"The Enigmatic Nuclear Localization of Tau: Elucidating its molecular nature, the signals promoting this localization, and the functional role at nucleoli"

Tesis
Entregada a la
Universidad de Chile
En cumplimiento parcial de los requisitos
para otorgar el grado de
Doctor en Ciencias con mención en Biología Molecular, Celular y Neurociencias

Facultad de Ciencias
por

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Agosto, 2006

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FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

INFORME DE APROBACION

TESIS DE DOCTORADO

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata.

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Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Biología Molecular Celular y Neurociencias, en el examen de Defensa de Tesis rendido el día 04 de Septiembre de 2006.

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I dedicate this PhD thesis to my mother Olga and to those who have given me the confidence, ability, love and encouragement to complete this work.

To those who share my universe and my time, the family and friends, those that are, those that were and, those that will be....

My name is Marcela Katherine Sjöberg Herrera. I was born in Santiago, Chile on 9 August 1977. My parents are Olga Herrera and Marcelo Sjöberg. I have one sister, Paula and two lovely nieces, Sofia and Valentina. My childhood took part in La Florida living in a house with my parents where my interest for the science began to arise. Between 1981 and 1995 I attended Maria Inmaculada School where I had the opportunity to learn german and after to travel on 1993 to Germany by student interchange, a fascinating experience living there for three months. In 1994 the visit of Dr. Jorge Allende to my school to speak about scientific careers helps me to decide my postulation to Biochemistry and Molecular Biotechnology Engineering at the University of Chile. By 1995 after finishing school, I went to University of Chile and studied Molecular Biotechnology Engineering at the Faculty of Sciences. It was a five-year degree, the four year of which I spent my time working in my undergraduate thesis, searching for mutations in the human growth hormone receptor, at the Human Molecular Genetics laboratory supervised by Dr. Pilar Carvallo at Catholic University. I obtained my engineering degree on 2002 while I was coursing the second year of the PhD program in Sciences, in Molecular and Cellular Biology and Neurosciences. I became so interested in the biomedicine field and neurosciences that I started to work related to Alzheimer disease in the laboratory of Dr. Ricardo Maccioni at the Faculty of Sciences. Then I decided to spend four month in Paris financed by a fellowship awarded by the Millennium Institute CBB, to continue with the research initiated at the laboratory of Dr. Maccioni, this time investigating the association of tau protein to satellite DNA under the supervision of Dr. Eliette Bonnefoy at University René Descartes-Paris 5. After return I decided to continue this research as my PhD thesis work supervised by Dr. Maccioni. As the PhD thesis work started to be concluded, beginning the year 2005, I took the opportunity, financed by my supervisor, to finish these studies performing Mass Spectrometry analysis of nuclear tau, at the laboratory of Dr. George Perry and Dr. Chu Chen at Case Western University in Cleveland Ohio, US.

ACKNOWLEDGEMENTS

I wish to thank the university professors who participate of my scientific formation during my undergraduate studies and during the PhD, for contributing to my scientific development and to give me the tools necessary to explore different areas in this huge universe.

I want to thank to my PhD thesis supervisor, Dr. Ricardo Maccioni who open the doors of his laboratory to me, given me the theoretical and material support for my thesis. Thanks for trust in me and supporting me financially to carry out fundamental experiments of this thesis in Paris and Cleveland. Thanks also for contribute to my scientific formation encouraging me to participate in international meetings.

I want to thank to my co-director Dr. Christian Gonzalez-Billault for his guidance and suggestions throughout the thesis project.

I would wish to thank the members of my thesis Committee for their guidance, disposition and appropriate suggestions, contributing to my scientific formation and doing of this PhD thesis a more rigorous work.

I would like to thank to Dr. Eliette Bonnefoy for trust in me and give me the opportunity to work at the laboratory of Régulation de la Transcription et Maladies Génétiques at Université René Descartes. Thanks for the strong theory and experimental support while I had performed experiments and during the preparation of the published paper associated to this thesis. Besides, I want to thank to Zeyni Mansuroglu that working at her laboratory helped me with immunoFISH confocal microscopy experiments for the publication of the manuscript, and to Elena A. Shestakova who teach me the immunoFISH technique, who I shared special moments and interesting conversations during my stay in Paris.

I would like to thank to Dr. George Perry for invited me to his laboratory at the Pathology Department in Case Western University, to perform Mass Spectrometry analysis and to Dr. Shu Chen for his theory and experimental support during those experiments. I wish to thank to friends that I meet at Cleveland, Kazuhiro Honda, Mike, Eliot, Ayuna, and Ana, for their company, advice and friendship.

I am also indebted to all my friends (Mario Vera, Daniela, Francisco Rivera, Francisco Remonsellez, Juan Pablo Muñoz, Christian Zambrano, Karel, Ariadna, Francisco Chavez, Pablo Muñoz, Gisella, Paula, Daniel, Elias, Ruben, Sergio, Hans and others) who were plentiful source of encouragement and who share with me their free time for scientific discussions and entertainment. I specially thank to Dr. Juan Pablo Muñoz for helping me during my thesis work and for informal discussions plentiful source of ideas and also for encouraging me to continue during difficult periods of my life.

Thanks to Dr. Carlos Jerez for open me the doors of his laboratory to learn about isoelectrofocusing. My special thanks to Francisco Remonsellez and Ricardo Nilo for helping me theoretically and technically with the bidimensional experiments.

Thanks to Dr. María Rosa Bono and particularly to Valesca for the Flow Citometer facilities and help me with the analysis of the data.

I want to thank CONICYT, for a three year PhD studentship and for a thesis fellowship. In addition, I would like to thank to the Scientific Initiative Milenio, particularly to the Milenium Institute for Advanced Studies in Cell Biology and Biotechnology (CBB) for support me with a studentship for the first PhD year and with a fellowship that allowed me to continue my research, initiated at the laboratory of my supervisor, in Paris which was crucial for the success of my thesis.

Finally and no less important, I want to thank to my parent, to my family and to God for be always to my side in this world and from heaven. I want specially acknowledge and thank the love and selfless attitude of my mother, Olga for teaching me to trust myself in spite of difficult moments and for be always there when I need. Thanks to my sister, Paula, for be unconditionally always ready to help me and for understand the different mode of life that I have chosen. I want to thank specially to my aunt Inés for be worry about me and for helping me whenever she could. Thanks to my couple, Adrian, for allow me to pursue my goal in finishing this thesis. To conclude I would like to mention that I really appreciate the patience, love, affection and unconditional support of each one of them.

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LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
AD	Alzheimer Disease
AT8	tau monoclonal antibody against phosphorylated residues Ser202/Thr205
bp	base pair
BrdU	Bromo-deoxyuridine
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
CAMPK II	Calcium-calmodulin protein kinase II
CB	Coomasie Blue
CBD	Corticobasal degeneration
CDC2	cell division cycle 2, G1 to S and G2 to M
CDK5	Cyclin-dependent kinase 5
cDNA	Complementary DNA
CNS	Central Nervous System
CRMP-2	Collapsin response mediated protein 2
C-terminal	carboxi terminal
dC	deoxi-cytosine
dG	deoxi-guanine
dI	deoxi-inosine
diMeH3K9	Histone H3 di-methylated at lysine 9
DMEM	Dulbeco Modified Eagle´s Medium
DNA	Desoxyribonucleotide acid
DNMT	DNA methyltransferase
dNTP	Desoxyribonuclotide-triphosphate
DS	Down´s Syndrome
E	Embryonal day
EMSA	Electrophoretic mobility shift assay
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridization
FTDP-17	Frontotemporal dementia linked to chromosome 17
g	gram
GCA	O-β-*N*-acetylglucosaminidase
GSK3B	Glycogen synthase kinase 3 beta
HMG	high mobility group of proteins
hnRNP	heteronuclear ribonucleoprotein
Höechst	bisbenzimidazole fluorescent compound
IEF	Isoelectrofocusing
kb	kilo base
kD	kilo Dalton

l	liter
MAP	Microtubule associated protein
MAP2c	Microtubule associated Protein 2c
MARK	Microtubule affinity-regulating kinase
MARK1	MAP/microtubule affinity-regulating kinase 1
MBD	Microtubule binding domain
mg	milligram
ml	milliliter
mRNA	messenger RNA
MT	microtubule
n	nano (10^{-9})
NFT	Neurofibrillary tangles
NGF	Neural Growth Factor
NLS	nuclear localization signal
NOR	Nucleolar Organizing Region
NPDPK	Non-proline directed protein kinase
N-terminal	amino terminal
°C	Centigrade
O-GlcNAcylation	O-linked N-acetylglucosaminylation
OGT	O-β- *N*-acetylglucosaminyltransferase
PBS	Phosphate buffered NaCl-solution
PCR	Polymerase chain reaction
PDPK	Proline directed protein kinase
pH	Negative Logarithm of the H^+ion concentration
PHF	Paired Helical Filaments
PI	Propidium Iodide
pI	Isoelectric point
PiD	Pick´s disease
Pin1	human peptidyl-prolyl cis-trans isomerase
PK	Protein kinase
PKA	Protein kinase A
PKC	Protein kinase C
pKi67	proliferation marker
PNS	Peripheral Nervous System
PP	Protein phosphatase
PSP	Supranuclear palsy
rDNA	ribosomal desoxyribonucleotide acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress-activated protein kinase
SCG10	Superior cervical ganglia protein 10
SD	Standard deviation
SP1	Promoter-specific transcription factor
ssDNA	single strand DNA

T	timidine
Tau-1	tau monoclonal antibody against dephosphorylated S202
Tau-5	tau monoclonal antibody against residues 210-241
TE	Tris-EDTA buffer
Tris	Tris-(hydroxymethyl)-aminomethane
UBF	Upstream Binding Factor
UTR	Untranslated region
ΣTZ	Streptozotocin
α-MEM	Alpha modified Eagles Medium
α-sat	human alphoid satellite DNA sequences
γ-sat	murine gamma satellite DNA sequences
μ	micro (10^{-6})

ABSTRACT

The complex nature of the cytoskeleton requires the presence of several proteins to modulate their maintenance and dynamics. Among them, the tau protein is one of the best studied because it plays a key role in the organization and integrity of the neuronal cytoskeleton, and because it is involved in neurodegenerative diseases, mainly Alzheimer's disease.

Tau belongs to the family of microtubule-associated proteins and in humans is codified by a single gene located on chromosome 17. Developmentally regulated alternative splicing generates six isoforms that are differentially distributed in neuronal subpopulations. These isoforms share a variable NH2-terminal domain, a constant central domain containing a proline rich region that includes several phosphorylation sites, and a carboxy-terminal domain where the microtubule binding repeats are located. Tau promotes tubulin polymerization and modulates the stability of microtubules, participating in processes such as neuronal polarity and axonal outgrowth. This function is mainly regulated by Ser/Thr phosphorylations, however last findings suggest that O-glycosylation is likely to be involved. The functions of tau are associated with an axonal and somatodendritic localization in neurons, however it has been also found associated with ribosomes and interacting with plasma membrane. In 1990 a nuclear form of tau was found to be associated with Nucleolar Organizing Regions of mitotic cells and latter on was also described in nuclei isolated from Alzheimer's disease and control brains. Further reports demonstrated the association of tau with the chromosome scaffold, as well as the ability of tau to interact with DNA. To date the significance of nuclear tau is unclear, however the evidence points out to an involvement of tau in the nucleolar dynamic organization of rRNA genes.

The present thesis proposes that tau proteins interact with specific DNA sequences and that a particular subpopulation of it localize in the nucleus of neuronal cells, in a cell proliferation and posttranslational modification dependent way. The study was focused on the nuclear tau localization, the analysis of its association with specific DNA sequences, its molecular characterization and the search of signals related

to AD that could be involved in its localization. The analysis of the nuclear tau localization in cell lines as well as in dividing primary culture cells using immunofluorescence techniques demonstrated that nuclear tau is specifically localized at nucleolar structures identified by the nucleolar protein UBF/NOR-90. Tau was also observed at the internal periphery of nucleoli partially co-localizing with the nucleolar protein nucleolin and human AT-rich α-satellite DNA sequences organized as constitutive heterochromatin. In a non-dividing cell model, E18 rat hippocampal neurons, was observed that tau localization shifted from mainly nuclear at day 1 of culture to mainly cytoplasmic and axonal at day 3 and 5, together with development of neuronal polarity. Searching for a specific association of tau to nucleolar DNA sequences, it was demonstrated that tau not only co-localizes with but also specifically binds to AT-rich satellite DNA sequences apparently through the recognition of AT-rich DNA stretches. Then, the nuclear tau localized in neuroblastoma cells was characterized from the molecular point of view. Immunoblot analyses of nuclear fractions revealed that the subpopulation of nuclear tau was conformed by five species with molecular weight close to 30, 55, 64, 69, 74 kD. Isoelectrofocusing analysis followed by immunoblot, showed that these bands segregate into two subpopulations of nuclear tau variants, one with a more basic character sharing isolelectric points between 8.5-9.5, and another group with isoelectric points between 7.0-8.0. Following the characterization of nuclear tau, the levels of the protein were evaluated in nuclear fractions, throughout the cell cycle and was demonstrated that the increase of cells in phase S concomitant with the decrease on the percentage of cells in G1 phase, is related with an increase in the nuclear levels of tau. Observation that is consistent with the idea that proliferating and cell cycle signals would modulate the nuclear tau levels in a functional mode. Besides the effect of post-translational modifications related to AD over the nuclear tau levels was evaluated, finding that they increase in response to β-N-acetylglucosaminidase and GSK3β inhibition, suggesting a relationship between the levels of O-GlcNAcylation, the basic character of some nuclear tau variants and the nuclear localization of this protein. Conversely nuclear tau levels did not change in response to cdk5 inhibition.

These results allow for the first time to propose a functional role for nuclear tau in relation with the nucleolar organization of cromatin and/or heterocromatization of a portion of RNA genes. Besides they suggest that during the course of the Alzheimer's disease, an aberrant nuclear tau could be affecting the nucleolar organization in proliferating cell and/or in neurons considering that tau has been found in nuclei of neurons from AD patients. In this thesis is proposed that tau isoforms, of basic character, posttranslationally modified by O-GlcNAcylation and modulated by cell cycle signals, localize at the nucleoli of proliferating cells. By this way and through the interaction with satellite DNA sequences, it would participate in mechanisms of chromatin organization at nucleolar level and/or epigenetic control of rRNA genes.

RESUMEN

La compleja naturaleza del citoesqueleto requiere de diversas proteínas que modulan su preservación y dinámica. Entre ellas, la proteína tau es una de las mejor estudiadas debido a que juega un papel clave en la organización e integridad del citoesqueleto neuronal y esta involucrada en la génesis de enfermedades neurodegenerativas, tales como el Alzheimer.

La proteína tau pertenece a la familia de proteínas asociadas a microtúbulos (MAPs) y en humanos esta codificada por un solo gen localizado en el cromosoma 17. Durante el desarrollo seis isoformas son generadas por procesamiento alternativo, las cuales se expresan diferencialmente en subpoblaciones neuronales. Estas isoformas están constituidas por un dominio NH2 terminal variable, un dominio central rico en prolinas, el cual contiene diversos sitios de fosforilación y un dominio COOH terminal donde se localizan repetidos de unión a microtúbulos. Tau promueve la polimerización de tubulina y modula la estabilidad de los microtúbulos, participando en procesos, tales como polaridad neuronal y crecimiento axonal. Estas funciones son reguladas por fosforilación, sin embargo, recientes hallazgos sugieren que la O-GlcNAc glicosilación también estaría involucrada en la modulación de sus funciones.

Las funciones de tau están asociadas con una localización neuronal axonal y somatodendrítica, sin embargo, también se le ha descrito asociada a ribosomas y membrana plasmática. En 1990 una forma nuclear de tau fue descrita asociada a Regiones Organizadoras Nucleolares (NOR) en células mitóticas y posteriormente fue también descrita en núcleos aislados de células provenientes de cerebros de individuos controles y de pacientes con Alzheimer. Subsecuentemente se describió la asociación de tau con el *scaffold* cromosómico así como también la capacidad de esta de interactuar con DNA. Hasta la fecha el significado de la presencia de tau en el núcleo es poco claro, sin embargo, las evidencias apuntan a que tau esta involucrada en la organizacion dinamica de genes rRNA a nivel nucleolar.

Esta tesis propone que la proteína tau interactúa con secuencias específicas de DNA y que una sub-población de esta se localiza en el núcleo de modo dependiente de

la proliferación celular y de modificaciones post-traduccionales. El estudio se enfocó en la localización nuclear de tau, su asociación con secuencias de ADN específicas, su caracterización molecular y en la búsqueda de señales relacionadas con la enfermedad de Alzheimer que podrían estar involucradas en dicha localización. La localización de tau nuclear en líneas celulares y en cultivos primarios de células en división por técnicas de inmunofluorescencia, demostró que la tau nuclear esta localizada específicamente en estructuras nucleolares, identificadas por la proteína nucleolar UBF/NOR-90. Tau fue además observada en la periferia interna del nucleolo, parcialmente co-localizando con la proteína nucleolar nucleolina y las secuencias α-satélites humanas ricas en AT, organizadas como heterocromatina constitutiva. En un modelo celular en reposo proliferativo, neuronas de hipocampo de rata E18, se observó que la localización de tau cambia de principalmente nuclear al día 1 de cultivo, a principalmente citoplásmica y axonal al día 3 y 5, en paralelo con el desarrollo de polaridad neuronal. Investigando acerca de una asociación específica de tau a secuencias de DNA nucleolares, se demostró que tau co-localiza parcialmente y se une específicamente a secuencias de DNA α-satélite ricas en AT, aparentemente a través del reconocimiento de secuencias ricas en AT. Posteriormente la tau observada en núcleo de células de neuroblastoma, fue caracterizada de un punto de vista molecular. Análisis de inmunodetección en fracciones nucleares revelaron que la subpoblación de tau nuclear esta constituida por cinco especies con tamaños moleculares cercanos a 30, 55, 64, 69, 74 kD respectivamente. Análisis mediante isoelectroenfoque seguido de inmunodetección, mostraron que estas especies migran en dos subpoblaciones de proteínas, una de carácter mas básico con puntos isoeléctricos entre 8.5-9.5 y otra con puntos isoeléctricos entre 7.0-8.0. Una vez caracterizada la tau nuclear, se evaluaron los niveles de esta proteína en fracciones nucleares a través del ciclo celular, demostrándose que incremento de células en fase S concomitante con la disminución del porcentaje de células en fase G1 se correlaciona con un incrementan los niveles de tau nuclear. Esta observacion es consistente con la idea de que señales de proliferación y del ciclo celular modularían los niveles de tau nuclear de un modo funcional. Adicionalmente se evaluó el efecto de modificaciones post-traduccionales relacionadas con la enfermedad de

Alzheimer, en los niveles de tau nuclear, encontrándose que estos incrementan en respuesta a la inhibición de β-N-acetilglucosaminidasa y GSK3β, sugiriendo una relación entre los niveles de O-GlcNAc glicosilación, el carácter básico de algunas variantes de tau y la localización nuclear de esta proteína. Por el contrario los niveles de tau nuclear no variaron en respuesta a la inhibición de la quinasa cdk5.

Estos resultados permiten por primera vez proponer un papel funcional para la tau nuclear en relación a la organización nucleolar de la cromatina y/o heterocromatinización de una fracción de genes rRNA. Además, sugieren que durante el curso de la enfermedad de Alzheimer, una población de tau nuclear anómala podría estar afectando la organización nucleolar, de células en proliferación y/o neuronas considerando que esta ha sido observada en núcleos de neuronas de pacientes con AD. En esta tesis se postula que isoformas de tau, de carácter básico, post-traduccionalmente modificadas por O-GlcNAcylación, y moduladas por señales del ciclo celular, se localizan en el nucleolo de células en proliferación. De este modo y a través de la interacción con secuencias de DNA satélite, participaría en mecanismos de organización de la cromatina nucleolar y/o de control epigenético de genes rRNA.

INTRODUCTION

In eukaryotic cells, a functionally organized cytoskeleton and the appropriate assembly of their components are essential for processes as important as the cell division as well as for cytoplasmic integrity. The cytoskeleton is a three-dimensional network of interconnected filaments constituted by: microtubules, actin filaments and intermediate filaments (Porter, 1984) (Fig. 1). The protein-protein interactions participating in the structure of the cytoskeleton network correspond to: i) homologous associations, such as the oligomerization of actin to form microfilaments or actin filaments, ii) heterologous associations, like the assembly of α-β tubulin heterodimers to form microtubules, and iii) interactions between these polymers and other proteins. Furthermore, macromolecular interactions between these polymers and cellular organelles, including mitochrondrias, centrosomes, lysosomes, nuclei and plasma membrane, determine the precise structural and functional organization of living cells (Maccioni, 1986; Maccioni and Cambiazo, 1995; Ramirez et al., 1999).

The cytoskeleton of microtubules coordinates morpho-physiologic changes such as cell shape, mechanical strength, locomotion, intracellular transport of membranous organelles and chromosome segregation during mitosis (Joshi, 1998; Machesky and Bornens, 2003). The dynamic behavior of microtubules is exquisitely regulated, both temporally and spatially (Akhmanova and Hoogenraad, 2005). In neurons, where morphologic changes play a critical role in process such as differentiation and migration, the microtubule dynamic is essential for cell polarity development, axonal

Cytoskeleton filaments

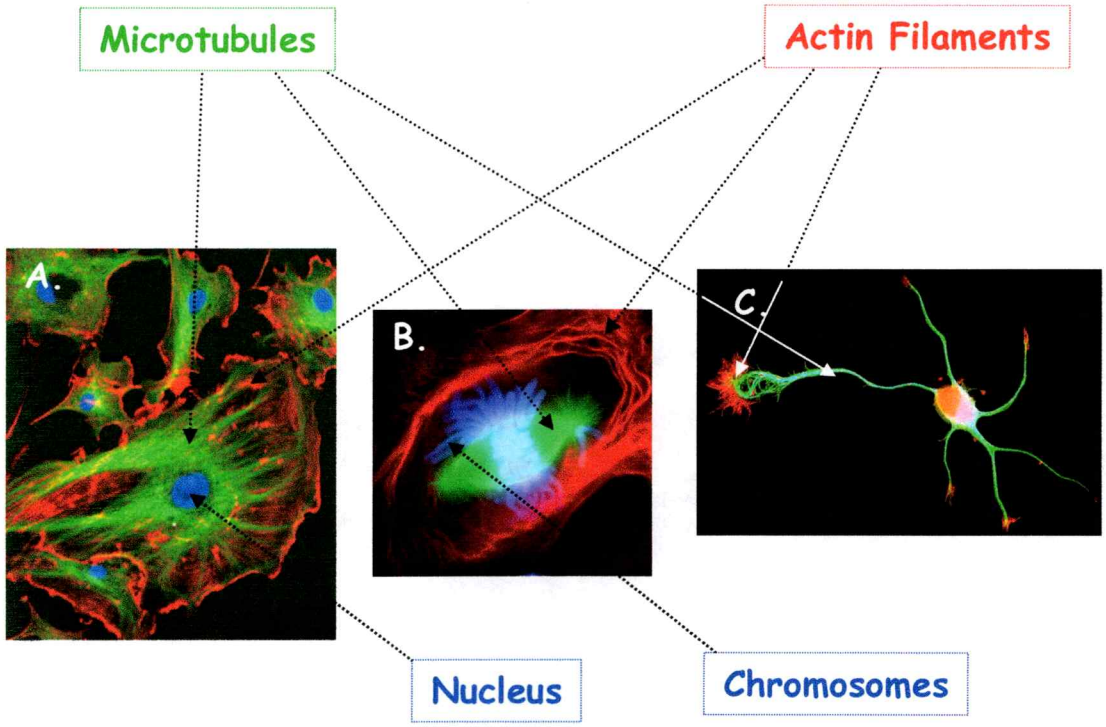


Figure 1. ***The filaments of the cytoskeleton.*** The immunoflourescent images show the distribution of the different filaments of the cytoskeleton in endothelial cells (A), in a lung cell arrested in metaphase, with the chromosomes lined up on the metaphase plate (B) and in a developing cortical neuron (C). Fluorescently labeled microtubules are stained with an antibody against β-tubulin (green), the actin filaments are stained with phalloidin (red) and the nucleus and chromosomes are stained with Höechst (blue).Images are from:http://en.wikipedia.org/wiki/Cytoskeleton; http://gnosticalturpitude.org/wordpress/?p=38; http://www.anatomy.wisc.edu/faculty_kalil.html.

outgrowth, cell signaling, adhesion, organization of cellular organelles and metabolic output (Machesky and Bornens, 2003).

The assembly and dynamics of microtubules is modulated by several microtubules associated proteins (MAPs). Different animal species and tissues contain variable sets of MAPs, and the cell type specificity of these proteins may account for their differential expression in each type of cell (Maccioni, 1986; Maccioni and Cambiazo, 1995). Neurons possess an specially broad range of MAPs, including the high molecular weight proteins such as MAP-1 and MAP-2; the low molecular weight proteins such as MAP-2c and tau; and others such as SCG10, MAP1b, and CRMP-2 (Bunker et al., 2006; Riederer et al., 1997). The presence of many neuronal proteins to control different aspects of microtubule dynamics, suggests that a fine tuning regulation is crucial for development and maintenance of neuronal function.

I. Tau, from the gene to the protein

Tau is one of the best characterized neuronal MAP, promotes tubulin polymerization by lowering the critical concentration of tubulin required for microtubule assembly and suppressing microtubule depolymerization (Weingarten et al., 1975).

I.1 Tau gene

The human tau protein is codified by a single copy gene constituted by 16 exons numbered from -1 to 14, located on chromosome 17 at band position 17q21 (Himmler, 1989; Neve et al., 1986) (Fig. 2A). Restriction analysis and sequencing reveals that tau

gene contains two CpG islands, one associated with the promoter region, resembling neuronal-specific promoter and other one associated with exon 9 (Andreadis et al., 1995). The promoter region also shows a TATA less sequence expected to be related to the presence of multiple initiation sites. Three SP1-binding sites are found close to the first transcription initiation site. The SP1-binding sites are important in directing transcription initiation in TATA-less promoters (Andreadis et al., 1996) and are suggested to control neural specific expression of tau (Heicklen-Klein and Ginzburg, 2000).

Among the 16 exons, the -1 and 14 corresponds to the 5` and 3` untranslated region of tau mRNA respectively (Goedert et al., 1989a; Goedert et al., 1989b). Exon 1 is part of the promoter and is transcribed but not translated. Exon 1 together exons 4, 5, 7, 9, 11, 12 and 13 are constitutive, whereas exons 2, 3, 4A, 6, 8 and 10 are regulated by alternative splicing (Andreadis, 2005). Exons 2 and 3 are two cassette exons near the N-terminus of tau, whose differential inclusion produces a set of three possible combinations (2-3-, 2+3-, 2+3+), since exon 3 has never been found without exon 2 (Goedert et al., 1989a). Exon 4A gives rise to the longest 9kb tau mRNA, which besides contains exon 2, 3, 10. It shares a high homology degree in human, bovine and rodent species (Couchie et al., 1992; Goedert et al., 1992b). Exon 6 contains cryptic splicing sites, corresponding to the two additional 3′splice sites beyond its canonical one, which causes frame shifts generating tau mRNA lacking the remaining 3` exons and thus C-truncated tau molecules (Luo et al., 2004b). Exon 10 known as "cassette exon 10", introduces by alternative splicing an additional "repeat" sequence (R2) into the microtubule binding domain (MBD) of tau, the other repeats are codified by exons 9,

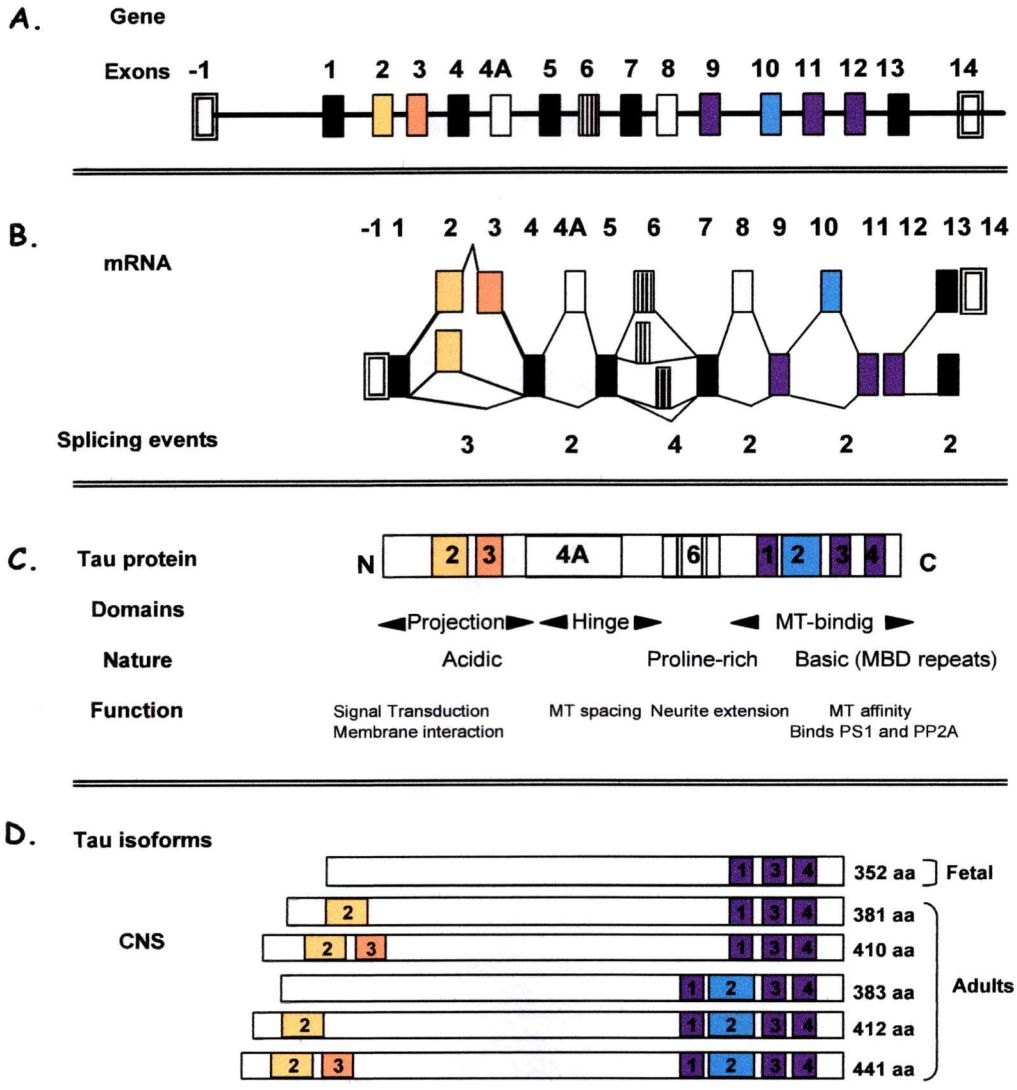


Figure 2. ***Tau gene structure, mRNA species, protein domains and isoforms.*** (A) The human tau gene located on chromosome 17 at position q21, spans over 110kb. It is organized in 16 exons numbered from –1 to 14. Exons –1 and 14 correspond to the 5′and 3′ untranslated regions of tau mRNA, respectively. The start codon is located on exon 1 and two stop codon are located one in the intron between exon 13 and 14 and the second in exon 14. Exons 1, 4, 5, 7, 9, 11, 12, 13 are constitutive; exons 2, 3, 4A, 6, 8 and 10 are regulated. (B) Schematic representation of tau mRNAS and the splicing events of regulated exons. (C) Tau protein domains and functions. (D) The six tau isoforms expressed either in fetal and adult CNS. (Adapted from *Sergeant N., et al 2005; Andreadis A., 2005*).

isoforms expressed either in fetal and adult CNS. (Adapted from *Sergeant N., et al 2005; Andreadis A., 2005*).
11, 12, each one a microtubule binding repeat (R1, R3, R4) (Himmler et al., 1989).

Concerning the tissue distribution, exons 2, 3 and 10 of tau are regulated in adult CNS and become constitutive in PNS (Gao et al., 2000; Goedert et al., 1989a; Goedert et al., 1989b; Kosik et al., 1989). Tau isoforms containing exon 3 are prevalent in skeletal muscle, minor in central nervous system (CNS) and absent in spinal cord (Wei and Andreadis, 1998). Exons 4A together with exon 8 are skipped in CNS. Exon 4A is restricted to retina and peripheral nervous system (PNS) (Couchie et al., 1992; Georgieff et al., 1993; Goedert et al., 1992b). Exon 6 is found in fetal and adult human tau mRNA (Wei and Andreadis, 1998). Despite in adulthood it persists in the CNS (Himmler et al., 1989), it disappears from the PNS (Georgieff et al., 1993). Table 1 summarizes the distribution of tau regulated exons in central and peripheral nervous system during development (Andreadis, 2005).

The alternative splicing regulation of tau regulated exons is modulated by at least 21 splicing factors (Table 2), *cis* regulatory elements, the cellular environment and the local concentration of *trans*-factors (Andreadis, 2005). Each splicing variants influence specific functions of the protein: exons 2 and 3 modulate interactions with the axonal membrane (Brandt et al., 1995), exon 4A alters microtubule spacing (Chen et al., 1992), exon 6 influences neurite elongation (Luo et al., 2004a) and exon 10 increases affinity to microtubules (Mandelkow et al., 1995).

