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Caractérisation de l'interaction des protéines associées aux microtubules, MAP2 et Tau avec les organelles membranaires et le rôle de ces protéines dans le maintien de la structure de ces organelles

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RÉSUMÉ

Les neurones ont une morphologie polaire complexe qui détermine leur fonction. Ces cellules polaires émettent deux types de prolongements: l'axone et les dendrites. Ces deux compartiments cytoplasmiques se distinguent par leur rôle, leur morphologie, leur composition moléculaire mais aussi au niveau de leur contenu en organelles membranaires. Au niveau moléculaire, un des exemples les plus éloquents est la présence de protéines associées aux microtubules différentes dans ces deux compartiments cellulaires. Ainsi, la protéine MAP2 est somato-dendritique alors que la protéine Tau est enrichie dans l'axone. Pour ce qui est des organelles membranaires, le réticulum endoplasmique rugueux (RER) est retrouvé dans le corps cellulaire et les dendrites. Dans le cadre de nos études nous avons voulu déterminer la contribution des protéines associées aux microtubules (MAP2 et Tau) dans la ségrégation des organelles membranaires au cours du développement neuronal. Nos recherches ont permis de caractériser une nouvelle interaction entre MAP2 et le RER. De plus, nous avons montré que MAP2 interagit *in vivo* et *in vitro* avec la p63, une protéine intégrale de type II du RER et que cette interaction serait médiée par le domaine de projection de MAP2. Au cours de la différenciation neuronale *in vitro*, nos résultats ont montré que la p63 et MAP2 coségrègent dans le compartiment somato-dendritique. En outre, un essai de reconstitution *in vitro* a permis de conclure que la protéine MAP2 permet la liaison des microtubules au réticulum endoplasmique. Nos résultats indiquent que MAP2 pourait jouer un rôle dans la distribution préférentielle du RER dans le compartiment somato-dendritique en le liant aux microtubules. Dans une autre étude, nous avons examiné les interactions membranaires de la protéine Tau. Cette étude a révélé que Tau interagit les membranes golgiennes dans les neurones. Chez certains patients atteints de démences fronto-temporales, des

mutations dans le gène codant pour la protéine Tau ont été identifiées. Dans ces conditions pathologiques, Tau devient hyperphosphorylée, se détache des microtubules, perd sa distribution axonale et s'auto-agrège dans le compartiment somato-dendritique. Nous avons voulu déterminer les effets des mutations dans Tau, associées aux démences fronto-temporales, sur l'organisation de l'appareil de Golgi. Une étude quantitative et qualitative a montré que la surexpression de la forme humaine normale et de certaines formes humaines mutantes de Tau (P301L, V337M et R406W), dans des cultures primaires des neurones de l'hippocampe, induisent une fragmentation de l'appareil de Golgi. Nous avons également étudié la morphologie de l'appareil de Golgi dans une lignée de souris transgéniques, les souris JNPL3 qui surexpriment la mutation P301L. Dans les motoneurones de la moelle épinière de ces souris transgéniques, nous avons observé une fragmentation significative de l'appareil de Golgi. Notre étude a également révélé que cette altération serait spécifique à cette organelle et a lieu de façon précoce chez les souris transgéniques asymptomatiques. Ainsi, l'ensemble de nos résultats suggèrent un nouveau rôle de MAP2 et Tau dans la polarisation et le maintien de la structure des composants membranaires dans les neurones.

Mots-clés: Neurone, polarité neuronale, microtubules, MAP2, Tau, réticulum endoplasmique rugueux, appareil de Golgi, fragmentation, démences fronto-temporales.

SUMMARY

Neurons have a highly complex polarized morphology that contributes to their function. These polarized cells elaborate two different types of processes: axon and dendrites. These processes can be distinguished not only by their role, morphology, and molecular composition but also by their content in membranous organelles. At the molecular level, dendrites and axon contain different microtubules associated proteins (MAPs): MAP2 in dendrites and Tau in the axon. The rough endoplasmic reticulum (RER) is found only in the somatodendritic compartment. In the present thesis, we investigated the contribution of the neuronal MAPs in membranous organelles segregation and distribution in neurons. A subcellular fractionation analysis of adult rat brain revealed that MAP2 was found enriched in a subfraction containing the RER markers, ribophorin and p63. We also showed that MAP2 interacts *in vivo* and in vitro with p63, an integral RER membrane protein. This interaction involves the projection domain of MAP2. Furthermore, in an in vitro reconstitution assay, MAP2 microtubules and not Tau microtubules were observed to bind to RER membranes. These results suggest and thereby could participate in the differential distribution of RER membranes within a neuron during the establishment of neuronal polarity.

