas durante 2 horas en presencia de 100 µM de H_2O_2, en a es posible observar que en presencia de 100 µM de H_2O_2, la marca para AT-8 disminuye, lo que se correlaciona con un aumento en la marca para Tau-1 manteniéndose invariables los niveles totales de la proteína tau (Tau-5). En B se observa un análisis por inmunocitoquimica de neuronas hipocampales tratadas de igual manera que en A. En esta figura se confirman los resultados mostrados en A para los cambios en la fosforilacion de tau.

Descubrimientos previos de nuestro laboratorio han demostrado que desregulaciones en la actividad de la enzima cdk-5 son claves en la vía neurodegenerativa mediada por el péptido β-amiloide (Alvarez y cols. 1999, 2001,

revisado en Maccioni y cols, 2001 a,b). Además, otros estudios lograron demostrar que la activación del complejo cdk-5/p35 puede estar mediada por la fosforilación de la tirosina 15 de la proteína quinasa cdk5, la cual podría ser responsable de la sobreactivación anómala de esta proteína quinasa (Álvarez y cols. 2001; Zukerberg y cols. 2000). En este contexto, decidimos analizar los niveles de Cdk5 y de fosfo-Cdk5 (Tyr^{15}) en neuronas tratadas con hierro. Para esto realizamos estudios de *western-blot*, en los cuales aplicamos concentraciones equivalentes de proteínas en los geles electroforéticos previo a la inmunodetección. Como se observa en la Figura 10 los niveles de ambos estados de cdk5 permanecieron inalterados luego de los tratamientos.Figura 10.- Niveles de cdk-5 y cdk-5/P permanecen inalterados luego de tratamientos con Fe.

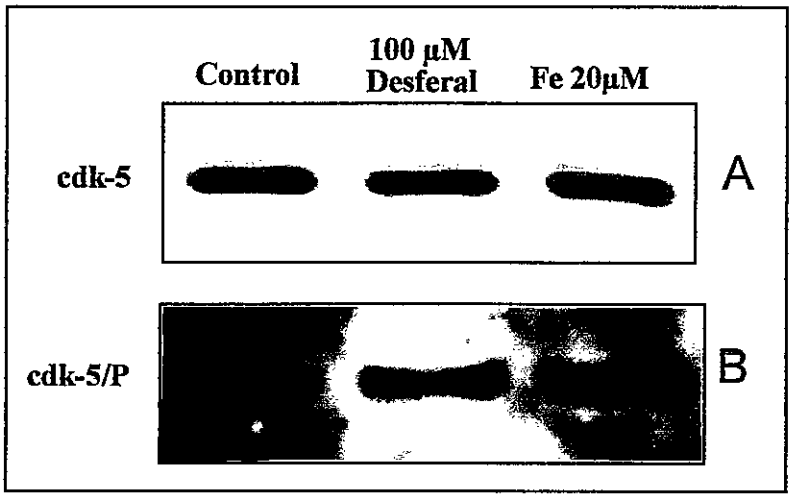

Figura 10. Niveles de cdk5 y cdk5-P permanecen inalterados luego de tratamientos con hierro. 50µg de extractos hipocampales de células incubados en ausencia de Fe (Control) y en presencia de 100µM de Desferal o 20µM de Fe durante 24 horas, fueron analizadas por técnicas de *western-blot* utilizando anticuerpos fosfoespecíficos que muestran que no hay variación en los niveles de cdk-5 (A) y de cdk-5 fosforilada en Tyr 15 (B).

Durante los últimos años, un número cada vez mayor de investigaciones se han centrado en estudiar el rol que cumplen las células gliales en el control de los procesos neuronales que guían a la neurodegeneracion. Esto debido a que las células gliales juegan un rol crítico en la homeostasis necesaria para la integridad neuronal. En este contexto, decidimos analizar el posible rol modulador de la glia en la alteración de los estados de fosforilación de la proteína tau inducidos por tratamientos con hierro. Para esto montamos un sistema de cultivos mixtos (co-cultivo neurona/glia), el cual se muestra en la Figura 11 A, en el cual las neuronas (flechas rojas) se encuentran en contacto directo con las células gliales (flechas verdes). De esta manera las neuronas son sensibles a recibir estímulos desde el medio, a través de factores solubles secretados por la glia y a través del contacto directo entre estos dos tipos celulares

Figura 11. La presencia de células gliales modula la respuesta neuronal frente a los tratamientos con Fe.

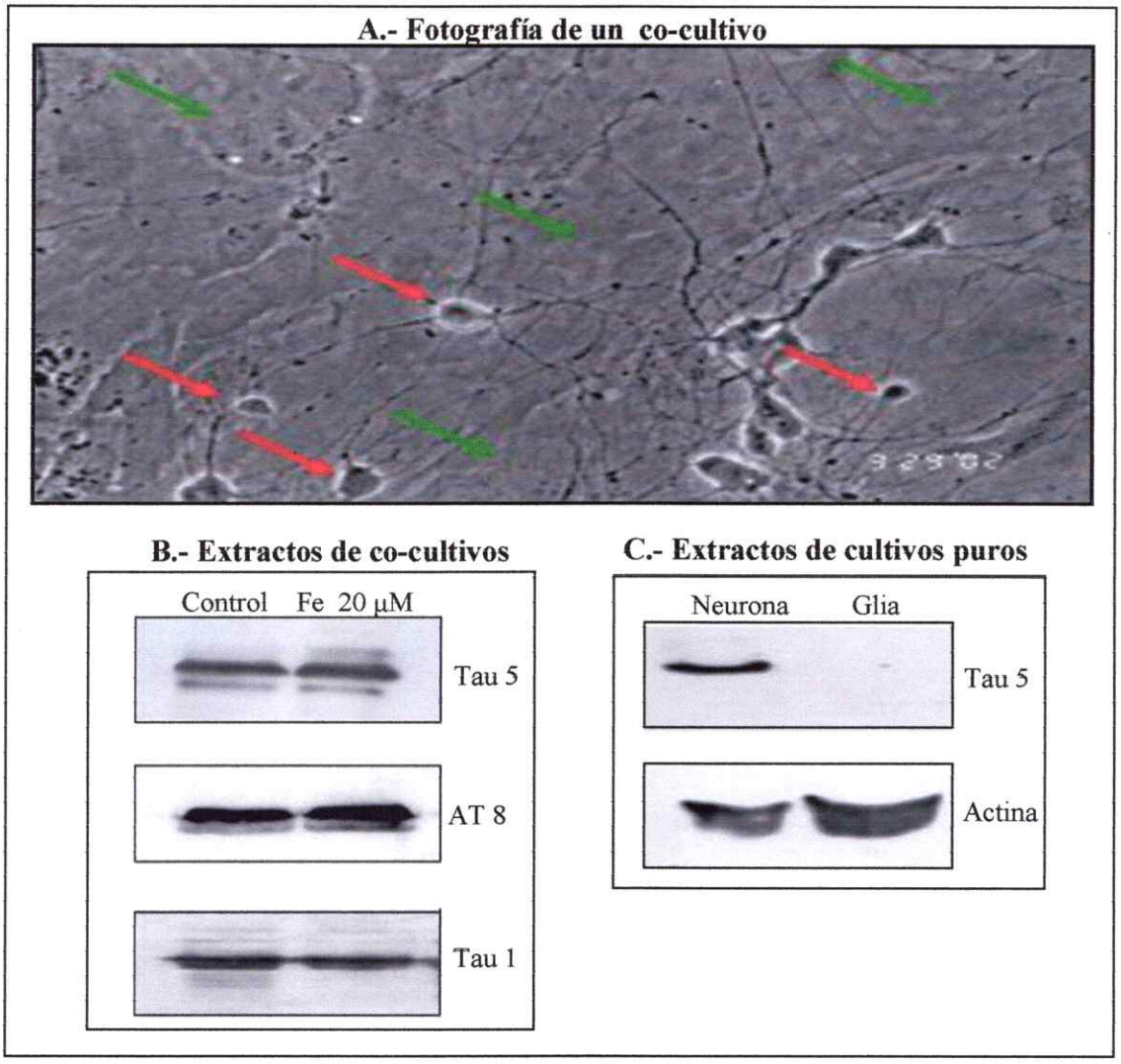

Figura 11. La presencia de células gliales modula la respuesta neuronal. En A se observa un sistema de cultivos mixtos en el cual neuronas (flechas rojas) y glias (flechas verdes) crecieron durante 4 días. B. Los cultivos fueron estimuladas con Fe-citrato 20 µM y se compararon 50 µg de extractos tratados con extractos controles los que por un análisis de *western-blot* no arrojaron diferencias en los estados de fosforilación de tau analizados con el uso de los anticuerpos Tau-5, AT-8 y Tau-1. En C se muestra que en 50 µg de extracto neuronal se observa proteína tau, no así en 50 µg de extracto puro de Glia.

En la Figura 11B es posible observar que iguales tratamientos con hierro que los realizados en cultivos de neuronas en ausencia de células gliales, no fueron capaces de producir cambios en los estados de fosforilación de la proteína tau. La Figura 11 C muestra un control de carga en el cual se observa que la contribución de la proteína tau glial al extracto total no podría alterar de manera importante los resultados obtenidos para un extracto de proteínas de un cultivo mixto. Así, analizados de manera integral, los resultados mostrados en la Figura 11, estos nos sugieren que las células gliales juegan un rol clave en la modulación de la respuesta neuronal frente a los estímulos realizados con hierro.

En base a los resultados obtenidos en esta parte de la tesis y de acuerdo con el marco teórico y antecedentes entregados. Estos nos llevan a plantear la representación esquemática que se muestra en la Figura 12. En esta, se observan los fenómenos que podrían estar ocurriendo en la neurona en presencia de hierro en el ambiente. En la vía 1, el hierro entraría a la neurona, y se acumularía provocando estrés oxidativo (vía 2). Este estrés induciría cambios en los estados de fosforilación de la proteína tau (vía 3) los cuales pueden afectar la estabilidad y dinámica de los microtúbulos, lo que podría provocar una alteración funcional en el citoesqueleto. La proteína tau al perder el patrón de fosforilación adecuado para su óptima y modulada interacción con el citoesqueleto, se alteraría la dinámica y estructura del citoesqueleto lo que podría provocar disfunción neuronal (vía 4). Ello es consistente con la observación que la disfunción neuronal es el proceso prioritario en la vía hacia la neurodegeneración, y que muerte neuronal sólo ocurre en estados avanzados de la EA.

12.- Esquema general

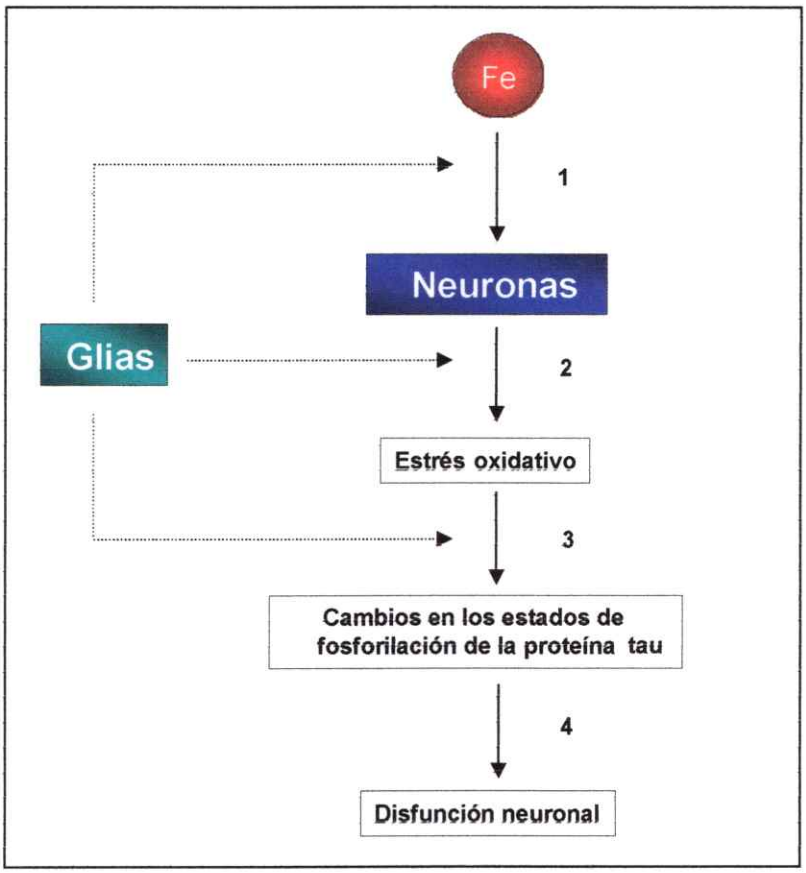

Figura 12. En esta figura se esquematizan los procesos que ocurrirían en la neurona. En 1 el hierro entra, luego (2) provoca estrés oxidativo el cual provoca cambios en los estados de fosforilación de la proteína tau (3) induciendo disfunción neuronal. (4). Este fenómeno estaría modulo por células gliales.

Por otra parte, las células gliales podrían regular este fenómeno en a lo menos 3 etapas, las cuales se esquematizan con flechas punteadas, y siguieren una regulación al nivel de la incorporación de hierro en la neurona, la mantención del estado redox neuronal y/o una intervención que involucre directa o indirectamente al mecanismo molecular que conlleva a la fosforilación y desfosforilación de la proteína tau en los epítopos involucrados.

3.2 ESTUDIOS DE LA HOMEOSTASIS DEL HIERRO BASADA EN DETERMNINACIONES EN LCR DE SUJETOS SENILES CON DIFERENTES GRADOS DE DAÑO COGNITIVO

Como complemento a los estudios en cultivo celular y con el objeto de evaluar las alteraciones en los niveles de hierro que pudiesen contribuir a explicar alteraciones patologícas como las que se observan en EA, se analizaron los niveles de hierro total y hierro redox activo en muestras de LCR de distintos grupos de pacientes (ver Materiales y Métodos). El análisis, por espectroscopia de absorción atómica, del Fe total contenido en el LCR de personas con diferentes grados de deterioro cognitivo mostró un amplio rango de concentraciones (Ver tabla 2) que fue desde 0.297 μM hasta 1.846 μM (Promedio: 0.818; Error estándar: 0.069; N=31). No se encontraron diferencias significativas entre los niveles totales de Fe y el grado de demencia. El análisis de las muestras arrojó los siguientes resultados. (Promedio ± Error estándar) 0.85 ± 0.16 μM; n = 10; para el grupo de pacientes normales (N); 0.790 ± 0.12 μM; n =9 para el grupo con Desorden Cognitivo Temprano (DC-T); 0.627 ± 0.06 μM; n =4 para el grupo con Desorden Cognitivo Avanzado (DC-A); 1.014 ± 0.28 μM; n=3 para el grupo Alzheimer en estadíos tempranos (Alzh-T) y 0.760 ± 0.15 μM; N=5; para el grupo Alzheimer avanzado (Alzh-A), respectivamente.

Figura 13.- Niveles totales de Fe en LCR no se encuentran alterados en demencias.

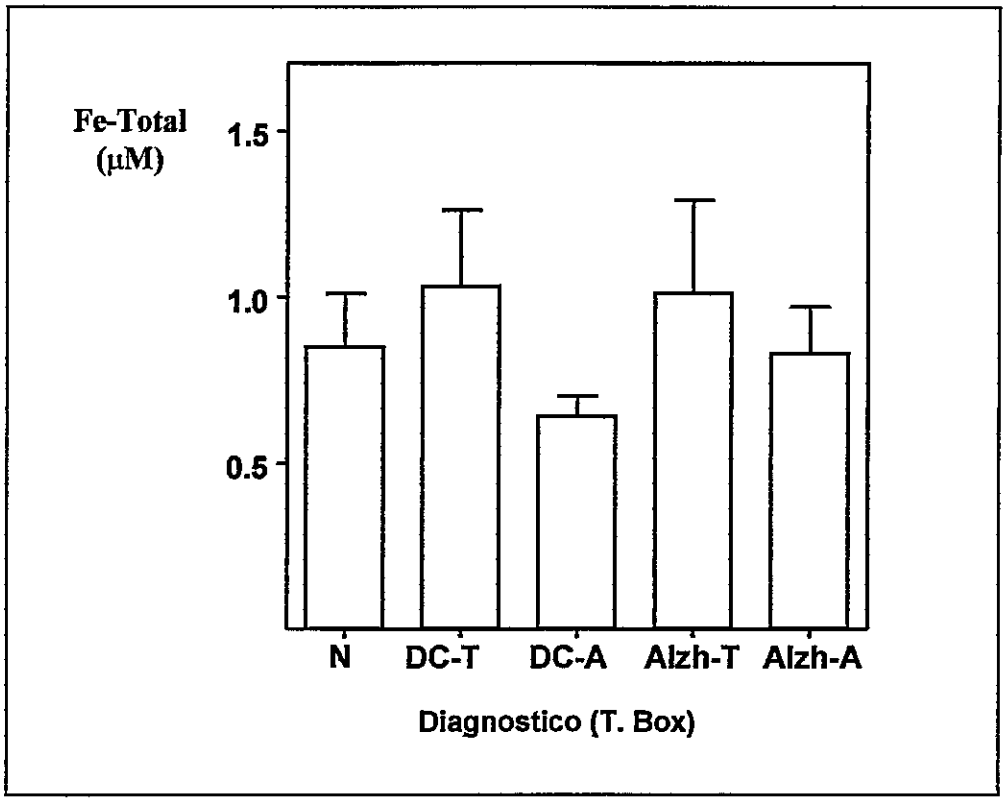

Figura 13. Niveles de Fe total en LCR obtenido de 37 individuos; 10 normales (N), 9 Desorden Cognitivo Temprano (DC-T), 10 Desorden Cognitivo Avanzado (DC-A), 3 Alzheimer Temprano (Alzh-T) y 5 pacientes con Alzheimer Avanzado (Alzh-A), fueron analizados por espectroscopia de absorción atómica. Los resultados no arrojaron diferencias significativas entre los grupos analizados.