Table 1.- *Distribution of tau regulated exons in human tissues*

Exon	Fetal CNS	Adult CNS	Adult PNS
2	no	yes, regulated	yes, constitutive
3	no	minor, regulated	yes, constitutive
4A	no	no	yes, constitutive
6	yes	yes, regulated	yes, regulated
10	no	yes, regulated	yes, constitutive

Ref. Andreadis et al., 2005

Table 2.- *Splicing factors on tau regulated exons and the effect over each one.*

Regulator	Exon		
	2	3	10
SRp20	N	I	(I)
ASF (SRp30a)	N	I	(I)
SRp30c	I	I	I
SC35 (SRp30b)	(I)	I	(I)
SRp40	(I)	•	(I)
9G8	N	nd	I
SRp55	I	A	I
SRp75	N	A	I
U2AF	N	•	I
PTB	I	•	I
NPTB	A	nd	N
hnRNPA1	N	I	N
hnRNPG	N	nd	I
SWAP	N	I	I
Htra2beta1	I	I	A
Nova1	A	I	I
KSRP	(I)	I	I
SLM1	A	A	(I)
SLM2	A	A	(I)
CELF3	(I)	I?	A
CELF4	A	I?	A

A= activator, I= inhibitor, N= no efect, ( ) = weak action, nd = not determined, • = construct or cell type specific.
Ref. Andreadis A. et al., 2005

I.2 Tau mRNAs

Three transcripts of 2, 6, and 9 kb are produced from the tau gene, which are differentially expressed in the nervous system, depending upon the stage of neuronal maturation and the neuron's type, however all contains exons 2, 3, and 10 (Goedert et al., 1992b; Goedert et al., 1989a; Goedert et al., 1989b; Nunez and Fischer, 1997; Wang et al., 1993) (Fig. 2B). The 2 kb mRNA targets to the nucleus and cytoplasm and is found in neuronal and nonneuronal tissues (Wang et al., 1993). It is not originated from further processing of the 6 kb mRNA containing the entire coding region of human tau as determined by RT-PCR and cDNA sequencing. The differential size with the 6 kb mRNA resides in the 3`untranslated region (UTR), where there are two polyadenylation sites separated by ~4kbp (Andreadis, 2005; Sadot et al., 1994). Two different 3'UTR which result from this choice may dictate the localization and/or stability of their respective mRNA (Andreadis, 2005; Behar et al., 1995; Kanai and Hirokawa, 1995; Sadot et al., 1994). The 6 kb transcript is found in abundance in CNS and encodes axonal tau (Drubin et al., 1984; Kosik et al., 1989; Neve et al., 1986), whereas the 9 kb transcript containing the extra exon 4A, inserted between exon 4 and 5, is restricted to retina and PNS giving rise to the biggest tau isoform (Andreadis, 2005; Ashman et al., 1992; Nunez and Fischer, 1997).

Even when one promoter for the tau gene has been mapped directly upstream of the exon -1 (Andreadis et al., 1995; Andreadis et al., 1996; Sadot et al., 1994), differences suggest that more than one promoter region is involved in the tissue specificity and in the response to transcription and growth factors (Andreadis, 2005). For example the 2 kb transcript is ubiquitous, whereas the 6 kb and 9 kb are both

restricted to neuronal tissues (Andreadis et al., 1996; Wang et al., 1993). Moreover the 6kb transcript is responsive to NGF whereas the 9kb is not (Drubin et al., 1988). The presence of regulated exons in tau mRNAs as well as the tissue distribution of those messenger are summarized in Table 3 (Andreadis, 2005).

Table 3.- ***Tau exon distribution in tau mRNAs***

mRNA (kb)	Compartment	Tissue	Exons
2	cytoplasmatic/ nuclear	ubiquitous	2, 3, 10 regulated
6	Axonal	CNS and PNS	2, 3, 6, 10 regulated
9	axonal	retina and PNS	2, 3, 4A, 10 constitutive 6 regulated,

Ref. Andreadis A., 2005

I.3 Tau protein

Tau is structurally a tripartite molecule, constituted by an animo-terminal (N-terminal) "projection domain", a constant central domain containing a "proline rich region" and a carboxy-terminal (C-terminal) microtubule binding domain (MBD) (Fig. 2C).

The "projection domain" has different lengths depending on the presence or absence of two inserts of 29 or 58 amino acids, encoded by exon 2 and 3 respectively (Goedert et al., 1989a; Himmler et al., 1989). Those inserts give a highly acidic character to this region, due to the amino acid composition of them. This domain determinates the spacing between microtubules in axon and may increase axonal

diameter as it is observed in peripheral neurons, which often project a very long axon with a large diameter, according to the expression of the "big tau" isoform, which contains the N-terminal sequence encoded by exon 4A (Chen et al., 1992). On the other hand, this domain establish interactions with spectrin and actin filaments (Carlier et al., 1984; Henriquez et al., 1995), interconnecting microtubules with cytoskeletal components such as neurofilaments (Miyata et al., 1986), and thus restricting the flexibility of the microtubules (Matus, 1990). This domain also mediates interactions of microtubules with the neural plasma membrane (Brandt et al., 1995; Hirokawa et al., 1988).

The proline rich region, that follows the N-terminal part of tau, has been involved in the binding to SH3 domains of src-family non-receptor tyrosine kinases, such as fyn (Hwang et al., 1996). In fact, recent studies determined that human tau binds to Fyn and is phosphorylated at Tyr18 and 29 (Lee et al., 2004). This region is also likely involved in the interaction with phospholipase C-γ (PLC-γ) isozymes. I*n vitro* studies demonstrate that tau complex specifically with the SH3 domain of PLC-γ and enhance its activity in the presence of unsaturated fatty acids such as arachidonic acid, suggesting the participation of tau in the signal transduction pathway involving PLC-γ (Hwang et al., 1996; Jenkins and Johnson, 1998).

The C-terminal domain of tau contains the microtubule-binding repeats, corresponding to three or four copies of a highly conserved 18 amino-acid repeat (R) (Goedert et al., 1989b; Himmler et al., 1989; Lee et al., 1989), separated from each other by less conserved 13- or 14- amino-acid inter-repeat domain. The 18 amino-acid

repeats binds to microtubules through a flexible array of distributed weak sites (Butner and Kirschner, 1991; Lee et al., 1989). It has been shown that adult tau isoforms containing the four repeats (R1-R4) are more efficient promoting the microtubule assembly than the fetal isoform containing three repeats (R1, R3, R4) (Butner and Kirschner, 1991; Goedert and Jakes, 1990; Gustke et al., 1994). Interestingly the peptide 274KVQIINKK281, located in the inter-region between repeats 1 and 2 (R1-R2 inter-region), unique to the adult tau isoform, has been shown to be the most potent inductor of microtubule polymerization, and thus is responsible for differences in the binding affinities between 3R and 4R-tau (Goode and Feinstein, 1994). Recent evidences support a role for the MBD in the modulation of the phosphorylation state of tau proteins, since a direct and competitive binding has been demonstrated between microtubules and the protein phosphatase 2A (PP2A) through the MBD (residues 224-236 of the longest isoform) of tau (Sontag et al., 1999). In addition, it has been found that the repeat domain alone does not interact with cellular microtubules, whereas the inter-repeat regions bind tightly to this region, without affecting the microtubule spatial arrangement (Preuss et al., 1997).

In human brain, the result of combinatory alternative splicing are a family of six tau isoforms which range from 352 to 441 amino acids (Fig. 2D). Their molecular weight is ranging from 45 kDa to 68 kDa, when resolved on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, besides the "big tau" isoform of 110 kDa (Boyne et al., 1995; Drubin et al., 1988; Mavilia et al., 1993; Oblinger et al., 1991). This tau isoforms are differentially expressed during development and differ from each other by the presence or absence of N-terminal inserts (Goedert et al., 1989a;

Goedert et al., 1989b; Himmler et al., 1989) together the presence of either the three (3R) or four (4R) C-terminal microtubule binding repeats. No more than one tau isoform has been found in human brain during fetal stages, which is characterized by absence of N-terminal inserts and three C-terminal repeats, whereas during adulthood six tau isoforms are expressed, containing either one or two N-terminal inserts together three or four C-terminal repeats (Goedert and Jakes, 1990; Kosik et al., 1989).

II. Post-translational modifications in tau proteins

Tau isoforms experiment different post-translational modifications, that determinate and/or modulate their function and intracellular distribution (Avila et al., 2004; Gong et al., 2005). Among them, the phosphorylation of tau, has been widely studied because it regulates the function of tau, however O-GlcNAcylation, is taking relevance because it seems to modulate the phosphorylation of tau in a site-specific fashion (Liu et al., 2004).

II.1 Phosphorylation

Tau proteins bind to the microtubules through the MBD, however the phosphorylation on specific sites can interfere in the binding of tau to microtubules in such a way that phosphorylated tau proteins are less effective on microtubule polymerization than the unphosphorylated forms (Biernat et al., 1993). Furthermore intramolecular interactions between the heptapeptide 224KKVAVVR230, located in the proline rich region and the repeats in the MBD, have an effect on the affinity of tau to microtubules (Goode et al., 1997).

At least 30 phosphorylation sites have been described in tau, among 80 putative Ser or Thr phosphorylation sites of the longest tau isoform (441 amino acids) (Fig. 3). Most of them occur on Ser-Pro and Thr-Pro motives (Buee et al., 2000; Hasegawa et al., 1992; Lovestone and Reynolds, 1997; Morishima-Kawashima et al., 1995) placed outside the microtubule-binding domain with the exception of Ser262 (R1), Ser285 (R1-R2 interrepeat), Ser305 (R2-R3 interrepeat), Ser324 (R3), Ser352 (R4) and Ser356 (R4) (Roder et al., 1997; Seubert et al., 1995). Isolated tau proteins show varying degrees of phosphorylation, and the site and degree of phosphorylation are modulated by several protein kinases and phosphatases.

The kinases involved in the site-specific phosphorylation of tau can be either, proline-directed protein kinases (PDPK), or non proline-directed protein kinases (NPDPK). Most oh them are PDPKs, which include the mitogen activated protein kinase (MAP) (Drewes et al., 1992; Vulliet et al., 1992), tau tubulin kinase (Takahashi et al., 1995), cyclin-dependent kinases including cdc2 and cdk5 (Baumann et al., 1993; Liu et al., 1995). The stress-activated protein kinases (SAP kinases) (Buee-Scherrer and Goedert, 2002; Jenkins et al., 2000), as well as the protein kinases PKN and PKC have been also involved in tau phosphorylation. PKN and PKC phosphorylate tau predominantly at specific sites located in the MBDs, which leads to disruption of the microtubule array (Taniguchi et al., 2001). However the action of PKN can also reduce the *in vivo* phosphorylation of tau at epitopes recognized by the phospho-dependent antibodies AT8, AT180 and AT270 (Fig. 3). On the other hand, the glycogen synthase

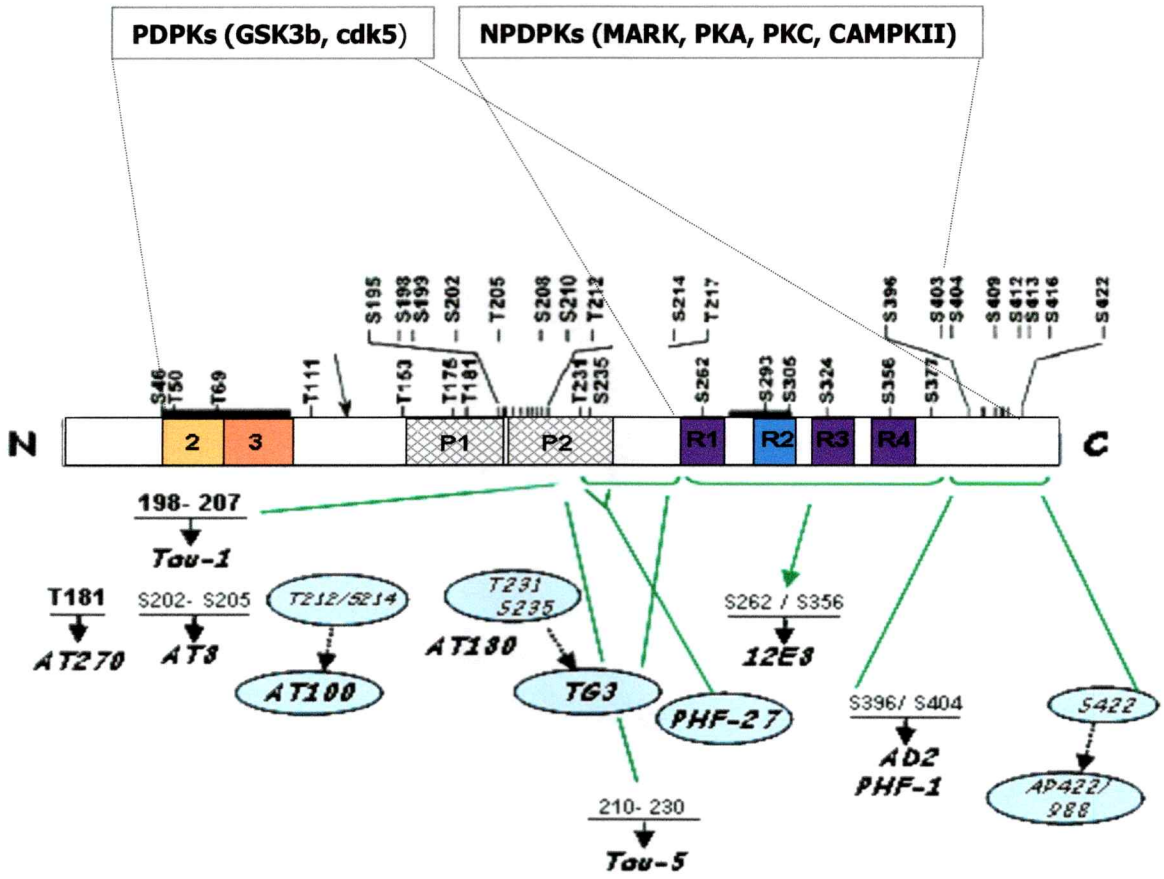


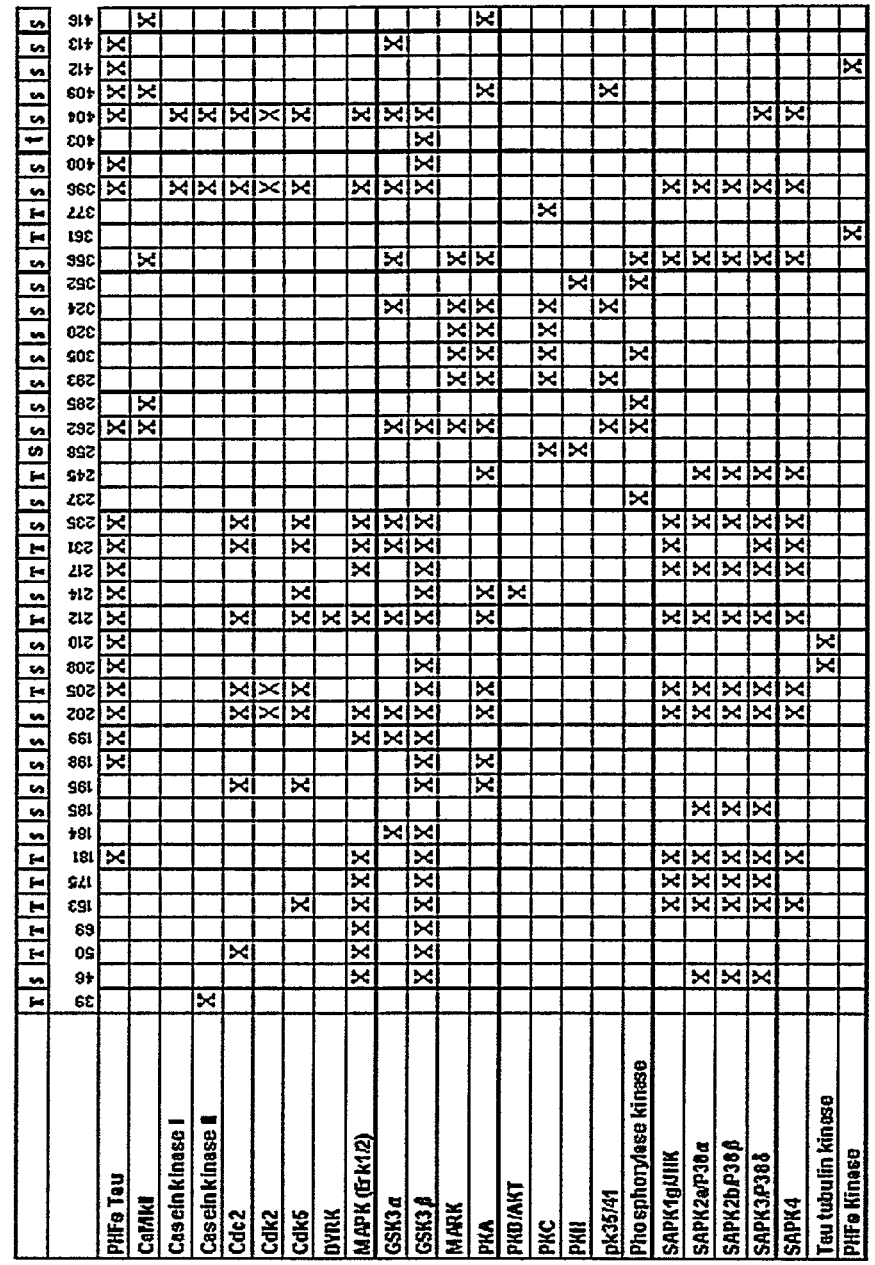
Figure 3. ***Phosphorylation sites in tau, kinases and anti-tau antibodies epitopes.*** The phosphorylation Ser/Thr sites alongside tau are denote in the upper side of the diagram of tau. In boxes upper side, the target region of non-proline directed protein kinases (NPDPK) and proline directed protein kinases (PDPK). P1, P2 represent the proline rich region of the central domain. Schematic representation of specific phosphorylation sites (indicated as peptidic sequence underlined) of the longest tau isoform (2+3+10+). Hyperphosphorylations sites are grouped in two clusters located on both sides of the microtubules binding domain with the exception of Ser262/ Ser356. Phosphorylation dependent antibodies (in italics) have been developed for some sites. AD-specific antibodies are circled. (Image adapted from Delobel et al., 2002; Gomez-Ramos et al., 2004; Morishima-Kawashima et al., 1995).

kinase 3 beta (GSK3β) phosphorylates tau in both non-Ser/Thr-Pro and Ser/Thr-Pro sites (Planel et al., 2002). In addition non-Ser/Thr-Pro sites can be phosphorylated by the microtubule-affinity regulating kinase (MARK) (Drewes et al., 1997), the Ca2+/calmodulin-dependent protein kinase II (CAMPKII) (Steiner et al., 1990), the cyclin-AMP-dependent kinase (PKA) (Litersky and Johnson, 1992) and by casein kinase I and II (Greenwood et al., 1994). The different kinases involved in tau phosphorylation *in vivo* and the specific sites where they act are summarized in **Table 4** (Buee et al., 2000).

In general, increases in tau phosphorylation correlates inversely with its ability to bind and stabilize microtubules, however, the extent to which phosphorylation affects this capacity is largely dependent on which sites are phosphorylated and also on the action of phosphatases over tau. In this sense, tau proteins can be rapidly dephosphorylated by endogenous phosphatases (PP).

The PPs have many direct or indirect physiological effects, and counter-balance the actions of kinases. They can associate directly or indirectly with microtubules (Dudek and Johnson, 1995; Liao et al., 1998; Sontag et al., 1999). For example, it has been demonstrated that tau acts as a link between the phosphatase-1 (PP1) and tubulin (Liao et al., 1998), whereas the phosphatase-2A (PP2A) is directly linked to the microtubules through ionic interactions (Sontag et al., 1999). *In vitro*, the purified PP-1, PP-2A and PP-2B (calcineurin) can dephosphorylate tau proteins (Goedert et al., 1995a; Goto et al., 1985; Yamamoto et al., 1988). Furthermore studies reported by Ono et al 1995 demonstrate that inhibiting PP-2A results in tau phosphorylation at sites different

Table 4.- ***Kinases involved in the site specific phosphorylation of tau protein***



Ref. Buee L., et al. (2000)

from the target sites of PP2B (Ono et al., 1995), suggesting that both phosphatases act over specific sites of tau proteins. A novel phosphatase, the protein phosphatase 5 (PP5), has been shown to dephosphorylate recombinant tau previously phosphorylated with the cAMP-dependent protein kinase and GSK3β. The activity of PP5 toward tau is comparable to those reported with other substrates, and the expression of active PP5 in PC12 cells results in a reduced tau phosphorylation, suggesting that PP5 can dephosphorylate tau at the cellular level (Gong et al., 2004).

II.2 O-GlcNAcylation

Complex N-and O-linked glycosylation occurs on membrane-bound or secreted proteins that are synthesized in the endoplasmic reticulum and Golgi complex of mammalian cells. In contrast to different N- and O-linked glycans, which luminal or extra cellular localization restricts their potential for dynamic responsiveness to signals, the monosaccharide β-N acetylglucosamine (GlcNAc) is the only one that is located in the nucleus and cytoplasm, modifying nucleocytoplasmic proteins in a dynamic and reversible way. This abundant post-translational modification know as O-GlcNAcylation, consist in the covalent linkage of a single moiety of D-N-acetylglucosamine attached in β-linkage to the side chain of specific serine and threonine residues present in nuclear and cytoplasmic proteins (Comer and Hart, 1999; Hart et al., 1996). Both the addition and removal of GlcNAc moieties are catalyzed by only two nucleocytoplasmic enzymes, the O-β-N-acetylglucosaminyltranferase (OGT) and the O-β-N-acetylglucosaminidase (GCA), respectively (Dong and Hart, 1994;

Haltiwanger et al., 1992; Iyer and Hart, 2003) (Fig. 4). O-GlcNAc moieties have been identified in different proteins including transcription factors, kinases and phosphatases, nuclear pore proteins, oncogene products, tumor suppressors, intermediate filaments and cytoskeletal proteins such as MAP2 and tau (Arnold et al., 1996; Lefebvre et al., 2003a; Lefebvre et al., 2003b; Wells et al., 2001).

On several of these proteins, O-GlcNAc and O-phosphate alternatively occupy the same or adjacent sites. In tau, the O-GlcNAc sites are less abundant than the phopsphorylation ones, and it has been described that in certain cases both modifications occupy the same site, concluding that O-GlcNAcylation regulates in a site-specific mode tau phosphorylation, negatively in most of the sites (Liu et al., 2004). It may have opposite effects, mediating the interaction level between tau and tubulin, therefore modulating the tau functions. On the other hand O-GlcNAcylation may also play a role in subcellular localization and degradation of tau proteins, indeed a reduction of O-GlcNAc incorporation was correlated with a decrease in nuclear tau levels (Lefebvre et al., 2003b).

III. Tau function and tauopathies

Tau regulates the dynamic stability and orientation of microtubules (Drubin and Kirschner, 1986), participating in process such as the assembly of microtubules over the centrosome, in axonal transport and in neuronal polarity during axonal elongation (Drechsel et al., 1992; Esmaeli-Azad et al., 1994; Trinczek et al., 1995). Tau proteins are mainly expressed in neurons of different animal species (Binder et al., 1985), in oligodendrocytes (Black et al., 1996; LoPresti et al., 1995; Papasozomenos and Binder,

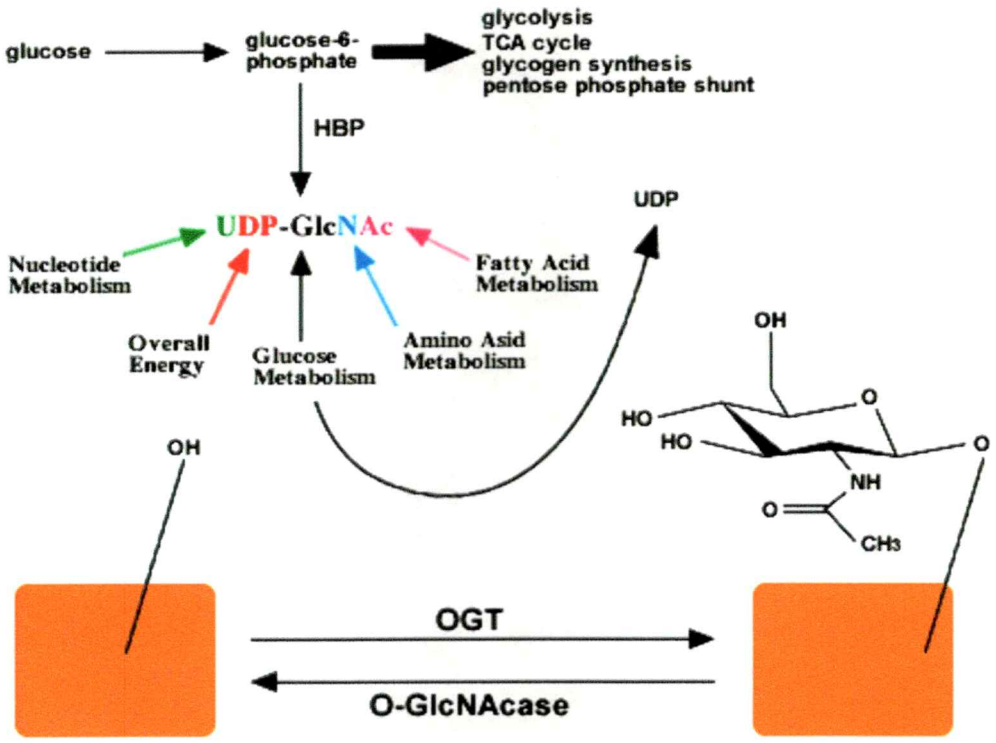


Figure 4. ***Enzymes of O-GlcNAc cycling.*** UDP-GlcNAc, the metabolic precursor of O-GlcNAc, is derived from glucose 6-phosphate (G6P), glutamine (Gln) and UDP through the actions of enzymes in the hexosamine biosynthetic pathway (HBP). Once G6P is converted to fructose-6-phosphate (F6P), the amino group of Gln is transferred to F6P to form glutamate and glucosamine-6-phosphate (GlcN6P). Then, GlcN6P can be either: (1) N-acetylated, converted to GlcNAc-1-phosphate and then coupled to UDP; or (2) converted to GlcN1P, acetylated and coupled to UDP. Consistent with O-GlcNAc being dynamic and inducible and in contrast to phosphorylation regulation, only two nucleocytoplasmic enzymes participates in the regulation of O-GlcNacylation. O-GlcNAc transferase (OGT) mediates the attachment and O-GlcNAcase (GCA) the removal of O-GlcNAc moieties from proteins (orange rectangles) (Image is from Iyer and Hart, 2003).

1987) and in glial cells under pathological conditions (Chin and Goldman, 1996). Tau mRNA and proteins are also present in several peripheral tissues including heart, kidney, lung, muscle (Wei and Andreadis, 1998), pancreas and testis as well as in fibroblast (Gu et al., 1996; Ingelson et al., 1996; Vanier et al., 1998).

Intracellulary tau can play a mediating role in the interactions between microtubules and actin filaments (Correas et al., 1990; Cross et al., 1993; Henriquez et al., 1995). It can also interact with the cellular centrosoma (Cross et al., 1996; Lu and Wood, 1993), with cytoskeleton filaments like vimentin (Capote and Maccioni, 1998), with calmodulin (Padilla et al., 1990), spectrin (Carlier et al., 1984), α-synuclein (Jensen et al., 1999) and with the phosphatases PP1 and PP2A (Lee et al., 1998; Sontag et al., 1999).

In CNS the main tau distribution corresponds to axons of mature and growing neurons (Kempf et al., 1996) and the distal end of growing neurons (Black et al., 1996). Besides tau has been observed in association with ribosomes in somatodendritic compartments of neurons and glial cells (Kowall and Kosik, 1987) and in nuclei of neuronal and non-neuronal cells (Wang et al., 1993). Others studies have found evidence that tau could acts as mediator of the interactions between microtubules and the neural plasma membrane (Brandt et al., 1995), participating in the signal transduction pathway related to phopholipase C-γ (PLC-γ) (Hwang et al., 1996) and to the non-receptor tyrosine kinases of the src-family, fyn (Lee et al., 1998).

The ability of tau to regulate microtubule dynamics depends on the balance between different tau isoforms, its structural integrity and the complex combinatorial

pattern of phosphorylation (Buee et al., 2000; Stoothoff and Johnson, 2005). The binding of tau to microtubules together with the variability of tau polypeptides are expected to have different and specific functions (Binder et al., 1985; Maccioni and Cambiazo, 1995).

While normal tau functioning is required for proper development of the nervous system, tau dysfunction has been correlated with neurodegerative diseases. Studies with cellular and animal models of tau pathology, conclude that neuronal dysfunction and neuronal loss may be attributed to a reduced tau functionality as consequence of dominant mutations (Barghorn and Mandelkow, 2002; Gamblin et al., 2000; Hong et al., 1998), aberrant phosphorylation patterns (Lovestone and Reynolds, 1997), tau cleavage or aggregation (Amadoro et al., 2006) and to alterations in the levels of isoforms expression (Panda et al., 1995) (Fig. 5A).

Approximately 20 mutations have been described in the tau gene, which can be classified in two groups where commonly an increase in the level of cytosolic tau and/or a change in 4R/3R ratio is observed. The first group includes mutations that affect the alternative splicing of exon 10, leading to a deregulation in the ratio of 4R to 3R isoforms. The second group represents mutations located in the vicinity of microtubule binding domains that decrease tau-microtubule interactions.

Hyperphosphorylation of tau in specific sites reduces its affinity to microtubules, underlying the formation of paired helical filaments (PHF), intra-neuronal aggregates of abnormal tau (Fig. 5B) (Buee et al., 2000). The PHFs are the responsible of brain lesions responsible of the process know as neurofibrillary degeneration, since the PHF are the major component of the neurofibrillary tangles (NFT), structures observed in

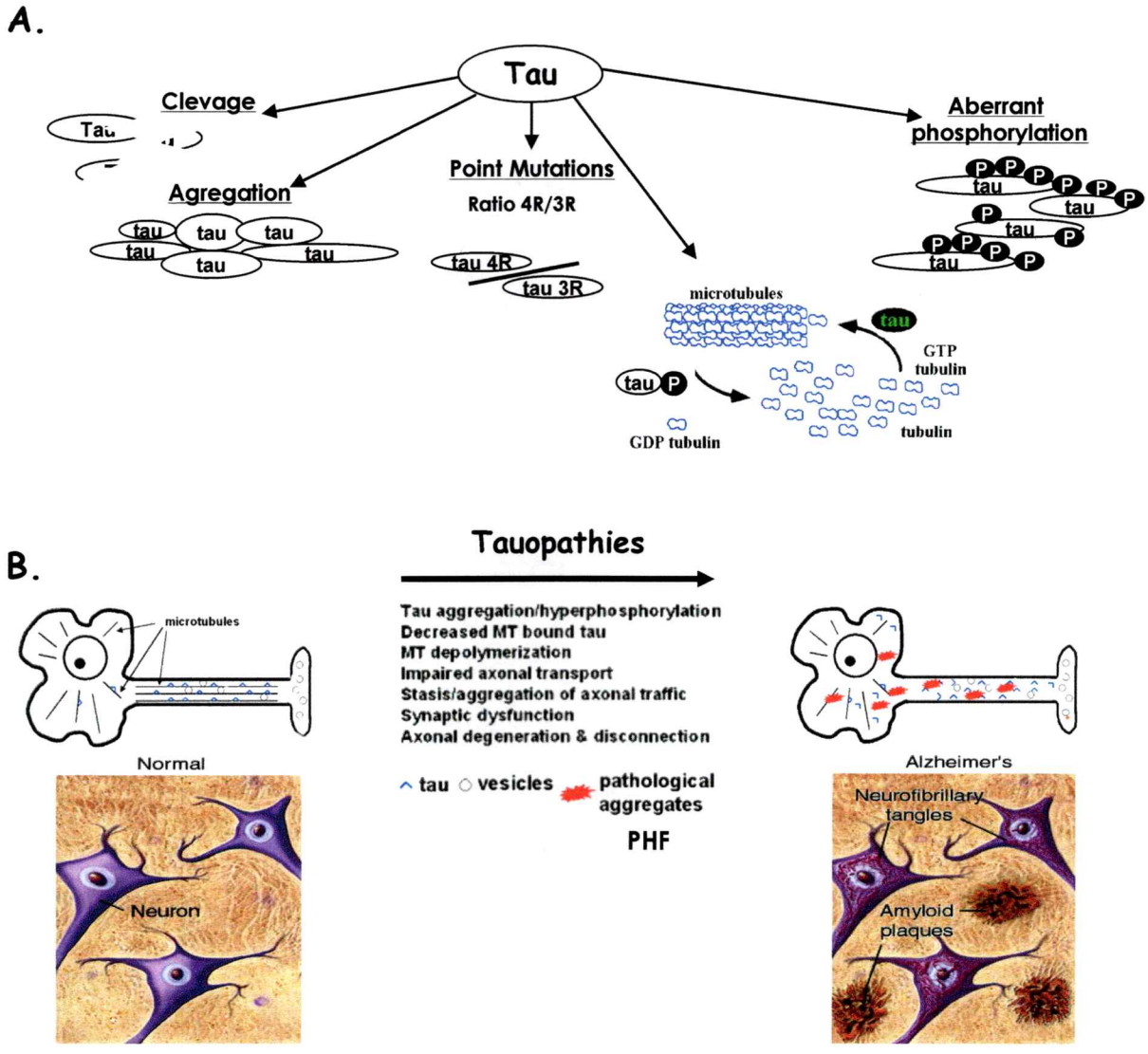


Figure 5. ***Scheme of tau dysfunction and tauophaties responsible of neurodegerative diseases.*** (A) Schematic representation of factors involved in tau dysfuntionality such as tau cleavage or aggregation; dominant tau mutations causing alterations in the ratio 4R/3R of isoforms expression; and aberrant phosphorylation patterns. (B) The reduction on the affinity of tau for microtubules, as a consequence of mutations or hyperphosphorylation of tau in specific sites, triggers the process of neurofibrillary degeneration. The formation of intra-neuronal aggregates of abnormal tau, the paired helical filaments (PHF), are the major component of the neurofibrillary tangles (NFT), the hallmark of "tauopathies". Image adapted from: http://www.uphs.upenn.edu/news/News_Releases/dec04/paclitaxel.htm.

neurodegenerated brains (Mandelkow et al., 1995). The NTF are the hallmark of several neurodegenerative diseases grouped as "tauopathies", among which are the progressive supranuclear palsy (PSP), Pick's disease (PiD), corticobasal degeneration (CBD), frontotemporal dementia, parkinsonism linked to chromosome 17 (FTDP-17) and Alzheimer disease (AD) (Fig. 5B) (Buee et al., 2000; Ingram and Spillantini, 2002). Deposits similar to those found in Alzheimer's can be found in some other brain disorders like amyotrophic lateral sclerosis (Lou Gehrig's disease), and Down's syndrome (DS) (Wisniewski et al., 1985)

A disease-specific biochemical bar-code has been described due to the aggregation of specific sets of tau isoforms (Fig. 6) (Buee et al., 2000), enabling a molecular characterization of tautophaties and group them in classes (Sergeant et al., 2005). Isoelectrofocusing analysis reveals that PHF-tau proteins are more acidic than normal tau isolated from biopsy-derived human brain, and antibodies recognizing unphosphorylated epitopes show that PHF-tau is phosphorylated at physiologically regulated sites (Sergeant et al., 1995). Few phosphorylation-dependent antibodies such as AT100 (Matsuo et al., 1994), AP422/988 (Bussiere et al., 1999), PHF-27 (Hoffmann et al., 1997) or the TG3/MC antibodies (Vincent et al., 1996) only detect PHF-tau, demonstrating the presence of specific abnormal phosphorylation sites (Fig. 3, blue circles). These phophorylation sites are besides conformation-dependent epitopes with the exception of Ser422. Until now a correlation between hyperphosphorylation, abnormal phosphorylation, tau aggregation and severity of symptoms exists, however it remains to determine whether the phosphorylations are a cause or a consequence in the aggregation process.