Moreover, in our subfractionation analysis, we found that Tau was present in a subfraction highly enriched in Golgi markers. In pathological conditions such as Alzheimer disease or fronto-temporal dementias (FTDs), the neuronal cytoskeleton is destabilized and neuronal polarity is lost. Genetic screening studies revealed that in some patients suffering from FTDs a mutation in Tau gene is present. In all these pathological conditions Tau is hyperphosphorylated, detaches from microtubules and looses its polarized distribution. Thus, we aimed to determine the effect of Tau mutations

associated with FTDs phenotype on the organisation of Golgi apparatus (GA) organization. Our study showed that overexpression of wild type or mutant forms (P301L, V337M and R406W) of human Tau induces a fragmentation of GA in primary rat hippocampal neurons. A similar phenotype was also observed in JNPL3 a transgenic model of FTDs. Fragmentation of GA was observed in a significant number of motor neurons from the spinal cord of JNPL3 transgenic mice. The alteration of GA was specific (no major changes in the organization of RER) and was an early event during neurodegeneration process in these transgenic mice. Indeed, in some transgenic animals, GA fragmentation occurred in asymptomatic mice even before the formation of neurofibrillary tangles. Collectively these results suggest a new role of the neuronal MAPs, MAP2 and Tau, in the polarization and in maintenance of the shape of the RER and the GA, respectively.

Keywords: Neuron, neuronal polarity, microtubules, MAP2, Tau, rough endoplasmic reticulum, Golgi apparatus, fragmentation, Fronto-temporal dementias.

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LISTE DES ABBRÉVIATIONS

a.a.....acides aminés

Aβ .....Amyloïde β

ABP..... «Actin-Binding-Protein»

ALS..... .....«Amyotrophic lateral sclerosis»

APP .....«Amyloid Precursor Protein»

ATP.....Adenosine triphosphate

CamKII .....«Calcium-calmodulin-dependent kinase II»

CDK.....«Cyclin-dependant Kinase»

CLASP.....« CLIP-associated protein»

CLIMP.....«Cytoskeleton-linking membrane protein»

CLIP .....«Cytosolic linker protein»

DMAP-85.....«Drosophila Microtubule Associated Protein-85»

ERKs .....«Extracellular signal regulated kinase»

FTDs .....«Fronto-Temporal Dementias»

FTDP-17....«FrontoTemporal Dementias with parkinsonism linked to chromosome 17»

GFAP.....«Glial Fibrillary Acid Protein»

Gsk3β.....Glycogène synthétase kinase 3β

GTP.....Guanosine Triphosphate

HMWMAP2 .....«High Molecular Weight MAP2»

IR .....«Inter-Repeat»

LMWMAP2 .....«Low Molecular Weight MAP2»

LTD.....«Long-term Depression»

LTP.....«Long-term potentiation»

MAPs.....«Microtubule-associated Proteins»

MARKs.....«Microtubule-affinity regulating kinase»

MARTA.....«MAP2-RNA Trans acting Protein»

Mt.....domaine de liaison aux microtubules

NFs.....Neurofilaments

NFTs .....«Neurofibrillary Tangles»

NMDA.....N-methyl-D-aspartate

PEM.....Pipes-EGTA-$MgCl_2$

PKA.....Protéine kinase A

PKC.....Protein kinase C

PMSF.....Phenyl-methyl-sulfonyl-fluoride

PNC.....Système Nerveux Périphérique

PP.....Protéine Phosphatase

ProC..... Domaine de projection de MAP2c

PRR.....Région Riche en Proline

RII.....sous-unité RII de la PKA

RE.....Réticulum endoplasmique

RER.....Réticulum Endoplasmique Rugueux

SAPKs.....«Stress-Activated-Protein-Kinase»

SM..... «Smooth membranes»

SNC.....Système Nerveux Central

VDAC .....«Voltage-dependent-anion channel»

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1. Introduction

1.1 Le neurone

Les neurones, unités fonctionnelles du système nerveux, sont responsables de recevoir, d'intégrer et de transmettre les influx nerveux. La fonction des neurones est étroitement liée à leur morphologie. En effet, ces cellules polaires développent deux types de prolongements cytoplasmiques: les dendrites qui reçoivent l'influx nerveux et l'axone qui le transmet.