Los niveles de transferrina (Tf) que se han descrito para el LCR son de 0.24µM (Symons y Gutteridge, 1998) de esta manera Tf en el LCR esta muy cerca de su punto de saturación o saturada, con lo que podría existir una fracción de Fe libre no unido a Tf presente. Por esto quisimos analizar los niveles de Fe redox activo en los diferentes grupos en estudio.

Figura 14. -Correlación positiva entre Fe redox-activo y etapas tempranas del proceso neurodegenerativo.

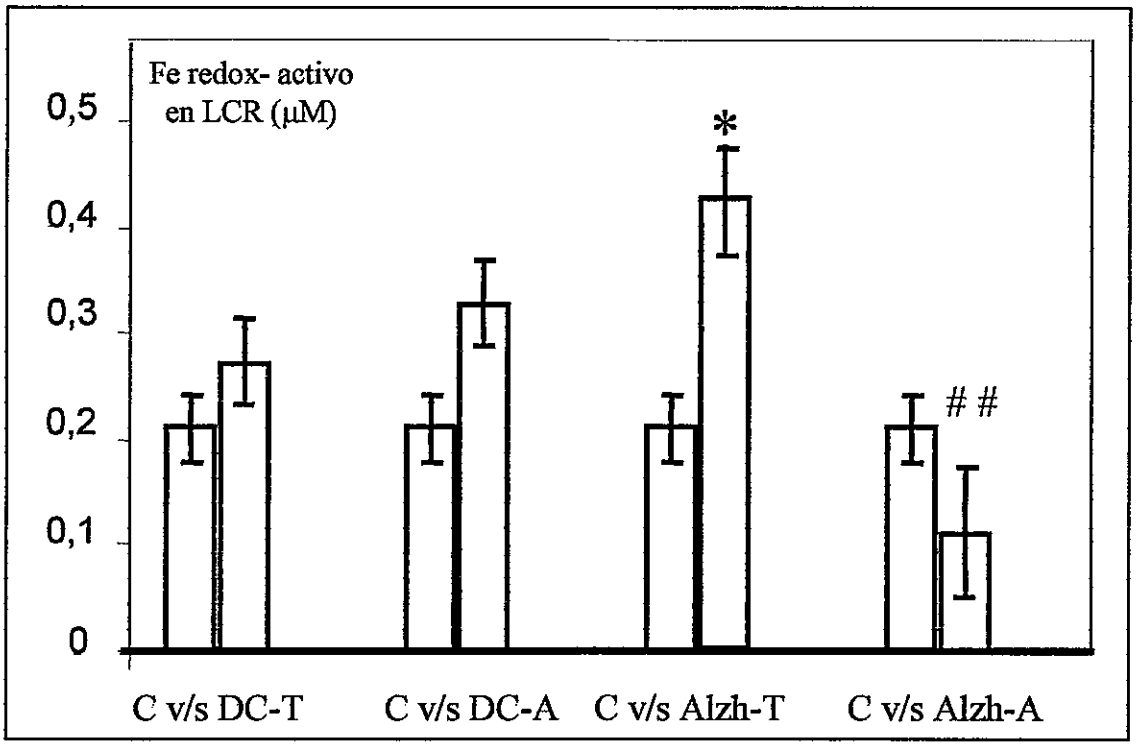

Figura 14: Niveles de hierro redox activo en LCR. Hierro redox activo fue determinado en 46 sujetos, los cuales de acuerdo a su deterioro cognitivo, se agruparon en las categorías de sujetos: 13 normales, 9 con deterioro cognitivo temprano, 11 con deterioro cognitivo avanzado, 5 Alzh-T y 8 con Alzh-A. Diferencias significativas se encontraron ente los sujetos normales y con EA temprana (*) y entre estos últimos y los sujetos con Alzh-A (# #). *, $P < 0.05$, # # $P < 0.01$, sugún test de Dunett.

Los valores de Fe redox-activo (Ver tabla 1) en el LCR de personas con un estatus cognitivo normal arrojaron valores de 0.210 ± 0.03 μM, n= 13; (Promedio ± Error estándar). En DC-T y DC-A, los niveles de Fe redox activo no fueron significativamente mayores que en los individuos controles (0.272 ± 0.04 μM, N= 18, 0.328 ± 0.04 μM, N= 11). Por otro lado las muestras de LCR obtenidos de individuos con Alzheimer mostraron un patrón de distribución bifásico en donde las muestras obtenidas de individuos con Alzh-T presentan un nivel de Fe redox activo significativamente mayor

al control (0.424 ± 0.05 μM, n= 5; P< 0.05) y el grupo de individuos con Alzh-A muestra un nivel de Fe redox activo muy bajo, siendo significativamente menor al grupo Alzh-T (0.112 ± 0.06 μM, N= 8; P< 0.01). Según datos arrojados por el test de Dunett.

Figura 15.- Correlación negativa entre Fe redox-activo en LCR y daño cognitivo en personas con EA.

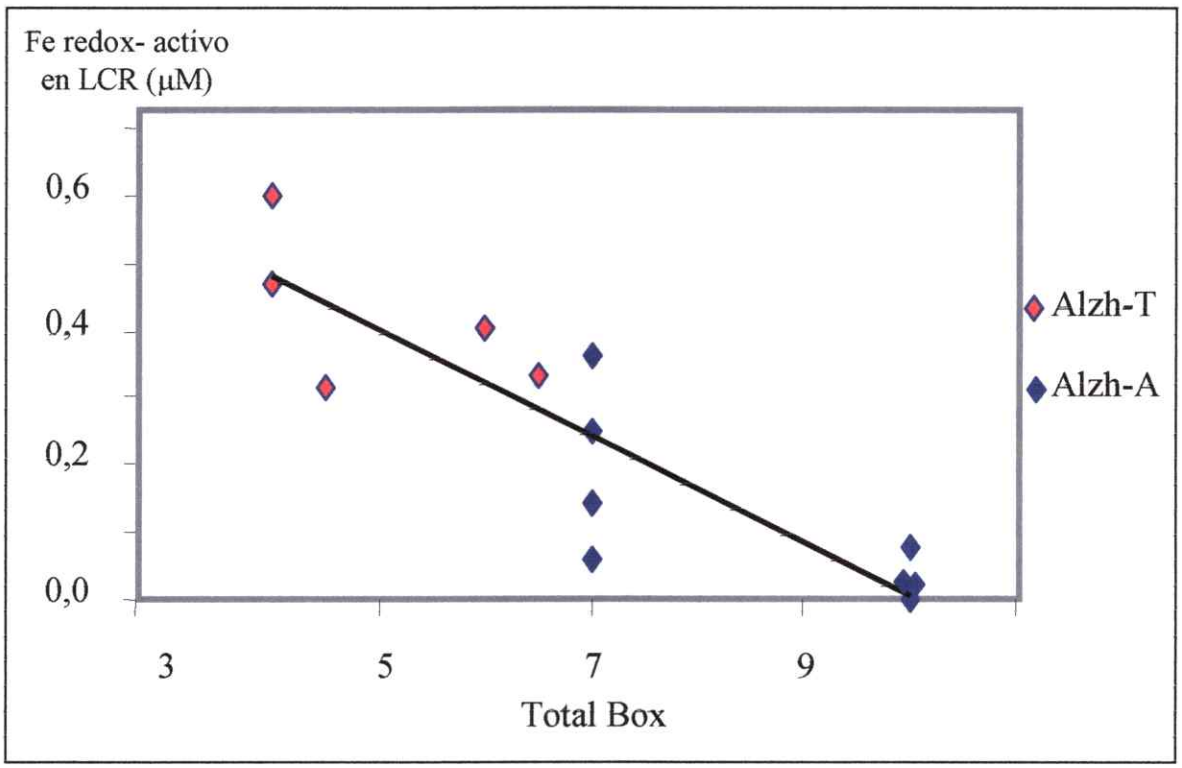

Figura 15. Correlacion entre los niveles de hierro redox activo y el grado de demencia en pacientes de EA. Los valores de hierro redox activo se garficaron respecto al grado de demencia en pacientes con EA, T.Box ≥ 4, Se encontro una fuerte correlacion negativa (r^2: 0.784; pendiente: -9.24).

Un análisis posterior de los datos revela que existe una fuerte correlación inversa entre el grado de demencia de los pacientes con EA y los niveles de Fe redox activo en LCR (Ver tabla 3). Este hecho sugiere que durante el transcurso de la patología el Fe redox activo es neutralizado por algún mecanismo.

TABLA 1:

Fe redox-activo según diagnostico (μM)

Normal	DC-T	DC-A	Alzh-T	Alzh-A
0,365	0,298	0,244	0,602	0,249
0,092	0,110	0,371	0,470	0,142
0,330	0,710	0,140	0,316	0,059
0,199	0,260	0,235	0,404	0,364
0,181	0,140	0,263	0,330	0,000
0,149	0,548	0,263		0,000
0,215	0,414	0,604		0,000
0,327	0,192	0,239		0,080
0,030	0,110	0,402		
0,130	0,100	0,375		
0,101	0,152	0,466		
0,320	0,303			
0,291	0,343			
	0,190			
	0,190			
	0,592			
	0,189			
	0,050			
13	18	11	5	8
0,210	0,272	0,328	0,424	0,112
0,03	0,04	0,04	0,05	0,06

n	
Prom	
EE	

TABLA 2:

Fe total en LCR según diagnostico (μM)

	Normal	DC-T	DC-A	Alzh-T	Alzh-A
	0,315	0,500	0,509	1,565	0,468
	0,363	0,571	0,530	0,840	0,529
	0,434	0,530	0,691	0,636	0,876
	0,466	0,619	0,776		1,104
	0,706	0,646			1,206
	0,846	0,697			0,837
	1,045	0,891			0,297
	1,142	1,032			
	1,334	1,624			
	1,846				
n	10	9	4	3	5
Prom	0,850	0,790	0,627	1,014	0,760
EE	0,16	0,12	0,06	0,28	0,15

TABLA 3:

Niveles de Fe redox activo (μM), según grado de demencia (T.Box)

Total Box	Fe redox activo
4	0,602
4	0,470
4,5	0,316
6	0,404
6,5	0,330
7	0,249
7	0,142
7	0,059
7	0,364
10	0,000
10	0,000
10	0,000
10	0,080

IV. DISCUSIÓN

En este trabajo hacemos una contribución para establecer el posible papel que podría jugar el hierro, como inductor de estrés oxidativo en el proceso neurodegenerativo que lleva a la adquisición de la enfermedad de Alzheimer. Para esto se analizo el efecto de tratamientos de sobrecarga de hierro y H_2O_2 sobre los estados de fosforilación de la proteína tau, en neuronas de hipocampo en cultivo. Además, realizamos un estudio clínico que nos permitió correlacionar los niveles y estados del Fe en LCR con la patología de la EA.

La serie de experimentos realizada en cultivos neuronales nos permite concluir que en respuesta a la exposición de neuronas de hipocampo de embrión de rata, a diferentes tiempos y concentraciones de Fe estas lo acumulan como se observa en la Figura 5. Una vez dentro de las células el Fe es capaz de inducir daño oxidativo, probablemente, por vía de la reacción de Fénton. Esta reacción química es una reacción cuya cinética es de primer orden y responde a la ley de acción de masas, de manera que al aumentar los niveles de Fe aumenta también la producción de especies reactivas de oxigeno (EROs). Lo mismo ocurre si exponemos las neuronas a tratamientos con H_2O_2 el cual a través de esta misma vía, es capaz de inducir daño oxidativo en el interior de la neurona. Este daño oxidativo fue evidenciado por la detección de aductos de 4-HNE sobre lisinas de las proteínas (ver Figura 7). Estos aductos se ven aumentados con los tratamientos con Fe lo que confirma que esta acumulación citoplasmática de Fe, efectivamente es capaz de inducir daño oxidativo en neuronas en cultivo.

Por otra parte, la formación de aductos de 4-HNE ha sido detectada en numerosas patologías neurodegenerativas, encontrándose la subunidad pesada de los neurofilamentos modificada con 4-HNE en la esclerosis lateral amiotrofica (Wataya y cols. 2002). Además, en la enfermedad de Alzheimer la proteína tau puede ser modificada por este aducto, lo que se ha podido observar en cortes de tejido cerebral *post-mortem* derivados de personas con EA (Takeda y cols. 2000).

El estrés oxidativo neuronal inducido por tratamientos con Fe o con H_2O_2 produce además una disminución en la fosforilación de la proteína tau, en epítopos característicos de la enfermedad de Alzheimer. La fosforilación de estos epítopos ha demostrado no ser dependiente de la actividad de gsk3β, pero si de la actividad de cdk-5 (Álvarez y cols. 1999, 2001; Maccioni y cols. 2001 b,c) cuya desregulación juega un rol critico en los mecanismos moleculares que gatillan la neurotoxidad mediada por el péptido β-Amiloide (Álvarez y cols 1999) en la EA. Cuando analizamos los niveles de la proteína quinasa cdk-5 y de su forma activa fosforilada no observamos cambios en las concentraciones citoplasmáticas, lo cual sugiere que esta modificación en los patrones de fosforilación de la proteína tau, inducida por estrés oxidativo, esta dada por cambios en la actividad de esta quinasa y/o por cambios en la actividad de fosfatasas vinculadas con este fenómeno. Con posterioridad a la obtención de estos resultados y con el objetivo de esclarecer el mecanismo molecular que lleva a una hipofosforilacion de la proteína tau en condiciones de estrés oxidativo continuamos esta investigación y en base a la evidencia experimental pudimos concluir que el mecanismo responsable de este fenómeno es una sobreactivacion de la actividad de la proteína fosfatasa 1 (PP1), la cual

esta dada por un aumento en la fosforilacion del inhibidor-2, el cual al fosforilarse deja de actuar como inhibidor de la PP1. Para mayor detalle ver anexo "Publicaciones" en Zambrano y cols. 2004.

Esta modificación en los patrones de fosforilación de la proteína tau podría alterar la dinámica de los microtúbulos ya que ésta, se encuentra finamente regulada por la acción de proteínas de la familia de las MAP's (Maccioni y cols. 1989; para revisión ver Maccioni y Cambiazo, 1995). Para el caso de la proteína tau, su asociación a los microtúbulos se vería disminuida tanto por una hiperfosforilación como por una hipofosforilación de su estructura (Medina y cols. 1995). Este hecho tiene una gran relevancia puesto que al disminuir la afinidad de la proteína tau por los microtúbulos, ésta se encontraría mayormente libre en el citoplasma lo que la hace susceptible de sufrir otro tipo de modificaciones las cuales posteriormente podrían inducir procesos de agregación que lleven a la formación de los ONF.

Los datos de la Figura 11 llevan a concluir que este proceso es claramente modulable por células gliales, las que podrían estar actuando en muchos niveles diferentes. Entre éstos, regulando la incorporación de hierro en las neuronas a través de factores solubles secretados por la glia al medio, los que eventualmente podrían alterar la homeostasis neuronal de este metal. Por otra parte, podría existir una contribución importante de la glia al nivel de una modulación en el balance redox neuronal por vía de un suministro vectorial de glutatión reducido hacia la neurona. Así, en estas condiciones y por la acción de elementos anti oxidantes, los fenómenos de acumulación de hierro neuronal no serian capaces de inducir daño oxidativo.

El análisis integrado de los resultados obtenidos en nuestro modelo nos permiten postular que, durante el envejecimiento una de las muchas cosas que ocurren de manera normal es una acumulación de hierro. Esta provocaría un aumento en el daño oxidativo neuronal, el que tendría como uno de sus blancos a la proteína tau la cual vería afectado su patrón de fosforilación. Esto podría provocar una disfunción celular a nivel del citoesqueleto y contribuyendo además, con los fenómenos de agregación de la proteína tau que lleven a la formación de los ONF.