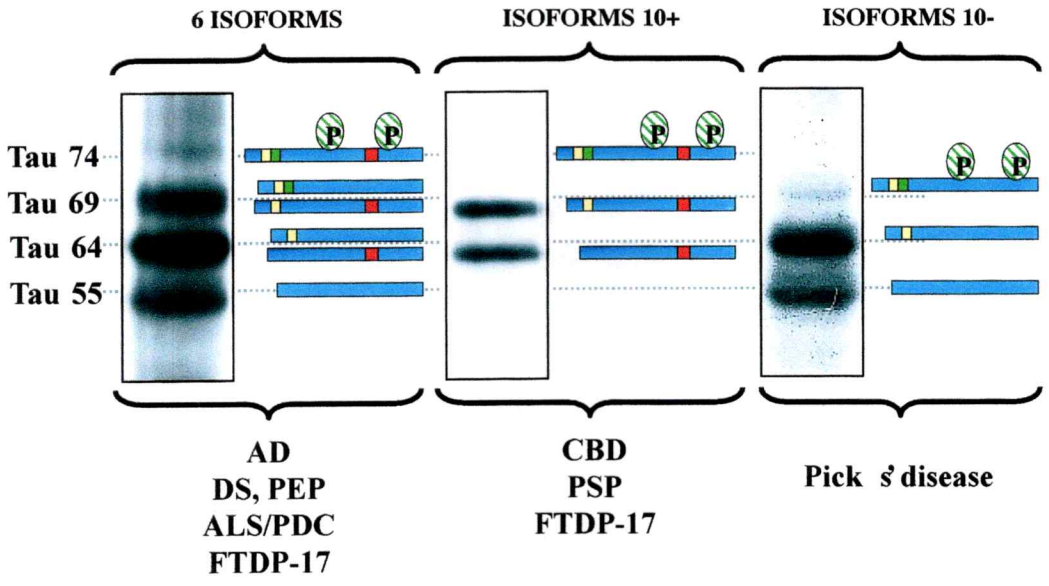


Figure 6. ***The bar code classification of taupathies***. Schematic representation of tau isoforms composition (right of each frame) and immunodetection using the phosphorylation-dependent monoclonal antibody AD2. The six tau isoforms are involved in the formation of the typical AD-triplet with the minor tau74 variant. This pattern is also described in Down syndrome (DS), post-encephalitic Parkinsonism (PEP), ALS/PDC guamanian syndrome (ALS/PDC) and some families with FTDP-17 (left panel). The typical PSP/CBD doublet tau 64, 69 is related to the aggregation of hyperphosphorylated tau isoforms with exon 10. The FTDP-17 families with mutations in exon 10 or intron 10 exhibit the same profile (middle panel). Hyperphosphorylated tau proteins without exon 10 aggregated in Pick's disease are detected as a tau55, 64 doublet (right panel). Color codes are: exon 2 (yellow box) and 3 (green box) in the amino-terminal part, in combination with either three (R1, R3 and R4) or four (R1–R4) repeat-regions (red boxes) in the carboxy-terminal part (image from Buee et al., 2000).

IV. Knockout and transgenic mice

IV.1 Tau knockout mice

The suppression of MAPs in cultured neuronal cells by using antisense nucleotides has implicated these proteins in microtubule stabilization and organization (Caceres and Kosik, 1990). In tau knockout mice, whereas axonal elongation was not affected, microtubule stability was decreased in some axons, thus showing phenotypic but no obvious functional effects. Indeed those animals showed an increase in microtubule associated protein 1A, which may compensate for the function, suggesting that neuronal MAPs may be functionally redundant (Harada et al., 1994). On the other hand tau null mice exhibit muscle weakness and learning defects (Ikegami et al., 2000). The hippocampal neurons do not mature properly as measured by axonal and neuritic extensions (Dawson et al., 2001). Those observations suggest that tau play an important role in the development and maintenance of the nervous system. Both studies showed that axonal growth and axonal diameter are particularly affected, and it is attributed to the length of the N-terminal region with or without the inserts encoded by exon 2 and 3.

IV.2 Tau transgenic mice

The expression of the longest human brain tau isoform (441 amino-acid isoform) in transgenic mice, under the control of the human Thy-1 promoter (Seki et al., 1985), has been performed in order to elucidate the role of tau in the pathogenesis of AD. In the second transgenic line tau mRNA levels were up to 5-fold higher than the endogenous murine tau mRNA levels. *In situ* hybridization on tissues sections from brain and immunohistochemical analysis, showed that human tau mRNA was highly

expressed and that it was only detected in a small percentage of nerve cells (Gotz et al., 1995). Interestingly, as in AD a prominent somato-dendritic staining was observed in addition to axonal staining, and tau was found hyperphosphorylated at some sites that are characteristic of tau from AD patient brains.

V. Nuclear tau

Tau, as well as other eukaryotic proteins, seems to display a functional diversity. Even when it has been described as a MAP, ultrastructural localization studies identified tau associated with ribosomes at somatodendritic compartments in certain areas of the central nervous system (Papasozomenos and Su, 1991). Later on, another non-microtubular localization of tau was identified in the nucleus of neuronal and non-neuronal cells (Lu and Wood, 1993), also displaying a nuclear localization in nucleoli of HeLa cells, non-transformed human fibroblasts and lymphoblasts (Thurston et al., 1996) and in nuclei isolated from human brain (Brady et al., 1995). Whereas in mitotic cells, nuclear tau was associated with the Nucleolar Organizer Regions (NORs), in interphase cells tau was localized at the dense fibrillar regions (Loomis et al., 1990; Thurston et al., 1996). Furthermore, it was described in the nuclei of human Huh-7 hepatoma cells (Cross et al., 2000). Purification of nuclear tau from Huh-7 hepatoma cells as well as from human fibroblasts demonstrated that nuclear tau from non neuronal cells share with neuronal tau the capacity to promote tubulin polymerization *in vitro*.

Some tau species have also been described associated with chromatin fractions, sharing a similar phosphorylation pattern with the cytoplasmic tau (Greenwood and Johnson, 1995). More recently it has been demonstrated that tau can bind to DNA in an

aggregation-dependent and a phosphorylation-independent way (Hua and He, 2002), to double- and single-strands DNA (Hua et al., 2003; Krylova et al., 2005). This interaction protects *in vitro* DNA from denaturation (Hua and He, 2003). Some of the most recent observations describe the capacity of tau protein to induce a conformational change on the DNA at a ratio of one tau molecule per 700 bp of DNA. This observation suggests that the mass ratio of tau to DNA is important during the complex formation (Qu et al., 2004).

VI. A short overview of the nucleolus

The nucleus in eukaryotic cells is functionally compartmentalized. The best example of such a compartmentilization is the nucleolus an structure characterized by its high density and chemical composition distinct from the surrounding nucleoplasm and known to be site of ribosomal biogenesis (Sullivan et al., 2001) (Fig. 7A). The nucleolus of higher eukaryotes contains three major morphological components: the fibrillar centers, the dense fibrillar component and the granular component, each one related to the process of ribosome formation (Carmo-Fonseca et al., 2000). The fibrillar centers harbor several hundred copies of rRNA genes, arranged in tandem at chromosomal loci termed nucleolar-organizing regions, NORs. NORs are present in the short arms of acrocentric chromosomes (13, 14, 15, 21, 22) and correspond to arrays of GC-rich ribosomal DNA (rDNA) repeats immediately juxtaposed to the sequences that

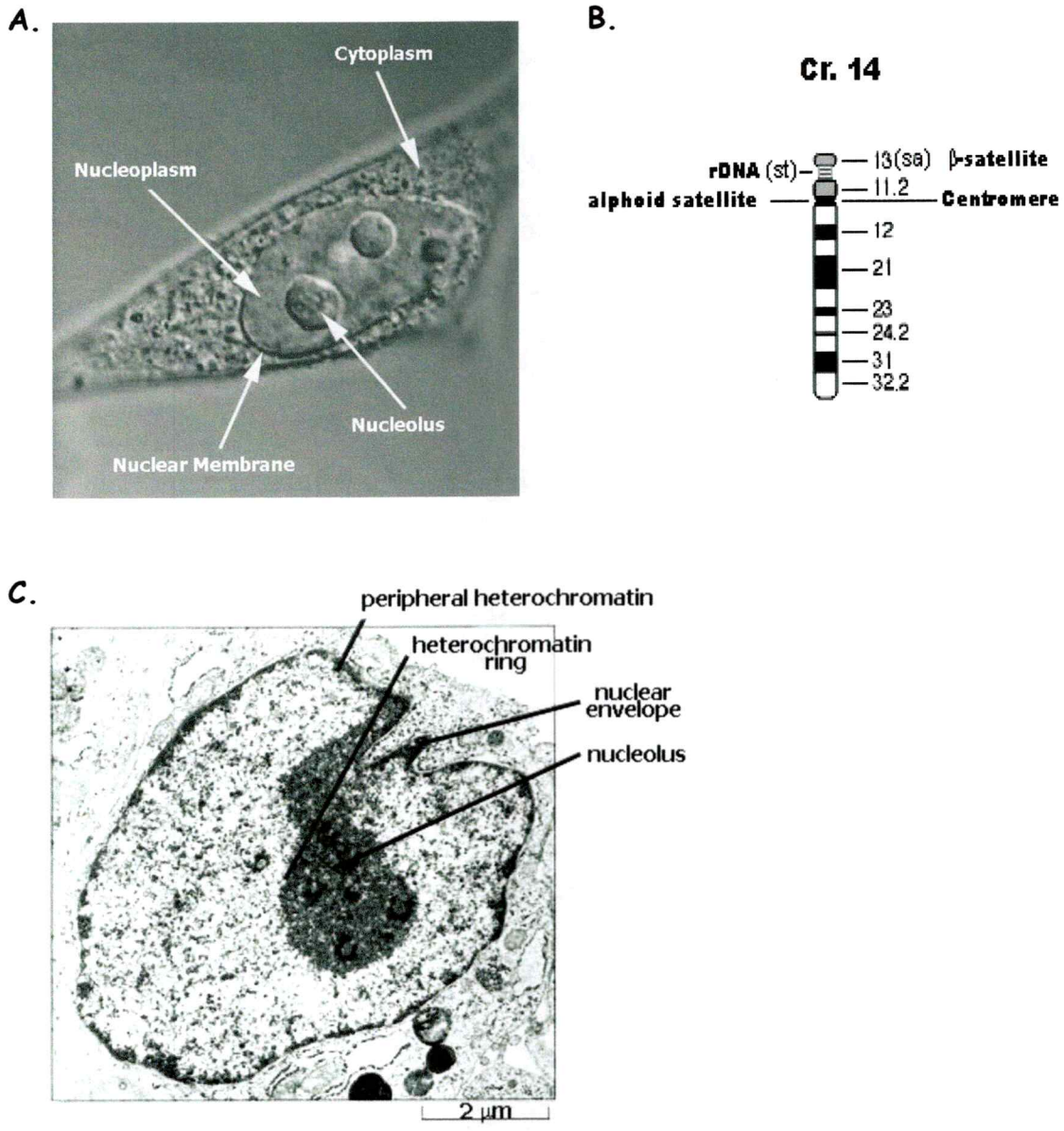


Figure 7. ***The nucleolus, acrocentric chromosomes and satellite DNA.*** (A) Representation of the cell nucleus and their parts: the nuclear membrane; the nucleoplasm and the nucleolus. (B) Schematic representation of acrocentric chromosome 14. The centromere and chromosome location of rDNA genes and centromeric alphoid (α)-satellite DNA sequences. (C) "*Peripheral heterochromatin*" and "*heterochromatin ring*" distribution at the nucleus and nucleolus respectively. (Adapted from http://www.personal.dundee.ac.uk/~jesleema/jesnucleolidic.html)

constitute the centromeres of acrocentric chromosomes (Fig. 7B). Centromeres of mammalian chromosomes are a specialized locus responsible for chromosome segregation during mitosis and meiosis (Rudd and Willard, 2004). They consist of tandemly repeated satellite DNA sequences (α-satellite in human cells and γ-satellite in murine cells), organized as constitutive heterochromatin (Csink and Henikoff, 1998; Karpen and Allshire, 1997; Murphy and Karpen, 1998). The distribution and organization of these centromeric sequences as well as that of active and inactive rDNA at the nucleolar structure does not occur in a random fashion. Variations are observed depending on the cell type, cell metabolic activity as well as stages of the cell cycle. In neuronal cells, centromeres are found clustered and attached as large aggregates to the surface of the nucleolus, so that the majority of neuronal nucleoli contain a characteristic ring of nuclear heterochromatin. Large arrays of inactive methylated rDNA repeats have been observed clustered inside the nucleoli at its periphery, close to the ring of centromeric heterochromatin (Akhmanova and Hoogenraad, 2005; Manuelidis, 1984) (Fig. 7C). Although the link between the nucleolus and centromeric heterochromatin has not been clearly defined, it has been suggested that centromeric perinucleolar heterochromatin and the associated proteins play an important role in the regulation of the nucleolar structure (Carmo-Fonseca et al., 2000).

At present it is generally accepted that the nucleolar compartment results from coalescence of transcription units with consequent recruitment of processing factors by the nascent RNA. But what causes coalescence of the rRNA genes in the first place?

Recent results reveal an involvement of chromatin-silencing proteins in the formation of the nucleolar compartment.

In this study to get an insight into the functionality of nuclear tau, non-neuronal cells lines were used to characterize and analyze the nuclear tau localization with respect to nucleolar proteins and pericentromeric heterochromatin. The specific association of tau with pericentromeric satellite DNA sequences is further investigated to elucidate relevant interactions of tau at nucleolar level. Thus, considering the participation of tau in functions related to the CNS and its involvements in tauopathies such as AD, the human neuronal model SHSY5Y is used to molecularly characterize the nuclear tau subpopulation, to evaluate changes in nuclear tau levels during the cell cycle and to analyze factors linked to AD involved in the mechanism of nuclear targeting.

HYPOTHESIS

Tau proteins associate with specific DNA sequences and a subpopulation of them localize in the nucleus of neuronal cells, in a proliferation and posttranslational modification dependent way.

OBJECTIVES

GENERAL OBJECTIVE 1

The analysis of the nuclear tau distribution and evaluation of the interaction with specific DNA sequences.

Specific objective 1: To investigate the nuclear distribution of tau respect nucleolar proteins and heterochromatin, in cycling and differentiated cells, by using neuronal and non-neuronal cell models, based on immunofluorescence localization analysis and inmunoFISH analysis.

Specific objective 2: To investigate the interaction between tau and satellite DNA sequences that conform centromeric and pericentromeric heterochromatin at nucleoli.

Specific objective 3: To investigate the specificity of interaction between tau and human and murine satellite DNA sequences.

GENERAL OBJECTIVE 2

The molecular characterization of the nuclear tau subpopulation of SHSY5Y cells and analysis of the nuclear tau levels through the cell cycle.

Specific objective 1: To characterize the variants composing the nuclear subpopulation of tau from a molecular point of view.

Specific objective 2: To study the nuclear tau levels during the cell cycle.

GENERAL OBJECTIVE 3

To investigate factors related to the pathogenesis of AD participating in the nuclear localization of tau.

Specific objective 1: To investigate the participation of the nucleocytoplasmatic protein, Pin1, in the nuclear localization of tau.

Specific objective 2: To investigate the participation of kinases cdk5 and GSK3β in the nuclear localization of tau.

Specific objective 3: To evaluate the participation of O-GlcNAcylation in the nuclear localization of tau.

MATERIALS AND METHODS

I Cell Cultures

Cell cultures were maintained at 37°C in a humidified incubator with 5% CO2 atmosphere. Cells were treated with 0,25% - 0.53mM Trypsin-EDTA (Gibco-Merck), to remove them from culture plates, then resuspended in complete medium and plated either on glass coverslips 48 hours before use for immunofluorescence analysis or seeded (10000 cells/cm2) for amplification and further analysis.

1) **Cell line HeLa (ATCC U.S.A. #CCL-2)**: HeLa cells derived from human cervix epithelial adenocarcinoma were grown in monolayer, in Dulbecco's modified Eagle's culture medium (DMEM) (Gibco) supplemented with 5% (v/v) of fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 50 U/ml of penicillin and 0.5 mg/ml of streptomycin.

2) **Cell line GM03440B**: The non-transformed cell line GM03440B of human skin fibroblasts, was grown in Minimum Essential Medium (MEM) (Gibco) and supplemented with 15% (v/v) FBS, 0.1 mM of MEM-Non-Essential Amino-Acids solution (Gibco), 2mM L-glutamine, 50 U/ml of penicillin and 50 µg/ml of streptomycin.

3) **Lymphoblastes**: Human lymphoblastes were grown in RPMI 1640 medium (Gibco) supplemented 15% (v/v) FBS (Sigma Chemical Co.), 20 mM HEPES, 2 mM L-glutamine, 50 U/ml of penicillin, 50 µg/ml of streptomycin and 1 mM sodium pyruvate.

4) **Primary culture of hippocampal neurons:** The primary culture of hippocampal neurons was performed following the protocol described by Banker (Banker and Cowan, 1977). Briefly hippocampi from Sprague-Dawley rats at embryonic day 18 (E18) were dissected in Ca^{2+}/Mg^{2+} -free Hank's balanced salt solution (HBSS, Gibco 14180-020) with 10 mM HEPES, pH 7.4, and 0.5% glucose . After rinsed twice with HBSS by allowing for the tissue to settle to the bottom of the tube, the tissue was resuspended in HBSS containing 0,25% Trypsin-EDTA and incubated for 5 minutes at 37°C. After three rinses with HBSS the tissue was mechanically dissociated in MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate and 2 mMl-glutamine (MEM-10) by gentle passage through pasteur pipettes. Undisrupted tissue fragments were allowed to settle, cells in suspension sere transferred to a new tube and viable cells were counted using 0.2% trypan blue. Cells were initially plated in MEM-10 media. Three hours after, media MEM-10 was removed and serum-free medium N2/MEM (MEM supplemented with 2 mM L-glutamine, 750 mg/l glucose, 100 µM putrescine, 20 nM progesterone, 30 nM selenium dioxide, 100 µg/ml transferrin, 5 µg/ml insulin, 1 mM sodium pyruvate and 0.1% ovalbumin) was added.

5) **Cell line SH-SY5Y (ATCC U.S.A.#CRL-2266)**: SH-SY5Y human brain neuroblastoma cells were seeded at 30,000 cells/cm^2 in MEM (Gibco) supplemented with F12 nutrient mixture (MEN/F12) (Gibco), 10% (v/v) of FBS, 1mM MEM-Non Essential Amino Acids Solution (Gibco) and 100 U/mL of penicillin, 100 µg/ml streptomycin and anphotericin B presents in the 100X solution of

Antibiotic/Antimycotic (Gibco). For differentiation cells were cultured in growth medium containing 10μM trans-retinoic acid (Calbiochem 554720). This treatment involved several passages (at least two passages) since retinoic acid alone did not suppress cell proliferation. For molecular characterization of nuclear tau cells were growth up to 70% confluence in 75 cm2 or T175 cm2 flask. For nuclear protein extracts and subsequently immunoblot analysis of synchronized, control and treated cells they were grown in three 100-mm diameter plates for each point.

II. Antibodies

The phosphorylation-dependent tau mouse monoclonal antibodies used in this study include: Tau-1, Tau-5 (generous gift from Dr. Lester Binder of Northwestern University, Chicago) and AT8.

Tau-1 binds amino acids 198-207 in the human tau, only when they are dephosphorylated (Szendrei et al., 1993). Tau-1 binding is occluded when this epitope is phosphorylated at Ser^{202}, as it is in AD (Liu et al., 1993).

Tau-5 reacts with residues 210-241 in the proline rich domain of tau protein, and was used to determine tau levels independent of the phosphorylation state (Thurston et al., 1996).

AT8 recognizes phosphorylated Ser 202/Thr 205 (Pierce MN1020) (Goedert et al., 1995b).

PHF1 recognizes phospho-S396 and S404 (generous gift from Dr P. Davies) (Greenberg et al., 1992).

The antibody used to identify centromeric heterochromatin was the rabbit polyclonal antibody dimethyl H3K9 recognizing lysine 9 dimethylated in histone H3 (Upstate 07-212).

The antibodies used to identify nucleoli through nucleolar proteins were, the rabbit polyclonal antibody, nucleolin C23 against the protein nucleolin (Santa Cruz Biotechnology, sc-13057) and the human autoantibody anti-NOR90 recognizing the upstream binding factor (UBF) (a generous gift of Dr Hung Tseng, of University of Pennsylvania, Philadelphia) (Chan et al., 1991).

The antibody used to normalize the levels of tau protein respect the total protein loaded in nuclear extracts of control and treated cells was the mouse monoclonal anti-β–actin (Sigma A-5441) recognizing the protein β-actin.

The purity of the nuclear fractions was analyzed by the nuclear marker, the protein histone H1 and the cytoplasm marker, the protein β-tubulin. Both were recognized by the rabbit polyclonal anti-histone H1 (Santa Cruz Biotecnologies SC10806) and the mouse monoclonal anti β-tubulin (Sigma T-0198) antibodies respectively.

The immunodetection of the protein Pin1 was performed with the polyclonal antibody Pin1 (SantaCruz Biotechnology SC15340).

The label of cell nuclei in immunofluorescent experiments was perform with two probes, Hoechst 33258 fluorescent DNA dye or 50 μg/ml propidium iodide (PI).

III. Fluorescent in situ hybridization coupled to immunofluorescence (immunoFISH3D)

For the immunofluorescence procedure, cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes, then in methanol at -20°C for 10 minutes. After fixation, cells were permeabilized with 0.1% Triton in PBS for 5 minutes, washed twice with PBS and blocked in PBS- 5% of bovine serum albumin (BSA) for 45 minutes. Cells were then incubated with primary antibody-containing blocking solution for 2 hour at room temperature. The following primary antibodies mixes were used: mouse Tau-1 (1:100), human Nor90 (1:50), mouse Tau-1/ rabbit dimethyl H3K9 (1:500) and mouse Tau-1/ rabbit nucleolin (1:100). Conditions of nucleolin immunolocalization were those described by Barboule et al. (Barboule et al., 2005). Cells were fixed with formaldehyde 3.7% for 15 minutes. After washing with PBS, cells were permeabilized with 0.2% Triton-X100 in PBS for 5 minutes, washed and incubated with methanol 100% for 10 minutes at –20°C. After extensive wash, coverslips were blocked in PBS-5% BSA for 45 minutes and incubated with the primary antibody-containing blocking solution, Tau-1 and C23, for 2 hour at room temperature. After incubation with the primary antibodies, four washing steps were performed and cells were incubated with the secondary antibody diluted in blocking solution for 1 hour. The secondary antibodies used were Alexa 488 goat anti-mouse IgG 1:200 (Molecular Probes), CY3 goat anti-rabbit IgG 1:200 (Chemicon International) and FITC goat anti-human IgG 1:100 for Nor-90 antibody (Tian et al., 2001).

The immunoabsortion assay to test antibody specificity was performed incubating by 1 hour, the monoclonal antibody Tau-1, diluted 1:100 in PBS-5%BSA, with two different concentrations of bovine brain purified tau protein (7 ng/µl and 15 ng/µl), previous to the step of incubation with the primary antibody, in the immunoflourescence procedure.

ImmunoFISH was carried out essentially as described by Robert-Fortel et al. (Robert-Fortel et al., 1993) with minor modifications. Cells were treated with the same immunofluorescent procedure described at the beginning of this section, until the incubation with the primary antibody Tau-1 for 2 hours. After that cell were washed twice and post-fixed with 4% paraformaldehyde in PBS for 3 minutes, permeabilized in 0.1% Triton in PBS for another 3 minutes, treated with 0.1 M Tris–HCl, pH 7.0 for 2 minutes and washed with 2x SSC twice for 2 minutes. Cells were dehydrated in 70, 80, 90 and 100% ethanol at 4°C for 2 minutes each and dried. Then cells were treated with 100 mg/ml RNAase A for 45 minutes at 37°C, washed, dehydrated, dried and subjected to in situ hybridization. Cells were hybridized with 50 ng of α-satellite p82H plasmid, a generous gift of Dr. Mariano Rocchi (University of Bari, Italy), directly labeled by nick translation (Amersham-Pharmacia, Nick Translation kit N5500) with FluoRED (rhodamine 4-dUTP RNP2122). The probe diluted in 75% formamide, 10% dextran sulfate, 2x SSC, 2.5 µg single-stranded DNA from salmon sperm (ssDNA) (Boehringer) (final volume 50 µl) was denatured at 95°C for 5 minutes. Hybridization was performed on slides for 5 minutes at 80°C (to denature DNA in cells) and then overnight at 37°C. After hybridization, coverslips were washed once in 2X SSC for 30 minutes at 37°C,

then in 1x SSC for 30 minutes at room temperature, and in 0.5x SSC for another 30 minutes at room temperature.

Nuclear counterstaining for immunofluorescence as well as for inmmunofish treatment was done with either Hoechst 33258 (1μg/ml bisbenzimide in PBS-3%FBS, Sigma) or PI (50 μg/ml), after the incubation with the secondary antibody or the hybridization step. Finally after wash 3 times with PBS, samples were mounted on slides using Fluor Save ^{TM}Reagent (Calbiochem 345789).

IV. Confocal microscopy analysis

The cells were observed with a Leica-DMRBE microscope with TCS 4D confocal head. The merged images were analyzed by the Scanware (LeicaLasertechnik GmbH) or Image J programs. Double-labeled pixels were identified from pixel fluorograms as described by Demandolx and Davoust (1997). Double-labeled pixels were displayed in white on the co-localization overlay images.

V. Purification of bovine brain microtubule associated tau protein

Microtubule associated tau protein was purified from fresh bovine brain, following the procedure of Grundke- Iqbal et al. (1986) with some modifications (Farias et al., 1992). Brain tissue was homogenized at 4°C in buffer 0.1 M Mes, pH 6.8, 1 M glycerol, 1 mM MgCl2 in a volume equivalent to tissue weight, in the presence of

protease inhibitors (10 μg/ml pepstatin, 10 μg/ml leupeptin, 100 μg/ml PMSF and 1 μg/ml aprotinin). The homogenates were centrifuged at 42,000 g for 30 minutes at 4°C to sediment cell debris. The supernatant was adjusted to 1 mM GTP, 2.5 M glycerol, 0.5 mM EGTA, 1 mM MgCl2 plus protease inhibitors, and incubated at 37°C in a thermoregulated bath for 1 hour. Microtubules were pelleted at 42,000 g for 30 minutes at 30°C, and the supernatant was discarded. Microtubular pellets were resuspended at 4°C using a glass homogenizer, in buffer 0.1 M Mes, pH 2.7, 1 mM EGTA, 0.5 mM MgCl2, 1 mM EDTA, 0.75 mM NaCl and 2mM DTT. The solution was boiled 5 minutes, and then centrifuged at 42,000 g for 30 minutes at 4°C. The supernatant was adjusted to 2.5% of perchloric acid and then centrifuged at 42,000 g for 30 minutes at 4°C. The supernatant was finally dialyzed against buffer 2.5 mM Tris-HCl, pH 7.6, in dialysis membranes at 4°C for 24 hours. The protein was concentrated using the system ultrafiltration cell (Amicon® models 8050 and 8003), with the appropriate membranes. The protein concentration was evaluated by Bio-Rad Protein Assay.

VI. Gel retardation assays

100 to 400 ng of purified tau protein were incubated with 0.5 μg of ssDNA, as a competitor, in 20 μl (final volume) of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 10% glycerol, and 5 mM dithiothreitol for 10 minutes at room temperature, before adding the corresponding 5'32P-labeled probe (0.05 pmol). Two satellite DNA fragments were used as labeled probes. These DNA fragments were: (i) a murine γ-

satellite DNA of 936 bp (four copies of 234 bp γ-satellite repeat) obtained by digestion of the plasmid PBS (a generous gift from Dr. Niall Dillon), with the restriction enzyme EcoRI, and (ii) a fragment of human α-satellite DNA of 700 bp generated by PCR amplification with the primers αSATsense (5′-GGAAACGGGAATTCCTTCACATAAAGAT-3`) and αSATantisense (5`-TCTCTCTAGGGATCCTGGAAGATACTCC-3`), using the p82H plasmid as template. The PCR conditions were as follows: 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute and 1 cycle of 72°C for 10 min. After adding the labeled probe, the mixture was incubated at room temperature for 15 minutes. Electrophoresis was carried out in an 8% polyacrylamide gel in 0.25X Tris-borate-EDTA. For the competition experiments 400 ng of purified tau protein were incubated with 25-, 50- or 75-fold excess of the corresponding unlabeled DNA fragments during 10 minutes. As a competitor DNA we used either: (i) a restriction fragment of 600 bp (f-ECFP), of random sequence, obtained by digestion of the plasmid pECFP with the enzyme PvuII; or (ii) sonicated poly dI/dC sequences; or (iii) sonicated poly dG/dC sequences; or (iv) the α and γ-satellite DNA unlabeled fragments. Each fragment of DNA was purified from agarose gel and ethanol precipitated. The labeling of the DNA fragments (5pM,) previously dephosphorylated with alkaline phosphatase (Promega), was performed at the 5′end with [γ32P] dATP (3000 Ci/mmol, Perkin Elmer) with the enzyme T4 polynucleotide kinase (Promega) (5-10 units/μl) at 37°C for 30 minutes. After labeling the probes were phenol extracted, ethanol precipitated and resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, to be ready for use. Afterwards,

the 5`32P-labeled γ and α-satellite DNA probe were added to the tau/unlabeled competitor DNA mix, and incubated for 10 minutes at room temperature, the electrophoresis was carried out. The EMSA gels were dried and the radioactivity was visualized using a PhosphorImager. The radioactivity of each lane was further quantified by the computer program ImageQuant and was expressed as a value of density CNT/mm^2, representing the total intensity of all the pixels in the volume divided by the area of the volume.

VII. Gel retardation assays followed by immunoblotting

Gel retardation assays were carried out as described above, except that 100 and 200 ng of purified tau were incubated with 0.5 μg of ssDNA, and 5 pmol of unlabeled γ-satellite DNA probe. After the electrophoresis, the gels were soaked in transfer buffer (50 mM Tris-HCl, 40 mM Glycine, 1 mM SDS and 20% methanol) for 40 minutes at room temperature, before being subjected to Western blotting with the primary antibody Tau-1 (1:1000).

MOLECULAR CHARACTERIZATION OF NUCLEAR TAU

VIII. Isolation of nuclei and nuclear protein extracts

Nuclei of SHSY5Y cells were isolated from cells cultured in 100-mm plates washed three times in PBS buffer at room temperature, removed from the plates or flask with a rubber policeman into PBS and pelleted by centrifugation at 700g for 5 minutes

at 4°C. The PBS was aspirated and the cells were lysed gently resuspending the cellular pellet in 100 µl of sucrose buffer per $1x10^7$ cells (0.32 M sucrose, 10 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$, 2 mM MgOAc, 0.1 mM EDTA, 0.1% NP-40, 1mM DTT, 0.5 mM PMSF). The lysis procedure was checked by microscopy to confirm the cellular lysis and that the nuclei remain intact, thus the cells were centrifuged at 500g for 5 minutes at 4°C. The supernatant was transferred to a new tube and stored as cytoplasmic fraction at –80°C. The pellet containing crude nuclei was washed once with 0.5 ml of sucrose buffer and twice with 1 ml of sucrose buffer without NP-40, aspirating the supernatant and centrifuging at 500g for 5 minutes at 4°C, after each washing step. The nuclei were suspended in 30 µl per $1x10^7$ cells of hypotonic, low salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% glycerol v/v, 0.5 mM DTT, 0.5 mM PMSF) by finger vortexing to swell the nuclei. Then was slowly added and mixed with the equal volume of high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 800 mM KCl, 0.2 mM EDTA, 25% glycerol v/v, 1% NP-40, 0.5 mM DTT, 0.5 mM PMSF) to extract the nucleoplasm into the buffer while the nuclear envelope stays intact and retains the genomic DNA. The sample was incubated for 30-45 minutes at 4°C on a shaker rotator and then centrifuged at 14000g for 15 minutes at 4°C. The supernatant corresponding to the nuclear proteins extract was transferred to a new tube, quantified and stored at –70°C. All the buffers contained protease and phosphate inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10µg/ml leupeptin, 10 µg/ml aprotinin and 5 µg/ml pepstatin A). Protein concentration in cytoplasmic and nuclear fractions was determined by BioRad method and a BSA standard curve. After

determinate the protein concentration, 20-25 ug of nuclear proteins were mixed with sample buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 2.5% 2-mercaptoethanol and 0.05% bromophenol blue) and boiled for further electrophoresis and immunoblot analysis.

IX. Immunoblot analysis

20-25 ug of nuclear proteins were loaded for each sample in 10%-12% SDS-polyacrylamide (acrylamide-bisacrylamide (29:1)) gels (SDS-PAGE) and electrophoresis was perform at 120 V for 2 hours. Next, proteins content in the gel were transferred to a nitrocellulose membrane (BioRad 1620115) at 100 V, 350 mA for 1 hour at 4°C. The membrane was blocked using 5% non-fat dry milk in PBS. After blocking, membranes were incubated overnight at 4°C with the antibodies against tau, Tau –1, Tau-5 and AT8, diluted 1:1000 in PBS-1% BSA. Membranes were also immunoblotted with anti β-tubulin (1:20000) and anti-histone H1 (1:1000) to verify the purity of the nuclear fractions, and with anti-β–actin (1:5000) to normalize the levels of protein tau respect the total protein. After incubation with primary antibodies, blots were washed three times for 10 minutes with PBS-0,1% Tween20 and then incubated with secondary antibody for 1 hour at room temperature. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse IgG (Calbiochem 401215) and goat anti-rabbit IgG (Calbiochem 401315) diluted 1:5000 in PBS-1%BSA. The membrane was developed by a chemiluminescence assay (Western Lightning Chemiluminescence Reagent Plus, Perking Elmer NEL104).

To identify the purity of bovine brain tau protein preparations, the samples were analyzed by 12% SDS-PAGE. Gels were stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes for the immunoblotting. After blocking the non–specific binding sites with 5% non-fat dry milk in PBS, the membranes were incubated overnight at 4°C with the primary antibody Tau-5 1:1000 in PBS-1% BSA. Then the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody, rabbit anti-mouse diluted 1:5000 in PBS-1%BSA and developed using chemiluminescence.