L'acquisition de cette morphologie au cours du développement et son maintien à l'âge adulte sont déterminés par des facteurs intrinsèques et des facteurs extrinsèques. Parmi les facteurs extrinsèques, on retrouve l'activité neuronale (Toni et al. 1999; Marrone and Petit 2002) ainsi que les facteurs trophiques (McAllister et al. 1995; Labelle and Leclerc 2000). Par ailleurs, les éléments du cytosquelette neuronal, soit les microtubules, les microfilaments d'actine et les neurofilaments, fonctionnent comme déterminants intrinsèques qui, d'une part, supportent la croissance des prolongements neuronaux au cours du développement, et d'autre part, consolident la morphologie neuronale à l'âge adulte (Ludin and Matus 1993). Dans des neurones matures, des changements morphologiques peuvent aussi avoir lieu. De tels changements morphologiques ont été corrélés aux processus d'apprentissage et de mémoire (Marrone and Petit 2002).

Il est difficile d'étudier les mécanismes moléculaires qui conduisent à la polarité neuronale *in vivo*. Pour cette raison, des modèles expérimentaux alternatifs ont été élaborés. La culture primaire de neurones, à basse densité, représente un bon modèle pour étudier la différenciation neuronale puisqu'elle se produit selon une séquence similaire à celle obsevée *in vivo* (Banker and Goslin 1998). En effet, dans ces cultures, la différenciation

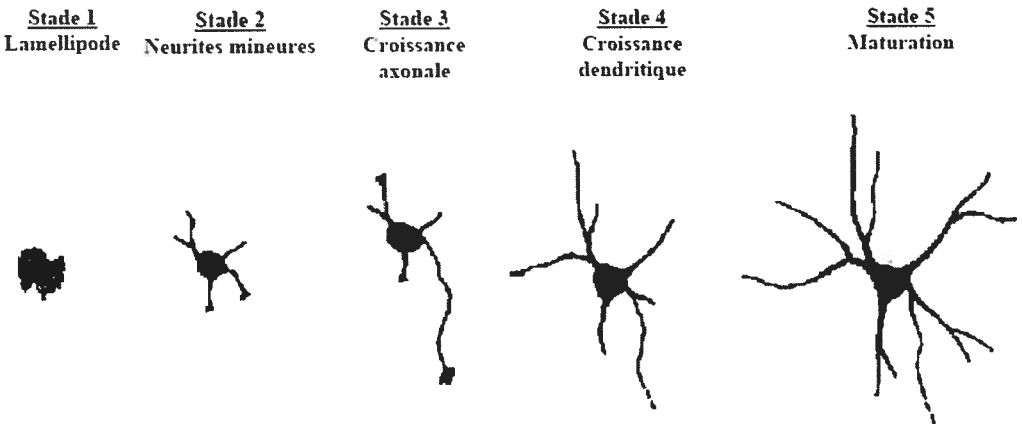
neuronale suit une séquence d'événements stéréotypés avec des étapes intermédiaires distinctes aboutissant à l'établissement de dendrites et d'axones bien différenciés et fonctionnels (Banker and Goslin 1998).

1.2. Les étapes de différenciation neuronale *in vitro*

Quelques heures après leur mise en culture, les cellules initialement apolaires et sphériques développent une lamellipode qui par la suite se fragmente en petits prolongements appelés neurites mineures. Une de ces neurites s'allonge plus rapidement que les autres et devient l'axone. Par la suite, les autres neurites mineures se développent et se différencient en dendrites. Enfin, la phase de maturation des neurones se caractérise par la formation de contacts synaptiques et des épines dendritiques (figure1).

Figure 1: Représentation schématique de l'établissement de la polarité neuronale dans les neurones de l'hippocampe en culture.

Les neurones de l'hippocampe en culture acquièrent leur morphologie neuronale polaire en suivant différents stades de développement bien définis. Lors du premier stade, immédiatement après la mise en culture, on assiste à la formation de lamellipodes. Dans les heures qui suivent, au stade 2, plusieurs prolongements courts (~20 μm) non différenciés se forment: ce sont les neurites mineures. Au stade 3, la polarité neuronale, devient plus évidente. En effet, une des neurites mineures s'allonge plus rapidement que les autres et acquière les caractéristiques morphologiques de l'axone. Par la suite, au stade 4, les autres neurites mineures se différencient en dendrites. Sept jours après leur mise en culture, l'axone et les dendrites continuent leur maturation (Dotti et al. 1988).