Debido a que la acumulación de Fe ha sido considerada como un factor importante en la etiología de diferentes desordenes neurodegenerativos (Ke y cols. 2003; Perry y cols. 2002; Youdim y cols. 1993) es por ello que nos interesó medir los niveles de este metal en el sistema nervioso central. De esta manera, en la segunda parte de esta tesis nos orientamos a analizar la biodisponibilidad de Fe en diferentes estados del proceso neurodegenerativo, como elemento esencial para entender el rol de este metal en estos procesos. Para responder a este punto, en esta tesis analizamos muestras de LCR de sujetos seniles con diferentes grados de perdida en sus capacidades cognitivas. En estas muestras medimos los niveles totales de Fe y los niveles de Fe capaz de catalizar la reacción de Fénton, es decir el Fe redox activo.

Los niveles totales de Fe mostraron un amplio rango de valores desde 0.297 μM hasta 1.846 μM. El origen de esta enorme variación es desconocida, sin embargo podría deberse a diferencias en los hábitos alimenticios, en las condiciones ambientales o en diferencias genéticas. No encontramos diferencias significativas ente los niveles de Fe total y el grado de demencia. Es por ello que quisimos analizar los niveles de Fe que se

encuentra en forma redox activo. Aumentos en los niveles de esta fracción de Fe, han sido correlacionados con la aparición de productos de oxidación y disminución de la capacidad antioxidante del plasma (Cighetti y cols. 2002). Sin embargo, no existen datos disponibles de los niveles de Fe redox-activo en LCR y su relación con alteraciones cognitivas.

En este trabajo encontramos importantes cantidades de Fe redox activo en LCR obtenido de personas normales y con daño cognitivo. Un hecho importante fue determinar que los niveles de Fe redox activo son independientes de los niveles de Fe total, contrariamente a lo esperado los niveles de Fe total y redox activo mostraron estar independientemente regulados. Nuestros valores para personas normales (0.210 ± 0.03 μM) están muy cercanos a los descritos previamente (0.55 ± 0.27 μM) utilizando un ensayo de basado en bleomicina (Symons y Gutteridge, 1998).

Los niveles de ascorbato determinados en LCR son de alrededor de 160 μM (Riber y cols. 1993). Así, bajo estas condiciones, la forma predominante de Fe en LCR seria Fe^{+2} la cual es directamente incorporada al interior celular por el transportador DMT1 (Gunshin y cols. 1997; Arredondo y cols. 2003) en un proceso conocido como incorporación de Fe no unido a transferrina. De esta manera las regiones cerebrales que presenten este transportador podrían ser particularmente sensibles a la acumulación de este metal por esta via. Interesantemente una de las regiones que presenta una mayor densidad del transportador DMT1 es el hipocampo (Gunshing y cols., 1997), la región mas afectada en EA.

El Fe redox activo se encuentra significativamente aumentado en las primeras etapas del deterioro cognitivo. Un aumento de este estado del Fe podría promover daño neuronal por diferentes vías por ejemplo con favoreciendo la formación de las PS, en donde se ha encontrado concentraciones de 1mM de Fe (Bishop y Robinson, 2003). Además, este Fe redox activo podrir actuar en conjunto con el péptido β-amiloide, induciendo un ciclo redox. (Huang y cols.1999). Como ya se mencionó anteriormente, un aumento en los niveles de Fe redox activo podría generar una acumulación de este metal en ciertas regiones del cerebro, lo que podría provocar un aumento en la carga oxidativa neuronal que genere daño oxidativo en la célula. (Nuñez-Millacura y cols. 2002; Youdím 2004). Además, del potencial daño que tendría este Fe redox activo en las neuronas, este podría actuar a cualquier nivel en el sistema nervioso central. De esta manera, el Fe redox activo podría jugar un rol critico al afectar a las células gliales las cuales cumplen un rol fundamental en el control de muchos procesos fisiológicos y patológicos en el Sistema nervioso central. (Schousboe 2003; Hirsh y cols. 2003). De esta manera aumentos en los niveles de Fe redox activo en el LCR podrían resultar en una serie de eventos que lleven a un deterioro en la función celular induciendo neurodegeneración.

Los niveles de Fe redox activo de las muestras de LCR de individuos con EA, muestran una distribución bifásica. En el grupo de pacientes con EA temprana se observan valores promedio significativamente mayores que las muestras de los individuos control. Además, en muestras de pacientes con EA avanzada los valores de Fe redox activo arrojan promedios significativamente menores a los controles. Este

hecho sugiere que durante el transcurso de la enfermedad se gatilla un mecanismo que permite reducir los niveles de Fe redox activo en el LCR y por ende su toxicidad. Este mecanismo podría ser explicado de una manera similar a la hipótesis del "*entombment*" del péptido β-amiloide (Cuajunco y cols. 2000). Esta hipótesis plantea que el sistema formaría las PS como un esfuerzo final para neutralizar la toxicidad del péptido. Así, además se neutralizarían los efectos neurotóxicos de este Fe redox activo.

Finalmente, quisiera destacar que esta tesis constituye un aporte al caudal de estudios que pretenden dilucidar cuales son los mecanismos moleculares involucrados en los procesos neurodegenerativos que ocurren tanto de manera patológica en la EA o naturalmente durante el envejecimiento. Cabe destacar que la contribución fundamental reside en el haber logrado evaluar las relaciones entre la toxicidad de los efectos pro-oxidativos del hierro con los mecanismo de neurodegeneración mediados por cambios en la proteína tau, una etapa clave en la patogénesis de la enfermedad de Alzheimer (Maccioni y cols, 2001 a,b). Por otra parte, el haber encontrado una relación entre los cambios en los niveles de hierro en el LCR y el deterioro cognitivo en la EA apoyan la hipótesis de que el hierro podría jugar un rol critico en la EA.

Existe clara evidencia que este deterioro cognitivo es un fenómeno gatillado por muchos factores dentro de los cuales el envejecimiento es un factor clave, y en donde alteraciones en las concentraciones de los metales redox activos y la acumulación neuronal de Fe podrían jugar un rol relevante. Esto nos permite plantear, en esta tesis el posible uso de una vía alternativa de terapia para la EA, con el uso farmacológico de

moléculas quelantes de hierro, capaces de atravesar la barrera hematoencefálica que actúen como antioxidantes en el sistema nervioso central.

Por ultimo, este trabajo es solo una aproximación al análisis experimental y biomédico de la patología de EA, por lo cual este tema requeriría en el futuro mayores estudios en modelos animales y en seres humanos. Esto debido a que nuestros modelos no nos permiten extrapolar con total claridad los procesos reales que ocurren en el cerebro humano durante el envejecimiento y la génesis de la enfermedad de Alzheimer, aunque entregan avenidas de estudio sobre las bases biológicas de esta patología.

V. CONCLUSIONES

1.- Los niveles intracelulares de hierro, en cultivos primarios de hipocampo, dependen tanto de los niveles de este metal en el medio de cultivo celular como del tiempo de exposición a este.

2.- Elevadas concentraciones de hierro en los cultivos de células hipocampales, generan daño oxidativo intracelular.

3.- En presencia de hierro, la proteína tau se encuentra hipofosforilada, respecto de la situación control, en epítopos de tipo Alzheimer, lo cual podría dar cuenta de los cambios que ocurren en etapas tempranas de esta patología. La estimulación de neuronas de hipocampo en cultivo con otro agente que genere daño oxidativo, H_2O_2, induce los mismos cambios en los estados de fosforilación en la proteína tau que los tratamientos con hierro.

4.- La hipofosforilacion de estos epítopos no está dada por cambios en los niveles de cdk-5 ni su fosforilación en la tirosina 15.

5.- La presencia de células gliales inhibe la hipofosforilacion de tau inducida por hierro, en un sistema de cultivos mixtos de neurona-glia.

6.- No existe correlación entre el grado de demencia de individuos seniles y los niveles de hierro totales en muestras de LCR. Sin embargo, existe una interesante correlación entre el grado de deterioro cognitivo y la fracción de hierro redox activa en LCR. Además existe una correlación inversa entre el deterioro cognitivo de un paciente que sufre de EA y los niveles de hierro redox activo en LCR.

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VII. PUBLICACIONES

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Iron-induced oxidative stress modify tau phosphorylation patterns in hippocampal cell cultures

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Abstract

Oxidative stress phenomena have been related with the onset of neurodegenerative diseases. Particularly in Alzheimer Disease (AD), oxygen reactive species (ROS) and its derivatives can be found in brain samples of postmortem AD patients. However, the mechanisms by which oxygen reactive species can alter neuronal function are still not elucidated. There is a growing amount of evidence pointing to a role for mitochondrial damage as the source of free radicals involved in oxidative stress. Among the species that participate in the production of oxygen reactive radicals, transition metals are one of the most important. Several reports have implicated the involvement of redox-active metals with the onset of different neurodegenerative diseases such as Alzheimer's Disease (AD), Progressive Supranuclear Palsy (PSP), Amyotrophic Lateral Sclerosis (ALS) and Parkinson's Disease (PD). On the other hand, our previous studies have indicated that Aβ-induced deregulation of the protein kinase Cdk5 associated with tau protein hyperphosphorylation constitute a critical pathway toward neurodegeneration. In the current paper we have shown that iron induces an imbalance in the function of Cdk5/p25 system of hippocampal neurons, resulting in a marked decrease in tau phosphorylation at the typical Alzheimer's epitopes. The loss of phosphorylated tau epitopes correlated with an increase in 4-hydroxy-nonenal (HNE) adducts revealing damage by oxidative stress. This effects on tau phosphorylation patterns seems to be a consequence of a decrease in the Cdk5/p25 complex activity that appears to result from a depletion of the activator p25, a mechanism in which calcium transients could be implicated.

Introduction

Oxidative stress can be defined as the loss of the balance between the systems that produce reactive oxygen species (ROS) and the antioxidant machinery. The production of ROS is the result of oxidative processes affecting cellular and biochemical integrity in neuronal cells. This imbalance occurs in several human neurodegenerative diseases and in animal models that mimics brain disorders (Markesberry 1997; Butterfield *et al.* 2001; Varadarajan *et al.* 2000). Neurodegenerative diseases involve protein aggregation, phenomena such as those occurring in Alzheimer's disease (AD) (Smith *et al.* 2000; Joseph *et al.* 2001; Maccioni *et al.* 2001a, b), Huntington's disease (HD) (Deckel 2001; Sayre *et al.* 2001), amyotrophic lateral sclerosis (ALS) (Estevez *et al.* 1999; Julien 2001), prion disorders such as Creutzfeld-Jacob disease (CJD) (Brown 2001), and disorders with aggregated α-synuclein such as Parkinson's disease (PD) and frontotemporal dementia (FTD) (Goedert *et al.*

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2001). In addition to aggregated α-synuclein in PD and FTD, other protein aggregates have been detected. These included β-amyloid peptide and tau protein in AD, hungtintin in HD and prions in CJD. The relationship between oxidative stress and protein aggregation still remains to be elucidated. In human aging there is an increase in oxidative stress markers and damage to cellular proteins, DNA and lipids (Christen 2000). The central nervous system (CNS) appears to be particularly vulnerable to ROS damage. A number of factors that contribute to the high vulnerability of the CNS to oxidative damage include a decreased level of the natural antioxidant glutathione in neurons, membranes containing a high proportion of polyunsaturated fatty acids (Hazel & Williams 1990), and a relatively increased oxygen requirement due to the high metabolic activities of the brain (Benzi & Moretti 1995).

During the last 10 years an increasing number of papers have dealt with the relationships between the presence of transition metals and neurodegenerative disorders (reviewed in Lynch *et al.* 2000; Qian & Shen 2001). Multiple lines of evidence implicate redox-active transitions metals as mediator of oxidative stress and ROS production in neurodegenerative disorders (Perez *et al.* 1998; Sayre *et al.* 1999; Quintana *et al.* 2000). Several studies have been conducted to analyze the roles of Fe, Cu, Mn and Zn in the production of oxygen and nitrogen reactive species, and oxidative stress damage to cells as related to neurodegeneration. Particularly iron has been implicated in the ethiopathology of several degenerative diseases of neuronal systems such as Alzheimer disease (Lovell *et al.* 1998), Parkinson (Jellinger 1999), progressive supranuclear palsy (PSP) (Perez *et al.* 1998) and cataracts (Goldstein *et al.* 2000). Iron is primarily stored in ferritin, although elevated levels of iron seem not to correlate with an increase in ferritin levels or the transport protein transferrin in Alzheimer's patients (Fischer *et al.* 1997). In this paper we analyze the changes in the levels of Cdk5 and p25 in hippocampal neuronal cells and tau hyperphosphorylation patterns in response to acute iron treatments. Our results indicate that iron-mediated oxidative stress induces oxidative stress markers such as HNE adducts and hemoxygenase-1, as compared with untreated neuronal cells and control cells treated with the iron chelating agent deferoxamine mesylate (Deferal). Furthermore, iron does not modify the intraneuronal level of Cdk5, even though a decreased pool of p25 was observed. The phosphorylation at Tyr-15 in Cdk5 was also assessed, revealing that iron did not produce any effect on the phophorylation dynamics. Interestingly, iron treatment of hippocampal cells with 20 μM Fe produced a significant decrease in the exposure of Alzheimer's type epitopes as analyzed with PHF-1 and AT-8 antibodies. The data suggests that the pathway involved in iron-mediated oxidative damage could involve different mechanisms than those implicated for β-amyloid induced oxidative stress.

Materials and methods

Primary cell cultures

Hippocampal neuron cell cultures were prepared from E18.5 rat embryos (Banker & Cowan 1977). Briefly, the hippocampus was dissected and then incubated in 0.25% trypsin-EDTA during 10 min at 37 °C. After trypsin digestion the tissue was washed with HBSS (GIBCO-BRL) solution and then disaggregated using a fire polished Pasteur pipette. Neurons were plated over poly-L-lysine coated coverslips at a 5,000 cells cm^2 for immunofluorescence experiments and 15,000 cells cm^2 for Western blots analyses. Cultures were maintained in 10% bovine serum until 3 h after plating, when the culture medium was replaced with medium containing the N2 supplement (GIBCO-BRL) (Bottenstein & Sato 1979). Cells were maintained in culture for 5 days, and the N2 medium was replaced every 48 h.

Iron treatment

Iron was supplied as iron citrate at 20 μM in a medium containing N2 supplement. For iron chelating experiments the drug Desferal (deferoxamine mesylate) (Sigma) was supplemented at 100 μM in a medium containing N2 supplement. Iron and iron-chelating treatments were performed during 24 h.

Immunoblots

After iron treatments, neurons were homogenized in RIPA buffer and the protein concentration determined by using the Bradford analyses (Bradford 1976). Equal quantity of each sample was resolved into 10% PAGE-SDS gels (Laemmli 1974). After transfer onto nitrocellulose membranes, samples were blocked in 5% non-fat dry milk and then incubated with the primary antibodies for 2 h at room temperature, or overnight at 4 °C. After three washing steps with PBS-Tween

(0.05%), membranes were incubated with peroxidase-conjugated secondary antibodies (Sigma). Finally, detection was performed using the chemiluminiscence system (ECL, Amersham Pharmacia) and samples were analyzed in a molecular imager FX (Biorad) of the Millennium Institute CBB core facility. We used the following primary antibodies: AT8 that recognize a phosphorylated epitope on tau protein; Tau-1 that recognize an unphosphorylated epitope of tau; PHF1 that recognize an Alzheimer's type phosphorylated epitope on tau protein, and Tau5 that recognize a normal phosphorylation epitope on tau protein conformation. A β-actin antibody (Sigma) was used to normalize the amount of protein loaded on each well.

Immunoflourescences

Cell cultures were fixed in 4% paraformaldehyde/4% sucrose during 15 min at 37 °C. After fixation, samples were permeabilized with 0.2% Triton X-100 at room temperature during 5 min. Samples were then washed three times in PBS and blocked with 5% BSA during 1 h at room temperature. Primary antibodies were diluted in 1% BSA and incubated in a wet chamber for 2 h at room temperature or overnight at 4 °C. After three washings with PBS, preparations were incubated with fluorescein or rhodamine-conjugated secondary antibodies (Sigma) during 1 h at room temperature (Capote & Maccioni 1998). Finally, samples were washed with PBS and mounted with Prolong mounting media (Molecular Probes). Additionally, F-actin was detected with rhodamine-conjugated Phalloidin at 5 μg ml (Sigma). Images were obtained from a Zeiss confocal microscope.

Results and discussion

Overall morphology of iron-treated hippocampal neurons

We first analyzed the effect of iron treatment on the general morphological features and cytoskeleton organization in cultured hippocampal cells. For such purpose, neuronal cultures maintained for 4 days *in vitro* (DIV) were incubated with increasing concentrations of iron ranging from 0 to 80 μM (data not shown). This study indicated that the optimal working iron concentration to be used for subsequent experiments was 20 μM, which is in the physiological range of iron concentration. Hippocampal neurons were at stage three of brain development by the time of iron administration (Dotti *et al.* 1988). There were not significant morphological differences between iron-treated and control cells, as analyzed through immunofluorescence using a monoclonal antitubulin FITC conjugated antibody and staining with rodhamine-Phalloidin for actin filaments (data not shown). Thus, cytoskeleton staining of microtubules and actin microfilaments showed no variations between treated and control groups. Iron does not appear to produce major changes in the cytostructure, therefore funcional aspects of iron-treated neurons (Maccioni *et al.* 2001b) was investigated in the context of the experiments described below.