The levels of tau determined by immunoblot analysis in each assay were quantified, expressed as a proportion of actin levels and normalized to controls. Results are means±s.e.m. from four or three independent experiments

X. *Two dimensional IEF/SDS gel electrophoresis*

Two-dimensional electrophoresis was performed as described supplier with minor modifications using PROTEIN IEF cell (Bio-Rad). The reagents Urea, CHAPS, iodoacetamide, and immobilized pH gradient (IPG) strips (pI3-10, 11 cm long) were purchased from Bio-Rad. DL-dithiothreitol (DTT) and Thiourea were purchased from Invitrogene and Sigma respectively.

For the first dimension isoelectric focusing, 150 µg of nuclear extracts (~75 µl) of SHSY5Y were either: (i) resuspended in 5–fold volume of isoelectrofocusing (IEF) buffer (8M Urea, 2 M thiourea, 2% CHAPS, 100 mM DTT, 0.27% ampholine pH 3-10 plus 0.13 % ampholine pH 5-7 and trace amounts of bromophenol blue) and incubated

for 30 minutes at room temperature, or (ii) precipitated by 4-fold volume of chilled absolute methanol at –70°C for 2 hours, followed by centrifugation at 14000g for 15 minutes at 4°C. The pellet was resuspended in 10% trichloroacetic acid for 2 hours at room temperature and then centrifuged at 16000g for 15 minutes. The pellet was dried and resuspended in 200 µl of rehydration buffer (7M Urea, 2 M thiourea, 2% CHAPS, 0.5% ampholine pH 3-10 and trace amounts of bromophenol blue). In the first case the sample was loaded onto the immobilized pH gradient (IPG) strip for active rehydration at 50 V for 14 hours. In the second case the sample was loaded onto the immobilized pH gradient (IPG) strip for passive rehydration overnight. The rehydrated IPG strips were focused for 15 minutes at 250 V and then for about 40 kVh. For the second dimension SDS-PAGE the focused IPG strips were equilibrated for 15 minutes each in equilibration buffer 1 (6 M Urea , 2% SDS, 20% glycerol, 2% DTT and 0.375 M Tris-HCl, pH 8.8), then in equilibration buffer 2 (6 M Urea , 2% SDS, 20% glycerol, 2.5% iodoacetamide and 0.375 M Tris-HCl, pH 8.8) and finally in blotting/running buffer (25 mM Tris-base, 190 mM Glycine, 1%SDS). The equilibrated strips were loaded onto 12% Tris-tricine SDS-PAGE gels (Bio-Rad Criterion gels 345-0107). Electrophoresis was conducted at 120 V. Proteins on the SDS-PAGE gels were either electrotransferred onto nitrocellulose membrane at 35 V overnight at 4°C or Coomasie stained (CB) with the staining solution (10% Brilliant Blue Coomaise, 50% absolute methanol, 10% acetic acid). The membranes were blocked with 5% nonfat milk in TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl, pH 7.6) 1 hour at room temperature prior to incubation with antibodies against tau.

XI. Immunoprecipitation of nuclear tau

Nuclear tau variants were immunoaffinity-purified from nuclear protein extract following the instructions of the Sieze X Protein G Immunoprecipitation kit (Pierce 45210) to avoid the presence of free IgG and others proteins in the sample. Between 200–250 μg of nuclear protein extracts of SHSY5Y cells were added to protein A-Sepharose beads cross-linked with disuccinimidyl suberate (DSS) to the antibodies Tau-1 and Tau-5 and incubated in a rotator overnight at 4°C. The beads were washed with buffer and eluted with protein sample buffer. Therefore the eluted proteins were analyzed by immunoblotting and CB staining (Parkin et al., 2002).

XII. Cellular synchronization

SHSY5Y cell were synchronized at the G1/ S border by aphidicolin arrest (Solovei et al., 2004). Cells were grown at 70% confluence and transferred to serum deprived medium, for 24 hours. Thereafter to block DNA polymerase (Ikegami et al., 1978) cells were transferred to full medium supplemented with 10% FCS plus 1 μg/ml aphidicolin (Calbiochem 178273). After 12 hours cells were release from the block, washed three times and transferred to medium with 10% FCS. Two procedures were employed in cell synchrony experiments.

First, the degree of synchronization and duration of cell cycle stages was evaluated by propidium idodide (PI) DNA staining, followed by Flow Cytometri analysis. PI, although not very specific (it stains all double-stranded regions of both DNA and RNA by intercalating between the stacked bases of the double helix) and not able to penetrate an intact cell membrane, has the advantage of absorbing 488 nm light and then fluorescing at wavelengths above 570 nm. This means that, in the presence of RNase, propidium iodide can be used as a DNA stain in cytometers with low-power argon lasers. The flow cytometric analysis of red fluorescence intensity for each cell, result in a DNA flow histogram, that provide a snapshot of the proportion of different kinds of nuclei present at particular moment. Therefore some nuclei will contain a 2C amount of DNA (either G0 or G1 cells), a 4C amount of DNA (G2 or M cells), and some different amounts of DNA that span the range between those two populations. The method used to analyze the DNA histograms was the *peaks reflect method.* The synchrony analysis was performed as follow: $5x10^5$ SHSY5Y cells were rescue from the rest of the harvested cells at the collection step, at different times after release from the block by aphidicolin. This amount of cells were resuspended in 1 ml of PBS-2%FBS and centrifuged at 2500 rpm for 5 minutes at 4°C. The cells in the pellet (50 µl) were fixed with 0.5 ml of ethanol 70% for 10 minutes on ice, thus completed to 1 ml with PBS-2%PBS and finally centrifuged at 3000 rpm for 6 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 0.5 ml of PI/RNAase solution (0.05 mg/ml PI in PBS plus 0.02% RNAase (DNAase free)) always in dark conditions to avoid PI fluorescence loss. The samples were then analyzed using a

FACScan flow cytometer (Becton-Dickinson) equipped with a 5-watt argon ion laser. The fluorescence of PI-stained nuclei was excited at 488 nm, and histograms of the number of cells versus linear integrated red fluorescence were recorded for 50,000 nuclei/sample. DNA histograms were analyzed using the Cell Quest and WinDi computer software and used to calculate the proportions of cells in different phases of the cell cycle. Results shown are mean of three experiments ± SD.

Second, to evaluate the changes in the nuclear tau levels through the cell cycle three 100-mm plates were used at each time point to prepare a nuclear protein extract for further immunoblot processing.

XIII. Recombinant Pin1 protein and pull down assay.

His-Pin1 was gift from Dr. M. Vincent (Universite Laval, Quebec, Canada). His-Pin1 fusion protein was expressed in E. Coli BL21 cells and purified with histidine-binding beads (Novagen) according to the manufacturer's instructions. Briefly, E. Coli BL21 was grown at 37°C until OD of 0.6, when 0.5 mM of IPTG was added. Cells were incubated at 28°C for 3 hours and then centrifuged at 1500 g for 15 minutes at 4°C. The pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole pH 8.0) in a proportion of 4 ml per 100 ml of bacterial culture. Then lysozime (1 mg/ml) was added and the mix was incubated for 30 minutes at 4°C. Thus the sample was sonicated 6 cycles of 10 seconds at power 40 (Ultrasonic Processor). The sample was centrifuged at 10000 g for 30 minutes at 4°C and the supernatant was incubated

with 50% histidine-binding beads (Novagene) in a proportion of 2 ml per 100 ml of bacterial culture, for 2 hours at 4°C. The mix was centrifuged at 500 g for 30 minutes at 4°C and the pellet was washed three times in washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH8.0). Finally the protein Pin1-His was eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH8.0).

Pull down experiments were performed with 300 µg of nuclear and cytoplasmic extract diluted in buffer CHAPS (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% CHAPS, 0.2% NaF, and protease inhibitors in absence of EDTA) and pre-incubated with histidine-binding beads for 30 minutes at 4°C, as a control to avoid unspecific interactions between tau and the resin. The sample was centrifuged at 500 g for 5 minutes at 4°C and the supernatant was incubated with the Pin 1-histidine-binding beads for 2 hours at 4°C. The resin was washed three times with buffer CHAPS containing increased concentrations of imidazole (10 mM, 35 mM and 60 mM). The fraction of tau protein bound to Pin 1 was diluted in loading buffer and analyzed by immunoblot.

XIV. Cellular Treatments

SHSY5Y cells were treated with 5 µg/ml and 10 µg/ml of Juglone (Calbiochem 420120) an inhibitor of Pin-1 protein, to elucidate the role of this protein in the nuclear tau localization.

SHSY5Y cells were treated with inhibitors of kinases and O-β-N-acetylglucosaminidase responsible of tau phosphorylation and tau O-GlcNAcylation to

elucidate the role of these tau modifications in its nuclear localization. The proteins inhibited were: (a) the glycogen synthase kinase 3 beta (GSK3β) inhibited by treating the cells with 10 mM LiCl supplemented with 5 mM myo-inositol for 24 hours (Davies et al., 2000); (b) the neuronal Cdc2-like kinase, cdk5, inhibited by treating the cells with 10 μM Roscovitin for 24 hours; (c) the β-NAcetylglucosaminidase inhibited by treating the cells with 10 mM Streptozotocin (Calbiochem 572201) for 3 hours. This compound inhibit the activity of O-β-N-acetylglucosaminidase therefore the levels of O-GlcNAc groups in proteins increase; (d) the phosphatase 2A inhibited by treating the cells with 0.1 μM okadaic acid (OA) for 3 hours (Gong et al., 2000). All treatments were performed in triplicates or more in independent experiments for statistical analysis. Data from the different experiments were analyzed by 1-way ANOVA and Student's t test. Values were consider statistically significant at $p<0.05$.

RESULTS

I. Nuclear distribution of tau species in proliferative and differentiated SHSY5Y cells.

SHSY5Y cells have been used by a number of groups as an experimental cellular model to study AD-like tau phosphorylation. It has been previously described that tau from undifferentiated SHSY5Y cells is found hyperphosphorylated when grown at low (5%) serum concentration, leading to decreased microtubule binding (Tanaka et al., 1995). In contrast, phosphorylation of tau decreases under serum-rich medium conditions (15% FCS) and concomitantly, its microtubule binding increases. Under differentiation conditions, whereas the degree of tau phosphorylation increases at some sites and decreases at others, the total tau levels increase between 2-3 fold, maintaining the microtubule binding activity and therefore enhancing the microtubule stability in neurites. Under these conditions, only 37% of the total tau binds to microtubules and more than 60% of the cytosolic tau remains localized in a perinuclear border and in certain process of the cell body (Haque et al., 1999).

Considering these preliminary data SHSY5Y cells were used first to confirm the nuclear the distribution of tau protein in this model and then to analyze the effect of differentiation over nuclear tau localization. The distribution of tau, as reveled by Tau-5 staining, was homogeneous through all the cell body under proliferative conditions (10% FCS) (Figs. 8A and B). In contrast, the distribution of tau was predominantly nuclear, as reveled by AT8 and Tau-1 staining, showing a differential pattern in each case.

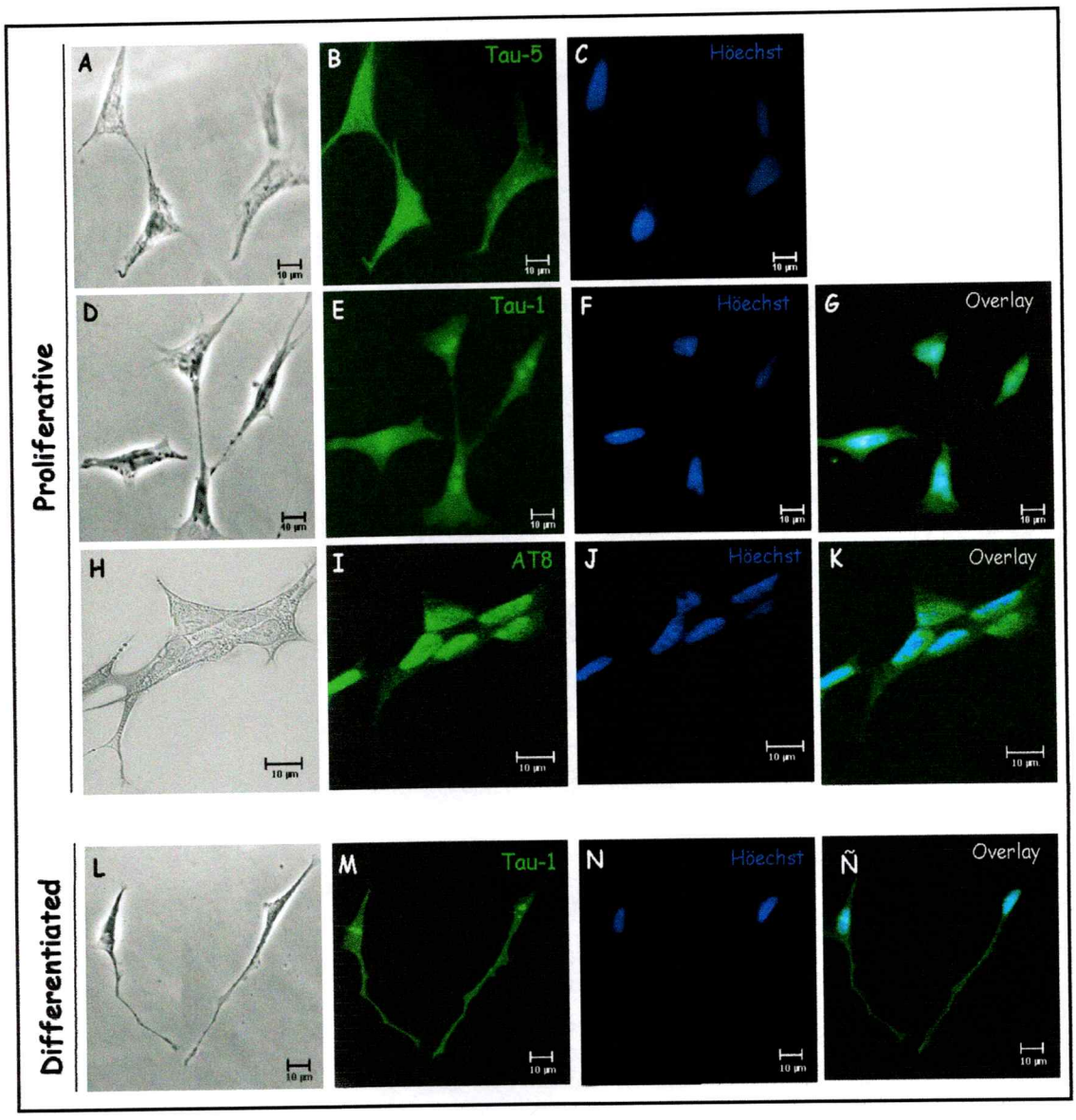


Figure 8. ***Subcellular distribution of tau protein in SHSY5Y neuroblastoma cells.*** SHSY5Y cells were grown in MEM-F12 under normal serum conditions (10% FBS), and differentiated conditions (10 µM *trans*-retinoic acid, 10% FBS, 4-5days) on coverslips and further fixed for immunofluorescence staining of tau with the monoclonal antibodies Tau-5 (B), Tau-1 (E) and AT8 (H). Phase contrast images show morphological differences between normal cycling (A, D, H) and differentiated cells (L). Immunofluorescent distribution of tau (green) and of nuclei stained with Höechst (blue, C, F, J, N). Overlay images show colocalization of tau and nuclei (cyan, right panel) after Tau-1 and AT8 labeling (G, K, O). While Tau-5 displays a diffuse cytoplasmic labeling, AT8 labeling is mainly nucleoplasmic and Tau-1 labeling is diffuse in cytoplasm and punctually specific in nuclei. Under normal and differentiated conditions the staining pattern of Tau-1 was conserved. Scale bar =10 µm

Most of the tau reactive to Tau-1 appears as diffuse in the cytoplasm and as bright fluorescent specs in certain areas of the nucleus (Fig. 8E). The tau reactive to AT8 labeled the entire nucleus (Fig. 8H), except for the specific regions labeled with Tau-1 that correspond to dense bodies inside the nucleus observed in the phase contrast image (Fig. 8A, D, L). The Hoechst nuclear staining showed the cell nuclei for each case (Figs. 8C, F, J, N). In order to corroborate that the nuclear tau staining was specific, different concentrations of purified tau protein were pre-incubated with the antibody Tau-1 in immunoabsorption assays. When pre-incubated with different concentrations of tau, Tau-1 nuclear staining disappeared gradually, suggesting that the antibody is indeed recognizing nuclear tau (Fig. 9A, B, and C). At the highest concentration of tau (15 ng/μl) the nuclear staining of tau was abolished (Fig. 9C), indicating that Tau-1 is recognizing specifically tau proteins. This result establishes the nuclear localization of tau, particularly in dense bodies of the SHSY5Y cell nuclei.

According to the previous description of tau associated to NOR of mitotic chromosomes (Thurston et al., 1996), the nature of those dense bodies was attributed to be nucleolar. In humans, the basic transcription machinery for rDNA contains, at least two transcription factors in addition to polymerase I, of which , the upstream binding factor (UBF) and the SL-1 complex are known. The UBF presents an exclusive nucleolar distribution and therefore was used to define whether those dense bodies represent nucleoli. Immunofluorescence experiments with the antibody anti-NOR 90 recognizing UBF allowed the identification of nucleoli in SHSY5Y cells (Fig. 10A) which corresponds to the same dense bodies stained with Tau-1 (Fig. 10B).

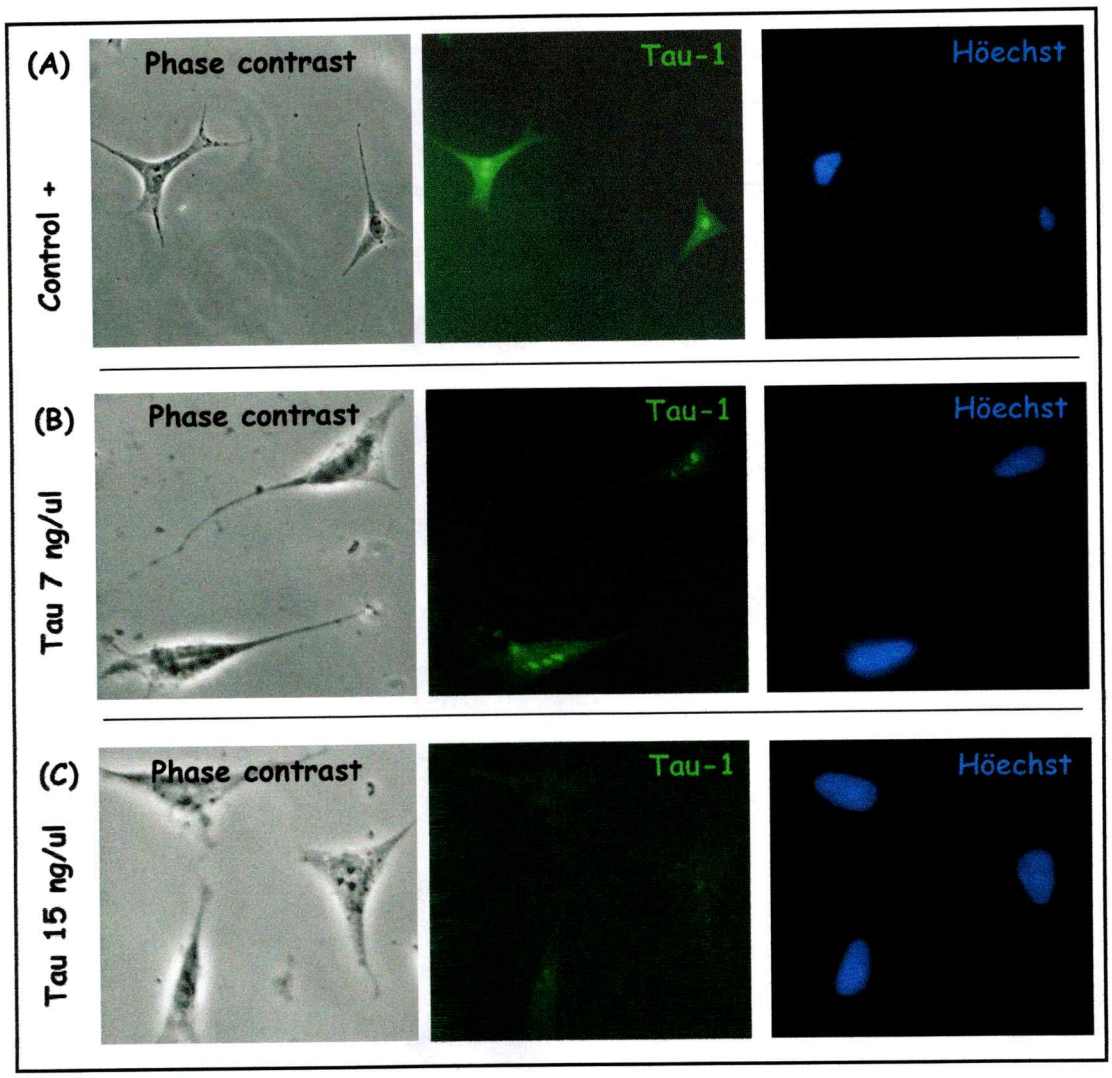


Figure 9. ***Immunoabsortion assay for Tau-1 immunofluorescence***. Interaction specificity analysis of Tau-1 by immunoabsorption assay, using purified tau protein. Phase contrast images of cells in A, B and C (left panels). (A) SHSY5Y cells were grown in MEM-F12 (10% FBS) on coverslips and then incubated with Tau-1 (1:100) followed by fluorescein-conjugated secondary antibody. (B) Same as A, but the incubation with the primary monoclonal antibody Tau-1 (1:100) was performed in the presence of 7 ng/µl of tau, both previously co-incubated for 1 hour. (C) Same as B, but using Tau-1 (1:100) plus 15 ng/µl of tau. The nuclear tau immunostaining disappear gradually establishing absence of unspecific interaction for Tau-1.

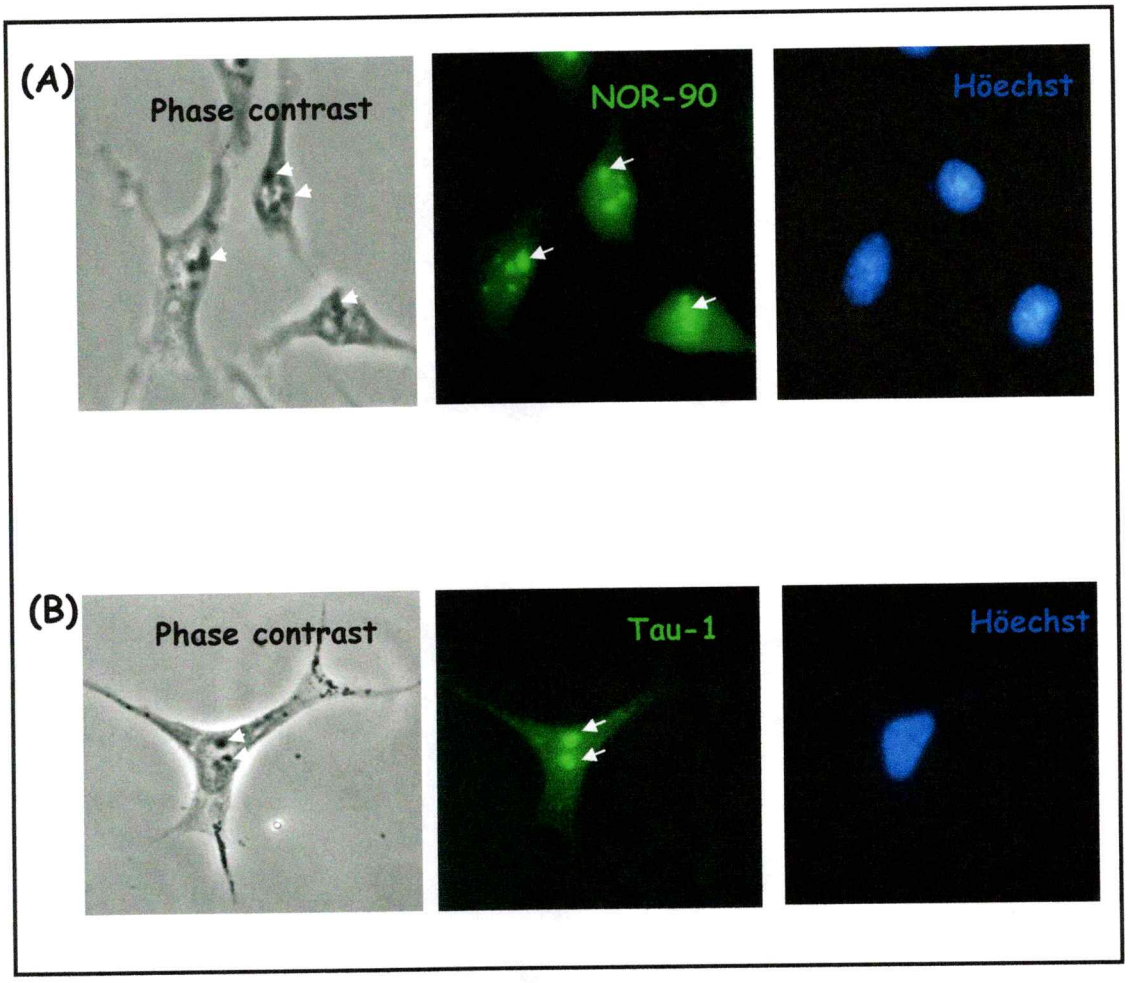


Figure 10. ***Immunofluorescence localization of nucleoli in SHSY5Y cells.*** SHSY5Y cells were grown in MEM-F12 (10% FBS) on coverslips and further fixed for immunofluorescence staining with: (A) the monoclonal anti-**UBF** human autoantibody NOR-90 (1:50) that label nucleoli (green, middle panel) and (B) the monoclonal antibody Tau-1 (1:100) (green, middle panel). Phase contrast images of cells in fluorescent images (A, B left panel). Arrowheads in phase contrast images indicate nuclear dense dark bodies as nucleoli. Arrows in fluorescent images indicate the nucleoli and Tau-1 concentrated at the same region.

In conclusion, the localization of tau in SHSY5Y cells under normal undifferentiated conditions is both cytoplasmic and nuclear. Specifically and according with preliminary observations in different cellular models, tau species unphosphorylated at Tau-1 epitope are distributed in the nucleoli, whereas tau species phosphorylated at AT8 epitope exhibit a nucleoplasmic distribution.

To test whether the nuclear tau localization is exclusive of proliferating cells, or is also a property of the differentiated phenotype, SHSY5Y cells were differentiated with retinoic acid. The differentiation was evidenced by morphological changes on SHSY5Y cells, such as well defined long processes observed by light microscopy (Fig. 8L). The distribution of tau revealed by Tau-1 staining, considerably labeled the neuritic processes remaining diffuse in the cytoplasm while persisting in nucleolar structures (Fig. 8M). Given that this treatment did not suppress cell proliferation at all, the nuclear tau distribution was tested in neurons and glial cells. Primary culture of hippocampal neurons with some glial cells obtained from rats at E18 were used to test the immunolocalization of tau species with Tau-1. Immunofluorescent tau localization in glial cells revealed a faint cytoplasm labeling and an intense nucleolar tau staining (Fig. 11D). On the other hand, immunofluorescent tau localization in hippocampal neurons showed at day 1 a strong nuclear Tau-1 staining and weak staining on processes (Fig. 11A). Latter on, between days 3-5 of the process of neuronal polarity development, Tau-1 immunofluorescence shifted from mostly nuclear to cytoplasmic staining also major and minor processes (Fig. 11B, C).

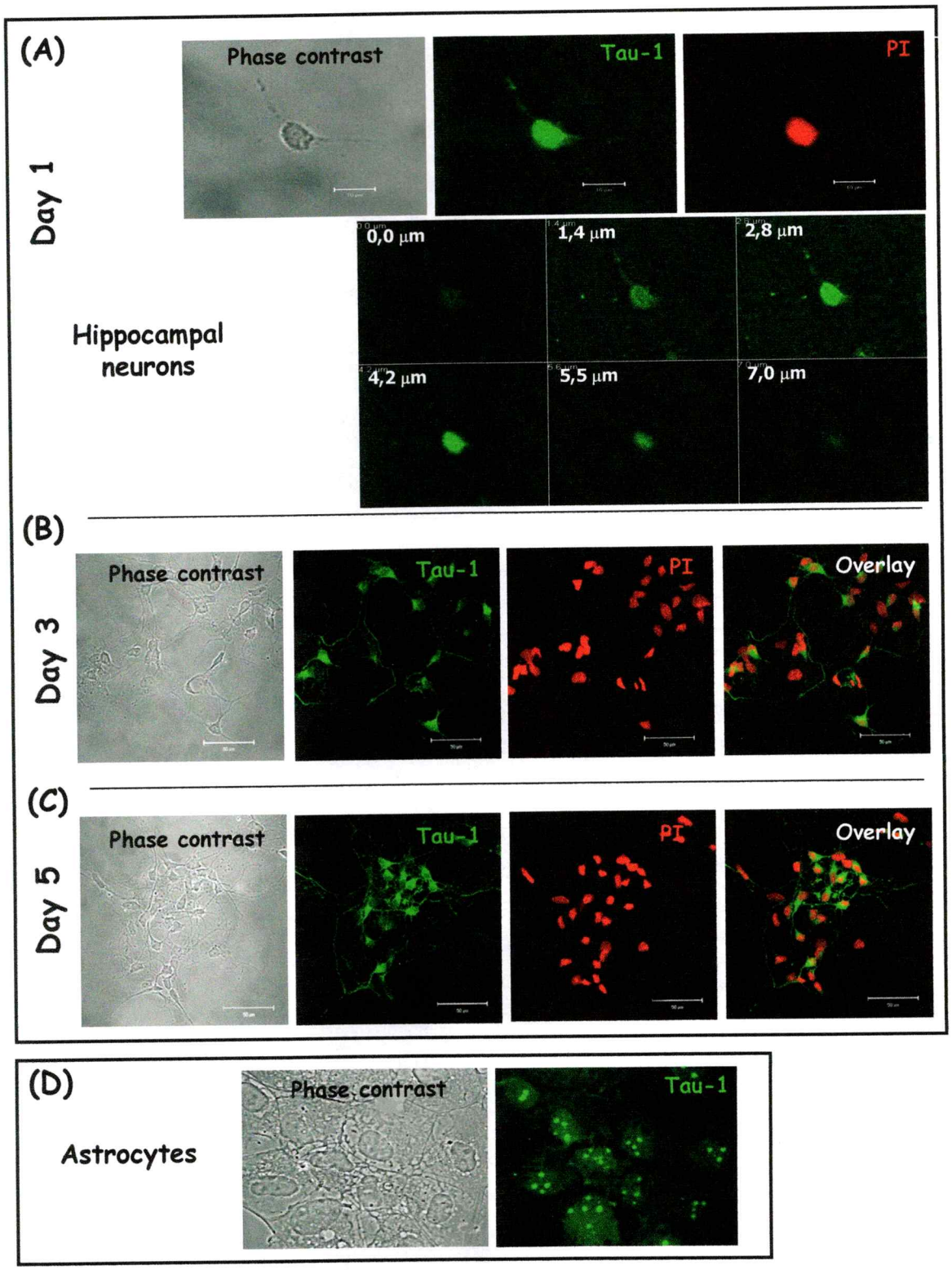

(A)
Day 1
Phase contrast
Tau-1
PI
Hippocampal neurons
0,0 μm
1,4 μm
2,8 μm
4,2 μm
5,5 μm
7,0 μm
(B)
Day 3
Phase contrast
Tau-1
PI
Overlay
(C)
Day 5
Phase contrast
Tau-1
PI
Overlay
(D)
Astrocytes
Phase contrast
Tau-1

Figure 11. ***Subcellular distribution of tau in hippocampal neurons and glial cells.*** Primary cultures of hippocampal neurons and glial cells from E18 rats were grown in MEM-10%FBS on coverslips and further fixed for immunofluorescence staining. Immunofluorescence localization of tau with the monoclonal antibody Tau-1 (green) was performed in: (A) hippocampal neurons at day 1 of culture; (B) in hippocampal neurons at day 3 of culture; (C) in hippocampal neurons at day 5 of culture; and (D) in astrocytes. Nuclei were labeled with 50 μg/ml of PI (red). Overlay images of Tau-1 and PI are in the right panel. Images illustrate main nuclear tau distribution at day 1. The serial optical sections of Tau-1 labeling obtained from confocal microscopy, at 2,8 μm and 4,2 μm corroborated nuclear tau labeling (A, lower panel). Overlay images show absence of nuclear tau localization and main labeling of cytoplasm and process (axonal process) at day 3 and 5. Phase contrast images (left panel) show morphological differences between hippocampal neurons at day 1, 3 and 5 of neuronal polarity development. Immunofluorescence images of astrocytes also show intense nuclear tau labeling specifically at nucleolar structures, as shown in figure 10. Scale bar = 50 μm.

Such change was quantified by measuring the fluorescence intensity in the nuclei and the cytoplasm in 50 cells for each one of these days. The shift of tau immunolocalization correlated with the increase of cytoplasmic fluorescence intensity and also with the decrease of nuclear fluorescence intensity at days 3 and 5 as compared to day 1 (Fig. 12 right panel).

In conclusion, a subpopulation of tau proteins reactive to Tau-1, which suggest their unphosphorylation at epitope 189-207, localizes in the nucleus, more specifically at the nucleolus of SHSY5Y neuroblastoma and glial cells. In post-mitotic hippocampal neurons, tau proteins reactive to Tau-1 are prominently found in nuclei just before of the neuronal polarity development. However as soon as neurons progress through the establishment of neuronal polarity, tau distributes in the cytoplasm and processes, probably because such proteins are required for axonal elongation among others morfo-physiological changes.

II. Nuclear tau localizes at the border of the nucleolus in non-neuronal cells.

In order to analyze the nuclear distribution of tau in non-neuronal cells, immunofluorescence co-localization experiments were performed in human fibroblasts and HeLa cells. Results obtained with Tau-1 and the antibody directed against nucleolar protein nucleolin confirmed the nucleolar localization of tau in human fibroblasts and HeLa cells (Fig. 13). As previously described (Dranovsky et al., 2001), nucleolin appeared concentrated at the internal periphery of the nucleoli of interphase cells.

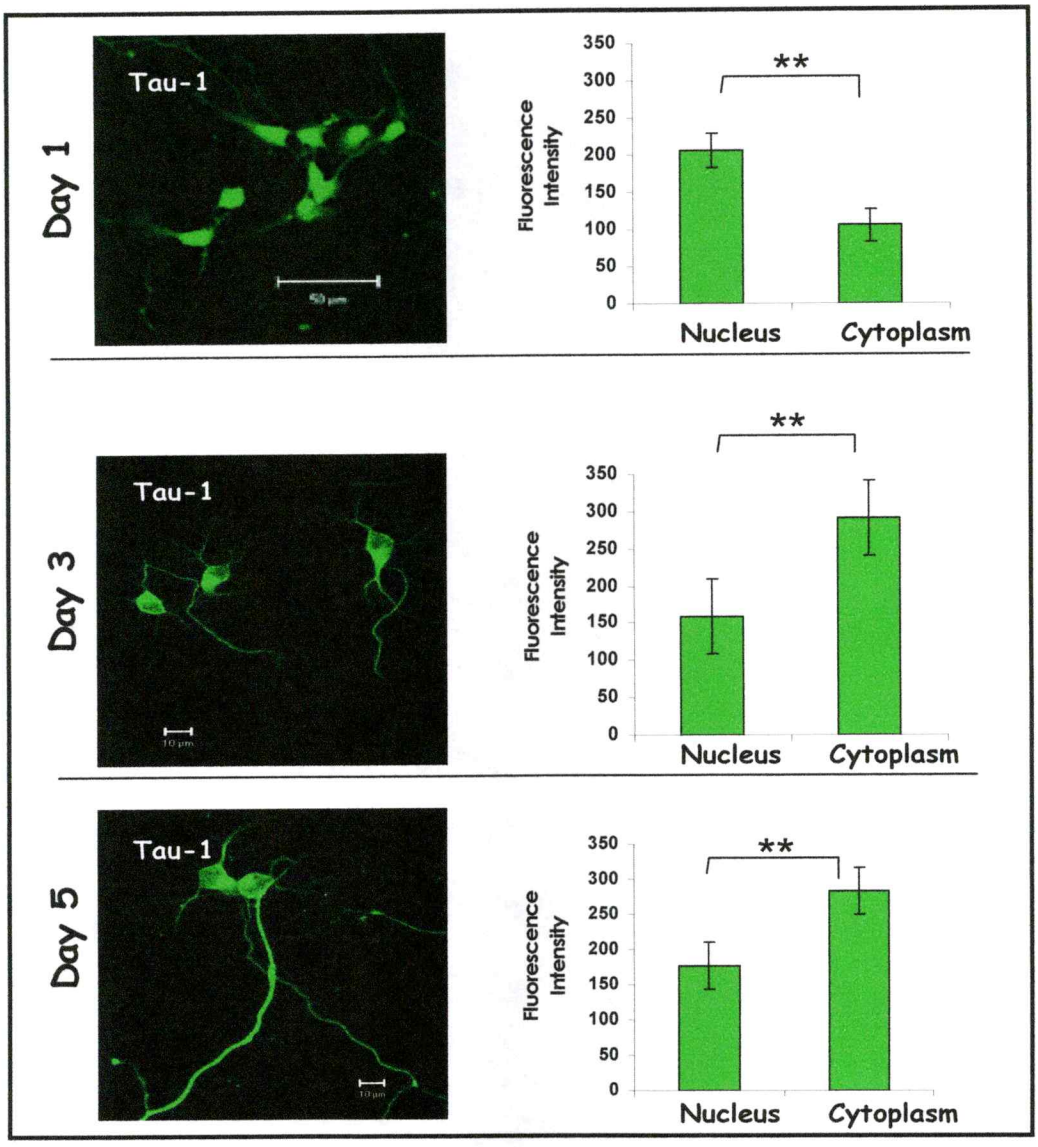


Figure 12. ***Subcellular distribution of tau protein in hippocampal neurons.*** Quantification of Tau-1 fluorescence intensity for 50 neurons, at day 1, 3, and 5 of culture and neuronal polarity development. The values of fluorescence intensity are expressed in arbitrary units and as means ± SEM; ** $\mathbf{p}<0.01$. Scale bar = 50 μm (day 1) and 10 μm (day 3 and 5). Statistical analyses were performed with Student´s T test.

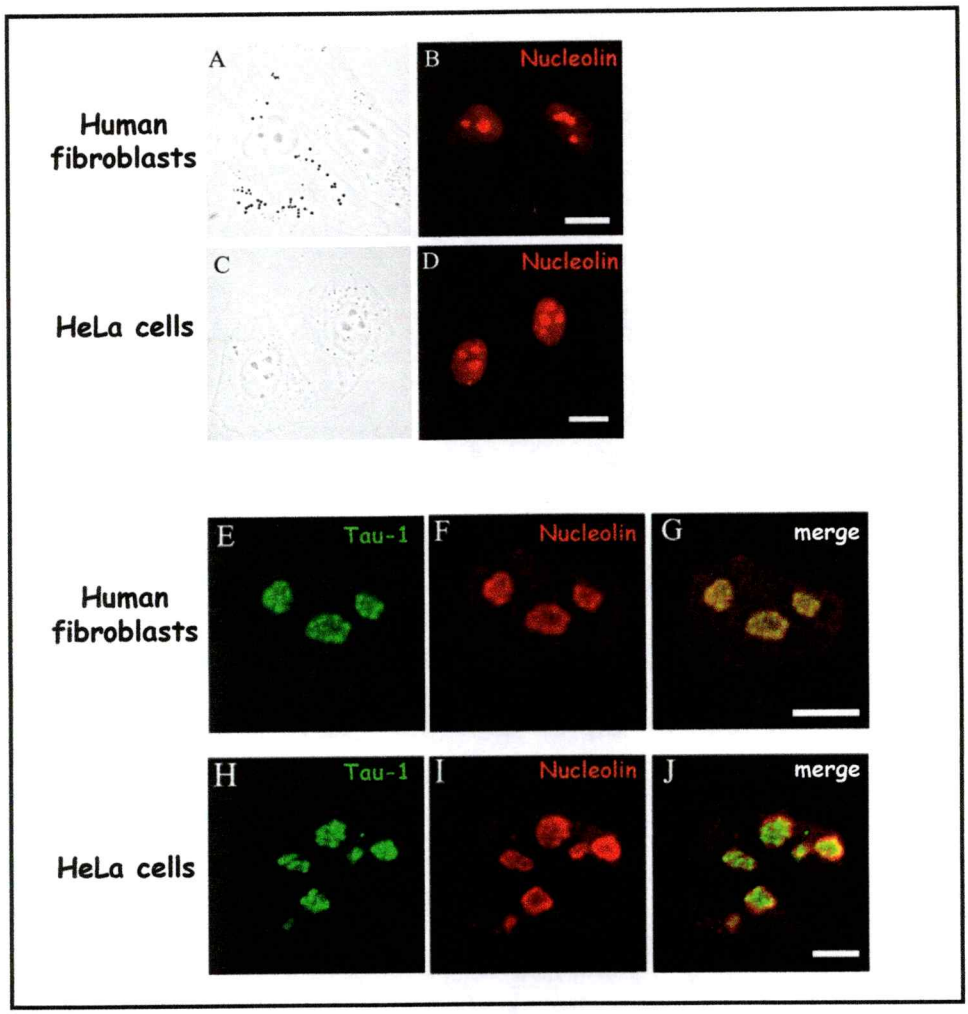


Figure 13. ***Nuclear tau partially co-localizes with nucleolin.*** Co-localization of endogenous tau with nucleolar protein nucleolin was analyzed by immunofluorescent technique and confocal microscopy in human skin fibroblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Upper panels (A and B), show phase contrast image (A) and nucleolin distribution (B) in human fibroblasts. Lower panels (C and D), show phase contrast image (C) and nucleolin distribution (D) in HeLa cells. Scale bar = 10 μm. Besides, left panels (E and H), correspond to tau distribution revealed with Tau-1 monoclonal antibody. Middle panels (F and I), show subnuclear nucleolin distribution detected with nucleolin C23 polyclonal antibody. Merged images of tau with nucleolin are shown on right panels for human fibroblasts (G) and HeLa cells (J). Scale bar = 5 μm.

In both cell types, a fraction of tau co-localized with nucleolin at the interior of the nucleolus (Fig. 13G and J). This co-localization is more significant at the interior periphery of the nucleolus especially in the case of non-dividing primary fibroblasts (Fig.13 E-G) as compared to proliferating HeLa cells (Fig.13 H-J).

Since in the nucleolus only the genes encoding ribosomal RNA (rDNA) are transcribed, supporting the function of ribosome biogenesis, the tandem repeated rDNA genes are organized at the nucleoli in such a way that a fraction of them is constitutively non-transcribed, conforming heterochromatin regions. It has been observed that the nucleolus of many large neurons is separated from the remainder of the nucleus by a characteristic shell of heterochromatin (Manuelidis and Borden, 1988), which serve as an attachment site for distinct inactive DNA. One of the typical marks related with centromeric heterochromatin associated to satellite DNA sequences is the lysine residue K9 of methylated histone H3 (Henikoff, 2000; Richards and Elgin, 2002; Sims et al., 2003). Anti-dimethyl H3K9 antibodies labeling constitutive heterochromatin are excluded from the center but not from the periphery of the nucleolus (Peters et al., 2003).

Immunofluorescent confocal microscopy was used to analyze the nuclear distribution of tau species, recognized by the Tau-1 antibody comparatively to dimethyl H3K9, in a non-neuronal cell model, human fibroblasts and HeLa cells in interphase. As shown in Fig. 14, tau was clustered as small dots in non-transformed fibroblasts, whereas it appeared as strong spots in transformed HeLa cells. As denoted by arrowheads, in both cases tau appeared to be predominantly present at the border of the nucleolus either partially overlapping (in the case of human fibroblasts) or in close

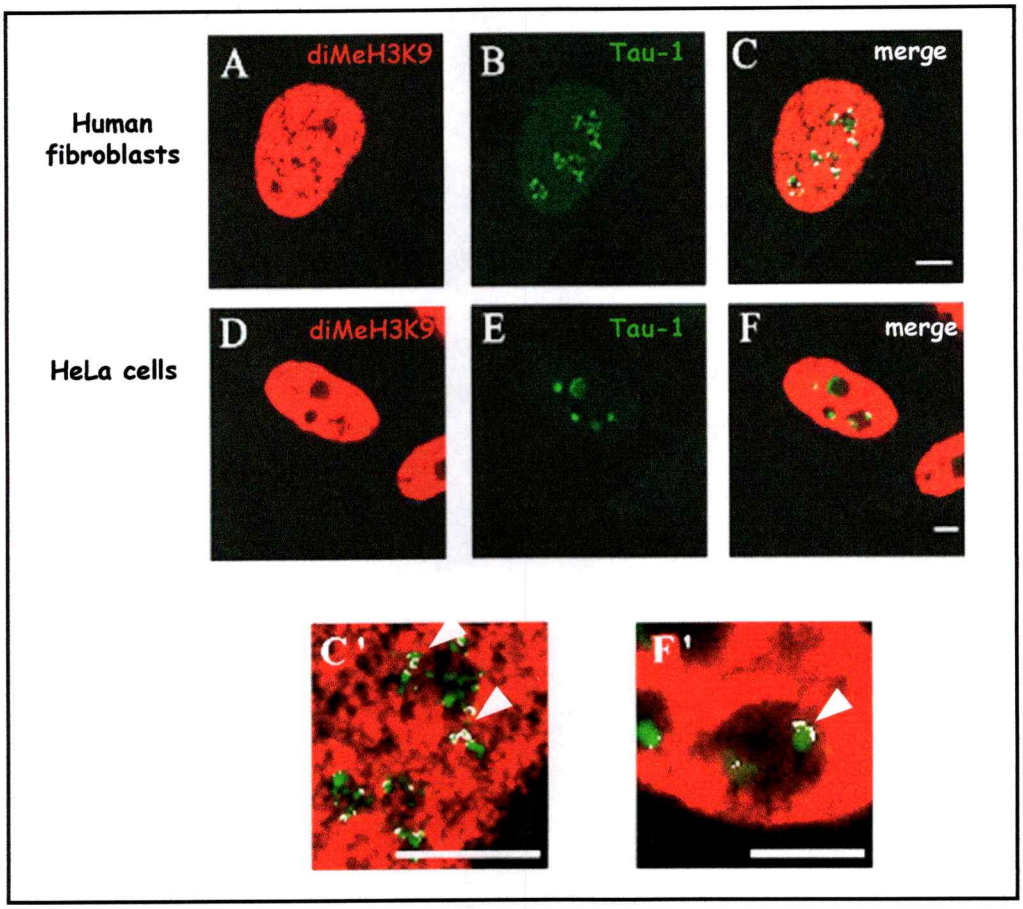


Figure 14. ***Nuclear tau localizes at the border of the nucleolus.*** Co-localization of endogenous tau with heterochromatin was analyzed by immunofluorescent technique and confocal microscopy in human skin fibroblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Left panels (A and D) correspond to diMeH3K9 distribution revealed with rabbit anti-diMeH3K9. Middle panels (B and E) show subnuclear tau distribution detected with the Tau-1 monoclonal antibody. Merged images of tau with diMeH3K9 are shown on right panels for human fibroblasts (C) and HeLa cells (F) with double-labeled pixels displayed in white. C' and F' are enlarged views of C and F and arrowheads indicate the overlapping between tau and diMeH3K9 at the neucleolus. Scale bar = 5 μm.

proximity to (in the case of HeLa cells) heterocromatin labeled by anti-dimethyl H3K9 antibodies.

In conclusion these observations indicate that in non-dividing human fibroblasts and HeLa cells, tau preferentially localize at the internal region of nucleoli, partially colocalizing in specific points with heterochromatin labeled by dimethyH3K9 at the border of nucleolus.

III. A fraction of nucleolar tau co-localizes with pericentromeric α-satellite DNA.

Alpha satellite is a repetitive DNA sequence present at the centromeric region of each normal human chromosome that consists of megabases of ~171-bp monomers arranged either in a highly homogeneous, multimeric organization or in a more heterogeneous monomeric form lacking this higher order periodicity (Warburton et al., 1996; Willard, 1991). In order to determine if the partial co-localization of tau with diMeH3K9 observed in human fibroblasts corresponds to a partial co-localization of tau with human α-satellite DNA, immunoFISH and confocal microscopy was used. Figure 15 shows the immunoFISH results obtained in human non-transformed fibroblasts (Fig. 15A-C), lymphoblasts (Fig. 15D-F) and transformed HeLa cells (Fig. 15G-I). As in Fig. 13 and 14, tau protein appeared not randomly distributed but displayed a spatial organization clustered as small dots in the case of non-transformed human fibroblasts and lymphoblasts, or as strong spots in the case of HeLa cells. In all cases a distinct relationship between tau and clusters of α-satellite labeled pericentromeric DNA was

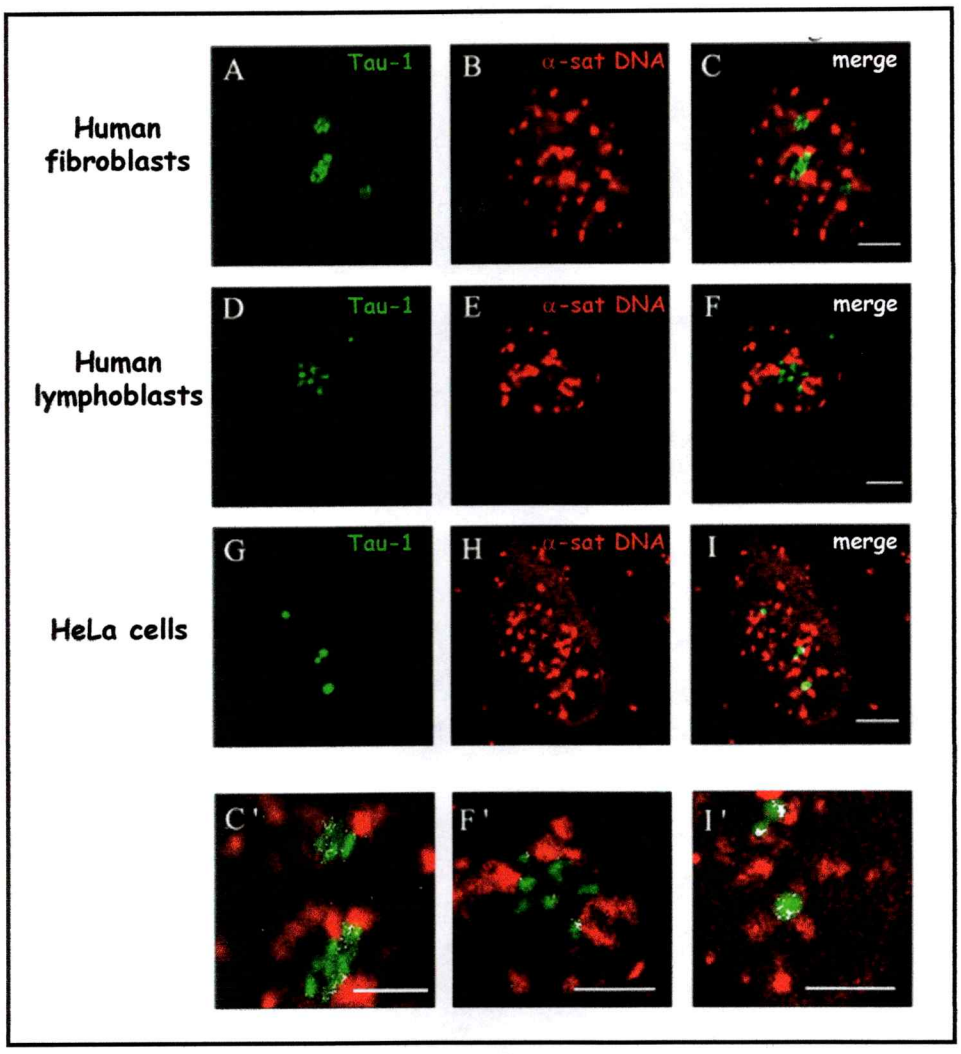


Figure 15. ***Tau partially co-localizes with pericentromeric α-satellite DNA.*** Co-localization of endogenous tau with pericentromeric α-satellite DNA was studied by immuno-FISH technique and confocal microscopy in human skin fibroblasts, human lymphoblasts and HeLa cells. Each row represents a single optical section of the same nucleus. Left panels (A, D and G) correspond to subnuclear tau distribution detected with the Tau-1 monoclonal antibody. Middle panels (B, E and H) show α-satellite DNA repeats distribution revealed by FISH using fluoRED labeled α-satellite p82H plasmid as a probe. Merged images of tau with pericentromeric α-satellite DNA are shown on right panels for human skin fibroblasts (C), human lymphoblasts (F) and HeLa cells (I) with double-labeled pixels displayed in white. C', F' and I' are enlarged views of C, F and I respectively. Scale bar 5 μm.

found, tau being systematically close to pericentromeric heterochromatin at the border of nucleoli, in non-transformed as well as transformed cells, partially overlapping with a portion of fluorescently labeled α-satellite DNAs.

Non-confocal conventional fluorescence microscopy images of human fibroblasts (Fig. 16A-E) or HeLa cells (Fig. 16F-J) are showed in Fig. 16. The merge images of Höescht and tau labeling (Fig. 16D, I) confirm the nucleolar localization of tau in these two cell types. In conclusion the merge images of tau and α-satellite DNA indicate that in all cases, nucleolar tau (green) appears to be at the center of a "group" of neighboring clusters of centromeric α-satellite sequences (red), with tau partially co-localizing with these sequences.

IV. Tau directly interacts with human α-satellite DNA sequences.

The previously described capacity of tau to interact with DNA (Hua and He, 2002; Hua et al., 2003; Krylova et al., 2005), the repetitive nucleolar distribution and the partial co-localization of tau protein with α-satellite DNA observed in figures 15 and 16, lead us to investigate the capacity of tau to directly bind and form protein-DNA complexes with human α-satellite DNA sequences. In an attempt to address this question, electrophoretic mobility shift assays were performed with purified tau protein and a 700 bp radioactively labeled DNA probe containing α-satellite sequences. The tau protein used in these gel shift experiments was prepared from normal bovine brain and purified as described in Materials and Methods.

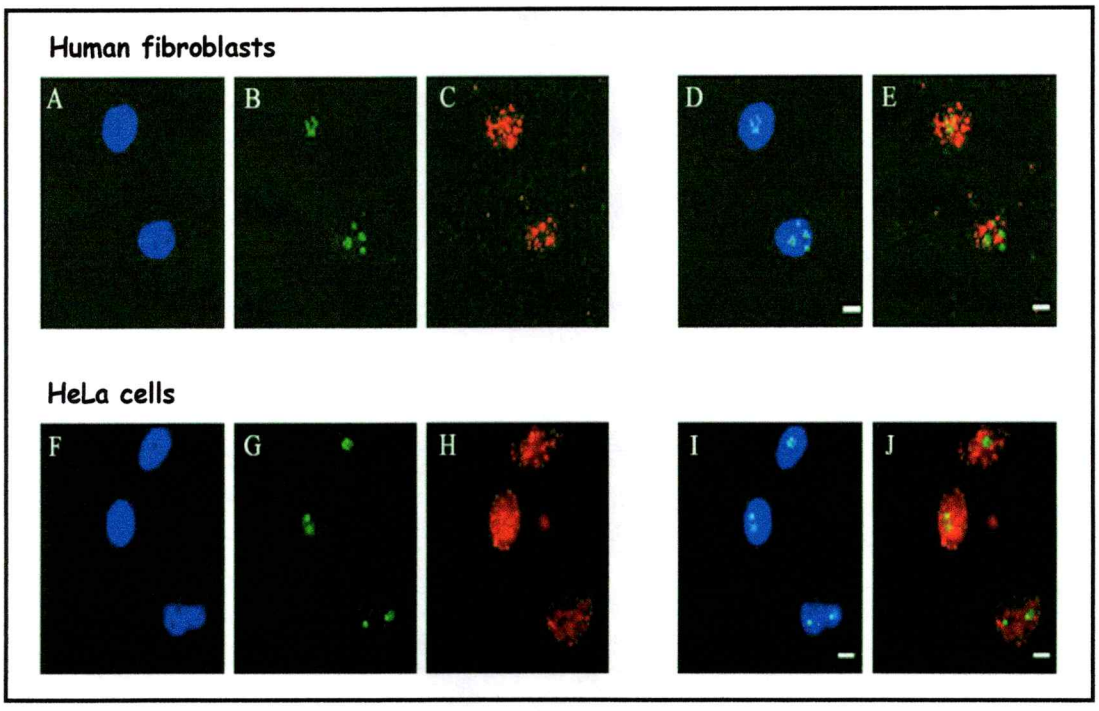
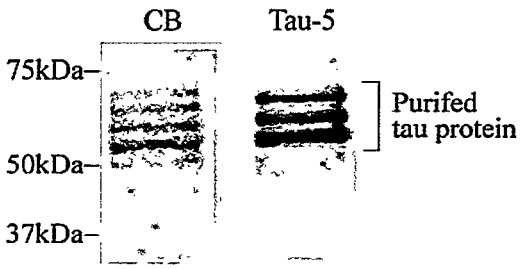


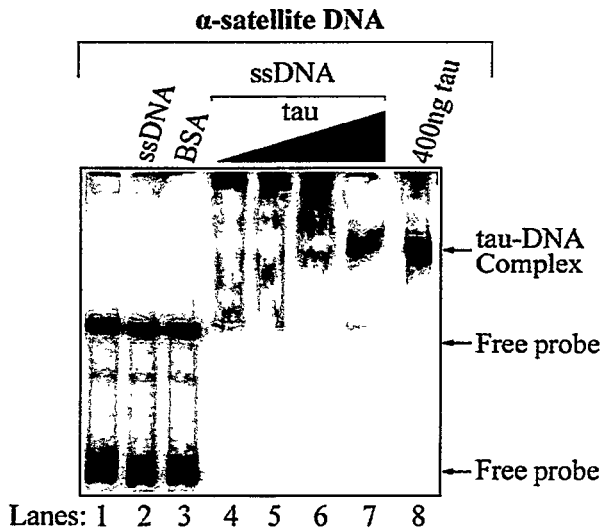
Figure 16. ***Tau localizes inside the nucleolus and partially co-localizes with pericentromeric α-satellite DNA.*** Conventional fluorescence microscopy was used to analyze total DNA distribution and co-localization of tau with α-satellite DNA in human skin fibroblasts and HeLa cells. Left panels (A and F) correspond to total DNA distribution in cells revealed with Höechst. Middles panels (B and G) show subnuclear tau distribution detected with the Tau-1 monoclonal antibody. Panels (C and H) correspond to α-satellite DNA repeats distribution revealed by FISH using α-satellite p82H plasmid as a probe. Merged images of tau with Höechst are shown on panels D (human skin fibroblasts) and I (HeLa cells) and merged images of tau with pericentromeric α-satellite DNA are shown on panels E (human skin fibroblasts) and J (HeLa cells). Scale bar 10 = μm.

The degree of purity of tau protein, specially the absence of contamination with other microtubule associated proteins (MAPS), was evaluated by SDS-PAGE followed by CB staining and Western blot. As shown in Fig. 17A, all the bands observed after CB of a SDS-PAGE correspond to tau protein as revealed with the Tau-5 monoclonal antibody (Fig. 17A). For EMSA experiments, four different concentrations of tau protein were used. Each one was incubated with a constant amount of radioactively labeled α-satellite probe, in the presence of an excess of unlabeled sonicated salmon sperm DNA as random non-sequence specific DNA competitor. A substantial retardation of the probe was observed in the presence of 100 ng of tau, corresponding to the lowest amount of tau used here (Fig. 17B, lane 4). At 100, 200 and 300 ng of tau, retardation of the free probe coincided with the formation of a tau/α-satellite complex that migrated as a smear. At 400 ng of tau, the shift of the probe was evident and the formation of a highly retarded tau/α–satellite complex was clearly observed (Fig 17B, lane 7). As a control, such a shift was not observed when the α-satellite DNA probe was incubated with equivalent amounts of BSA (Fig. 17B, lane 3). Also, the pattern of the formation of the tau/α-satellite complex remained unchanged regardless of the presence or absence of the unlabeled ssDNA as competitor (Fig 17B, compare lane 7 to lane 8). Overall, the results obtained here demonstrated that tau was able to form protein/DNA complexes with human α–satellite DNA sequences, and strongly suggest that tau could be interacting with these sequences with certain specificity.

(A)



(B)



Lane	Density CNT/mm²
1	6924,7
2	7063,9
3	6780,4
4	5797,9
5	5618,4
6	5932,3
7	6681,9
8	6755,2

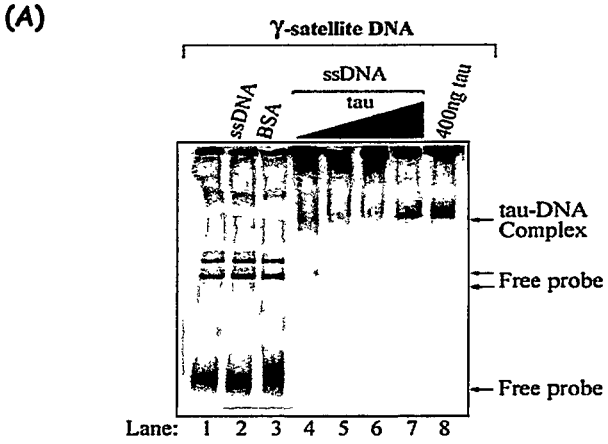
Figure 17. ***Tau protein associates with α-satellite DNA.*** The association of tau protein with α-satellite DNA was analyzed by electrophoretic mobility shift assay (EMSA). (A) Purified tau protein from bovine brain was resolved by 12% SDS-PAGE and then stained with Coomasie blue (CB) or immunodetected with Tau-5 monoclonal antibody to confirm the absence of contaminants. (B) Different concentrations of purified tau protein (100 ng, 200 ng, 300 ng and 400 ng) were incubated with the ^{32}P-labeled α-satellite DNA probe of 700 bp. The incubations were performed in the presence of 0.5 μg of unlabeled sonicated salmon sperm DNA (ssDNA) used as random non-sequence specific competitor DNA in 50 mM NaCl buffer. The EMSA gels were dried and radioactivity was visualized using a PhosphorImager. The radioactivity of each lane was further quantified by the computer program ImageQuant and expressed as a value of density CNT/mm2. The arrows indicate the shift observed for the labeled probe due to the interaction of the probe with tau and the free probe.

V. Tau forms protein/DNA complexes with murine γ-satellite DNA sequences.

Considering that tau was able to form protein/DNA complex with human α-satellite DNA sequences, we investigated the capacity of tau to bind to the structural related murine pericentromeric γ-satellite DNA sequences. Major murine satellite repeats (corresponding to γ-satellite sequences) share with the human α-satellite sequences the same centromere localization, the same (A+T)-rich base content and the same heterochromatin structure (Craig et al., 2003; Guenatri et al., 2004; Joseph et al., 1989; Choo, 1997). In order to investigate the capacity of tau protein to interact with murine γ-satellite DNA, EMSA experiments were performed under the same conditions as the one used in figure 17B, except that a 936 bp radioactively labeled γ-satellite DNA fragment, containing four γ–satellite repetitive units, was used as a probe instead of human α-satellite DNA.

As previously observed with the human α-satellite DNA, a substantial retardation of the murine γ-satellite DNA probe was observed at the lowest (100 ng) concentration of tau protein (Fig. 18A lane 4), coinciding with the formation of a smear of tau/α-satellite complex. The shift of the murine γ-satellite probe was total at 400 ng of tau (Fig. 18A lane 7). At this concentration, a highly retarded tau/γ–satellite complex was clearly formed regardless of the presence or absence of an excess of unlabeled ssDNA as competitor (Fig. 18A, compare lane 7 to lane 8).

In order to verify the presence of tau in the corresponding protein/DNA complex, we carried out an EMSA experiment followed by a Western blot. For this



Lane	Density CNT/mm²
1	6822,8
2	7235,3
3	6563,6
4	6626,7
5	5241,9
6	5238,1
7	5973,4
8	6978,4

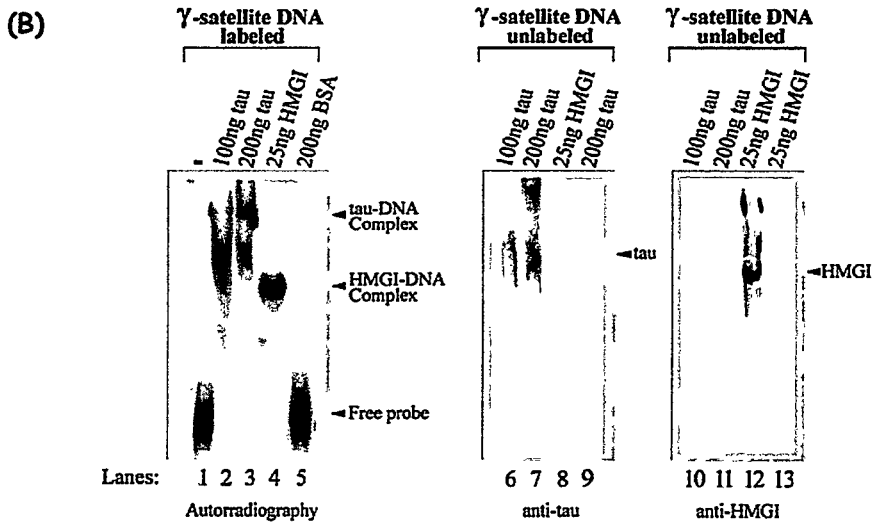


Figure 18. ***Association of tau protein with murine γ-satellite DNA.*** Electrophoretic mobility shift assays (EMSA) were performed to analyze the interaction of purified tau protein with murine γ-satellite DNA sequences. (A) Different concentrations of purified tau protein (100 ng, 200 ng, 300 ng and 400 ng) were incubated with the ^{32}P-labeled γ-satellite DNA probe of 936 bp in the presence of 0.5 μg of ssDNA as random non-sequence specific competitor DNA. The gels were dried and the radioactivity visualized by PhosphorImager was quantified using the computer program ImageQuant. The radioactivity of each lane is expressed as density CNT/mm2. (B) Indicated amounts of tau protein or HMGI protein were incubated with the γ-satellite DNA probe, either labeled (lanes 1 to 5) or unlabeled (lanes 6 to 13) in the presence of 0.5 μg of ssDNA unlabeled competitor. No satellite DNA was added in lanes 9 and 13. The complexes were resolved in a non-denaturing TBE-polyacrylamide gel. The gel was either, dried and autoradiographed (lanes 1 to 5) or transferred to a nitrocellulose membrane and immunobloted using either the Tau-5 monoclonal antibody (lanes 6 to 9) or an anti-HMGI polyclonal antibody (lanes 10 to 13). The arrows indicate the shift observed for the complexes due to the interaction with tau and the free-labeled probe.

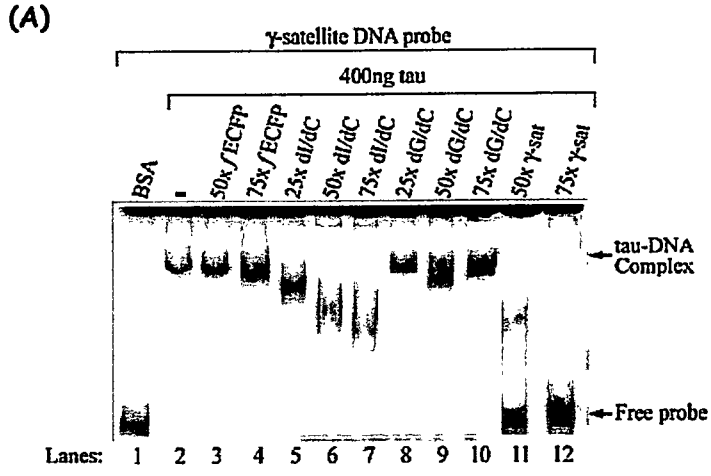
purpose, two concentrations of tau (100 and 200 ng) and one concentration of protein HMG-I were incubated either with the ^{32}P-labeled γ-satellite probe (Fig. 18B lanes 1 to 5), or with the the unlabeled γ-satellite probe (Fig. 18B lanes 6 to 13) in the presence of unlabeled ssDNA as in figure 17B. The monoclonal Tau-1 antibody revealed the presence of tau protein (Fig. 18B, lanes 6 and 7) in the tau/γ-satellite complex. This reaction was specific because nothing was revealed with the anti-tau antibody in lane 8 containing HMGI and γ–satellite DNA, or in lane 9 containing 200 ng of tau alone without DNA. By contrast, the polyclonal anti-HMGI antibody revealed the presence of HMGI in the HMGI/γ-satellite complex (Fig. 18B, lane 12). This reaction was also specific because no bands were revealed in lanes 10 and 11 containing tau protein and γ-satellite DNA or in lane 13 containing 25 ng of protein HMGI without DNA.