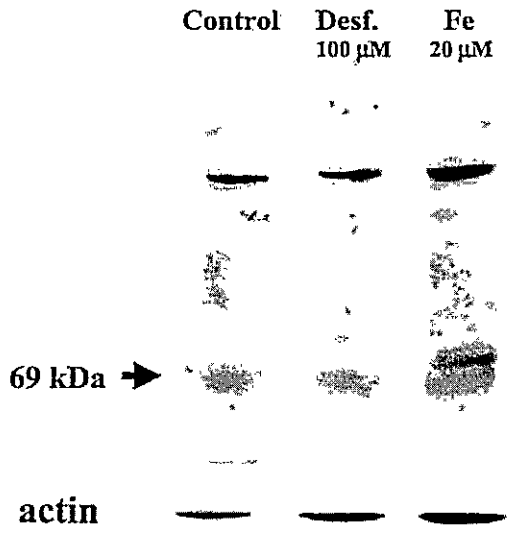

Fig. 1. Western blots of cells extracts from hippocampal neurons incubated in the presence or absence of iron. Neuronal cells were incubated in the absence of iron (control), or in the presence of 100 μM Deferal or 20 μM iron. Cells were homogenized and subjected to Western blot assays by using an anti-HNE adduct rabbit polyclonal antibody. The migration of actin as internal reference is indicated. A protein band of around 69 kDa was apparent after exposure of neuronal cells to iron.

A marker for lipid-peroxidation is increased in iron-treated neurons

It was of importance to analyze if iron-treatment produced an increase in the oxidative stress response within the hippocampal neurons. For such purpose we measured the levels of 4-HNE adducts. 4-HNE is an aldehyde product of lipid peroxidation that can damage primary neuron cell cultures (Mark *et al.* 1997), and can induce cross-linking of cytoskeletal proteins. As it is shown in the Figure 1, there is a marked increase in the amount of HNE-adducts in samples

derived from iron-treated cells. Controls using the iron chelating agent Desferal showed similar 4-HNE adducts levels as those observed in untreated controls, thus supporting the observation that the increase in HNE levels is due to iron overload. This increase in the amount of HNE-adducts found in treated cells also suggests that iron is triggering the oxidative stress response inside the neurons, and affecting lipids of the cell membrane. There is a vast amount of reports dealing with the importance of oxidative stress in the pathology of Alzheimer's disease (reviewed in Markesberry 1997; Smith *et al.* 2000). The results that imply free radical oxidative stress in AD includes: (i) increased levels of redox-active metals ion in AD brain; (ii) increased lipid peroxidation as detected by an increase in HNE adducts; (iii) increased protein, DNA and RNA oxidation, and upregulation of antioxidant enzymes; and (iv) extensive amounts of peroxynitrite and advanced glycation end products (AGE)-modifications. These studies point to the effect of iron treatment over hippocampal neurons in culture, due to an increase in oxidative stress of the cell. An increase in the amount of HNE-adducts in the iron-treated neurons support this conclusion. HNE-adducts are generated in response to lipid peroxidation induced by an increase in oxidative stress.

The levels of Cdk5 remained unchanged after iron treatment

Previous findings of our laboratory have implicated a deregulation of the Cdk5 enzyme in the pathway of Aβ-mediated neurotoxicity (Alvarez *et al.*, 1999, 2001). Studies have also revealed that an activation of the Cdk/p35 protein complex mediated by a single site Cdk5 phosphorylation is responsible for the anomalous overactivation of this protein kinase (Alvarez *et al.* 2001; Patrick *et al.* 2000). Additionally it has been shown that the generation of free radicals in neurons treated with Aβ may be important in the role of neurotoxicity (Behl *et al.* 1994; Harris *et al.* 1995; Mattson *et al.* 1995 a, b; Sagara *et al.* 1996). In this context, we decided to analyze the Cdk5 and the phospho-Cdk5 (at Tyr^{15}) levels (Zuckerberg *et al.* 2000) in the iron-treated cells. Looking at the total levels of this kinase we found no significant variations in the amount of Cdk5 (Figure 2A). Moreover, the levels of phosphorylated Cdk5 also remained unchanged as analyzed with antibody that recognize phosphorylated Tyr^{15} epitope on this protein (Figure 2B). However, when we analyzed the levels of p35, one of the neurospecific activators for Cdk5 we found that although p35 did not change its levels within the range of iron concentrations used, the expression of the soluble fragment p25 lacking the N-terminal p35 protein moiety decreased (Figure 2C). It has been reported that soluble p25 could act as a regulatory protein controlling Cdk5 activity (Patrick *et al.* 2000). Taken collectively these results suggest that iron-treatment of hippocampal cells produce a decrease in the activity of the Cdk5/p25 complex.

Iron alter the phosphorylation patterns of brain tau protein

Tau total levels were not altered in the cells treated with iron as shown with a phosphorylation independent antibody Tau-5 (Figure 3A). However, when we looked for the phosphorylated forms of the tau protein, we unexpectedly found a significant decrease in phosphorylated tau as shown with the AT8 antibody that recognize tau epitopes of Alzheimer's type (Figure 3B). Data of Figure 3 indicates that tau protein loses its hyperphosphorylation upon iron treatment. Studies have indicated that Cdk5 driven hyperphosphorylation occurs at residues Ser^{202}-Pro, Thr^{205}-Pro and Ser^{235}-Pro (Alvarez *et al.* 1999). Consistent with the latter result, the amount of tau protein in its hypophosphorylated form was increased in response to iron treatments, as shown with Tau1 antibody (Figure 3C). There are two generic reactions in which transition metals are related with some relevance to neurodegenerative disorders. First, a metal-protein association leading to protein aggregation; this reaction can involve redox-inert metal ions such as Zn^{2+}, or redox-active metal ions such as Cu^{2+} and Fe^{3+}. Second, metal-catalyzed protein oxidation leading to protein damage: this reaction involves a redox-active metal ion such as Cu^{2+}, Fe^{3+} or Mn^{2+} (Smith *et al.* 1997; Perry *et al.* 1998).

In the search for a mechanistic approach for the analysis of oxidative stress effects on tau phosphorylation patterns

Several reports have indicated that an imbalance in the oxidative stress cellular responses could be responsible for the hyperphosphorylation of cytoskeleton proteins involved in neurodegenerative diseases. This has been extensively analyzed with respect to the role of the microtubule-associated protein tau in the etiology and pathogenesis of Alzheimer's disease (Smith *et al.* 1998; Maccioni *et al.* 2001b). Nevertheless, there are

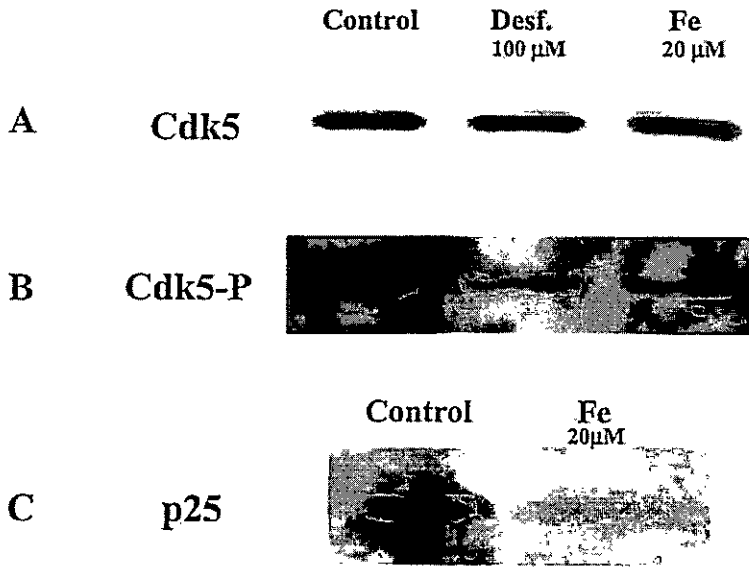

Fig. 2. Western blots of hippocampal extracts incubated in the presence or absence of iron. Neuronal cells were incubated in the absence of iron (control), and in the presence of 100 μM Deferal or 20 μM iron. A. Cells were homogenized and the high speed supernatants subjected to Western blots assays by using an anti-Cdk5 antibody clone C8 from Santa Cruz. B. Cell extracts were analyzed by using and anti-Cdk5 phosphorylated at Tyr15. C. Cell extracts of cells incubated in the presence and absence of iron were subjected to Western blot analysis for p25 using an anti-p25 monoclonal antibody.

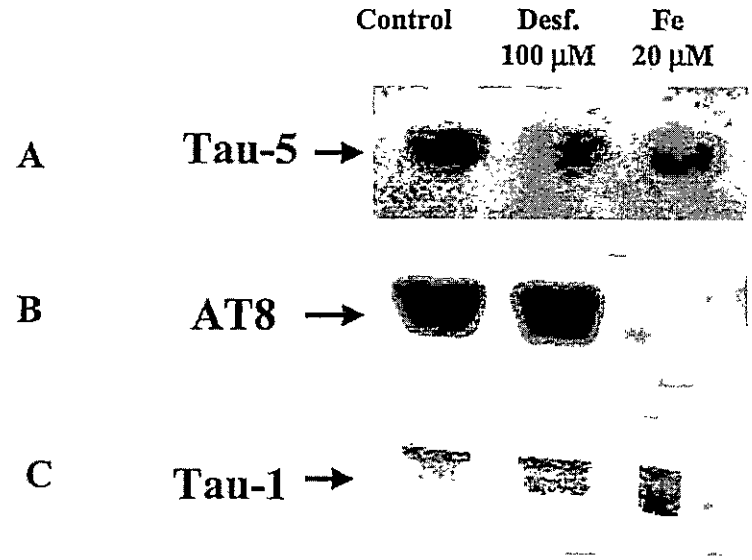

Fig. 3. Western blots of neuronal extracts incubated in the presence or absence of iron. Neuronal cells were incubated without iron (control), and in the presence of 100 μM Deferal or 20 μM iron. A. Hippocampal extracts treated and untreated with iron analyzed by Western blots using Tau-5 antibody which recognizes conformational epitopes for all tau variants. B. Cells were homogenized and the high speed supernatants subjected to Western blots assays by using the AT8 antibody that recognizes Azheimer's type tau epitopes. C. Cell extracts were analyzed by using Tau-1 antibody that recognizes unphosphorylated tau.

contradictory data on the phosphorylation state of tau protein in response to oxidative stress. Some reports point out an increase in the phosphorylation of tau in AD brains derived tissue (Takeda *et al.* 2000). On the other hand H_2O_2-induced oxidative stress has been shown to produce dephosphorylation of tau protein in rat primary neuronal cultures (Davis et al. 1997). An increase in the amount of dephosphorylated tau is also shown in neurons treated with glutamate (Anderton *et al.* 1995; Davis *et al.* 1995; Fleming & Johnson 1995) and ischaemia (Geddes *et al.* 1994; Schakelford & Nelson 1996). However the decrease of hyperphosphorylated tau protein is not dependent on an increase of GSK3β kinase levels (Davis *et al.* 1997). Thus, the increase of dephosphorylated tau isoforms upon oxidative stress effects is likely to depend on Cdk5, since AT8, PHF1 and Tau1 antibodies recognize mainly phosphoepitopes on tau that are catalyzed by kinases belonging to the proline directed protein kinases family. This could be the situation for the effects of iron observed in this study, since the depletion in p25 levels could account for the low levels of tau phosphorylation upon iron treatment. Therefore, the fine regulation and cross-talks of these kinases involved in the molecular events in the pathogenesis of Alzheimer's disease, needs further analysis (for review Maccioni *et al.* 2001b).

The fact that different oxidative stress treatments lead to contradictory results in the tau phosphorylation levels suggest that some of these differences could be related to variations of intracellular messengers in response to such treatments. Calcium ion could be one of the possible intracellular messengers involved in this response. Calcium is an important intracellular messenger for neuronal signaling pathways. Through variations in both the amplitude and frequency of intracellular calcium transients, the same calcium ion can elicit different responses. Alterations in intracellular calcium concentrations are clearly involved in modulating the phosphorylation state of tau protein *in situ*. However, results have been decidedly mixed, and there is little consensus as to the specific effects of elevating calcium intracellular concentration on tau phosphorylation. For example, acute treatment of primary neuronal cultures with calcium ionophores has been reported to increase (Mattson *et al.* 1991; Mattson *et al.* 1992) and decrease (Adamec *et al.* 1997) tau phosphorylation. The same mixed results have been found using human neuroblastoma cell lines, as ionophore treatment resulted in both increases (Shea *et al.* 1997) and decreases (Xie & Johnson 1998) in tau phosphorylation. Increasing intracellular calcium by activation of N-methyl-D-aspartate receptors has been shown to result in the dephosphorylation of tau in rat brain slices (Fleming & Johnson 1995) and cortical neuronal cultures (Adamec *et al.* 1997). In an elegant study, it was shown that the variations of the levels of tau phosphorylation were in fact dependent on the transient calcium concentration (Hartigan & Johnson 1999). Thus, a possible explanation for discrepancies between different studies dealing with the role of oxidative stress could be related with the overall modification of the intracellular calcium homeostasis. These differences could be mimicking some acute or chronic responses of the cellular machinery to oxidative stress (Figure 4).

What are the relationships between Aβ neurotoxicity and oxidative stress effects in neurodegeneration? In regard to β-amyloid effects in inducing deregulation of the Cdk5/p35 complex, Cdk5 activation increases hyperphosphorylated tau protein in neuronal cell cultures (Alvarez *et al.* 1999) and in a transgenic mice overexpressing tau protein (Gotz *et al.* 2001). Moreover, tau hyperphoshphorylation is also detected in a double mutant mouse overexpressing amyloid precursor protein and tau (Lewis *et al.* 2001). The effect of Aβ fibrils have been suggested to produce an increase in the oxidative stress (Behl *et al.* 1994; Harris *et al.*, 1995; Manelli & Puttfarcken 1995; Mattson 1995a, b; for review Maccioni *et al.* 2001b). Also, it has been reported that Aβ induced an increase in tau phosphorylation (Busciglio *et al.* 1995; Takashima *et al.* 1996 Alvarez *et al.* 1999).

Alzheimer's disease is characterized by the deposition of Aβ within the neocortex, associated with neuronal loss and oxidative stress. The deposition of Aβ is considered to be closely related to the primary pathogenesis of familial AD. Familial AD-linked mutations of amyloid precursor protein (APP), presenilin-1 and presenilin-2, increase both cerebral Aβ burden and Aβ1-42 production, underscoring the role that Aβ metabolism plays in AD pathogenesis. Furthermore, the deposition of Aβ in the neocortex of transgenic mice overexpressing Aβ is accompanied by many of other neuropathological features of AD including intraneuronal tau abnormalities and neuronal loss (Calhoun *et al.* 1998), as well as signs of oxidative damage similar to those observed in AD-affected brain (Smith *et al.* 1998). The length of the Aβ species is considered to be one important factor in AD pathogenesis as Aβ1-42, a minor free soluble species in biological fluids, is enriched in amyloid

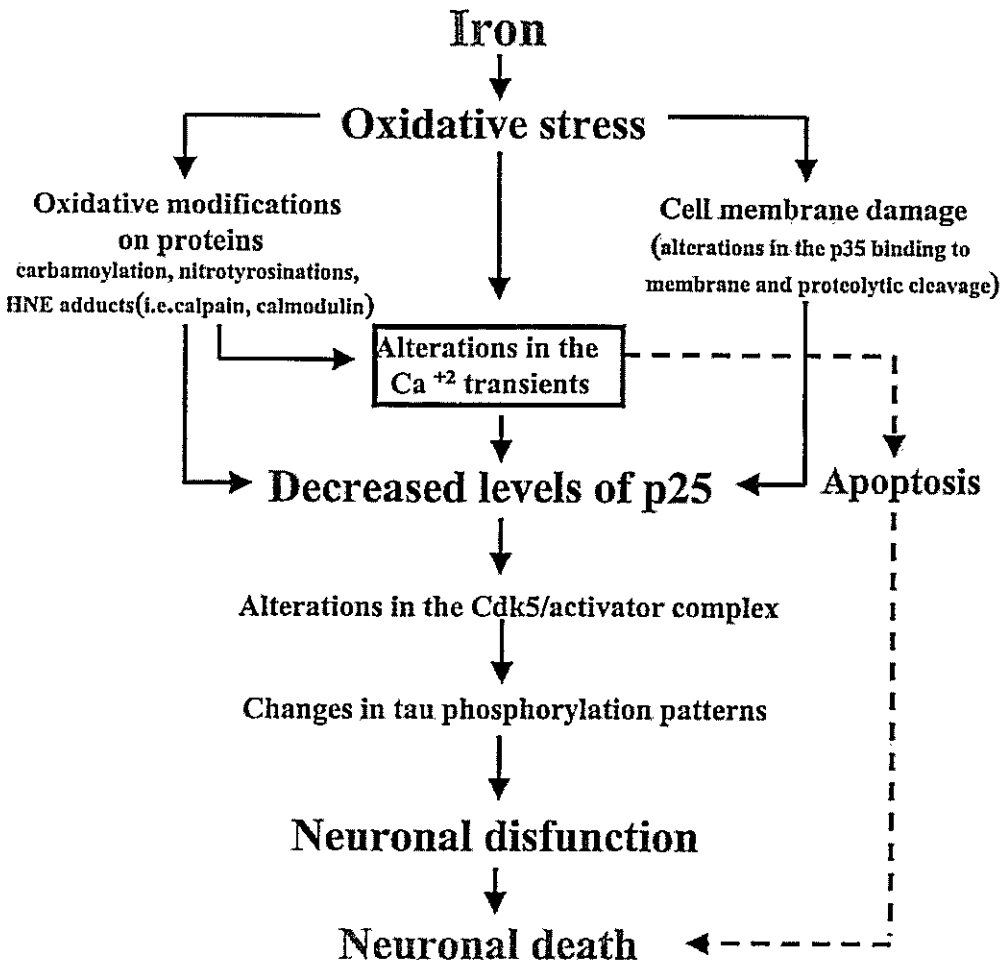

Fig. 4. Schematic representation of the action of oxidative stress via iron effects on neurodegeneration of hippocampal cells on the basis of data from these studies and previous investigations (for review Maccioni *et al.* 2001b). The mechanism considers the possible contribution of changes in calcium transient and the depletion in Cdk5 activators (i.e., p25) in the observed changes on phosphorylation patterns on brain tau protein.