VI. Tau-satellite DNA interaction is apparently mediated by AT-rich DNA sequences

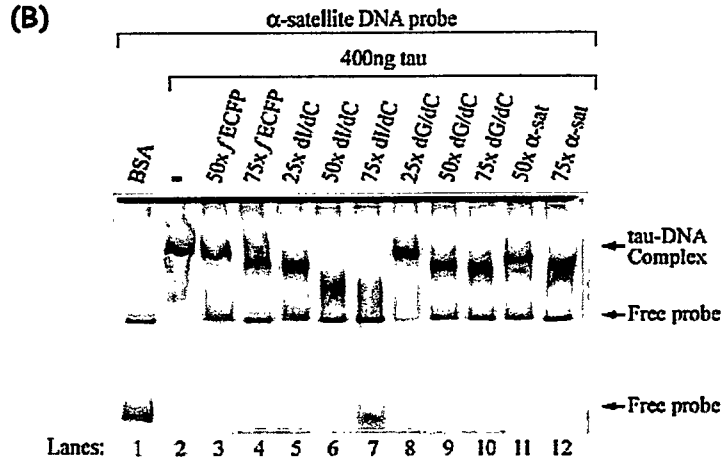
In order to ascertain the binding specificity of tau protein for these pericentromeric satellite DNA sequences, and to test the capacity of tau to bind other DNA fragments of the same size containing random sequences, competition experiments were carried out. In these experiments, a constant concentration of tau (400 ng), was incubated with an excess (25X, 50X or 75X excess) of different unlabeled DNA probes of equivalent sizes corresponding to: *f*-ECFP, sonicated poly dI/dC,

sonicated poly dG/dC, α-satellite and γ-satellite (see Materials and Methods). The corresponding unlabeled DNA probes were incubated with tau before adding the radioactively labeled γ-satellite (Fig. 19A) or α–satellite (Fig. 19B) probes. In Fig. 19A, are shown the competition experiments obtained for the tau/γ-satellite DNA complex. As expected, competition was observed in the presence of an excess of unlabeled γ-satellite DNA fragment (Fig. 19A, compare lane 2 without competitor with lanes 11 and 12 with competitor DNA). Under the same conditions, no competition was observed in the presence of an excess of unlabeled *f*-ECFP (Fig. H7A, lanes 3 and 4) or unlabeled sonicated poly dG/dC (Fig. 19A, lanes 8 to 10). However, competition was observed in the presence of an excess of unlabeled sonicated poly dI/dC (Fig. 19A, lanes 5 to 7), even though this competition was not as strong as the one obtained in the presence of unlabeled γ-satellite DNA. Very similar results were obtained during competition experiments obtained for the tau/α-satellite DNA complex (Fig. 19B, lanes 1 to 12).

Therefore, in contrast to poly dG/dC or *f*-ECFP DNA, only poly dI/dC and α-satellite DNA competed and displaced tau from tau/satellite DNA complexes. This result was reproducible in different assays, for tau complexes with both γ-satellite DNA and α-satellite DNA. The poly dI/dC polymer shares strong structural similarities with DNA rich in A-T bases (Lavery and Pullman, 1981). In conclusion the specific displacement of tau and satellite DNA complexes with an excess of poly dI/dC sequences indicate a particular affinity of tau protein for sequences with a high adenine (A) and thymidine (T) base content.



Lane	Density CNT/mm²
1	72201,3
2	74745,7
3	80573,3
4	91510,4
5	90041,0
6	85777,7
7	86408,5
8	76279,4
9	79662,9
10	79977,9
11	88094,0
12	93592,1



Lane	Density CNT/mm²
1	45844,0
2	59452,1
3	60560,0
4	74660,9
5	73206,1
6	76747,8
7	78039,2
8	56479,7
9	61289,6
10	69351,2
11	76345,8
12	70981,1

Figure 19. ***Tau protein specifically interacts with γ or α-satellite DNA sequences.*** (A) 400 ng of purified tau protein were incubated with 32P-labeled γ-satellite DNA probe in the absence (lane 2) or presence of an excess of unlabeled fECFD DNA fragment (lanes 3 and 4), unlabeled sonicated poly dI/dC (lanes 5 to 7), unlabeled sonicated poly dG/dC (lanes 8 to 10) or unlabeled γ-satellite DNA (lanes 11 to 12). (B) 400 ng of purified tau protein were incubated with 32P-labeled α-satellite DNA probe in the absence (lane 2) or presence of an excess of unlabeled fECFD DNA fragment (lanes 3 and 4), unlabeled sonicated poly dI/dC (lanes 5 to 7), unlabeled sonicated poly dG/dC (lanes 8 to 10) or unlabeled α-satellite DNA (lanes 11 to 12). The arrows indicate the shift observed for the complexes between the satellite probe and tau and the free-labeled probe at the bottom of the gel. The EMSA gels were dried and radioactivity was visualized using a PhosphorImager and quantified with the computer program ImageQuant.The radioactivity of each lane is expressed as a value of density CNT/mm2.

VII. Nuclear tau species of SHSY5Y cells.

The localization of tau in SHSY5Y cells examined using fluorescent microscopy demonstrated a particular and consistent nuclear staining pattern in neuronal, as well as in non-neuronal cells, in contrast to a diffuse and light cytoplasmic staining.

In order to characterize the tau species present in the nucleus of a neuronal model, nuclear and cytoplasmic fractions were prepared from SHSY5Y cells. Since it appears that the nuclear tau levels represent a small percentage of the total protein of the cell nucleus, a large sampling of SHSY5Y cultured cells was necessary: (i) to confirm the existence of nuclear tau species by immunoblot analysis with different antibodies against tau; (ii) to molecularly characterize the nuclear species of tau; and (iii) to investigate the isoelectric character of the nuclear tau species.

Based on immunoblot analysis, the same amount of cytoplasmic and nuclear proteins were loaded and tested by nuclear and cytoplasmic markers, to demonstrate the purity of each fraction and therefore to avoid the contamination of any detectable amount of cytoplasmic tau in nuclear fractions. The purity of nuclear fractions was demonstrated by the presence of the histone H1 marker and the absence of β-tubulin detection at the third wash of isolated nuclei (Fig. 20A), whereas the purity of cytoplasmic fractions was corroborated by the presence of β-tubulin and the absence of histone H1 (Fig. 20A).

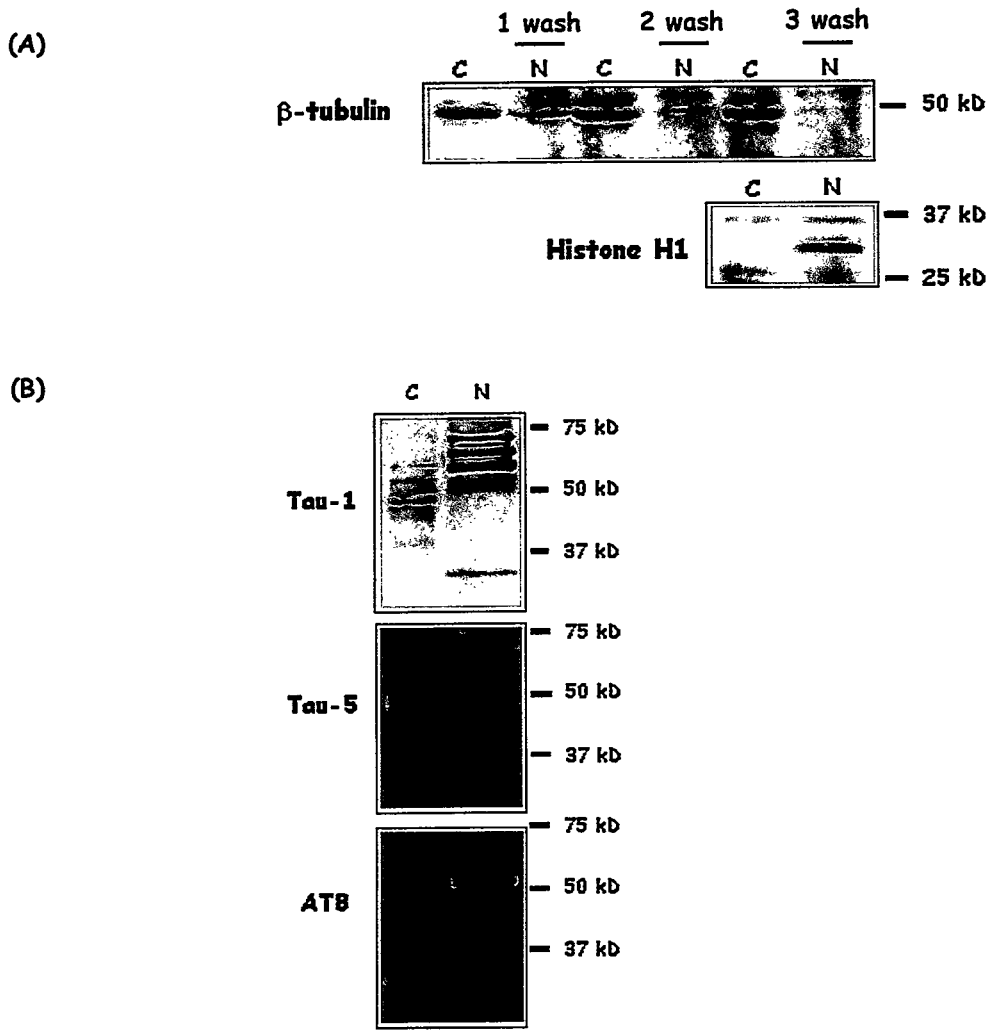


Figure 20. ***Characterization of nuclear tau proteins present in SHSY5Y neuroblastoma cells.*** SHSY5Y cells were fractionated into nuclear (N) and cytosol (C) fraction. 25 μg of cytosolic and nuclear protein were subjected to 10% SDS-PAGE and further immunoblot analysis. (A) Immunoblot analysis to assess the purity of the nuclear and cytoplasmic fractions (25 μg), with anti-β-tubulin, as cytoplasmic marker, and anti-Histone H1 as nuclear marker. At the third wash of nuclei, cytoplasmic contamination in the nuclear fraction was undetectable. (B) Immunodetection of tau proteins in 25 μg of pure nuclear and cytoplasm fractions, with the monoclonal antibodies Tau-1, Tau-5 and AT8. Whereas in cytosol a 50kD tau is predominant as shown by Tau-5, in nuclear fractions five tau species were detected; three main nuclear tau species migrating around 64, 69 and 74kD, as shown by Tau-1 and two others migrating at 30 kD and 55kD as is shown by Tau-5 and AT8 respectively.

Once the purity of both fractions was corroborated, the presence of tau proteins in the nuclear fraction was confirmed by immunoblot analysis using the tau antibodies Tau-1, Tau-5 and AT8. Four nuclear tau species were immunodetected with Tau–1, represented by the pattern of bands migrating between 50 and 75 kD and one band migrating at 30 kD (Fig 20B, upper panel). On the other hand, two nuclear tau species were immunodetected with Tau-5, represented by a predominant band around 30 kD and another less abundant at 55 kD, as compared to the cytoplasmic tau specie at 50 kD (Fig. 20B, middle panel compare C and N fraction). This is the predominant form and correspond to the standard tau detected in total cell extracts. In nuclear, as well as cytoplasm fractions, almost the same levels of tau protein were detected. Finally, two nuclear tau species were immunodetected with AT8, one major band at 55 kD and another less predominant at 30 kD (Fig. 20B, lower panel).

The results above indicate that the nuclear tau subpopulation of SHSY5Y cells is constituted by: (i) three main species with molecular weights around 64, 69 and 74 kD, which are reactive to Tau-1 and non-reactive to Tau-5, suggesting that they share the epitope 198-207 unphosphorylated at S^{202}, and apparently another modification interfering with the Tau-5 binding; (ii) a specie close to 30kD recognized by Tau-5 and representing a small nuclear tau specie; and (iii) a specie of 55 kD reactive to AT8, suggesting its phosphorylation at residues Ser202/Thr205. Besides, the higher levels of tau were found in nuclear fractions of SHSY5Y cells as compared to cytoplasmic fractions, which is consistent with the immunofluorescence results for each antibody.

VIII. Two-dimensional electrophoretic analysis of nuclear tau proteins: evidence for a basic and an acid subpopulation of nuclear tau.

Phosphorylation in combination with the type of isoform can modulate the properties of tau proteins. Therefore, given it key posttranslational modification role, phosphorylation may also be involved in nuclear tau targeting or function. Two-dimensional isoelectrofocusing (IEF) was carried out to investigate the isoelectric character of nuclear tau species, and therefore the level of phosphorylation. Nuclear extract of SHSY5Y were analyzed under two conditions (Materials and Methods, section X): (i) loading in the first dimension, the sample of nuclear proteins previously resuspended in IEF buffer, and (ii) loading in the first dimension, with the sample of nuclear proteins being previously concentrated, resuspended in rehydration buffer. Under the conditions (i), two dimensional IEF and immunoblots analysis with the antibodies Tau-1 (Fig. 21A), Tau-5 (Fig. 21B) and AT8 (Fig. 21C) resulted in a pattern of dots with molecular weights similar to the pattern of bands observed for each antibody in one dimensional gel, running between pI 8.0-9.0. The immunodetection evidenced a basic character for the nuclear tau species described before. The spots migrate around the same molecular weights mentioned before and showed a smear in the region of migration (Fig. 21A-C). This suggests the presence of modifications such as glycosilation, which is known to produce the aforementioned pattern in proteins separated by IEF gels. In order to improve the resolution of nuclear tau species in two-dimensional gels, the proteins in the sample were concentrated, the high concentration of salts was eliminated and the loading of the sample into the strip was performed by passive rehydration, which is recommended for basic proteins.

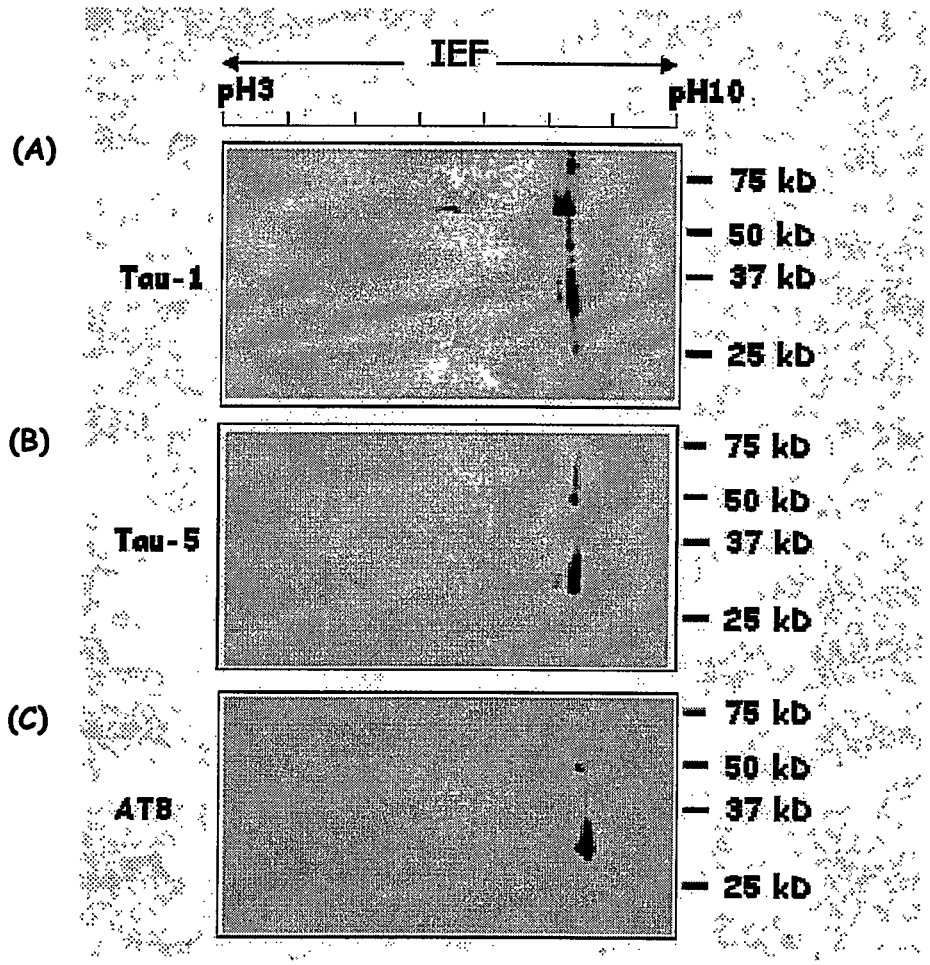


Figure 21. ***Two-dimensional electrophoretic analysis of nuclear tau proteins.*** SHSY5Y cells were fractionated into nuclear fraction and 150-200 µg of nuclear protein were subjected to IEF in strips IPG 3-10 (BioRad) using active rehydration at 50 V for 14 hours to evaluate net charge (pI) in nuclear tau species. The second dimension was perform in 10% SDS-PAGE gels and further immunoblot analysis were done with the antibodies Tau-1 (A), Tau-5 (B) and AT8 (C). Immunodetection of tau proteins with each antibody show a pattern of spots, migrating equivalent to the pattern of bands for each tau antibody. The nuclear tau species reveal a basic character as most of nuclear proteins do, and therefore a low degree of phosphorylation, migrating in the pI range 8.0-9.0.

The immunodetection with Tau-1 under improved conditions, allowed the detection of two major groups of nuclear tau species migrating between 50 kD and 75 kD. One group composed of five species (dots) with a basic isoelectric point, between pI 8.5- 9.5 and a second group composed of four species with a more acidic isoelectric point, between pI 7.0-8.0, (Fig. 22A).

The results showed before demonstrate that nuclei of SHSY5Y cells contain more than the five tau species, ranging between 30 kD and 75 kD. At least nine of these species are grouped in two subpopulations mainly differentiated by the pI. Considering the high degree of modifications that tau proteins can experiment as well as the effect that all together can have in the pI, it is possible conclude two main points: (i) the more acidic group, sharing a pI 7.0-8.0, correspond to nuclear tau species with molecular weights between 50-75 kD, maybe phosphorylated in a mayor extent but at different levels; (ii) the more basic group sharing a pI 8.5-9.5, correspond to nuclear tau species with molecular weights between 50-75 kD, probably less phosphorylated and possibly post-translational modified by O-GlcNAcylation which is know to give a basic character to modified proteins.

To characterize the primary structure and post-translational modifications of those nuclear tau species the proposal was to perform mass spectrometry analysis of each spot detected. As can be observed the correlation between the spots detected with Tau-1 and the spots observed in the CB stained IEF gel, was confusing and inexact (Fig. 22A, CB). To solve this problem nuclear tau species were purified by immunoaffinity from nuclear extracts of SHSY5Y cells, using the antibody Tau-1 (Materials and Methods, section XI). The immunodetection with Tau-1 revealed several bands in three

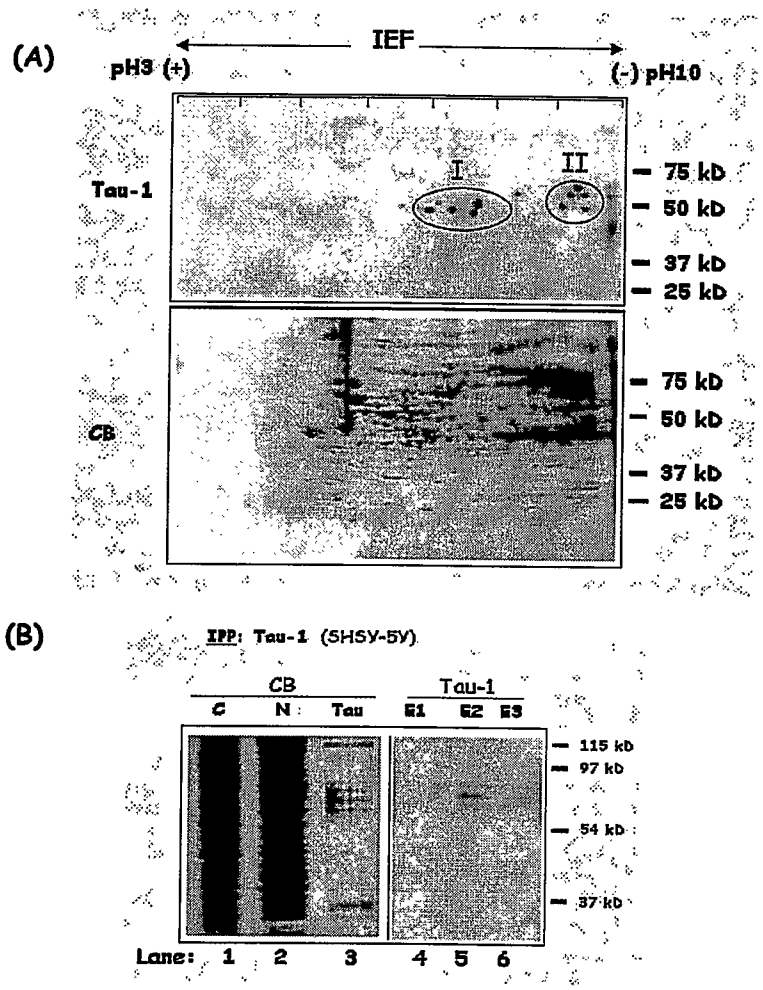


Figure 22. ***Improved isoelectrofocusing and immunopurification of nuclear tau proteins.*** SHSY5Y cells were fractionated into nuclear fraction and 300 μg of nuclear protein were subjected to methanol precipitation to eliminate the high salt concentration in the sample affecting the running during the IEF. The sample divided in two was loaded on two strips IPG 3-10 (BioRad) and passive rehydration for 14 hours was used to incorporate the sample in the strip and then maximum voltage for 7-8 hours until reach 40 kVh. The second dimension of both strips was performed in 10% SDS-PAGE. (A) The representative 2-D image of the immunoblot analysis with Tau-1 and the Coomasie blue (CB) stained gel after IEF. Two groups of nuclear tau proteins are show by a circle and indicated by (I) representing four nuclear tau species in the pI range of 7.0-8.0 and a second circle (II) representing five nuclear tau species in the pI range of 8.5-9.5. (B) Immunoaffinyti-purified tau from SHSY5Y cells was resolved by 10% SDS-PAGE and then stained with CB (lanes 3) or with the antibody Tau-1 (lanes 4-6) in three independent elution steps. Three tau bands between 54 kD and 97 kD are clear at the third elution step. As control lane 1: cytoplasm extract and lane 2: nuclear extract.

independent elutions of the immunoprecipitated nuclear tau (Fig. 22B, lanes 4-6). The Coomasie blue staining of immunopurified nuclear tau in gel, showed several bands, some migrating between 54 kD and 97 kD represent most probably nuclear tau species whereas the others possibly correspond to contaminants proteins (Fig. 22B, lane 3). Putative tau bands were extracted from the CB stained gel containing the sample of immunopurified nuclear tau, and digested for further mass spectrometry analysis, however the extraction does not allowed the adequate purity and amount for those analyses.

IX. Nuclear tau levels rise during G1 to S phase passage of the cell cycle.

Given the presence of tau in nuclei of cycling cells independent on the neuronal or non-neuronal origin, the following experiments were conducted to evaluate if the nuclear tau levels are cell cycle regulated as an approximation to delineate a putative function for nuclear tau. To address this issue, SHSY5Y cells were synchronized by aphidicolin treatment (Materials and Methods, section XII), an agent that inhibits DNA polymerase α, causing S-phase arrest. After a 12-h treatment with aphidicolin, neuroblastoma cells were blocked at the G1/S boundary. A fraction of cells was rescue at different points of time from 0 to 12 hours in order to cover the cell cycle, after remove the aphidicolin from the media and apply full medium to the cultured cells (Crociani et al., 2003) parallel to the procedure of nuclear extract preparation. The degree of cell synchronization and the timing of the cell cycle stages were analyzed and confirmed by fluorescence intensity of cells stained for DNA content using PI and

determined by flow cytometry. The flow method measured the proportion of cells that are in the process of making DNA and will not be affected by increases in the total number of cells. The histograms delineated nuclei with 2C amount of DNA (G1 phase), nuclei with 4C amount of DNA (G2/M phase) and nuclei with amounts of DNA between the two regions, that therefore caught in the process of synthesizing DNA (S phase). The flow histograms at each point of time showed that the cells enter slowly and asynchronously into S phase, so that after 8 h a major percentage of cells is in S phase and further progress to reach the S/G2 boundary from 12 h (Fig. 23). Even though SHSY5Y cells were synchronized at time 0, each cell progress throughout the cell cycle with different timings, a phenomenon that is evidenced at 12 hours where a fraction of cells are still in G1 phase as the DNA histograms shown.

The second aim was evaluate if the nuclear tau levels are cell cycle regulated. Therefore the remaining fraction of the harvested cells for flow cytometer analyses was used for nuclear fraction preparations at each point of time followed by immunoblot analysis with the antibodies Tau-1 and Tau-5. The nuclear tau levels revealed by quantification of Tau-1 immunodetection (considering the sum of the three bands), were almost the double at 4 and 7 hours compared to time 0 (Fig. 24A). On the other hand, the nuclear tau levels revealed by quantification of Tau-5 immunodetection (considering the band of 55kD) were over the double at 7 and 9 hours compared to time 0 (Fig. 24B). In both cases the nuclear tau levels of G1 cells (at time 0) were lower than the nuclear tau levels observed after remove the aphidicolin to stop the inhibition of DNA polymerase α leaving the cells progress through S phase. The highest nuclear tau levels

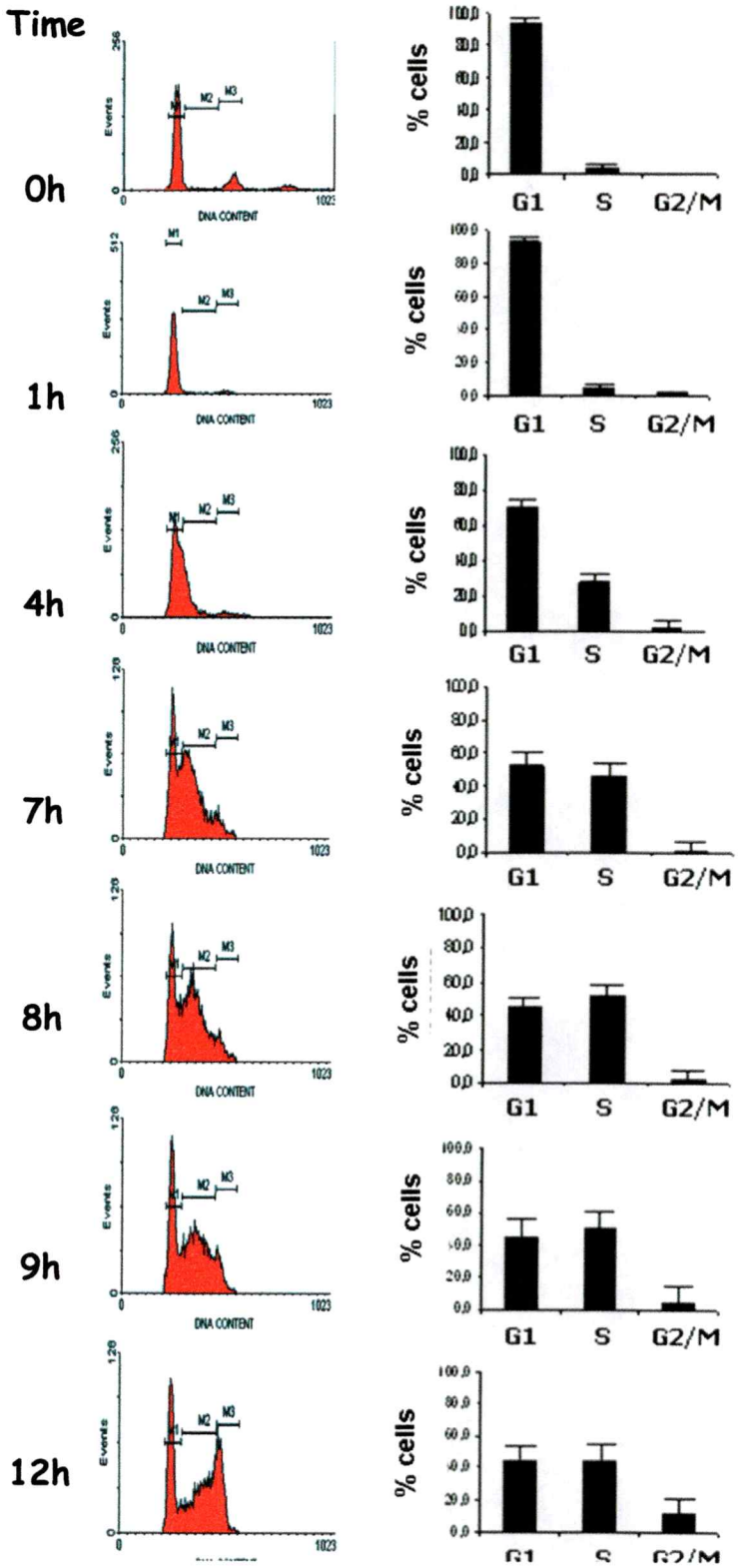


Figure 23. ***Cell cycle synchronization of SHSY5Y cells.*** SHSY5Y cells were synchronized at the border of G1/S phase by serum deprivation during 24 hours and treatment with 1ug/ml of aphidicolin for 12 hours. The degree of cell synchronization and the timing of the cell cycle stages was analyzed and confirmed by fluorescence intensity of cells stained for DNA content using PI and a flow cytometer. The flow histograms (right panel) showed the DNA content into the three cell cycle stages at each point of time. The histograms (left panel) for each point of time, delineated the fraction of SHSY5Y cell nuclei with 2C amount of DNA (G1 phase), nuclei with 4C amount of DNA (G2/M phase) and nuclei with amounts of DNA between the two regions, that caught in the process of synthesizing DNA (S phase).

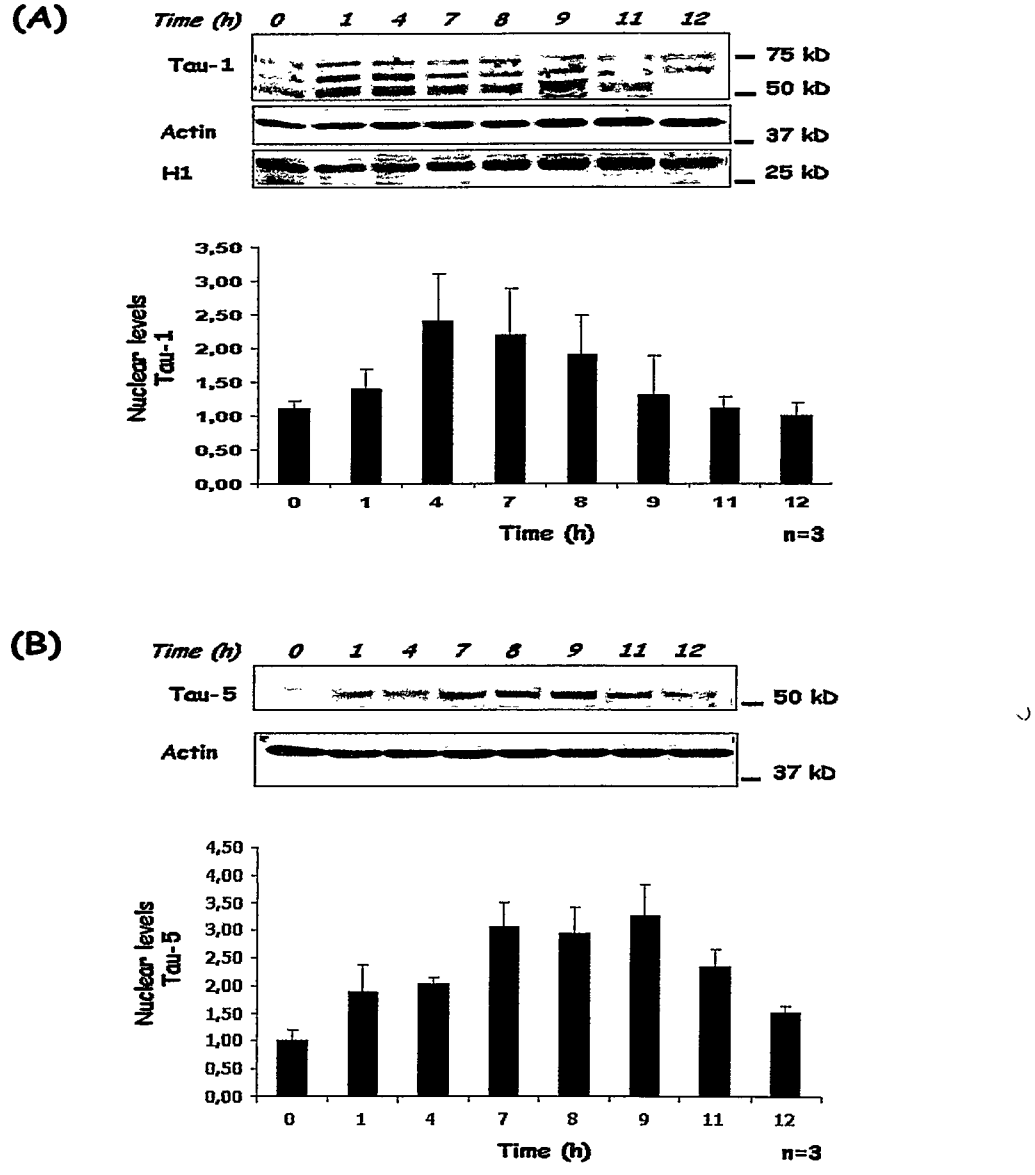


Figure 24. ***Analysis of nuclear tau levels during the cell cycle.*** SHSY5Y cells synchronized at the border of G1/S as previously describe, were fractionated into nuclear fractions at different times after release from the block by aphidicolin. 25 μg of the nuclear fractions prepared at each point of time, were resolved on 10% SDS-PAGE gels, transferred and immunobloted for β-actin, histone H1 and Tau-1 (A) and for β-actin and Tau-5 (B). Band intensities were normalized against b-actin loading controls for each sample and expressed in arbitrary units relative to the expression at time zero. According to flow cytometry analysis an increase in nuclear tau levels is shown from 4 to 9 hours during the S-phase of the cell cycle. Values at each point are expressed as means ± SEM; * $p<0.05$.

were observed when the proportion of cells in S phase began to increase concomitantly with a decrease in the percentage of cells in G1 phase between 4-9 hours. Latter on the nuclear tau levels decreased through the slow progress of the cells into the G2/M phase reaching similar levels to the observed in G1 phase (Fig. 24A, B).

Regardless the experimental procedure did not allow us to obtain better correlation amongst the several samples analysed because of the high standard deviation at each point of time, the results revealed a constant increase on the nuclear levels of tau during S phase, in three independent assays using both antibodies.

Interestingly the finding of an increase on the nuclear tau levels in cells stimulated to proliferate, concurrently with the increase of cells at S phase, suggest the involvement of nuclear tau in events taking place either during the G1 to S phase passing or during the DNA synthesis at nucleolar level, considering its nucleolar distribution.

X. Pin1 inhibition did not affect the nuclear localization of tau.

The nuclear localization of tau is a determining factor in its ability to develop a nuclear function. The import and export of macromolecules between the cytosol and the nucleus occurs through the nuclear pore complex (Duverger et al., 1995). Molecules smaller than 40 kD could reach the nucleus by direct passive diffusion (Duverger et al., 1996; Munkonge et al., 2003), however molecules larger than that are transported into the nucleus by a selective process consisting in the recognition of nuclear localization signals (NLS), a series of basic residues that mediates binding to a protein called either

a karyopherin or importin mediating the passage across the nuclear envelope (NE). NLSs can be divided into four groups (Boulikas, 1993), termed "simple", "bipartite" or "split", "nonpositive" and "highly basic" NLSs, the first two candidates are bipartite NLSs and the rest are simple NLSs. The sequences surrounding NLSs can also play a role in the nuclear localization of certain proteins.

An understanding of how tau protein is targeted to the nucleus would allow indentify a target to inhibit its translocation and therefore an approach to block its nuclear function. The mechanism by which tau is targeted to the nucleus is not clear since the amino acidic sequence lacks any classical NLS. Considering that some proteins rely on the complexation with other proteins in the cytoplasm for their nuclear localization, the possibility that tau binds to a protein bearing NLS for its transport to the nucleus was investigated.

Among the proteins that interact with tau, the human peptidyl-prolyl cis-trans isomerase (PPIase), Pin1 is a preferentially nuclear protein containing a NLS. The interaction between both proteins is mediated by the phosphorylated threonine 231 residue (Thr231), located upstream of the MBD in the proline rich region of tau, and the WW domain of Pin 1 (Lu et al., 1999). T231 can be phosphorylated by GSK3β, but only after phosphorylation of serine 235 by cdk5 (Goedert et al., 1994; Ishiguro et al., 1993). Very recently has been demonstrated that Pin1 also recognizes the phosphorylated Thr212-Pro213 suggesting that additional phospho-Ser/Thr-Pro could be substrates of Pin1 (Smet et al., 2004). It was considered a good candidate for nuclear tau targeting since it is a mitotic regulator, whose activity is required for normal entry

and progression through mitosis, in yeast and mammalian cells (Fujimori et al., 1999). Besides it interacts with a range of proteins that are phosphorylated prior to cell division (Shen et al., 1998), catalyzing the cis-trans isomerization of the phosphorylated Ser/Thr-Pro motif. Furthermore Pin1 also functions as a chaperone, modulating the assembly, folding, activity and transport of essential cellular proteins (Lu et al., 1996). Additionally, Pin1 has been implicated in AD through the regulation of amyloid precursor protein processing and amyloid-beta production (Pastorino et al., 2006).

To elucidate this issue, the first approach was to observe the localization of both proteins by immunoflourescence experiments. The results showed that Pin1 is distributed in the cell body and nucleus, partialy colocalizing with Tau-1 in certain areas restringed to the nucleus, suggesting the idea of a possible interaction between both proteins at the nuclear compartment (Fig. 25A). The presence of Pin1 in nuclear and cytoplasmic extracts was also confirmed by immunoblot assays. The levels of 18 kD Pin1, founded in SHSY5Y nuclear extracts, were lower than the cytoplasmic ones (Fig. 25B). Pull down experiments with the Pin1 protein coupled to histidine to investigate the interaction between the citoplasmic and nuclear tau with Pin1, showed a preferential associtation of Pin1 to cytoplasmic tau compared to nuclear tau. Thus, whereas 50 kD citoplasmic tau immunoprecipitated with Pin1, undetectable levels of nuclear tau were immunoprecipitated by Pin1 (Fig. 25C, compare lane 4 to 3). Considering the possibility that Pin1 binds tau in the cytoplasm for further nuclear transport leaving it free in the nucleoplasm, a second approach was inhibit Pin1 and to observe the effects over the nuclear tau levels. Treatments with the irreversibly inhibitor of Pin1, Juglone, at concentrations of 5 and 10 μg/ml for 3 hours, did not change considerably the nuclear

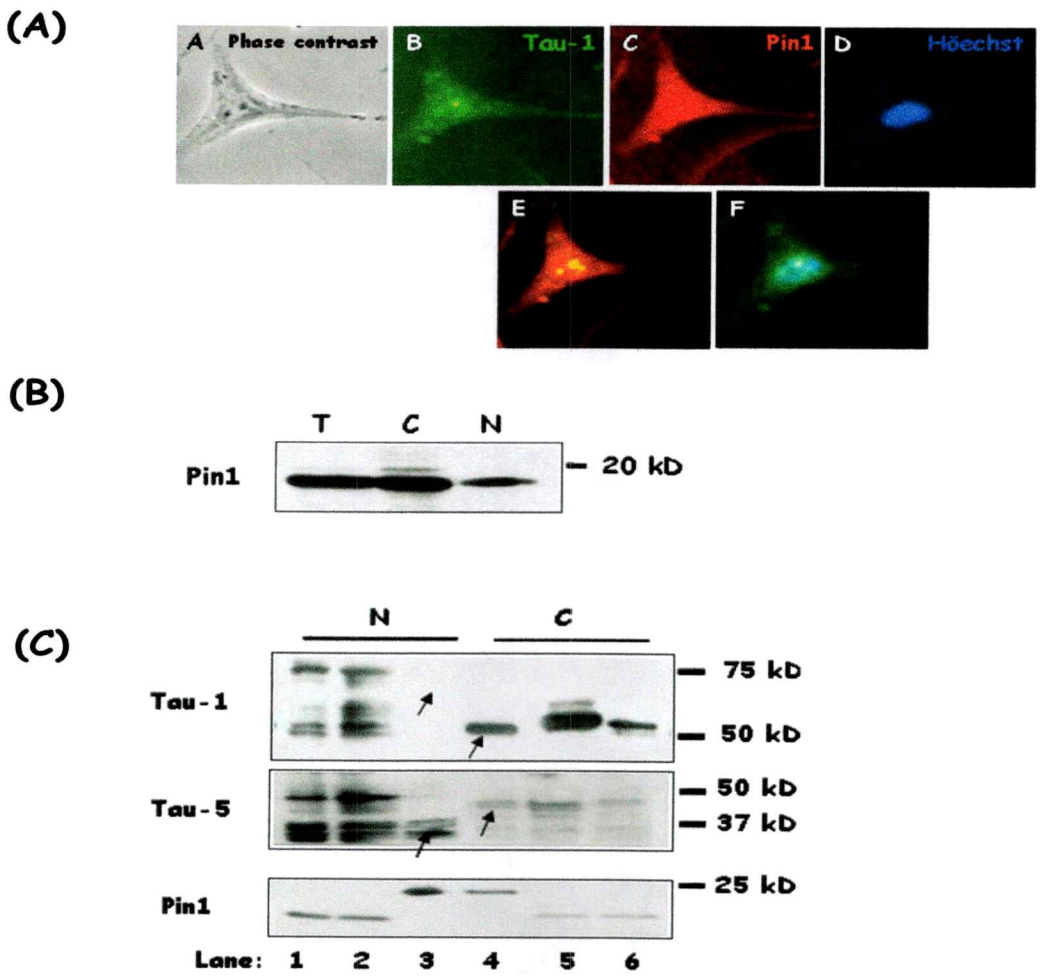


Figure 25. ***Participation of Pin1 in the nuclear tau localization.*** (A) SHSY5Y cells were grown under normal serum conditions for further co-localization (e) of endogenous nuclear tau (green, b) and Pin-1 (red, c) by immunofluorescent and confocal microscopy. Nuclear counterstaining with Höechst (blue, f). (B) SHSY5Y cells were fractionated in total (T), cytoplasm (C) and nuclear (N) extract. Immunoblott analysis of the Pin-1 level in 25 μg of protein for each extract, revealed that Pin1 is mainly concentrated at the cytoplasm. (C) Pin1-tau interaction analyzed by pull down assays using recombinant Pin1 coupled to histidine resin. 300 μg of the nuclear and cytoplasmic fractions were incubated with Pin1-His resin and the fraction of tau protein bound to Pin 1 was diluted in loading buffer and analyzed by immunoblott with Tau-1, tau-5 and Pin1. Lanes 1 and 6 represent nuclear and cytoplasmic extracts; lanes 2 and 5 the supernatant after pull down; and lanes 3 and 4 the fraction of nuclear and cytoplasmic tau bound to Pin1 respectively, indicated by arrows. Tau- 1 immunoblotting show a clear association of cytoplasmic tau to Pin1 from pull down assays. In the lower panel immunoblott corroborate the presence of endogenous Pin1 (lanes 1, 2, 5, 6) and recombinant Pin1-Histidine (lanes 3, 4).

tau levels, immunodetected with Tau-1 and Tau-5 (Fig. 26). At higher concentrations of inhibitor (20 μg/ml) and longer times of treatment (5 hours), it was observed an increase in the nuclear levels of tau, however it was concomitant with high levels of cell death, and therefore these results were not further evaluated.

Altogether this results do not allow to establish a directly involvement of Pin1 in the nuclear localization of tau, result that are in agreement with the observation that Pin1 play a major role restoring the ability of phosphorylated tau to promote microtubule assembly (Lu et al., 1999).

XI. AD related tau modifications affecting the nuclear levels.

Previous studies have demonstrated that tau protein is an integral component of nuclei isolated from AD brains (Brady et al., 1995). Besides, AD like hyperphosphorylation of tau protein has been showed in mitotically active cells (Delobel et al., 2002; Pope et al., 1994; Preuss et al., 1997). Furthermore the expression of cell cycle markers has been widely demonstrated, suggesting that cell proliferation signals are induced in response to neurodegeneration associated to AD.

The kinases cdk5 and GSK3β participates in the pathogenesis of AD (Alvarez et al., 1999; Ferreira et al., 1997), and as was mentioned in the section before, participates in the phosphorylation of tau at Thr231. A recent report, described the dense immunolabeling of heterochromatinic regions with the antibody TG-3, recognizing the phosphoepitope Thr 231, detecting an early pathology in AD

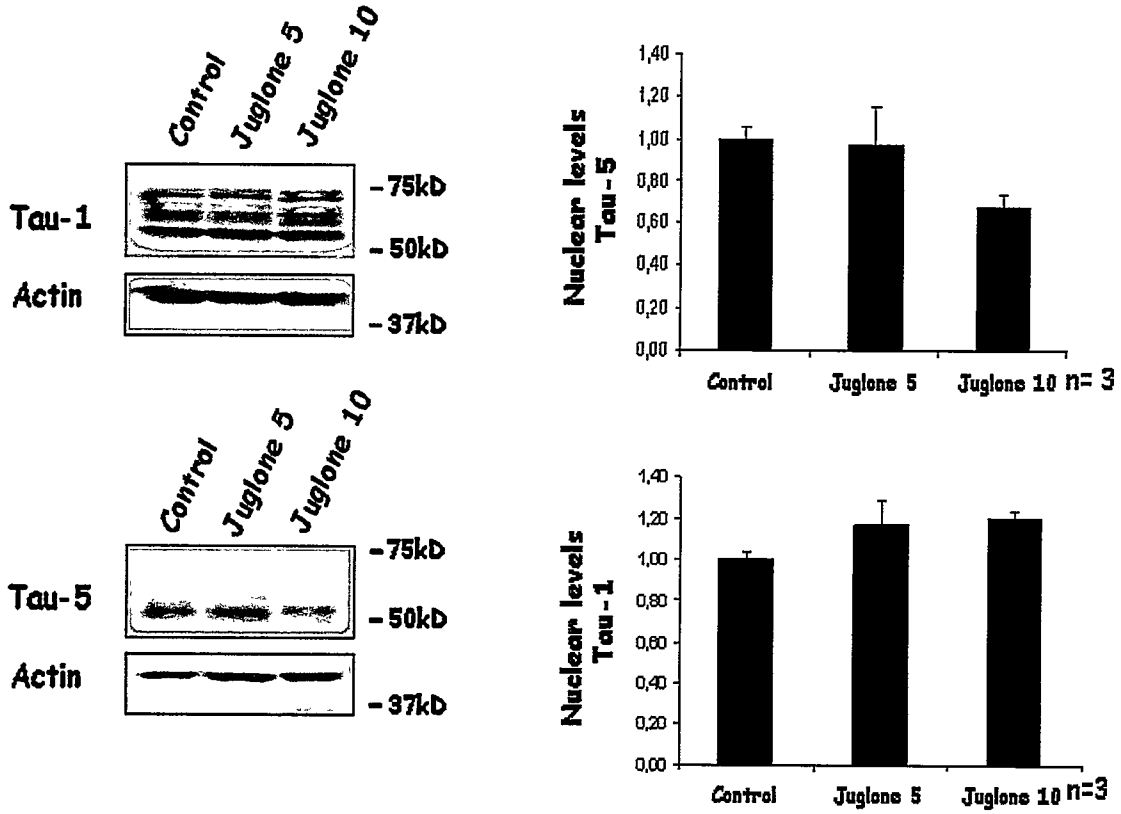


Figure 26. ***Nuclear tau levels in response to Pin1 inhibition.*** (A) SHSY5Y cells were grown under normal serum conditions and treated with 5 and 10 μg/ml of Juglone an irreversibly inhibitor of Pin1, for 3 hours. Nuclear fractions of each treatment were prepared and 25 μg of nuclear proteins were analyzed by immunoblott with Tau-1 (upper panel) and Tau-5 (lower panel). Inhibition of Pin1 did not change considerably the nuclear tau levels detected with both antibodies. Band intensities were normalized against b-actin loading controls for each sample and expressed in arbitrary units relative to the expression of nuclear tau in the control (untreated nuclear extracts). The values at each point are plotted and expressed as means ± SEM; * $p<0.05$. No significant differences were observed in the nuclear levels of tau between control and treated cells.

(Luna-Munoz et al., 2005). Taken together these findings, it was of interest to examine the involvement of modifications mediated by the kinases cdk5 and GSK3β, in the nuclear tau localization. SHSY5Y cells treated with the GSK3β inhibitor, LiCl (10 mM) for 24 hours, showed a significant increase on the nuclear levels of tau species compared to the control and quantified for Tau-1 as well as Tau-5 immunodetection (Fig. 27A, B Litium). In contrast, cells treated with the cdk5 inhibitor, Roscovitine (10 μM) for 24 hours, showed no significant changes in the nuclear levels of tau species compared to control cells and quantified for both Tau-1 and Tau-5 immunodetections (Fig. 27A, B Roscovitine).

On the other hand, the role for sugars as non-peptidic NLSs in numerous glycosilated cytosolic and nuclear proteins has been supported experimentally (Duverger et al., 1993; Duverger et al., 1995). Thus the potential role of O-linked β-N-acetylglucosamine tau modification, in the nuclear targeting was tested. SHSY5Y cells treated with the O-β-N-acetylglucosaminidase inhibitor, STZ (10 mM) for 3 hours, stimulating the level of O-GlcNAcylation of tau showed a significant increase in the nuclear levels of tau species compared to control cells and quantified for both Tau-1 and Tau-5 immunodetection, (Fig. 27A, B STZ). The results presented above suggest that while phosphorylation of tau by GSK3β negatively regulates the nuclear levels of tau species, O-GlcNAcylation do positively and thus may serve and function as a non-peptidic nuclear targeting signal.

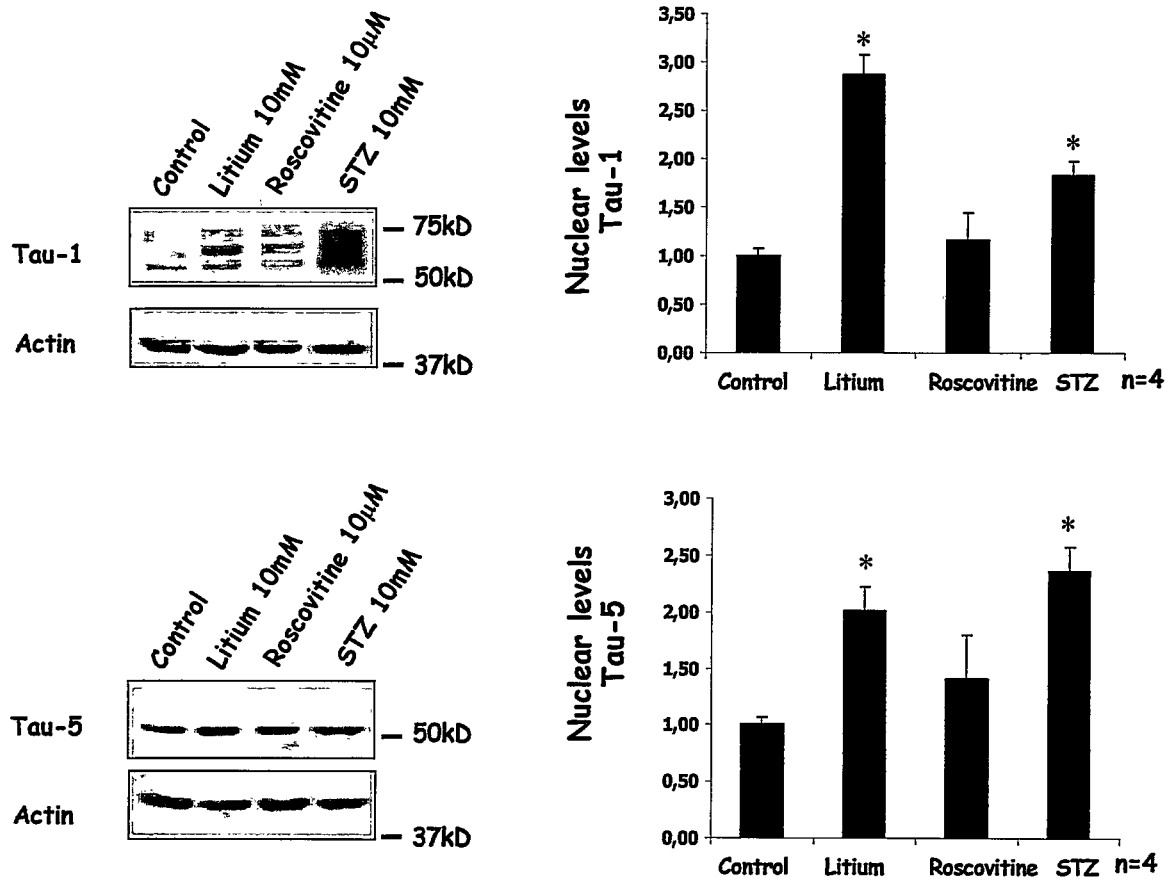


Figure 27. ***Nuclear tau levels in response to GSK3β, cdk5 and β-N-acetylglucosaminidase inhibition.*** Nuclear fractions of SHSY5Y cells were prepared from untreated cells (Lane 1: control) and treated cells. Lane 2: LiCl 10mM under the presence of myoinositol 5mM for 24 hours to inhibit the kinase GSK3β; lane 3: Roscovitine 10 mM for 24 hours to inhibit the kinase cdk5; and lane 4: streptozotocine (STZ) 10mM during 3 hours to inhibit the enzyme β-N-acetylglucosaminidase that removes the O-GlcNac groups from proteins. Immunodetection of nuclear levels of tau in 25 μg of nuclear protein extract with the antibody Tau-1 (A) and Tau-5 (B). Band intensities were normalized against b-actin loading controls for each sample and expressed in arbitrary units relative to the expression of the control. The normalized densitometry values are plotted as means ± SEM; * $p<0.05$.

DISCUSSION

Nowadays, it is well established that the microtubule associated tau protein participates in the cytoskeleton integrity, and that abnormally modified forms of tau are involved in the pathogenesis of Alzheimer disease (Maccioni and Cambiazo, 1995; Maccioni et al., 2001). The common function attributed to tau protein is the participation in neuronal polarity, the stabilization of microtubules and the control of the dynamic instability by affecting the polymerization rates of microtubules (Mitchison, 1992). However, as previously discussed in the Introduction, the function of tau protein at the nuclear level has not yet been established. The significance of the nuclear localization of tau has gained more relevance now on the basis of the findings on the capacity of tau to bind to DNA, a capacity that was originally suggested by Corces et al. (1980) when the effect of DNA in microtubule assembly was analyzed (Corces et al., 1980).

In this PhD thesis, different aspects related to the enigmatic nuclear tau protein were studied. The work was focused on the analysis of its nuclear distribution, binding to DNA, molecular characterization and in tau modifications associated to AD that could be relevant in this particular localization. The results support the earlier recognition of tau at nucleolar regions and showed the presence of at least five species composing the nuclear tau subpopulation in neuroblastoma cells as well as the ability of tau protein to interact with specific centromeric DNA sequences. Furthermore, it was demonstrated that the nuclear localization of tau, in neuronal and non-neuronal cells,

occurs preferentially in cells stimulated to proliferate and may be modulated by specific post-translational modifications. In this context different salient aspects will be discussed below.

I. Multiple tau subcellular localizations suggest additional roles for tau in normal cell physiology.

Bimodal localization of proteins makes difficult to define the role associated to each specific subcellular location for these proteins. Previous studies have suggested that tau may play non-microtubule-stabilizing roles in neurons. Until now it has been demonstrated that short tau isoforms allow plasticity of the cytoskeleton, while longer isoforms may preferentially play a role in its stabilization (Andreadis, 2005; Goedert et al., 1995a; Liao et al., 1998). Besides, given that the C-terminus of tau binds axonal microtubules while the N-terminus binds components of the neural plasma membrane, it has been suggested that tau functions as a linker protein (Hwang et al., 1996; Jenkins and Johnson, 1998; Lee et al., 2004; Williamson et al., 2002).

Initial studies concerning tau protein epitopes in nuclei, described its presence in JC and CG (human neuroblastoma cells), in CV-1 (African green monkey kidney cells), in WERI (human retinoblastoma cells), in WI-38 (human lung fibroblast) and in human macrophages (Loomis et al., 1990). Later on, microinjection experiments of labeled tau into Chinese hamster ovary cells (CHO), resulted in nucleolar and centrosomal targeting, suggesting that it can be localized in the nucleus to function in a different capacity than cytoplasmic tau, in a cellular model lacking tau proteins (Lu and Wood,

1993). Afterward, tau was associated with the fibrillar region of interphase nucleoli and the nucleolar organizing region of mitotic acrocentric chromosomes (Thurston et al., 1996; Wang et al., 1993). The nuclear localization of tau was supported in LA-N-5 neuroblastoma cells by fractionation studies, where fourteen percent of the total tau protein was extracted from the chromatin fraction (Greenwood and Johnson, 1995).

The results of partial, if not predominant nuclear tau localization observed herein, either in hippocampal cells just before the beginning of the development of neuronal polarity, in glial cells and in neuronal and nonneuronal cells, strengthen the possibility that tau functions at least in a bimodal manner, associated to the microtubular cytoskeleton and to the nucleus. Beyond this observation, the presence of nuclear tau mainly in cycling cells supports the idea that proliferative signals are involved and contribute to nuclear targeting of tau. Evidence sustaining this hypothesis comes from diverse studies. Brady et al. (1995) observed a punctuate and extranucleolar tau staining in nuclei isolated from frozen human brain, arguing that nuclei displaying nucleolar tau may be undergoing intense changes in rRNA synthesis, correlated with a cell cycle or the cell differentiation stage. On the other hand, Thurston et al., (1996) showed an increase in nuclear tau immunolocalization in lymphocytes stimulated to RNA and subsequent DNA synthesis with PHA (mucoprotein extracted from the red kidney bean, *Phaseolus vulgaris*), underlying an upregulation of nuclear tau in a cell stimulated to divide. Shea and Cressman (1998) described the rapid nuclear declination of a small tau isoform (26-30 kD) during differentiation, proposing that the role of nuclear tau isoforms may be obsolete following terminal differentiation of neurons and

that it could have a distinctive role in mitosis of neuronal precursors. Finally, and more remarkable is that nuclear tau has been also observed in AD brains, possibly due to the reactivation of the cell cycle markers that has been described in nerve cells with filamentous tau deposit. These include proteins involved in Go/G1 transition such as cyclin D, cdk4, cdk6, retinoblastoma protein, and inhibitors of cell cycle such as p15, p16, p18, p19; markers of G1 / S transition such as cyclin E and cdc25A; regulators of G2 / M such as cyclin B, cdc2 and cdc25B, polokinase, and p27Kip1; and some mitotic epitopes such as phosphorylated histone H3, PCNA, Ki67 that co-localize with hyperphosphorylated tau (Delobel et al., 2006).

Alternatively, some authors proposed a role of heat shock protein for tau (Papasozomenos, 1995) considering that heat shock proteins are known to migrate transiently into the nucleus, redistribute and exit into the cytoplasm following heat shock (Welch and Mizzen, 1988). In this sense, the nuclear localization of tau excluded from the nucleoli, was proposed to be in response to chronic stress underlying the pathology of AD. This proposal could explain the nuclear immunostaining differences observed herein in SHSY5Y cells, with the antibodies Tau-1 and AT8, since AT8 recognizes tau epitopes phosphorylated predominantly at NFT in AD (Augustinack et al., 2002).

An analysis of the tissue expression patterns of tau is summarized in figure 28, (obtained from the GeneCard web site). The findings mentioned before and the expression of tau protein in diverse tissues linked to the identification of nuclear tau in a

variety of cell lines from different origins, suggest a functional diversity for tau beyond the classical role associated to the neuronal cytoskeleton. In this regard, tau may be

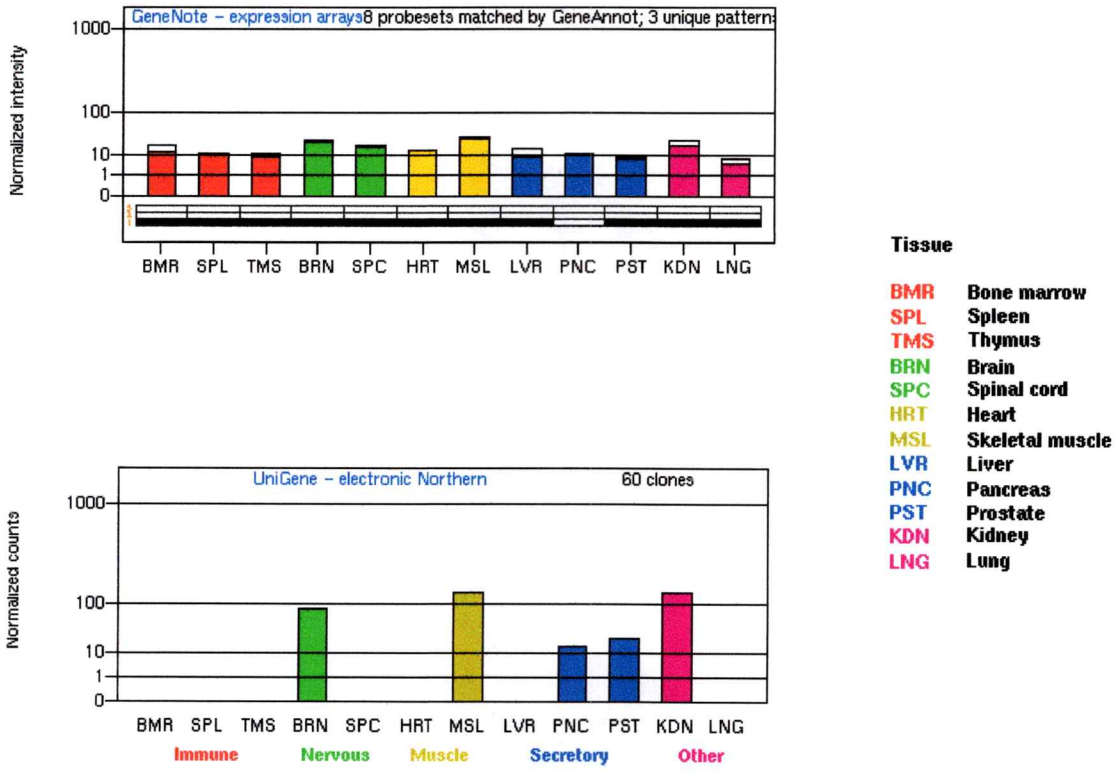


Figure 28. ***Expression of tau in Human Tissues*** (from the web page of genecard: http://www.genecards.org/cgi-bin/carddisp.pl?gene=MAPT&search=tau). Experimental results according to: a) GeneNote, probe sets-to-genes annotations according to 2GeneAnnot, 3GeneTide and b) Electronic Northern calculations according to data from UniGene (Build 188 Homo sapiens).

reasonably postulated as a housekeeping gene, if classification of housekeeping genes is mainly based on the number of tissues in which genes are expressed. Even more the profile of tau expression and the presence of multiple initiation sites in the promoter

region associated with the occurrence of TATA-less sequences, the absence of CAAT boxes and the abundance of G and C residues are characteristics common to housekeeping genes (Andreadis et al., 1996). However, the proposed working concept of housekeeping genes as ''those genes critical to the activities that must be carried out for successful completion of the "cell cycle'' (Warrington et al., 2000; Zhang and Li, 2004), together the fact that tau levels can fluctuate, indicate that those tau characteristics are not enough to hypothesize that idea and therefore it could be considered better as a pleiotropic protein than as a housekeeping.

II. Nuclear tau: a possible role in nucleolar organization

The nucleolus is the best characterized example of a large scale chromatin organization. The tandem organized human's ribosomal RNA (rRNA) genes at five different chromosomal locations, come together to form the nucleolus in G1 when rRNA transcription is activated following exit from mitosis (Grummt, 2003; Raska et al., 2004).

This work confirmed the nucleolar localization of tau in contact with the internal periphery of the nucleolus partially co-localizing with nucleolar protein nucleolin, by double fluorescence labeling of human primary fibroblasts and proliferating HeLa cells with NOR-90, anti-nucleolin and Tau-1 antibodies. This co-localization was almost total in non-dividing fibroblasts whereas it was only partial in dividing HeLa cells. As nucleolar tau, nucleolin localizes at the dense fibrillar component of nucleoli. Nucleolin interacts with nascent pre-rRNA transcripts as well as with several ribosomal proteins.

It has been implicated in chromatin structure, rDNA transcription, rRNA maturation, ribosome assembly and nucleo-cytoplasmic transport (Bouvet et al., 1998; Ginisty et al., 1999; Johansson et al., 2004; Roger et al., 2003). Nucleolin is characterized by a N-terminal half including multiple phosphorylation sites (Belenguer et al., 1990) linked on both sides to stretches of acidic amino acids such as glutamic and aspartic acid, a central domain constituted by four RNA-binding domains (RBD) and a C-terminal part including a glycine-arginine rich domain (RGG) (Angelov et al., 2006; Caizergues-Ferrer et al., 1989) to binds RNA non-specifically with low affinity (Ghisolfi et al., 1992).

Partial co-localization of tau with nucleolin suggests that both proteins could interact, reinforcing the hypothesis of a possible role for tau during nucleolar organization. Besides tau, although not as acidic as nucleolin, contains an acidic amino-terminus that could interact with histones and a basic carboxy-terminus, at the MBD that could interact with the acidic region of nucleolin or with DNA. In this regard co-immunoprecipitation experiments of tau with nucleolin and histones would allow to examinate those interactions.

Interestingly others finding emphasizes different functions for nucleolus rather than the main role of ribosomal biogenesis. Evidence reveal that it can function as a stress sensor for the cell, by down-regulating rRNA synthesis (Dillon, 2006; Rubbi and Milner, 2003). Stress conditions that inhibit RNA Pol I lead to perturbation of nucleolar structure and release of ribosomal proteins into the nucleoplasm. These proteins associate with and block the activity of MDM2 ubiquitin ligase, which is normally responsible for ubiquitination of p53. As a result, p53 is stabilized, triggering cell cycle

arrest and apoptosis (Mayer et al., 2005). Under cell stress nucleolin was reported to translocate from the nucleolus to the nucleoplasm by a p53-dependent mechanism, in which nucleolin is mobilized for transient replication inhibition and DNA repair following heat shock (Daniely et al., 2002). In this framework, the idea of tau as a heat shock protein may have sense since here was shown that phospho-tau epitopes (AT8) associated to AD which also is linked to cell stress, instead of a nucleolar staining presented a nucleoplasmic distribution, analogous to the nucleolin behavior under similar conditions.

According to this, the relevance of nucleolar tau could be questionable based on the results obtained using anti-sense strategies. Thurston et al. (1996) once had described the nucleolar localization of tau, reported that the nucleolar morphology remained unchanged after transient transfection experiments with tau anti-sense (Thurston et al., 1997). Nucleoli are complex structures whose formation are regulated during the cell cycle and require the participation of several factors. Therefore it would not be expected that transient knock-down of only one of the factors participating on nucleolar structure would induce strong morphologic changes at nucleoli. Similar observations have been made for cytoplasmic tau, in a tau deficient mice where while altered microtubule axonal organization and inhibition of neuronal maturation in primary hippocampal neurons was observed, any particular strong phenotype was displayed (Dawson et al., 2001; Harada et al., 1994). Defects in axonal elongation and neuronal migration were observed only after knocking out simultaneously tau and MAP1B protein (Takei et al., 2000). In this sense data obtained from pathological situations such as AD and DS, as well as from cell cycle stages analyses on tau, tau-

nucleolin interactions and their corresponding subcellular distributions would help to get an insight on the role of nucleolar tau on nucleolar organization and/or function.

An important role for heterochromatin during nucleolar formation has been suggested (Carmo-Fonseca et al., 2000). Centromeric heterochromatin displays a perinucleolar localization in human cells, especially in neuronal cells (Leger et al., 1994; Manuelidis, 1984; O'Keefe et al., 1992; Payen et al., 1998). A fraction of rRNA genes, that are maintained silenced in nucleoli of all cell types regardless of their metabolic activity, is organized as dense heterochromatin-like structures. They are located at a nucleolar region adjacent to the centromeric perinucleolar heterochromatin (Akhmanova et al., 2000; Carmo-Fonseca et al., 2000). Coalescence of clusters of rRNA genes from different chromosomes would be required for nucleolar formation and integrity in human cells (Mirre et al., 1980). The mechanism governing this coalescence remains undetermined, however a possible link between perinucleolar heterochromatin silencing proteins and nucleolar integrity has been proposed (Carmo-Fonseca et al., 2000). Proteins such as Drosophila Modulo (Perrin et al., 1999); (Perrin et al., 1998) and Polycomb (Dietzel et al., 1999) and mammalian proteins pKi-67 (Bridger et al., 1998) and ATRX (McDowell et al., 1999) are heterochromatin-associated proteins that have been reported being able to display a nucleolar or NOR localization and therefore could potentially participate in rDNA silencing. Nonetheless, with the exception of the murine homolog of pKi-67 (Starborg et al., 1996), none of

these proteins have been described as being able to interact with perinucleolar centromeric heterochromatin while being localized inside the nucleolus.