deposits. Many studies have now confirmed that Aβ is neurotoxic in cell culture. Hence, there is a compelling argument to considered Aβ deposition as a therapeutic target in AD. As for transition metals role in Aβ-mediated neurotoxicity it has been suggested that Cu^{2+} and Fe^{3+}, unlike Zn^{2+} induce greater Aβ aggregation under mildly acidic conditions such as those believed to occur in AD brain (Atwood *et al.* 1998). Significantly, the solubility of rat or mouse Aβ1-40 is unaffected by Zn (II) or Cu (II) at low micromolar concentrations. Apolipoprotein E can also modulates the precipitation of Aβ by Cu^{2+} and Zn^{2+}, which is important because ApoE isoforms segregate with the genetic risk for AD; inheritance of the ApoE4 isoform carries the greatest risk. The ApoE4 isoform is poorest in maintaining Aβ in a soluble form, as compared with the others isoforms (ApoE2 and ApoE3), whether the precipitating metal is Cu^{2+} or Zn^{2+} (Moir *et al.* 1999). It has been also reported that some Cu/Zn selective chelators enhance the solubilization of Aβ deposits from post-mortem AD brain samples, supporting the possibility that these metals could play a role in the assembly of the deposits (Atwood *et al.* 1998). However, metals could be playing more than this role. It has been also reported that Aβ is redox active, and reduces Cu (II) or Fe (III) and then produces H_2O_2 by electron transfer to O_2. The metal-reducing activity and H_2O_2 production of Aβ species is enhanced in human Aβ42 as compared with human Aβ40. Considering that neurodegeneration is a multifactorial process, these investigations suggest that oxidative stress and Aβ amyloid, through its different

aggregation forms, could induce different alterations in the signaling pathways of neurons. This could be exerted by either independent mechanisms or by concerted actions, and the potentiation of these signals appears to be critical for neuronal degeneration.

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Original Contribution

OXIDATIVE STRESS PROMOTES τ DEPHOSPHORYLATION IN NEURONAL CELLS: THE ROLES OF Cdk5 AND PP1

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Abstract—Oxidative stress has been demonstrated to produce modifications in several intracellular proteins that lead to alterations in their activities. Alzheimer's disease is related to an increase of oxidative stress markers, which may be an early event in the progression of the disease and neurofibrillary tangles formation. Abnormal phosphorylation of τ has been implicated in the etiopathogenesis of Alzheimer's disease. By using phospho-specific antibodies, we analyzed the changes in τ phosphorylation patterns after treatment of rat hippocampal and SHSY5Y human neuroblastoma cells with H_2O_2. We found that τ isoforms were hypophosphorylated at the Tau1 epitope after 2 h in the presence of H_2O_2. The decrease in the phosphorylation levels of τ protein were prevented by pretreatment with N-acetyl-L-cysteine. These changes were shown to depend on the activity of the cdk5/p35 complex, since a 3-fold increase in substrate phosphorylation and a 2-fold increase for the complex association were observed. Also, a decrease in the amount of inhibitor-2 bound to phosphatase PP1 was found in SHSY5Y cells under oxidative stress conditions. This decrease of inhibitor-2 bound to PP1 is due to an increased phosphorylation of the inhibitor-2 protein, thus leading to increased PP1 activity. Therefore, we propose that oxidative stress-induced activation of cdk5 leads to inhibitor-2 phosphorylation, relieving its inhibitory effect on PP1.

Keywords—Tau phosphorylation, Oxidative stress, Cyclin-dependent kinase 5, Protein phosphatase 1, Hydrogen peroxide, Alzheimer's disease, Free radicals

INTRODUCTION

Oxidative stress is a major pathological aspect in several neurodegenerative conditions such as stroke [1], spinal cord injury [2], multiple sclerosis [3], amyotrophic lateral sclerosis [4], Parkinson's disease [5], frontotemporal dementia [6], and Alzheimer's disease (AD) [7]. Oxidative stress results from the generation of reactive oxygen species (ROS) by the electron transport chain in the mitochondrion or another enzymatic system such as NADPH oxidase. AD is an aging-related pathology that is characterized by the presence of oxidative stress markers such as 8-hydroxyguanosine and hydroxynonenal adducts at early stages of the pathology [8]. Hydrogen peroxide (H_2O_2) is particularly toxic because it is considered to be a relatively stable compound among free radical species that can diffuse freely inside cells [9]. It can be detected in the rat brain at micromolar concentrations after ischemia/reperfusion [10]. Once inside the cell, it reacts with Fe^{2+} to give rise, via the Fenton reaction, to the highly reactive hydroxyl radical [11].

AD is characterized by the accumulation of senile plaques largely composed of the Aβ peptide within the neocortex. This deposition has been implicated in neuronal death and is related to oxidative stress. Also, the abnormal accumulation of the aberrantly phosphorylated microtubule-associated protein τ is another feature of the disease. Many efforts have been made to establish clear and univocal relationships between these two pathological hallmarks. In this regard, it has been indicated that

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Aβ can produce an increase in oxidative stress [7,12–18], and it has been reported that Aβ induces an increase in τ phosphorylation, indicating that Aβ neurotoxicity is mediated by τ modifications [19–22]. These effects of Aβ are overcome by inhibiting two proline-directed protein kinases, namely gsk3 and cdk5. Thus, Aβ neurotoxicity is diminished in neuronal cells incubated with butyrolactone I or cdk5 antisense oligonucleotides [21,22] and with lithium chloride [23]. On the other hand, the role of phosphatases in the regulation of τ phosphorylation patterns has been focused on PP1, PP2A, and PP2B (calcineurin). It has been shown that cdk5 is involved in the phosphorylation of the inhibitor-2 (I-2) of phosphatase PP1 at Thr^{72}, leading to its dissociation from the phosphatase and ultimately to its activation [24]. Despite some controversial results regarding the effect of oxidative stress on the phosphorylation levels of τ protein [25–27], it is clear that τ phosphorylation can be modified in response to such cellular insults. In this report, we describe the decrease of τ phosphorylation due to H_2O_2 being paralleled by an increase in the phosphorylation of I-2 and PP1 activity, most likely due to decreased association of I-2 to the enzyme. These changes depend on an increase in the cdk5 activity. Thus, at early stages of the oxidative stress exposure of neuronal cells, cdk5 could promote τ dephosphorylation of τ at the Tau1 epitope by activating PP1.

MATERIALS AND METHODS

Cell cultures

Hippocampal neuronal cell cultures were prepared from E18.5 rat embryos [28]. Briefly, hippocampi were dissected and then put into 0.25% trypsin-EDTA for 15 min at 37°C. After trypsin treatment, the tissue was washed with HBSS solution (Gibco BRL, Grand Island, NY, USA) and then disaggregated using a fire-polished Pasteur pipette. Neurons were plated on poly-L-lysine-coated coverslips at 5000 cells/cm^2 for immunofluorescence experiments and 15,000 cells/cm^2 for Western blot analyses. Cultures were maintained in 10% horse serum for 3 h after plating, when the culture medium was replaced with medium containing the N2 supplement (Gibco BRL) [29]. Cells were maintained in culture for 5 days, and the N2 medium was replaced every 48 h. Human neuroblastoma SHSY5Y cells were cultured in DMEM/10% FBS. Prior to the experiments with H_2O_2, the medium was replaced with DMEM without serum.

H_2O_2 treatment

H_2O_2 (Sigma Chemical Co., St. Louis, MO, USA) previously diluted with sterile PBS was added to cell cultures at 100 μM in serum-free medium containing an N2 supplement. Also, antioxidant treatments with N-acetyl-L-cysteine (NAC) were performed 30 min before the addition of H_2O_2 to prevent oxidative stress. All the treatments were done on neurons cultured during the 5 days. All reagents were prepared freshly before use.

Viability assays

Primary hippocampal neurons were seeded into 96-well culture plates coated with poly-lysine at 100 μg/ml. Different concentrations of H_2O_2, NAC, or a combination of both were added to the cells. MTT was added to all wells and further incubated overnight at room temperature in the dark [21]. The day after, neurons were lysed and absorbance at 590 nm was determined using a Metertech E960 spectrophotometer (Metertech Inc., Nankang, Taipei, Taiwan). MTT assays were performed in triplicate. Also, neuronal viability was assayed with the Mito-Capture kit (Calbiochem, San Diego, CA, USA) following the manufacturer's instructions.

Immunoblots

Neurons were homogenized in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin] and the protein concentration was determined by the Bradford analysis [30]. Equal aliquots of each sample were separated in 12% PAGE-SDS gels [31]. After transfer to nitrocellulose membranes, samples were blocked in 5% nonfat dry milk and incubated with primary antibodies for 2 h at room temperature or overnight at 4°C. After three washing steps with PBS, membranes were incubated with peroxidase-conjugated secondary antibodies (Sigma Chemical Co.). Finally, detection was performed using a chemiluminescence system (Amersham, Arlington Heights, IL, USA) and samples were analyzed in a Molecular Imager FX (Bio-Rad, Richmond, CA, USA). We used the following primary antibodies: AT8, which recognizes phosphorylated epitopes Ser^{202} and Thr^{205} on τ protein; Tau1, which recognizes the same unphosphorylated epitopes of τ; PHF1, which recognizes phosphorylated epitopes Ser^{396} and Thr^{404}; and Tau5, which recognizes an independent phosphorylation epitope on τ protein. The antibody against Michael's adducts of 4-hydroxynonenal-L-lysine was purchased from Calbiochem. Monoclonal anti-cdk5 and polyclonal anti-p35 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used according to the manufacturer's instructions. Monoclonal antibodies generated from hu-

man PP1 α and I-2 were purchased from BD Transduction Laboratories (San Jose, CA, USA).

Immunofluorescence

Cell cultures were fixed in 4% paraformaldehyde/4% sucrose for 15 min at 37°C. Subsequently, fixation samples were permeabilized with 0.2% Triton X-100 at room temperature for 5 min. Samples were then washed three times in PBS and blocked with 5% BSA for 1 h at room temperature. Primary antibodies were diluted in 1% BSA, 0.1% Triton X-100, and incubated in a humid chamber for 2 h at room temperature or overnight at 4°C. After washing three times with PBS, preparations were incubated with fluorescein or rhodamine-conjugated secondary antibodies (Sigma Chemical Co.) for 1 h at room temperature. Finally, samples were washed with PBS and mounted with Prolong mounting media (Molecular Probes, Eugene, OR, USA). Images were acquired with a Zeiss LSM confocal microscope, model META (Carl Zeiss, Göttingen, Germany).

Immunoprecipitation and cdk5 activity assays

Primary cultured neurons were plated at 5×10^5 cells/cm^2 on polylysine-coated 60 mm dishes. After treatments, cells were lysed in RIPA buffer; 100 μg of total protein extract were used for immunoprecipitation with an anti-cdk5 antibody (C8 antibody; Santa Cruz Biotechnology) at a final dilution of 1:100. Then, the antigen-antibody complex was captured with either agarose-protein A for polyclonal antibodies or agarose-protein G for monoclonal antibodies. For in vitro kinase assays, the immunoprecipitates were rinsed three times with RIPA buffer and once with kinase buffer (50 mM Hepes, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT). The rinsed agarose beads were incubated with kinase buffer containing 2.5 μg histone H1 plus 5 μCi [γ-^{32}P] ATP, in a final volume of 30 μl, for 30 min at 30°C. After incubation, the samples were analyzed by SDS-PAGE and autoradiography with a Molecular Imager FX (Bio-Rad).

In vitro phosphatase assay

To analyze PP1 activity, the enzyme was immunoprecipitated from both control and H_2O_2-treated SHSY5Y cells. Immunopurified PP1 was washed with phosphatase buffer containing 37.5 mM HCl-Tris (pH 7.5), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.01% Tween 20, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin protease inhibitors. The enzyme was then mixed with 50 μg/ml of purified τ from bovine brain in a final volume of 30 μl for 1 h at 30°C. The reaction was stopped by adding 4X Laemmli buffer and the samples were then analyzed for τ phosphorylation in the AT8 epitope.

Metabolic labeling of I-2 protein

SHSY5Y cells were incubated for 2 h with DMEM medium without phosphate, and then 200 μCi/ml of aqueous radiolabeled [^{32}P]-phosphate was added. After 1 h, 100 μM H_2O_2 was added to cells, while control cells were incubated with vehicle. Later on, the cells were collected for I-2 immunoprecipitation, separated by SDS-PAGE, and analyzed for $^{32}PO_4$ incorporation through a Molecular Imager FX (Bio-Rad).

RESULTS

Oxidative stress in hippocampal neurons and SHSY5Y cells

In the first set of experiments, we assessed the effect of oxidative stress induced by H_2O_2. For this purpose, we initially determined the number of live neurons after treatment with different concentrations of H_2O_2. We analyzed hippocampal neuronal viability for H_2O_2 concentrations ranging from 0 to 500 μM. At 100 μM H_2O_2, 84% of the cells were viable (Fig. 1B). Even at higher concentrations up to 500 μM, over 75% of the neurons were viable. For all subsequent experiments, we set the H_2O_2 concentration at 100 μM. To confirm that H_2O_2 treatment was not deleterious in our system, we analyzed cell viability by using the Mito-Capture assay. As indicated in Fig. 1A, there were no variations between control and treated cells. Finally, to confirm that oxidative stress response produced after H_2O_2 treatment leads to protein modifications implicated in the oxidative stress pathways, we determined the formation of 4-hydroxynonenal (4-HNE) adducts, a canonical post-translational modification of proteins after oxidative stress insults [32]. Thus, by using indirect immunofluorescence, we verified that hippocampal cells under oxidative stress showed an increase of 4-HNE adducts, particularly in those regions corresponding to the axon and minor processes (Fig. 1C). Taken together, these results indicate that the effects mediated by H_2O_2 indeed involve oxidative stress pathways. On the other hand, the concentration of H_2O_2 used was not deleterious for neuronal survival at working concentrations after 2 h of treatment. To discard the possibility that these effects were due to alterations in glial cells contaminating at our primary hippocampal cultures, we estimated the number of glial cells in our culture system. Using GFAP staining, we estimated that glial cells represented less than 5% of the total cell population (data not shown).