In this study using conventional and confocal fluorescence microscopy it was shown that in human cells, clusters of nucleolar tau are systematically surrounded by and partially co-localize with α-satellite pericentromeric heterochromatin. In mitotic chromosomes, tau associates with NOR regions of acrocentric chromosomes that contain rDNA immediately juxtaposed to pericentromeric sequences (Thurston et al., 1996). From these observations we arise the hypothesis that nucleolar tau could create a link between rDNA repeats and pericentromeric heterochromatin, and by doing so participate in rRNA gene silencing and/or nucleolar organization and integrity.

III. The implicances of tau binding AT-rich pericentromeric satellite DNA sequences.

The capacity of nuclear tau to associate and interact with pericentromeric α-satellite DNA sequences was analyzed by using fluorescence microscopy and gel retardation. Fluorescence confocal microscopy experiments have shown that nucleolar tau localized closely to perinucleolar heterochromatin, partially overlapped with clusters of pericentromeric α-satellite sequences in primary human fibroblasts and lymphoblast as well as in transformed HeLa cells. In vitro gel retardation experiments clearly demonstrate that tau had the capacity to directly bind to α-satellite DNA sequences. The interaction of tau with α-satellite DNA occurred even in the presence of high excess of

double-stranded sonicated salmon sperm DNA suggesting a potential sequence specificity of tau towards pericentromeric α-satellite sequences. Under the same conditions, tau also formed protein-DNA complexes with murine pericentromeric γ-satellite DNA sequences. Even though human α- and murine γ- satellite repeats do not share the same DNA sequence, both display a strong AT-rich base content. The affinity of tau towards AT-rich DNA was confirmed by the capacity of poly dI/dC polymers to fully compete for tau/α-satellite and tau/γ-satellite complexes whereas, under the same conditions, neither salmon sperm DNA nor random plasmid DNA fragment or poly dG/dC DNA were able to fully compete for these complexes. Synthetic poly dI/dC polymers have been described to share common structural properties with poly dA/dT molecules (Lavery and Pullman, 1981) so that proteins that bind DNA through the minor groove, displaying a marked preference for (A+T)-rich DNA fragments also display a high affinity for poly dI/dC polymers (Bailly et al., 1996; Brown and Anderson, 1986). Specificity for AT-rich DNA sequences has been previously described for several architectural chromatin-associated proteins such as HMG proteins, especially HMGI/Y (Bustin and Reeves, 1996) (Maher and Nathans, 1996) as well as for linker histone H1 (Kas et al., 1989).

That could also be the case for nucleolar tau protein when interacting with human and murine satellite DNA sequences. The basic MBD of tau could mediate the interaction with satellite DNA sequences and perhaps with rDNA. Interestingly, the high mobility group 1/2 of proteins (HMG1/2) can interact with DNA by non-sequence-specific DNA recognition. HMG1/2 proteins are considered modulators of the

chromatin structure (Bustin and Reeves, 1996) and interact directly with nucleosomes (Nightingale et al., 1996) to facilitate DNA recombination; repair, activation (Shykind et al., 1995) and repression (Ge and Roeder, 1994). Their affinity for DNA can be modulated by its basic C-terminal tail, ranging from micromolar to nanomolar (Churchill et al., 1999). This interaction consist in water-mediated hydrogen bonds between a Ser residue and the DNA bases, providing a mechanism by which these chromosomal proteins bind to DNA in the minor grove on the outside of a smoothly bent AT-rich DNA sequence (Murphy et al., 1999). Direct and water mediated hydrogen bonds to DNA bases are generally considered to be sequence specific interactions (Pabo and Sauer, 1992). Nevertheless it is well known that the DNA minor groove has more degeneracy in hydrogen bonding donor and acceptor positions among possible base pairs than the major grove (Murphy et al., 1999).

The tau-DNA interaction specificity demonstrated either for human or murine satellite DNA, strengthen the alternative that tau like as HMG proteins, could bind to these sequences through contacts between Ser or Thr residues and AT rich satellite DNA sequences, based on water mediated hydrogen bonds at the minor grove (Churchill et al., 1999).

IV. The molecular nature of nuclear tau species.

Identification of nuclear tau in cycling cells as well as in AD brains most likely due to reactivation of mitotic mechanisms prompts the question if nuclear tau levels are cell cycle-regulated and consequently which is the molecular nature of nuclear tau

proteins. The intricated panorama offered by the diverse tau subcellular distributions, and isoforms motivated the molecular characterization of the nuclear tau protein in SHSY5Y cells. Fractionation techniques allowed investigating and corroborating the existence of nuclear tau proteins using the antibodies Tau-1, Tau-5 and AT8. Among the nuclear tau proteins identified, three main species with molecular weights around 64, 69 and 74 kD were recognized by Tau-1, suggesting that they share the epitope S202 unphosphorylated and that they apparently include an additional modification interfering with Tau-5 binding. Besides two others nuclear tau species were also identified, one with molecular weight close to 55 kD reactive to AT8, suggesting its phosphorylation at residues Ser202/Thr205 and a small nuclear tau specie around 30 kD that was also concentrated in nuclei fractions. The small tau isoform (26-30 kD) was previously described within nuclei of these human neuroblastoma cells and the presence of N and C terminal epitopes confirmed that it is not derived by simple cleavage of larger tau isoforms (Shea and Cressman, 1998). The triplet of nuclear tau species sensitive to Tau-1, unreported in nuclei before, show strong similitude with the pattern of bands between 55 and 74 kD, previously described for PHF-tau preparations from brains of Alzheimer's disease and Down's syndrome patients (Fig. 6) (Goedert et al., 1992a). The interpretation of the pattern observed herein is difficult because there are six tau isoforms in normal brain and phosphorylation produce subtle changes in their mobility. However these could represent normally modified forms of tau because of Tau-1 staining, perhaps phosphorylated but at epitopes targets of cyclin dependent kinases such as cdc2 (considering the presence of nuclear tau in cycling cells) and/or of stress activated protein kinases. An additional experiment of enzymatic

dephosphorylation and deglycosilation would allow evaluating the contribution of both modifications to the molecular weight of each nuclear tau specie as Goedert et al (1992) observed for the three abnormal bands that align with the six unmodified tau isoforms (Fig. 29).

Moreover, the different nuclear tau species identified with Tau-1 and AT8 are congruent with the differences in the immunostaining patterns of each antibody, suggesting that differentially modified tau proteins can be localized in the nucleus, either in the nucleoplasm or at the nucleoli depending at least on the modification status.

Finally by both immunobloting with Tau-1, AT8, and Tau-5 and IEF experiments the isoelectric point of nuclear tau species was investigated. Nuclear tau species migrate at extremely basic pI and were not efficiently separated. This can be explained because basic proteins are difficult to resolve in 2D gels and additionally because the high salt concentration present in the buffer where nuclear proteins are maintained can interfere with the ionic force during the electrophoretic running. Therefore after elimination of salts content, the isoelectrofocusing and immunoblot analysis with Tau-1, resulted in the identification of two different groups of spots corresponding to nuclear tau species with isoelectric points between 8.5-9.5 and 7.0-8.0 respectively, and with molecular weights ranging between 50-75 kD.

It is well-known that the net charge of individual proteins depends on several factors, such as amino acid composition and posttranslational modifications including phosphorylation, dephosphorylation and glycosilation.

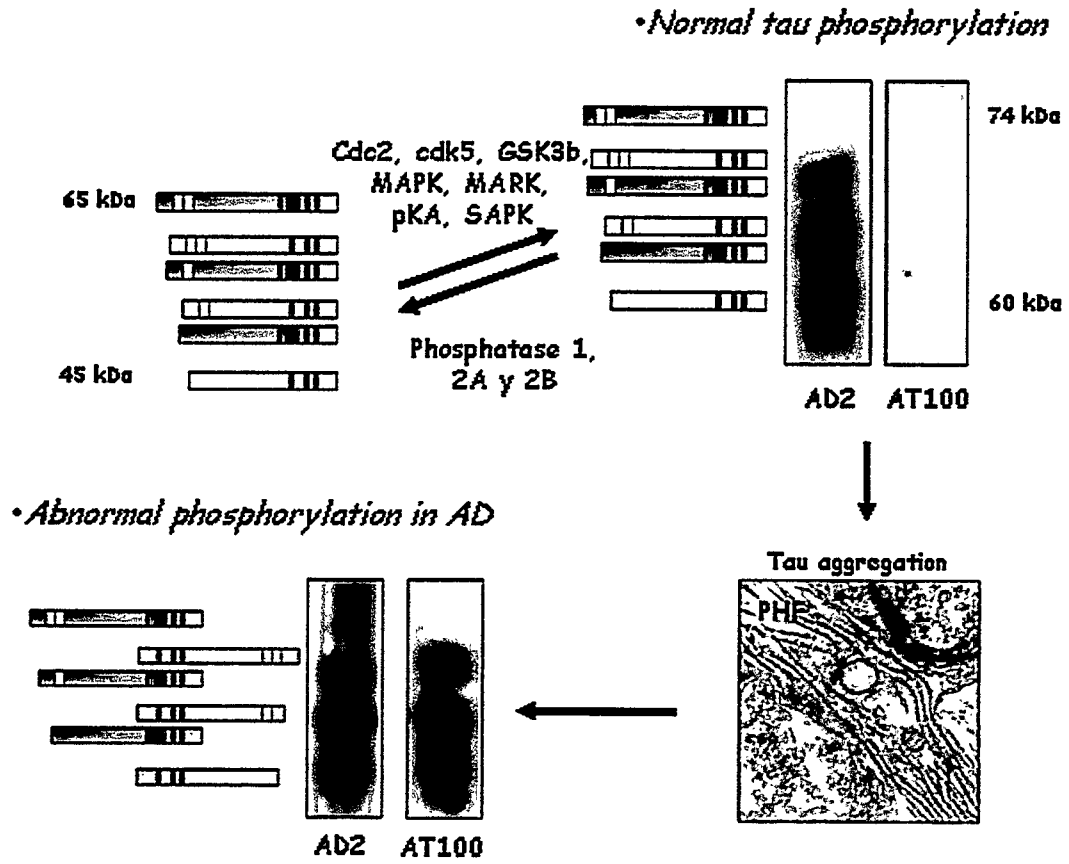


Image from Hamdane M. et al., 2003

Figure 29. ***Electrophoretic profiles of normal and pathological tau isoforms.*** Schematic representation of the modifications leading to Tau proteins aggregation in AD. In human brain, there is a balance phosphorylation–dephosphorylation of tau proteins allowing microtubule dynamics. Tau proteins are detected by anti-phospho-Tau antibodies but do not display Alzheimer-type epitopes including those of AT100 and TG-3. In the brains of patients exhibiting AD, tau proteins aggregate into paired helical filaments (PHF). Aggregated tau proteins are found abnormally phosphorylated within these filaments and exhibit Alzheimer-type epitopes (AT100/TG-3) (from Hadame et al., 2003).

Thus phosphorylation tends to increase the negative charge of a protein and therefore shift the protein's migration toward the anode (+), whereas dephosphorylation tends to decrease the negative charge of the protein and thus shifts the protein toward the cathode (-). On the other hand, the glycosilation tends to increase the positive charge of the protein and in certain cases when large chains of glycans are added, it modification can also increase lightly the molecular weight of the protein. The result is a shift of the protein toward the cathode in the first dimension and toward higher molecular weights in the second dimension.

Contrary to cytoplasmic tau-PFH proteins, those found in the nuclei show a basic character suggesting that they share a low degree of phosphorylation. These nuclear species as many nucleocytoplasmic proteins including transcription factors could be modified by O-GlcNAcylation, among other post-translational modifications, sustaining the basic pI. Besides the reduction in the molecular weight observed for basic nuclear tau species, can also suggest the presence of alternative glycosilations, like lysine glycation (Nacharaju et al., 1997) or another one, since moieties such as N-acetylglucosamine can be partially removed from proteins by methanol precipitation.

V. Hypothetic function of nucleolar tau in S phase of the cell cycle.

Since tau was found in nucleoli and share the ability to interact with α-satellite DNA, the idea of cell cycle regulation of nuclear tau levels was elucidated after molecular characterization of nuclear tau. The levels of the proteins were examined at

different times points after cell cycle synchronization. The data showed that over a base amount, the level of nuclear tau became almost double once the proportion of cells progressing into S phase begin to increase and are maintained until the cells begin to progress into G2/M phase. Although a high degree of synchronization was observed for the three independent assays at time 0 (G1), the procedure of synchronization allowed similar but not exactly the same timing of progress through different cell cycle phases. A plausible explanation resides on the behavior that cell populations have in front perturbations such as chemical synchronization. Helmstetter et al. (2003) explain this on the basis that this sort of protocols interrupts the normal progress of the cycle in cells that are at different cell cycle stages, inducing stress, and provoking metabolic alterations concomitant with a relative grade of synchrony that it is not conserved longer than two generations (Helmstetter et al., 2003). Even though the cellular synchronization was not optimal, the change and increase on the nuclear tau levels observed herein suggest first, that nuclear tau is modulated by proliferative and cell cycle signals and second, that nuclear tau participates in a functional process during the S phase or in the passage of G1 to S phase.

Conceptually, the cell cycle is divided into two main parts: 'mitosis' (M) and 'interphase' (G1, S, G2) (Fig. 30A). Studies have led to the consensus that mitosis is associated with extensive morphological changes delineating well defined stages while biochemical methods must be used to partition the interphase into the phases, G1, S, and G2. Further studies are proposed to elucidate changes in the nuclear tau levels and distribution using different approaches. The technique of combining BrdU labeling with in situ hybridization, and examination by confocal microscopy will allow the

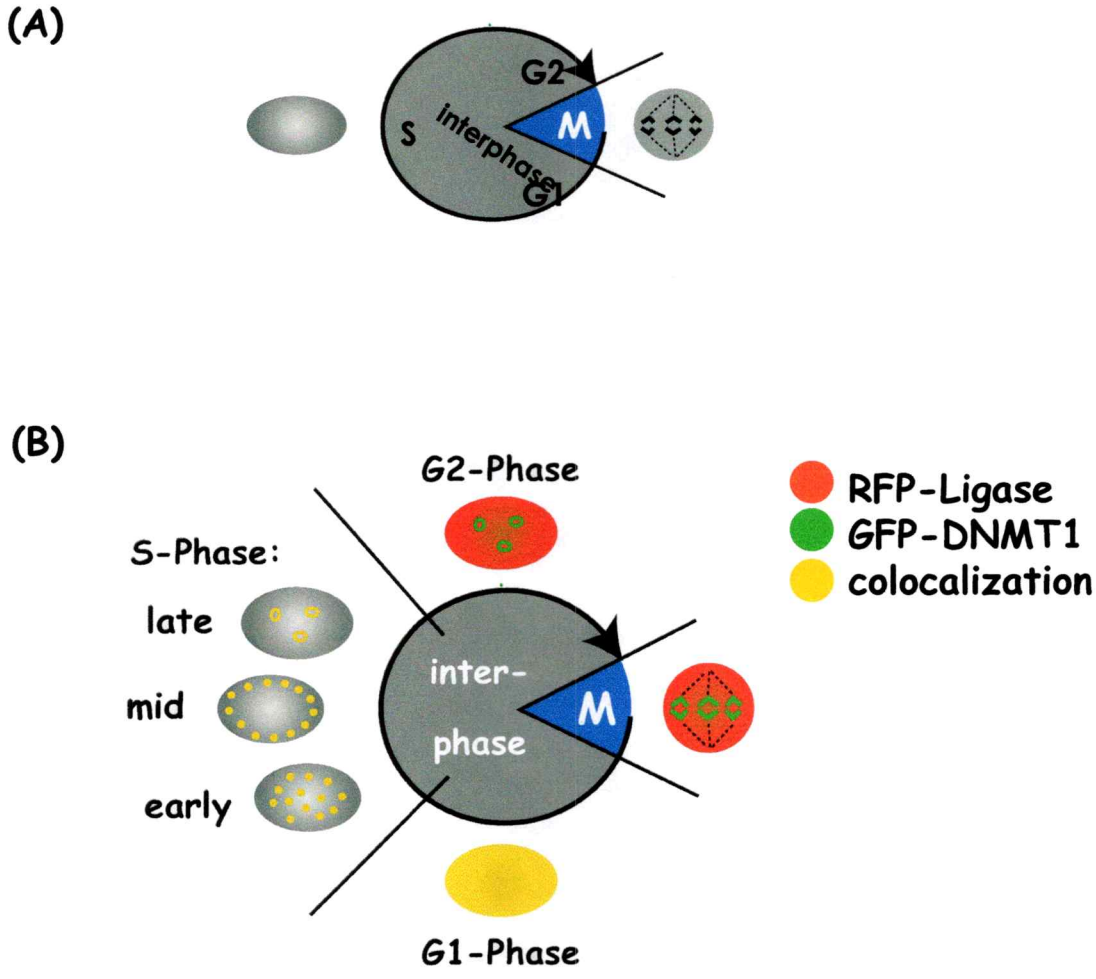


Figure 30. ***Cell cycle markers for live and fixed cells.*** (A) Only mitosis (M) and interphase can be identified in live cells, based on the cellular and nuclear morphology. (B) In live cells expressing RFP-Ligase (red), G1 and G2 phase can be identified by a diffused Ligase dsitribution at the nucleus, M phase because it is excluded from the chromatin and S phase by the different punctate pattern corresponding to replication sites (C) Expression of both GFP-DNMT1(green) and RFP-Ligase (red) allows identification for the cell cycle phases in fixed as well as live cells. Co-localization of RFP-Ligase and GFP-DNMT1 at RF during S phase is shown in yellow. During G2, only GFP-DNMT1 is bound to pericentric heterochromatin (shown as green "donut" shaped structures) while RFP-Ligase is diffused. During mitosis GFP-DNMT1 is at the chromatin. In G1, both RFP-Ligase and GFP-DNMT1 are diffused (shown as yellow) (from Easwaran et al 2005).

determination of the timing of replication of specific genes. rDNA probes can be hybridized in situ to BrdU and tau labeled cells. Moreover, Ligase I and Dnmt1 are also good markers to identify cell cycle stages since they show specific immunostaining patterns (Easwaran et al., 2005) (Fig. 30B), which linked to tau immunostaining, will provide accurate information about the cell cycle regulation of nuclear tau distribution.

The replication of eukaryotic chromosomes taking place throughout the phase of DNA synthesis occurs in a fashion that early and late replicating chromosomal domains appear to localize to distinct spatial compartments of the nucleus, at defined times during S phase. In general, transcriptionally active chromatin replicates early in S phase whereas transcriptionally inactive chromatin replicates at any interval during S phase (O'Keefe et al., 1992). The more condensed heterochromatin, which is typically found at centromeric regions, has been shown to replicate during late-S phase (Ten Hagen et al., 1990). However some differences have been observed. For example in mouse NIH3T3 cells, while the bulk pericentromeric heterochromatin replicates exclusively during mid to late S phase, centromeric DNA domains associated with constitutive kinetochore proteins are replicated throughout all stages of S phase (Weidtkamp-Peters et al., 2006). The location of a gene on a chromosome as well as the chromatin structure, are factors that affect its timing of replication (Calza et al., 1984; Dhar et al., 1989). In the interphase nuclei of human and mouse central nervous system cells, specific chromosome domains (centromeric alphoid repeats) have been shown to be organized in a reproducible manner (Manuelidis and Borden, 1988). Consequently with the data obtained from cell synchronization experiments, α-satellite DNA sequences,

localized to either the nucleolar surface and/or the nuclear periphery (peripheral heterochromatin), has been described to replicate during mid S-phase (O'Keefe et al., 1992) or late in S phase; with slightly different replication times for independent chromosomes (Ten Hagen et al., 1990). Thus the tau-satellite DNA association at the nucleolar periphery may be affected by the progress of the cell cycle.

Preliminary data describes the capacity of tau to bind single strand DNA and double strand DNA inducing its dissociation (Krylova et al., 2005). Since tau was always found in nucleoli just partially colocalizing with satellite DNA and dimethylH3K9 the interaction of tau with nucleolar components such as rDNA is suggested. Therefore tau may have the ability to interact with two different types of DNA. A hypothesis is that major levels of tau would be needed in the nucleolus during S phase to unfold the fraction of repressed rDNA sequences for further replication.

Based on data that describes the capacity of tau: (i) to induce conformational changes in the DNA forming super-coiled structures (Qu et al., 2004), and (ii) to prevent DNA from thermal denaturation and improve renaturation (Hua and He, 2003), an alternative hypothesis could also be propoused. In this nucleolar tau would stabilize the dsDNA helix of rDNA immediately after replication of them during S phase, participating in chromatin condensation. In this context the interaction of tau with satellite DNA would help to keep repressed a subset of rDNA, packaged into heterochromatin at regions in the nucleolar periphery, which in turns helps to avoid either unwanted recombination or failures in chromosome disjunction related to rDNA repeats. This hypothesis would be further supported by the localization of methylated inactive rDNA adjacent to perinucleolar heterochromatin (Akhmanova et al., 2000),

which suggests that rDNA genes, methylation and transcriptional repression are linked processes.

In both hypotheses nuclear tau, either abnormally modified or mutated would fail to perform those proposed physiological functions.

Neurons as postmitotic cells do not experiment DNA replication and chromosome segregation, thus the establishment of stable nuclear structures cover optimally the requirements of transcription and RNA processing. Therefore re-entry into the cell cycle is likely deleterious in terminally differentiated neurons and contribute to the biochemical abnormalities, such as hyperphosphorylated tau protein, oxidative stress and finally neuronal degeneration characteristic of the pathology of AD.

VI. Posttranslational modifications modulating the nuclear tau localization.

The mechanism by which some proteins targets to the nuclear compartment is not immediately evident from the analysis of the primary sequence. Mechanisms underlying the transit of tau to the nucleus are still unclear. Most nuclear localization signals (NLS) are short sequences of 8-10 basic amino acids. A strict consensus sequence for nuclear tau import has not been described and the three or four MBD has been proposed to represent a NLS. However transfection experiments in CHO cells, indicated that expression of tau containing those MBD is not enough to induce the nuclear targeting of tau (Wang et al., 1993).

Alternatively, nuclear targeting of tau has been hypothesized to be accomplished via a message targeting mechanism mediated by untranslated regions (UTR) of the tau

message. The targeting to the nucleus through UTR has been postulated for several mRNAs (Kloc et al., 2002; Singer, 1992). This idea suggests that differences in tau localization could relay in differential targeting catalyzed by UTR. Supporting this idea, transfection experiments of the 2-kb tau mRNA in CHO cells allowed to speculated that the UTR of the 2-kb mRNA in humans may serve to target the mRNA to regions of the cytoplasm, specifying both translation and posttranslational modifications specific for nuclear and non-microtubule cytoplasmic functions (Wang et al., 1993).

The work presented here evaluates the role of Pin1 in the nuclear localization of tau. We postulated to Pin1 as a candidate involved in the mechanism of nuclear targeting of tau, mainly because it is an important mitotic regulator that interacts with tau in a context that fits well with the hypothesis of nuclear tau localization exclusively in cycling cells. Pull down assays demonstrated that both proteins interact strongly in the cytoplasmic fractions. However despite this interaction could explain the further transport, inhibition experiments of Pin1 activity does not reveal a significant reduction on nuclear tau levels, discarding the direct involvement of Pin-1 in the mechanism underlaying its nuclear localization.

Conversely non-peptidic nuclear targeting signals may be provided by post-translational modifications, such as O-GlcNAcylation. Indeed O-GlcNAc moieties have been implicated in the nuclear targeting of signal-free neoglycoproteins (Duverger et al., 1995; Duverger et al., 1996). Besides, O-GlcNAcylation may occur reciprocally with O-phosphorylation. An example is the transcriptional regulator c-Myc, where point mutations of dominant sites of O-GlcNAc affects their nuclear transport (Kamemura and Hart, 2003). Also, a recent study demonstrated that O-GlcNAc moieties contribute

to nuclear localization of alpha4 phosphoprotein (Dauphinee et al., 2005), which lacks any classical or annotated NLS evident from the primary sequence (Cokol et al., 2000).

In SHSY5Y cells the selective inhibition of β-N-acetylglucosaminidase with STZ (Liu et al., 2000) shows that O-GlcNAcylation positively participates in the nuclear localization of tau. In addition the inhibition of GSK3β increased the nuclear levels of tau. A recent report demonstrated that tau O-GlcNAcylation negatively regulates its phosphorylation at Ser-199, Ser-202, Thr-205, Thr-212, Ser-214, Sr-262, and Ser-396 (Liu et al., 2004). Interestingly most of these residues are target sites of phosphorylation by GSK3β (Table 4), sugesting that a balance between both modifications could be a mechanism involved in the nuclear targeting of tau. These results also reveal that O-GlcNAcylation is implicated in the nuclear transport of tau and that it could functions as a non-peptidic NLS. Supporting this proposition is the fact that hyperphosphorylated tau proteins with a reciprocal reduction in O-GlcNAcylation, showed reduced nuclear transport (Lefebvre et al., 2003b).

Under the light of previous and the present results of this PhD thesis, a model summarizing the nuclear tau species, its post-translational modifications and their nucleolar functional interactions is proposed (Fig. 31A, B).

VII. Potential links between nucleolar tau, Alzheimer's disease and trisomy 21

Interaction of tau with DNA has been reported to be aggregation-dependent in a way that aggregated tau looses its capacity to interact with DNA (Hua and He, 2002).

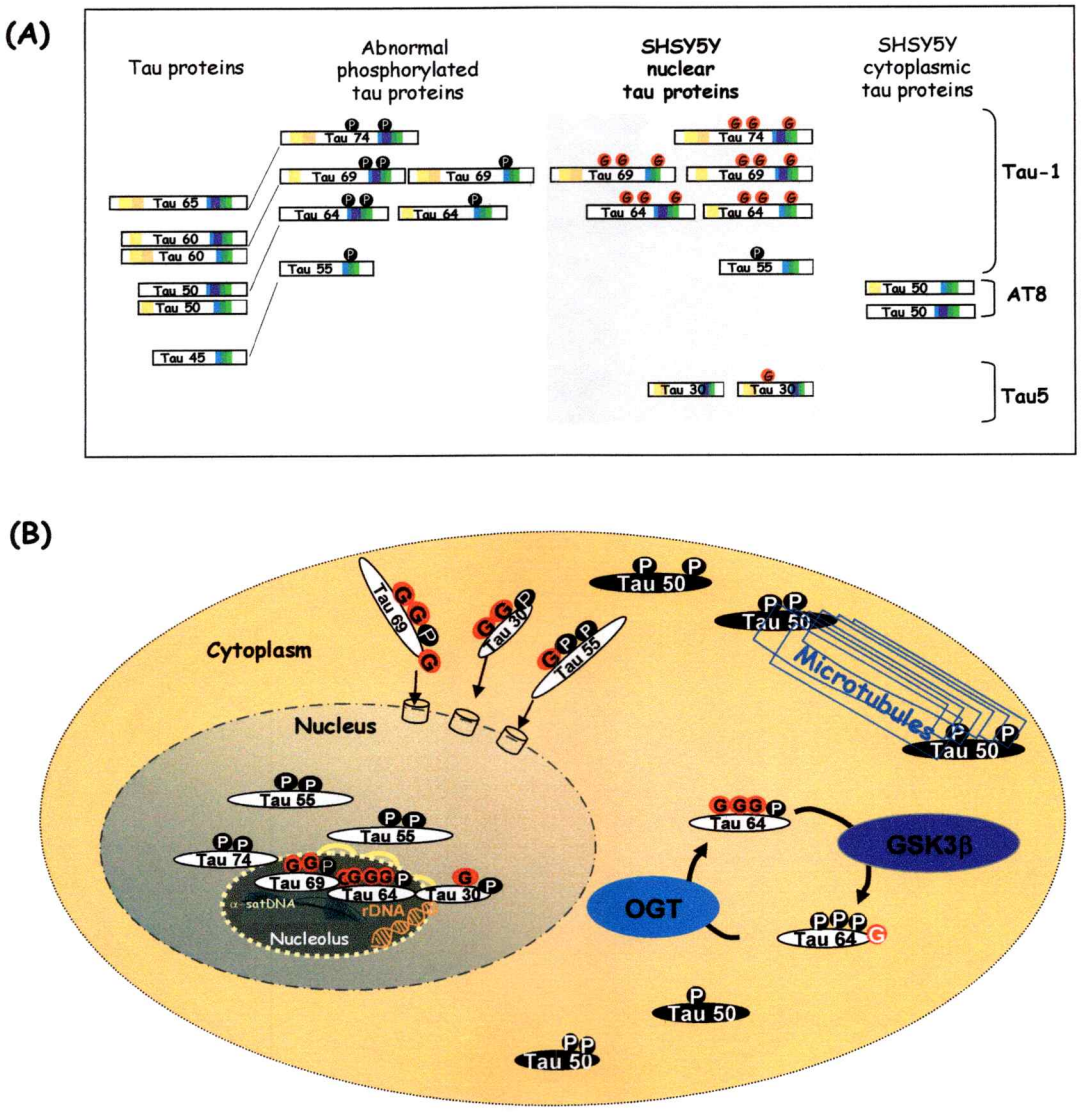


Figure 31. ***Schematic overview of the results.*** Representation of tau modifications leading to nuclear or cytoplasm localization. (A) The diagram depicts the nuclear and cytoplasm tau species found in SHSY5Y cells compared to the normal and abnormal tau proteins previously described by Hadame et al., 2003. (B) An schematic overview and model of the results. In the cytoplasm the specific tau species postulated to be modified by O-GlcNAc travel throw the nuclear pore complex to the nucleus. Some of them phosphorylated at AT8 epitope are localized in the nucleoplasm (Tau 55), whereas others (Tau 30, Tau 64, Tau69, Tau 74) localize at the nucleolus. At the nucleolus they would interact with satellite DNA localized at the nucleolar periphery and perhaps with rDNA and/or nucleolar proteins, participating in events that take place during the passage of G1 to S-phase or during S phase of the cell cycle.

Since aggregates of tau are formed during Alzheimer's disease (AD) it would be interesting to analyze the capacity of nucleolar tau to interact with pericentromeric α-satellite heterochromatin in AD neuronal cells. A strategy to solve and investigate the tau-DNA association *in vivo* would formaldehyde crosslinking followed by ligation and PCR analysis of ligated products (Orlando, 2000). Another alternative would be UV laser photofootprinting to lid with the problem of unspecific interactions resultant from formaldehyde crosslinking (Pashev et al., 1991).

The repetitive nature of a given DNA sequence could be a primary cause of its participation in heterochromatin, since repeated DNA is often repressed constitutively to avoid unwanted recombination events (Henikoff, 2000). In eukaryotes the fraction of repeated of rDNA genes that are not actively transcribed are constitutively represed and positioned in a particular region at the nucleoli. It has been suggested that the heterochromatin structure of silenced rRNA genes protects rDNA repeats from illegitimate recombination. In yeast, recombination of rDNA repeats induces cellular aging. As suggested by Akhamanova et al. (2000), such a phenomenon would have serious consequences in long life span cells such as neurons. A role of cellular aging in AD has been reported (Harman, 2002). A potential link could therefore exist between AD pathogenesis, tau aggregation-dependent disruption of tau/α–satellite interactions and cellular aging.

Nucleolar tau has been reported to localize in the short arms at the NOR regions of acrocentric chromosomes #13, 14, 15, 21 and 22 (Thurston et al., 1996). Nondisjunction of these chromosomes leads to trisomy, of which only trisomy 21 is

viable. Most patients with trisomy 21 or Down syndrome (DS) that live over their fourth decade develop Alzheimer disease (AD) (Wisniewski et al., 1985). Although the association between trisomy 21 and AD pathogenesis is not clear, the presumable reason is the lifelong over-expression of the amyloid precursor protein (APP) gene localized on chromosome 21, and the consequent overproduction of Aβ-amyloid in the brains of these patients. However another link between both pathologies could be related to nuclear tau, hence the incidence of trisomy 21 is enhanced in the offspring of families displaying familial AD (Wang et al., 1993 and references therein). Also preferential occurrence of chromosome 21 missegregation has been described in lymphocytes of AD patients (Migliore et al., 1999). The cause of nondisjunction of acrocentric chromosomes remains undetermined, however in human meiotic oocytes, NOR regions and satellite sequences from several acrocentric chromosomes (homologous and non-homologous) associate in a common nucleolus, conformation that could favors acrocentric chromosomal anomalies such as translocation and nondisjunction (Mirre et al., 1980). A role for tau during coalescence of NOR regions through the interaction with α-satellite DNA of acrocentric chromosomes, would imply a participation of abnormally modified nuclear tau during nondisjunction of acrocentric chromosome 21, establishing nucleolar tau as another potential molecular link between AD and trisomy 21.

Although the exact function of nuclear tau has not been clearly elucidated yet, the information supplied by the experimental results showed here, integrated with the

knowledge in related fields, shed light onto the molecular mechanisms underlying and modulating the nuclear tau localization and function. The particular nucleolar locatization, the specific association of tau to pericentromeric satellite DNA and the increase of nuclear tau levels when cells begin to progress from G1 to S phase of cell cycle, allow hypothesize that nuclear tau is likely to be either a modulator factor of the DNA structure and of the spatial organization of satellite DNA and possibly rDNA sequences at nucleoli or a factor involved in some aspect of rRNA synthesis, processing or transport before mitosis. In a cell prepared to undergo mitosis, interactions with histones throughout the nucleolus would occur to relax ribosomal chromatin. Participation of nucleolar tau in these interactions could be possible. Observations conducting this idea, suggest alternatively a role for tau in the conformation of the nucleolar structure and/or in heterochromatisation of a fraction of ribosomal genes. This idea is supported by the results of tau/nucleolin partial co-localization inside the nucleolus. Interestingly phosphorylated nucleolin alongside with abnormally phosphorylated tau, are both early markers for NFT during AD development both proteins being epitopes of the monoclonal antibody TG-3 generated against NFT found in AD (Dranovsky et al., 2001; Hamdane et al., 2003).

These findings integrated with previous published data shade new lights on the molecular nature of nuclear tau species and its postraslational modifications as well as on the understanding of its function and participation in events taking place during the phase of DNA synthesis.

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