Tau phosphorylation is modified by oxidative stress in hippocampal cells

Oxidative stress has been related to the onset of AD [7], and one of the pathological hallmarks for AD is the

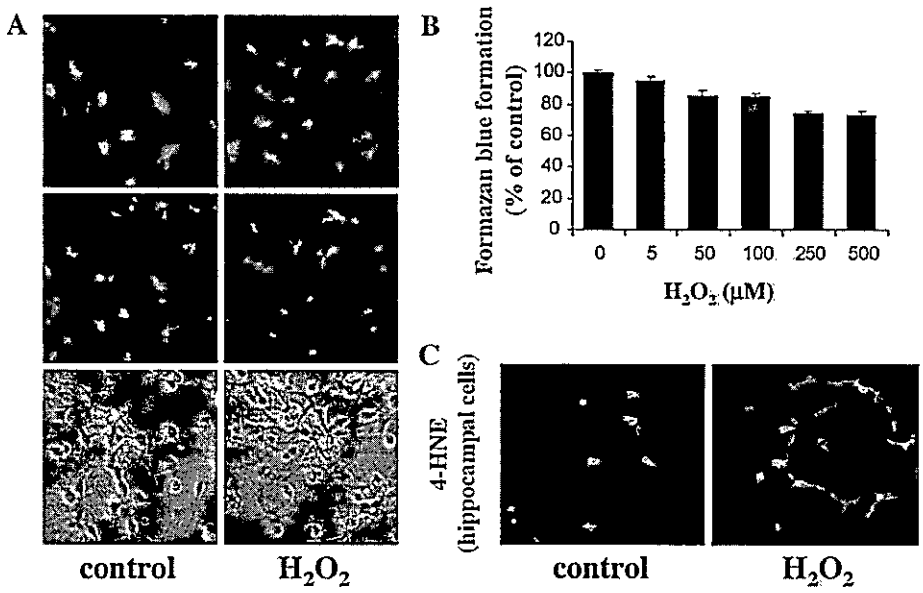

Fig. 1. Viability of hippocampal cells under oxidative stress conditions. (A) No differences in hippocampal neurons viability after treatment with H_2O_2 were evident, as assessed by the Mito-Capture assay. (B) The percentage of live hippocampal cells after treatment with different H_2O_2 concentrations, as assessed by the MTT assay. Based on these data, the working concentration for the subsequent experiments was set at 100 μM. (C) Immunofluorescence of 4-HNE adduct formation with ε-lysines in hippocampal cells treated with 100 μM H_2O_2 and the untreated controls (magnification ×40 in A and C).

accumulation of the abnormally hyperphosphorylated microtubule-associated protein τ. Previous findings from our laboratory indicated that Aβ peptide induced an imbalance in the amount of phosphorylated τ due to a deregulation of the cdk5/p35 complex [22]. Also, Aβ effects have been related to an increase in the oxidative stress response of neuronal cells [7,12–18]. Therefore, by using phosphospecific antibodies, we analyzed the relative amounts of phosphorylated and unphosphorylated τ epitopes AT8, PHF1, and Tau1 following H_2O_2 treatment. As indicated in Fig. 2A, hippocampal cells treated with 100 μM H_2O_2 exhibited a decrease in the amount of phosphorylated τ variants, as given by AT8 antibody. Consistent with this finding, a decrease in the PHF1 epitope was also verified (Fig. 2A). Concomitantly with these results, an increase in the amount of species recognized by Tau1 antibody, which reacts with unphosphorylated τ epitopes, was evidenced (Fig. 2A). These variations in the relative abundance of phosphorylated τ isoforms were not due to differences in the amount of total protein, since protein extracts incubated with the Tau5 were equivalent in all cases (Fig. 2A). Tau5 antibody recognizes conformational epitopes on total τ protein in a phosphorylation-independent way. These data were also confirmed in fixed neurons through indirect immunofluorescence. Thus, a decrease in the amount of phosphorylated τ forms was verified in H_2O_2-treated neurons, as given by AT8 antibody (Fig. 2B). In the same manner, an increase of unphosphorylated forms of τ protein was detected in H_2O_2-treated cells, as given by Tau1 antibody (Fig. 2B). No variations between control and treated cells were found with Tau5 (Fig. 2B) or by actin immunostaining of these cells (data not shown). Interestingly, the overall appearance of neurons seemed unchanged after H_2O_2 treatment. Altogether, these results indicate that H_2O_2 treatment produces a decrease in the phosphorylation levels of τ protein at epitopes that have been shown to be dependent on cdk5 activity.

Cdk5 activity is increased in neurons subjected to oxidative stress

Since AT8 and Tau1 recognize epitopes dependent on proline-directed protein kinases, a good candidate to assess such responses was the cdk5/p35 complex. We first analyzed the total levels of the kinase in protein extracts derived from control and H_2O_2-treated neurons. The levels of the protein kinase were unaltered in response to oxidative stress stimuli. Furthermore, the presence of the antioxidant NAC did not alter the total protein levels of cdk5 (Fig. 3A). Loading was verified by using actin as an internal control (Fig. 3A). We then estimated the amount of the kinase in the different experimental conditions and we found no variations between control and treated groups (Fig. 3B). As indicated above, our findings dealing with the levels of phosphorylated τ suggested that the activity of the cdk5/p35 complex could be decreased. Thus, we decided to analyze cdk5 activity in vitro. Surprisingly, immunoprecipitated cdk5 after H_2O_2 treatment was

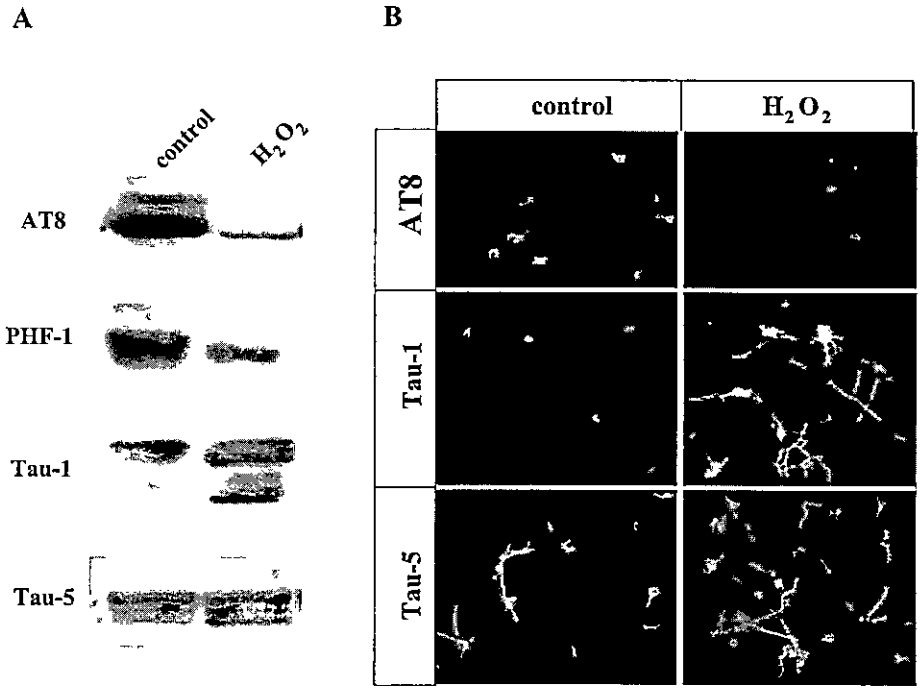

Fig. 2. Oxidative stress decreases τ phosphorylation. (A) Western blots of hippocampal neurons treated with 100 μM H_2O_2, showing that H_2O_2 induces a decrease in the phosphorylated forms of τ protein. AT8 (first panel), PHF-1 (second panel), and Tau1 (third panel) antibodies are phosphoepitope-specific antibodies used to detect either phosphorylated or unphosphorylated τ, respectively. Tau5 (fourth panel) recognizes total τ isoforms in a phosphorylation-independent manner. (B) Immunofluorescence of hippocampal neurons treated with 100 μM H_2O_2 reproduce the results obtained in the Western blot analyses. Thus, a decrease in AT8 (upper panel) staining concomitant with an increase in Tau1 (middle panel) staining is verified in hippocampal cells. In control experiments, no changes in Tau5 (lower panel) staining were detected.

found to be more active as shown by the phosphorylation of histone-1 (Fig. 3C). The activation of the kinase occurred even 1 h after H_2O_2 treatment (Fig. 3C). The presence of NAC again was able to prevent the cdk5 activation (Fig. 3C). We estimated the increase of cdk5 activity to be 3.5-fold compared to untreated controls (Fig. 3D). Since cdk5 activity depends on neurospecific activators binding, we then analyzed the expression profile of the neurospecific cdk5 activator p35. Overall, p35 and p25 protein levels were unchanged in neurons treated with H_2O_2, NAC, or both compounds (Fig. 3E). As in the previous experiments, actin was used as a loading control. The fact that we could not detect changes in the overall amount of p35 gave rise to the possibility that variations in the activity of the kinase was indeed due to changes in the affinity of the activator for the kinase after H_2O_2 treatment. To test this hypothesis, we performed cdk5 immunoprecipitation after H_2O_2 incubation, and then we estimated the amount of p35 bound to cdk5, leading to an active complex. Interestingly, an increase in the amount of p35 bound to cdk5 was verified in the H_2O_2 condition (Fig. 3F). This result suggests that H_2O_2 treatment induced an increase in the activity of cdk5 that depended on differential p35 binding to the enzyme.

Cdk5 activity is involved in the PP1-dependent dephosphorylation of τ

To evaluate the dependence of cdk5 activity on the response to oxidative stress in neural cells, we performed combined treatments of H_2O_2 and roscovitine, an inhibitor of cdk5. Hippocampal cells under this oxidative stress stimulus showed an increase in the unphosphorylated τ species at the Tau1 epitope (Figs. 2A and 4A). When hippocampal cells were exposed to H_2O_2 in combination with roscovitine, a decrease in the unphosphorylated forms of τ protein was observed, as given by Tau1 staining. These results suggest that, under these particular oxidative stress conditions, cdk5 might be mediating τ dephosphorylation rather than increasing τ phosphorylation (Fig. 4A). Quantification by densitometric analyses confirmed the Western blot data (Fig. 4C, $p < 0.05$). A possible explanation for this behavior might be that cdk5 plays a role in the control of a protein phosphatase activity responsible for τ dephosphorylation at the Tau1 epitope. To further examine this possibility, we combined treatment with H_2O_2 and 1 μM okadaic

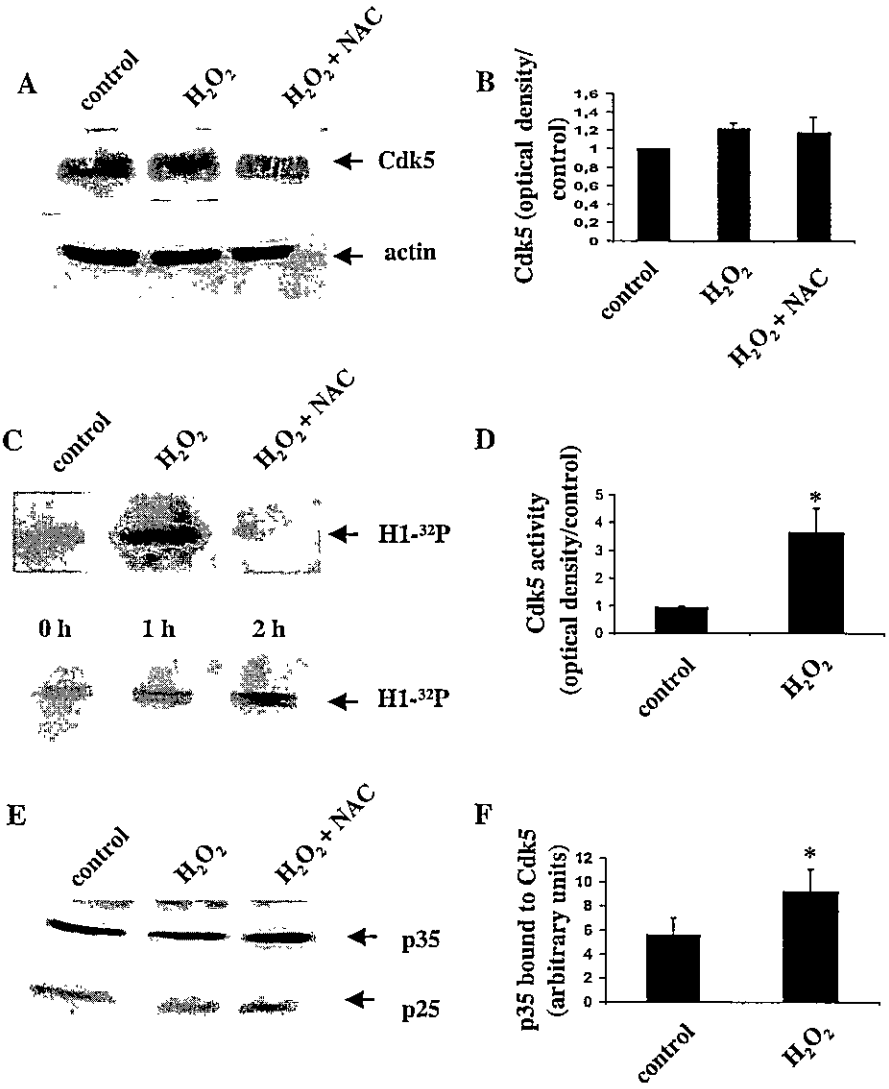

Fig. 3. Oxidative stress increases cdk5 activity. (A) Cdk5 protein levels were not modified in the presence of H_2O_2 or after the addition of H_2O_2 plus NAC, as assessed by Western blot analysis. Actin was used to normalize samples loading. (B) Densitometric analyses showing no significant variation in the overall levels of the kinase. (C) Cdk5 activity assay, using histone H1 as substrate, showed an increase in enzyme activity after 2 h of treatment with H_2O_2. The effect was further verified by autoradiography at shorter time intervals (1 h, lower panel). Also, the effect on cdk5 activity was reversed by adding NAC, as analyzed by autoradiography (upper panel). (D) Densitometric analyses showing a 3-fold induction in the cdk5 activity after H_2O_2 treatment ($n = 4$, $p < 0.05$). (E) p35 and p25 overall levels in neurons treated with H_2O_2. There were no variations in the amount of both p35 and p25 levels in control and treated cells. Also, treatment with H_2O_2 plus antioxidant NAC did not modify endogenous cdk5 activator levels. (F) Graph showing a variation in the amount of p35 bound to immunoprecipitated cdk5. Statistical analyses indicating that p35 binding to cdk5 is significantly increased after H_2O_2 treatment ($n = 4$, $p < 0.05$).

acid, an inhibitor of protein phosphatases PP1 and PP2A. Interestingly, okadaic acid treatment was able to reverse H_2O_2-induced dephosphorylation (Fig. 4B). Also, incubation of cells with 5 nM okadaic acid, a concentration described to be specific for PP2A inhibition, failed to reverse τ dephosphorylation, suggesting that PP1 could be the phosphatase responsible for τ dephosphorylation. Moreover, analogous treatment with cyclosporine A, a calcineurin inhibitor, did not affect τ dephosphorylation at the Tau1 epitope induced by H_2O_2 (Fig. 4B). These data indicate that τ dephosphorylation may be mediated by PP1 rather than PP2A and PP2B. Again, quantitative densitometric analyses clearly revealed the okadaic acid effects in reversing τ dephosphorylation at the Tau1 epitope (Fig. 4C).

It has been previously reported that cdk5 could be involved in the phosphorylation of the PP1 I-2, resulting in the loss of a negative regulation of the phosphatase due to dissociation of the inhibitor [24]. To confirm this possibility, we immunoprecipitated PP1 from human

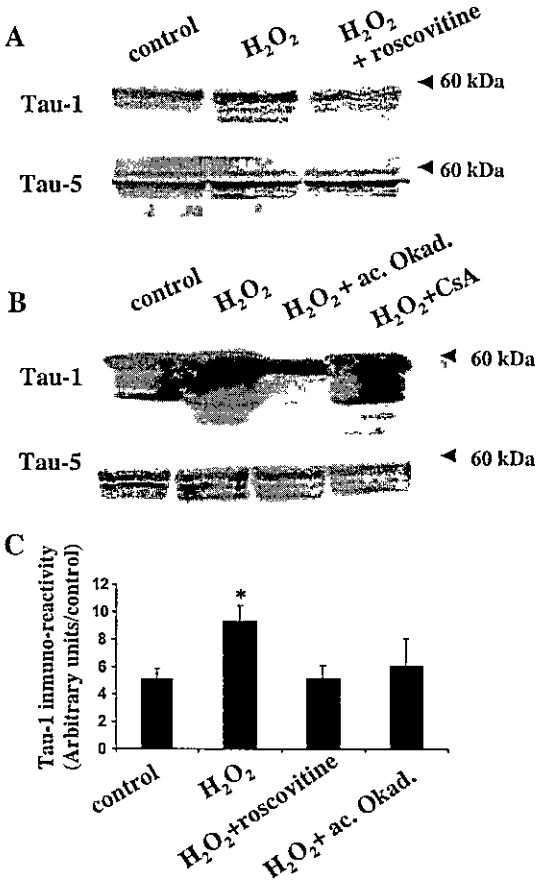

Fig. 4. Cdk5 and PP1 inhibitors prevent τ dephosphorylation. (A) Tau dephosphorylation induced by oxidative stress is reversed in the presence of the cdk5 inhibitor roscovitine. (B) Tau dephosphorylation induced by oxidative stress is also reversed in the presence of okadaic acid, an inhibitor of protein phosphatase PP1. On the other hand, the calcineurin inhibitor cyclosporine A did not reverse τ dephosphorylation. (C) Quantitative analyses indicating the significant increase of Tau1 staining in H_2O_2-treated neurons, and the reversion of the effect with both cdk5 and PP1 inhibitors ($p < 0.05$). Details are given in Materials and Methods.

SHSY5Y cells treated with H_2O_2 at different time intervals. Subsequently, we analyzed the amount of the I-2 pulled down by PP1 (Fig. 5). Notably, we found a significant decrease in the amount of I-2 associated with PP1 after H_2O_2 treatments. The time-dependent decrease occurred after 15 min of H_2O_2 treatment and continued for at least 2 h. Concomitant with a loss of PP1 inhibition, we verified an increase in the dephosphorylated forms of τ associated with the immunoprecipitated pellets containing PP1, as given by the Tau1 epitope antibody (Fig. 5A, Tau-1 P) and a decrease in the soluble forms of dephosphorylated τ (Fig. 5A, Tau-1 S). All the stated effects were verified without any substantial change in the PP1 levels (Fig. 5A). These results clearly suggest that PP1 activation in response to oxidative stress is most likely to depend on the release of I-2 from the I-2/PP1 complex. Quantitative analyses showed a 3-fold decrease in the amount of I-2 associated with PP1 after 2 h of H_2O_2 treatment (Fig. 5B).

To substantiate that activation of cdk5 after H_2O_2 treatment was paralleled by changes in the phosphorylation of I-2 and the increase in PP1 activity, we performed metabolic labeling experiments and an in vitro PP1 phosphatase assay. Fig. 6A shows that immunoprecipitated PP1 from H_2O_2-treated cells was more active, as given by a decrease in the phosphorylated τ at the AT8 epitope in an in vitro phosphatase assay. Concomitantly, this decrease of phosphorylated τ at the AT8 epitope was shown to be significant ($n = 3$, $p < 0.05$). An increase in PP1 activity should be paralleled by an increase in I-2 phosphorylation, leading to dissociation of the I-2/PP1 complex. Therefore, we estimated the changes in metabolic labeling of I-2 after H_2O_2 treatment. As shown in Fig. 6B, I-2 incorporated more $^{32}PO_4$ after H_2O_2 treatment, strengthening the idea that changes in the phosphorylation state of I-2 could be related to increased PP1 activity. Again, these results were confirmed to be significant when quantified ($n = 4$, $p < 0.05$). Altogether, these experiments clearly suggest that, after the addition of H_2O_2, cdk5 is activated and the activity of PP1 is increased. Thereaf-

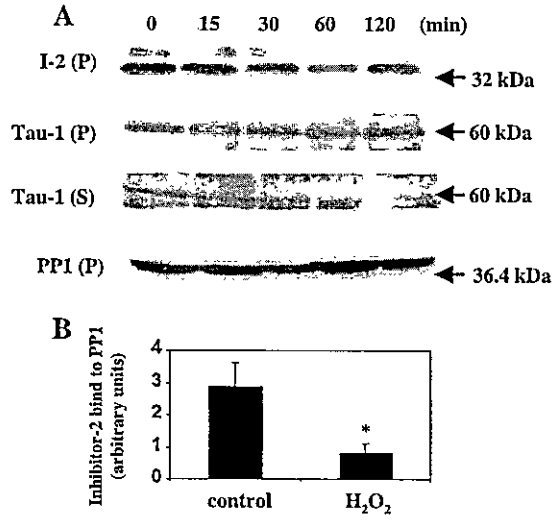

Fig. 5. I-2/PP1 complex was dissociated under oxidative stress stimuli. (A) Immunoprecipitated PP1 pellets were analyzed for the presence of either pulled-down I-2 or dephosphorylation at the Tau1 epitope. A time-dependent decrease in the I-2 association to PP1 was observed in neuronal cells treated with H_2O_2 (first panel). Concomitant with this, a time-dependent increase of dephosphorylated Tau1 epitope associated to PP1 was found (second panel). The increase of dephosphorylated τ (recognized by Tau1) associated with PP1 was paralleled by a decrease in the soluble fraction of dephosphorylated τ (third panel). As a control for the amount of PP1, Western blots using an antibody against catalytic subunit revealed no changes in the PP1 levels (fourth panel). (B) Quantitative analyses revealed that, after 2 h of H_2O_2 addition, there was a 3-fold reduction in the association of I-2 to PP1.

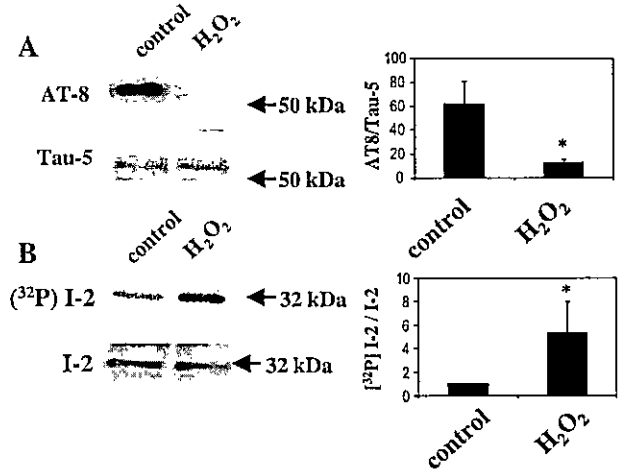

Fig. 6. In vitro phosphatase activity of PP1 and metabolic labeling of I-2 with $^{32}PO_4$ in SHSY5Y cells treated with H_2O_2. (A) In vitro phosphatase activity was performed by incubating immunopurified PP1 enzyme from both control and H_2O_2-soluble protein extracts. After immunopurification, the enzyme was mixed with pure τ protein derived from bovine brain, and the amount of phosphorylated τ at the AT8 epitope was determined (more details are given in Materials and Methods). The assay indicates that H_2O_2 treatment results in an increase in the activity of PP1 derived from cells, as evidenced by a decrease in AT8 labeling (left panel). Quantitative analyses showed the ratio between τ phosphorylated at the AT8 epitope and total τ levels, confirming Western blot results ($n = 3$, $p < 0.05$) (right panel). (B) Metabolic labeling of I-2 after H_2O_2 treatment, showing an increase in the amount of $^{32}PO_4$ incorporation to I-2 after oxidative stress stimuli. The difference was not due to changes in the protein overall levels (left panel). Quantitative analyses showed the ratio between [^{32}P] I-2 and total I-2, confirming an increase in the metabolic labeling of I-2 after H_2O_2 treatment ($n = 4$, $p < 0.05$) (right panel).

ter, the active phosphatase should be responsible for τ dephosphorylation at the Tau1 epitope.

DISCUSSION

In this study, we showed that neuronal oxidative stress induced by the addition of H_2O_2 produced a decrease in microtubule-associated protein τ phosphorylation at the AT8 and PHF1 epitopes. Oxidative stress induced by H_2O_2 also increased the amount of 4-HNE-modified proteins. The phosphorylation decrease in τ protein is most likely to be produced by an increased activity of PP1 triggered by the dissociation of its I-2. Cdk5 is a member of cyclin-dependent kinases that, among other activities, phosphorylates τ protein under Aβ stimulus in residues that are found abnormally phosphorylated in AD [21,22]. In our biological model, we observed an increase in the activity of cdk5 in neurons treated with H_2O_2 but a decrease in the phosphorylation of τ. Since no major changes were observed in p35 levels, we determined that the increase of cdk5 activity was produced by an increase in the amount of p35 bound to the enzyme. Alternatively, it might be possible that another activator could be participating in cdk5 activation [22]. According to the present data, the mechanism implicated in such a dephosphorylation considers an initial phosphorylation of the PP1 I-2. After its phosphorylation, the I-2 would be released from the complex, and PP1 could be then responsible for τ dephosphorylation.

Oxidative stress phenomena have been related to the onset of several neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) [4], Parkinson's disease [33], and AD [7,34]. The effects of such oxidative damage can be evaluated in terms of modifications in proteins, lipids, and nucleic acids. It has been shown that, in ALS, the heavy neurofilament subunit is modified with 4-HNE [35]. Similarly, in AD, τ protein can be modified by 4-HNE in vitro [32] and in AD-derived brain sections [36]. Oxidative stress can be produced in AD by the extracellular deposition of Aβ peptide [19]. In previous findings by our laboratory, we demonstrated that Aβ peptide also induced a deregulation of the cdk5/p35 complex in cell cultures [22]. Increased τ phosphorylation at the AT8 epitope was verified in those cells, and this response was inhibited by using pharmacological inhibition of the kinase with butyrolactone I or roscovitine [21]. Furthermore, we have recently described a cdk5 imbalance in a transgenic model that overexpress AβPP [37]. Many efforts have been made to analyze the relationships between the amyloid pathway and τ phosphorylation. In this respect, it has been proposed that the Aβ effect on neurons could be elicited by triggering oxidative stress responses [12,38] in vitro and in vivo [39,40]. However, other stimuli leading to an increase of the oxidative stress responses have seeded controversy in the τ phosphorylation issue. Thus, H_2O_2 has been described to produce a decrease in τ phosphorylation in primary cortical neurons [41].

In this study, we have implicated some of the kinase and phosphatase systems responsible for the decreased τ phosphorylation. The increase of unphosphorylated τ isoforms is most likely to depend on cdk5, since AT8 and Tau1 antibodies recognize mainly phosphoepitopes on τ that depend on kinases belonging to the proline-directed protein kinase family, and this decrease of phosphorylated τ species recognized by the aforementioned antibodies was not dependent on the activity of gsk3β levels [41].

These changes have also been described for neurons treated with excitotoxic levels of glutamate [42–44] and under other conditions, such as ischemia [45,46]. Moreover, a decrease of Alzheimer's-type τ phosphorylation was detected in hippocampal cells suffering another oxidative insult, namely iron overload [47]. The fact that different oxidative stress treatments lead to opposite results on τ phosphorylation suggests that differences could be related to certain variations in the intracellular

levels of second messengers. Thus, dephosphorylation of τ protein could indicate an initial response of cells against oxidative insults. In oligodendrocytes, it has been demonstrated that τ is dephosphorylated in response to in vivo oxidative insults such as stroke and head injury in humans [48] and rodents [49]. Tau protein in its dephosphorylated forms binds more efficiently to microtubules [50] and cell membranes [51]. Interestingly, protein phosphatase 1 can be targeted to microtubules upon binding to τ protein, suggesting that the control of τ phosphorylation by PP1 in our system might have relevant biological effects [52]. Our results along with previous reports suggest that an initial response of neurons after H_2O_2 treatment (an acute effect) would lead to τ dephosphorylation at the AT8 epitope, due to an increased PP1 activity through a mechanism involving phosphorylation of I-2. However, τ phosphorylation results from the balance of kinases and phosphatase activities. Thus, later on, chronic and cumulative oxidative stress conditions in combination with other factors (i.e., amyloid β or inflammatory factors) should favor cdk5/p35 activity on τ protein that, together with other kinases, could lead to increased τ phosphorylation. Thus, in this report we suggest a novel mechanism by which acute treatments with H_2O_2 might induce τ dephosphorylation.

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ABBREVIATIONS

4-HNE — 4-hydroxy-2-nonenal
I-2 — protein phosphatase 1 inhibitor 2
NAC — N-acetyl-L-cysteine
PP1 — protein phosphatase 1

Cognitive Impairment Correlates with Redox-active CSF Iron

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Running Title: Redox-active CSF iron and dementia.

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Keywords: Labile-iron, oxidative stress, redox-active metals, cerebrospinal fluid, dementia, Alzheimer´s disease.

Summary.

Background The presence of oxidative stress and iron accumulation constitutes a hallmark of neurodegenerative processes. However, the reactivity of CSF iron, and its possible correlation with the progression of the pathology is unknown. Here, we postulates that loss in cognitive function in Alzheimer's disease associates with changes in CSF reactive iron.

Methods. We compared CSF samples from 46 elders with different degrees of dementia. The individuals were rated using a semi-structured interview and divided into five different groups for the study: subjects with a Normal Cognitive Status (NCS), Early Mild Cognitive Impairment (E-MCI), Late Mild Cognitive Impairment (L-MCI), Early Alzheimer Disease (E-AD) and Late Alzheimer Disease (L-AD). The subjects in the study consisted of 13 NCS; 9 E-MCI; 11 L-MCI, 5 E-AD and 8 L-PAD. Total CSF iron was analyzed by atomic absorption spectrometry. Redox-active iron was analyzed by a novel fluorimetric assay. One-way ANOVA was used to test differences in mean values, and Dunnett's post-hoc test was used for comparisons (GraphPad InStat). Differences were considered significant if $P < 0.05$.

Findings Total CSF iron concentration varied from 0.32 μM to 1.85 μM (0.818 ± 0.069, mean ± SEM). No correlation was found between total iron and severity of dementia. Considerable amounts of iron were found in its redox-active state, and the amount of redox-active iron correlated with the extent cognitive impairment. Significantly higher levels than control were found in E-MCI, L-MCI and E-AD patients. L-AD patients showed a precipitous decrease in redox-active iron to levels close to nil.

Interpretation. CSF redox-active iron is a good indicator of the degree of dementia in early steps of AD. Moreover, CSF redox active iron may play a critical role in the establishment of the senile plaques. The drastic decrease in redox-active iron in L-AD patients may represent an ultimate effort of the central nervous system to minimize iron-associated toxicity.

Introduction.

Alzheimer's disease (AD) is the most common cause of dementia, and will reach epidemic proportions if no cure is found within the next decade. AD is also a major public health problem and one of the most costly diseases in modern society. In some countries with restrained economical resources, the elderly population is growing rapidly as life expectancy increases. The neuropathological features of AD are a gradual and widespread neuronal loss, the extraneuronal β-amyloid deposits or senile plaques (SP), alterations in cerebral blood vessels, and the presence of neurofibrillary tangles (NFTs) mainly composed by hiperphosphorylated tau protein (Maccioni et al., 2001).

Oxidative stress is a major pathological aspect of several neurodegenerative conditions and results from the generation of large amounts of reactive oxygen species (ROS), which can alter the structure of several molecules including proteins, lipids and nucleic acids, ultimately leading to cell degeneration and death.

Several reports have implicated redox-active transition metals such as iron and copper with the onset of different neurodegenerative disorders such as AD (Smith et al. 1997; Egaña et al. 2003) and Parkinson's disease (PD) (Faucheux et al. 2003; Youdim et al. 2004). High levels of both copper (Squitti et al. 2002) and iron (Ozcankaya et al. 2002) has been found in blood plasma of people with AD. Iron and copper catalyze the production of reactive oxygen species (ROS) via Fenton reaction, transforming the mild oxidant hydrogen peroxide in to hydroxyl radical ($HO^{\bullet}$), one of the most reactive species in nature (Symons & Gutteridge, 1998). The damage to molecules and tissues due to this reactivity has been related to neurodegenerative disorders (Perry et al. 2002; Gerlach et al. 1994; Sayre et al. 2000) and aging (Hirose et al. 2003; Zecca et al. 2001).

Alterations between bound and free iron play a pivotal role in the capacity of iron to induce oxidative stress (Kruszewski. 2003). In particular, evidence indicates that there is abnormal iron accumulation in the brain of AD (Andrasi et al. 2000, Smith et al. 1997) and PD (Berg et al. 2001) patients. Moreover, several studies strongly suggest that neurons from Alzheimer brains are subjected to strong oxidative load (Abe et al. 2002; Giasson et al. 2002). These observations suggest that iron accumulation play a crucial role in the etiopathogenesis of AD, through modification of the balance between pro-oxidant and anti-oxidant activities. The level of Fenton-reactive metals in CSF, and their correlation with

neurodegenerative disorders are unknown. To address this point, in this work we analyzed the levels of total iron and redox-active iron, in cerebrospinal fluid samples from elderly people with different degrees of dementia and correlated these levels with the severity of the dementia.

Methods

Patients

The study and the experimental protocols were approved by the Committee on Ethical Issues of the Faculty of Medicine, University of Chile, and all subjects provided informed consent prior to the initiation of the study. In the cases of demented subjects the informed consent for participation in the study was obtained also from their caregivers. Participants were recruited through the printed media and underwent a multistage screening procedure. To be included in the study, participants needed: (i) to be more than 60 years old; (ii) to be free of significant underlying medical, neurologic, or psychiatric illness, (iii) and to be willing to participate in the study procedures.

Neuropsychological battery of tests. The neuropsychological evaluation consisted in Neuropsychological Battery of CERAD that include: Folstein's MiniMental Test, Verbal Fluency, Boston Nomination Test (15 items), Learning Word List (10 items), and Praxis.

Application of semi-structured interview. Application of the CDR ratings were obtained using a semi-structured interview specially adapted by Daly et al (2000), from the previously validated original version of Hughes et al., (1982). This interview was specially adapted to be used with a population with very mild impairments. It includes a standardized medical, neurologic, and psychiatric examination, in addition to a semi-structured set of questions about functional status regarding Activities of Daily Living (ADL) and Instrumental Daily Living (IADL). A translation and Spanish adaptation of the English version of the semi-structured interview was used in a preliminary or pilot study. The subjects distributed across a range of cognitive function from no impairment to mild impairment, and their collaterals were evaluated by 3 independent interviewers. The

questions that the three interviewers considered were not well understood by the subjects or collaterals were modified according to the language skills of our population. The rating of the overall CDR scores and the ratings of the each six CDR domains were analyzed with a kappa index. Total box scores derived from CDR were obtained to evaluate the degree of cognitive impairment. A very high concordance was obtained.

These subjects underwent a comprehensive medical and neurologic examination to ascertain that they were free of any significant medical condition. The subjects could be using psychoactive medications, and disabilities and comorbid illnesses could be present, but the neurologists did not judge that these factors were causing clinically significant cognitive impairments. Once the interview was completed and rated, the subjects in the study were administered a neuropsychological battery. The CDR ratings were completed with the interviewers blinded to the results of the neuropsychological test. The written interviews were scored by a reviewer who made his own rating judgment, blinded to the interviewer. I Subjects were divided into five different groups for the study: subjects with a Normal Cognitive Status (NCS), Early Mild Cognitive Impairment (E-MCI), Late Mild Cognitive Impairment (L-MCI), Early Alzheimer Disease (E-AD) and Late Alzheimer Disease (L-AD). Score ranged from 0 for NCS individuals, 0.5-1.0 for E-MC, 1.5-3.0 for L-MCI, 4.0-6.5 for E-AD and 7.0-10 for the patients with L-AD. A weekly consensus conference was carried out among the interviewers in order to reach an agreement regarding the rating of each of the CDR subcategories. In this open discussion an explicit reference was made to CDR ratings coded by Daly et al. to be sure that the final rating of each of the CDR subcategories adhered as closely as possible to the CDR expanded criteria of Daly et al (2000).

CSF samples. Lumbar CSF samples were obtained by lumbar punctures performed early in the morning. CSF samples were stored into polypropylene tubes without preservative and frozen at the bedside on dry ice within minutes of withdrawal. Samples were kept at – 80° C.

Laboratory methods

Measurement of total iron. Cerebrospinal fluid samples were analyzed to obtain the total iron by AAS. 100 µl of CSF samples were mixed with 100 µl of 5% ultrapure nitric acid and incubated at 60 °C for 12 hrs, the digest was cooled, centrifuge at 12.000 g for 2 min and the supernatant diluted to 1 mL with 0.2% ultrapure nitric acid. The final sample was colorless and transparent. Fe contents were determined in an atomic absorption spectrometer with graphite furnace SIMAA 6100 (Perkin Elmer, Shelton CT). MR-CCHEN-002 (*Venus antiqua*) and DOlt-2 (*Dogfish liver*) preparations were used as reference materials to validate the mineral analyses. The results were expressed as a µg/L.

Measurement of redox-active iron. Redox active CSF iron was determined as described for labile plasma iron (Esposito et al. 2003). Quadruplicates of 20 µl of CSF were transferred to clear-bottom, 96-well plates (Maxisorp 96, Nunc, Rotskilde, Denmark). To two of the wells were added 180 µl of iron-free HEPES-buffered saline (HBS; HEPES 20 mM, NaCl 150 mM, pH 7.4) (pre-incubated at 37°C) containing 40 µM ascorbate and 50 µM DHR (dihydrorhodamine 123, dihydrochloride salt, Biotium, Hayward, CA, USA). To the two other wells was added 180 µl of the same solution containing 50 µM of the iron chelator deferiprone (L1, Apotex, Toronto, Ont., Canada). Immediately following reagent addition, the kinetics of fluorescence increase were followed at 37°C in a BMG Galaxy Fluostar microplate reader (BMG Lab Instruments, Germany) with a 485/538 nm excitation/emission filter pair, for 40 min, with readings every 2 min. The slopes (r) of DHR fluorescence intensity with time were calculated from measurements taken between 15-40 minutes and are given as F.U. min^{-1} (fluorescence units per min). The duplicate values of r in the presence and absence of L1, r_{L1} and r, respectively, were averaged and the LCSFI concentration (µM) was determined from calibration curves relating the difference in slopes with and without L1 against Fe concentration: LPI= $\Delta r/r_{st} = (r\text{-}r_{L1})/r_{st}$, where Δr and r_{st} denote the L1 sensitive component of r and the calibration factor relating Δr to the Fe concentration, respectively. Calibration curves were obtained by spiking plasma-like medium (PLM) or sera with Fe:NTA, 1:7 (mol:mol) to give final concentrations of 40 to 100 µM Fe, followed by serial dilution in PLM or in the same serum and incubation for 30

minutes at 37°C to allow binding of the Fe. Quadruplicates of 20 μL of these samples were assayed for labile iron as described above.

Statistical analysis. One-way ANOVA was used to test differences in mean values, and Dunnett's post-hoc test was used for comparisons (In Stat program from GraphPad).

Results

Total levels of Fe in CSF. Analysis of total CSFI by atomic absorption spectrometry showed a broad range of concentrations from 0.32 μM to 1.85 μM (0.817 ± 0.069, mean ± SEM). No correlation was found between total CSF iron and the severity of dementia (Figure 1), with values (mean ± SEM) of 0.85 ± 0.16, 0.69 ± 0.12, 0.63 ± 0.064; 1.02 ± 0.28 and 0.84 ± 0.15, for NCS, E-MCI, L-MCI, E-AD and L-AD, respectively.

Positive correlation between L-CSFI and early stages of the neurodegenerative process. Transferrin (Tf) levels of 0.24 μM has been reported for CSF (Symons & Gutteridge,1998). Thus, Tf in CSF should be close to saturation or saturated, and some reactive, non-transferrin bound, iron could be present. Thus, we analyzed the redox active CSF iron of the different groups under study. NCS individuals had redox active CSF iron of 0.210 ± 0.03 μM; (mean ± SEM; N= 13). Redox active CSF iron levels higher than control were found in E-MCI and, L-MCI individuals, 0.271 ± 0.044 μM; N= 9; and 0.327 ± 0.040 N= 11, respectively (Figure 2). AD patients showed a biphasic pattern. Compared to NCS individuals, E-AD individuals had significantly higher redox active CSF iron levels (0.424 ± 0.052 μM; N= 5), while L-AD individuals had significantly lower levels than L-AD (0.066 ± 0.058 μM; N= 6). These results indicate that redox active CSF iron is a good indicator of the degree of dementia in early and medium stages of Alzheimer's disease.

Negative correlation between redox-active CSF iron and cognitive damage in AD patients. Further analysis of redox-active CSF iron levels in AD patients revealed that the degree of dementia, quantified by the Total-Box score, was negatively correlated with redox active CSF iron (R^2: 0.80; slope: -9.24) (Figure 3). These results indicate that

development of the late stages of the pathology is accompanied by a progressive decline in redox active CSF iron in a paired-sample analysis we found no correlation between total CSF iron and redox-active iron (data not shown).

Discussion.

Iron accumulation has been implicated as an important factor in the etiology of several neurodegenerative disorders (Ke et al. 2003; Perry et al. 2002; Youdim et al. 1993). Thus, studies of iron availability at different stages of loss of cognitive functions are essential to understand the role of iron in neurodegenerative processes. To tthis end, in the present work we analyzed in CSF samples from elders with varied cognitive status damage total Fe levels, and evaluate the levels of Fe capable to catalyze the Fenton reaction by a novel redox-active iron assay.

Total Fe levels in CSF showed a broad range of values, ranging from 0.32 μM to 1.85 μM. The source of this variation is unknown, but it could be due to diet habits, environmental conditions or genetic factors. No correlation was found between total iron and the degree of dementia, so we decided to determine redox active CSF iron, i.e., the fraction of the total iron that was in a labile, Fenton-reactive form. The labile plasma iron pool correlates with the appearance of oxidation products and decreased plasma antioxidant capacity (Cighetti et al. 2002). However, no data was available linking redox active CSF iron in CSF and cognitive impairment.

Important quantities of CSF reactive iron was found both in normal and cognitive-impaired individuals. Redox-active CSF iron levels were independent of total CSF iron content. Hence, contrary to intuition, total and redox active CSF iron pools seems to be independently regulated. Our values for normal subjects (0.21 ± 0.03 μM) agree with a previously reported one of 0.55 ± 0.27 μM, determined using a bleomycin-based assay (Symons and Gutteridge, 1998).

The reported ascorbate concentration in CSF is about 160 μM (Reiber et al., 1993) therefore, the predominant labile Fe species in CSF should be in the Fe^{2+} form. Fe^{2+} is

readily available for directly incorporation into the cell by the Fe^{2+} transporter DMT1 (Gunshin et al., 1997; Arredondo et al., 2003), in a process known as non-transferrin bound iron uptake. Thus, brain cells which express DMT1, like hippocampus (Gunshin et al. 1997) should be particularly susceptible to iron accumulation.

Redox-active CSF iron significantly increased in the first stages of cognitive impairment. Increased redox-active CSF iron could promote neuronal damage by several ways. It could contribute to the formation of senile plaques, where 1 μM Fe has been found (Bishop and Robinson, 2003). In addition, redox-active CSF iron could act together with amyloid-β peptide to induce oxidative stress in an oxidative redox cycle. (Huang et al., 1999). As discussed above, increased redox-active CSF iron should also result in increased cell iron accumulation, with an outcome of increased oxidative stress and oxidative damage in neuronal cells (Núñez-Millacura et al. 2002; Youdim. 2004). Indeed, both iron accumulation and oxidative damage has been reported in hippocampus and *substancia nigra pars compacta* neurons from AD and PD patients (Smith et al. 1997; Berg et al. 2002; Zecca et al. 2001). Moreover, iron available for non-transferrin bound iron uptake could affect glial cells that play a key role in many physiological and pathological process of the central nervous system (Schousboe et al. 2003; Tarkowski 2002; Hirsch et al 2003). Thus, increased redox-active CSF iron should result in a series of events leading to deterioration of the redox capacity of cells and to neurodegeneration.

Redox-active CSF iron values in Alzheimer's disease patients distributed into two groups. The mean of the E-AD samples was significantly higher than NCS values while the mean from L-AD patients was lower than NCS. The latter decrease in redox-active CSF iron suggests the induction of mechanism to reduce reactive iron and its associated toxicity, in a way similar to the proposed "entombment" of toxic amyloid-β peptide into senile plaques (Cuajungco et al., 2000). Thus, the decrease in redox-active CSF iron may correspond to a final effort to decrease Fe-mediated oxidative stress in the brain.

In summary, a positive correlation between redox-active CSF iron and cognitive impairment was found in the early and medium stages of the neurodegenerative process

associated with Alzheimer's disease while a strong reduction was observed in advanced steps of the disease. Thus, redox-active CSF iron may be used as an early marker to assess the risk of dementia. These findings indicate that redox-active CSF iron should be considered a risk factor in the development of cognitive disorders. They also advocate for a strategy of iron chelation therapy to prevent the progress of the neurodegenerative disorders (Shachar et al., 2004).

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Figure legends.

Figure 1. Total Fe levels in CSF from NCS, MCI and PAD individuals. CSF from 37 subjects (10 NCS; 9 E-MCI; 10 L-MCI, 3 E-AD and 5 L-PAD) was analyzed for total iron by atomic absorption spectrometry. No significant differences were found between the groups.

Figure 2. Redox-active CSF iron levels in CSF from NCS, MCI and AD individuals. Redox-active CSF iron was determined in 46 subjects (13 NCS; 9 E-MCI; 11 L-MCI, 5 E-AD and 8 L-PAD) as redox-sensitive labile iron, as described in Material and Methods. Significant differences were found between L-AD individuals compared with NCS individuals ($P < 0.05$) * and compared with E-AD ($P < 0.01$) # #.

Figure 3. Correlation between redox-active CSF iron levels and the degree of dementia in Alzheimer's disease patients. Values of redox-active CSF iron were plotted against the Total Box score (score ≥4) of patients diagnosed as possible Alzheimer disease. A strong negative correlation (r: 0.89; slope: -9.24) was found.

Figure 1

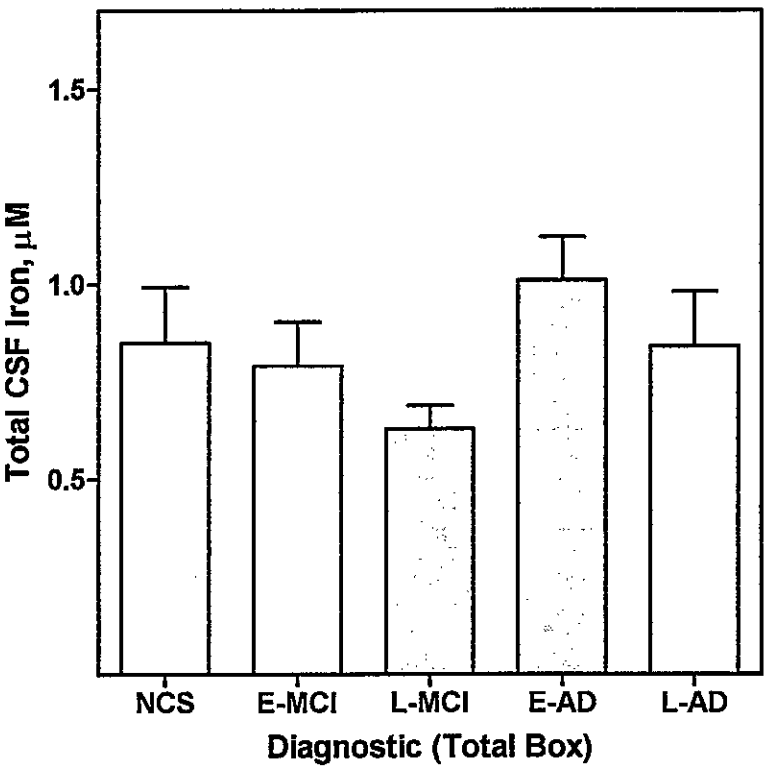

1.5
1.0
0.5
Total CSF Iron, µM
NCS
E-MCI
L-MCI
E-AD
L-AD
Diagnostic (Total Box)

Figure 2

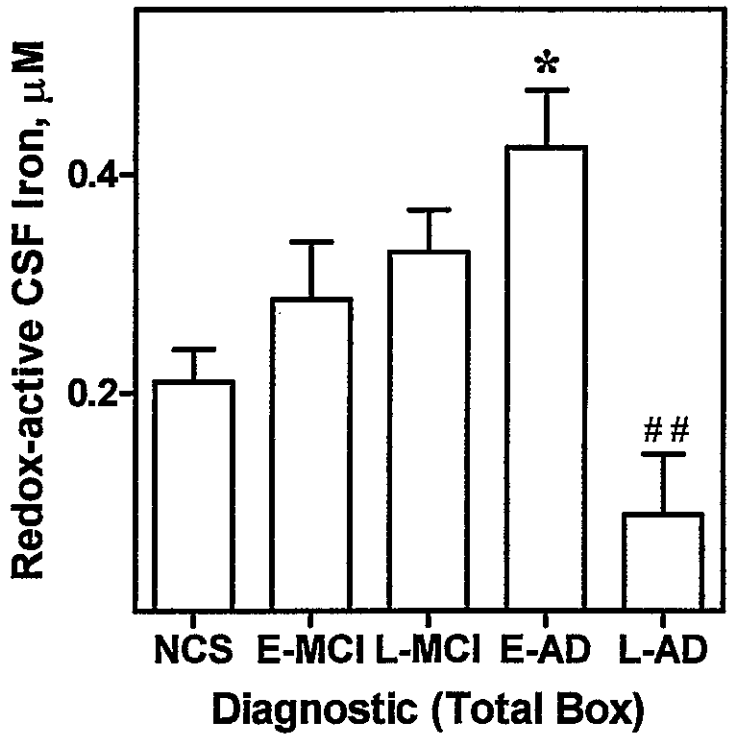
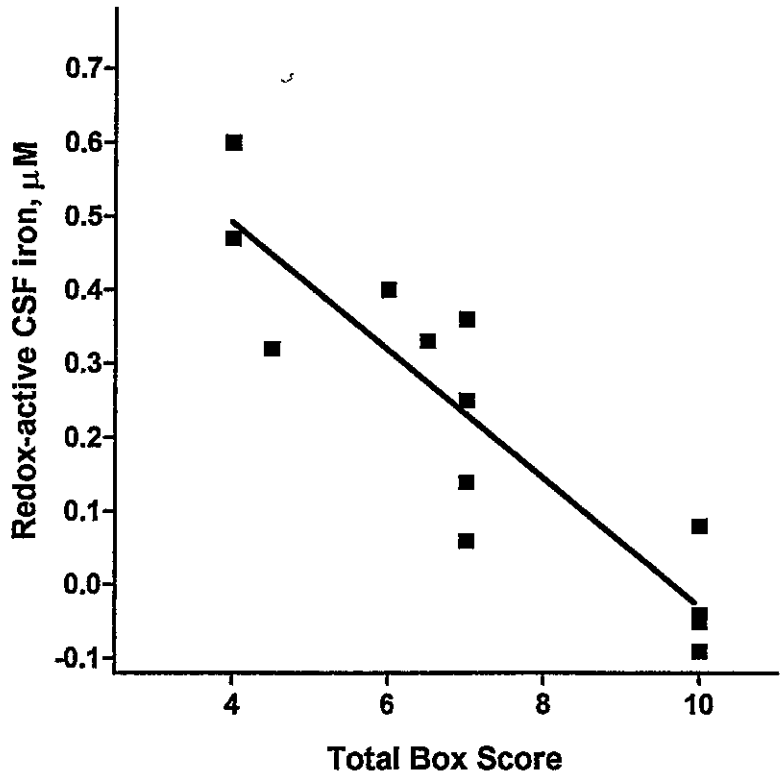

Redox-active CSF Iron, μM
0.4
0.2
*
##
NCS
E-MCI
L-MCI
E-AD
L-AD
Diagnostic (Total Box)

Figure 3


Redox-active CSF iron, μM
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
4
6
8
10
Total Box Score