Stade 1
Lamellipode
Stade 2
Neurites mineures
Stade 3
Croissance axonale
Stade 4
Croissance dendritique
Stade 5
Maturation
Jour 0,25
Jour 0,5
Jour 1,5
Jour 4
Jour 7

1.3. La polarité neuronale

Les deux compartiments cytoplasmiques neuronaux se distinguent par leur rôle mais aussi par des critères morphologiques, moléculaires et au niveau de leur composition en organelles membranaires.

Morphologiquement, les dendrites sont multiples, relativement courtes et ont un diamètre large à la base qui diminue vers l'extrémité du prolongement alors que l'axone, généralement unique et relativement long (peut atteindre jusqu'à un mètre), a un diamètre uniforme (Banker and Goslin 1998).

La composition moléculaire des dendrites et de l'axone est aussi différente. Ainsi, certains éléments du cytosquelette neuronal (Craig and Banker 1994; Scott and Luo 2001), des récepteurs de neurotransmetteurs et des canaux ioniques sont retrouvés exclusivement ou préférentiellement dans l'axone ou dans les dendrites (Bradke and Dotti 2000; Tang 2001; Fukata et al. 2002). Par exemple, les microtubules dans les deux types de prolongements, se distinguent à plusieurs égards. Les microtubules sont des structures filamenteuses dynamiques qui possèdent deux extrémités: une dite «positive» à croissance rapide alors que l'autre est dite «négative» à croissance lente (Hirokawa 1982; Laferriere et al. 1997). L'orientation des microtubules dans l'axone et dans la partie distale des dendrites est uniforme, avec les extrémités négatives des microtubules dirigées vers le corps cellulaire, alors que dans la partie proximale des dendrites, l'orientation des microtubules est mixte (Burton and Paige 1981; Baas et al. 1988). De plus, le nombre, l'espacement et la composition de ces microtubules sont différents dans les deux types de prolongements (Bartlett and Banker 1984a; Hirokawa 1991). Finalement, les dendrites et l'axone se différencient par les protéines associées aux microtubules qu'ils contiennent (Hirokawa 1982; Hirokawa 1991). Ainsi, la MAP de haut poids moléculaire

MAP2 est principalement localisée dans le compartiment somato-dendritique alors que la MAP Tau est particulièrement enrichie dans l'axone (Caceres et al. 1984; Hirokawa 1991). La présence de MAPs différentes dans ces deux types de prolongements serait responsable de la différence de l'espacement entre les microtubules dans l'axone (~20 nm) et dans les dendrites (~65 nm) (Kanai et al. 1989; Chen et al. 1992; Belanger et al. 2002).

Par ailleurs, une distribution appropriée des organelles membranaires dans les différents compartiments cellulaires est primordiale pour l'établissement de la polarité neuronale (Craig and Banker 1994; Scott and Luo 2001). En effet, certains éléments membranaires sont indétectables voire exclus de l'axone des neurones matures. C'est le cas de l'appareil de Golgi (Horton and Ehlers 2003a), de certaines vésicules golgiennes (Setou et al. 2000) ainsi que le réticulum endoplasmique rugueux (RER) (Deitch and Banker 1993). En contrepartie, on retrouve par exemple dans l'axone un enrichissement important de vésicules membranaires qui transportent la protéine précurseur de l'amyloïde (APP) (Koo 2002).

En plus de ces différences au niveau morphologique, de la composition des éléments cytosquelettiques et membranaires, la concentration de calcium intracellulaire est différente entre l'axone et les dendrites (Mattson et al. 1990). En effet, dans les cultures primaires des neurones de l'hippocampe, il existe un gradient de calcium intracellulaire de sorte que les concentrations sont plus importantes dans les dendrites que dans l'axone (Mattson et al. 1990; Mattson and Partin 1999).Une perturbation de ce gradient de calcium intracellulaire, par l'utilisation de l'ionophore de calcium (A23187), est corrélée avec la suppression de la polarité neuronale (Mattson et al. 1990).

La compréhension des mécanismes qui conduisent à l'élaboration de domaines subcellulaires ayant une morphologie, une composition moléculaire et des propriétés fonctionnelles distinctes est fondamentale. Comment la polarité neuronale est établie et maintenue tout au long de la «vie» du neurone? Comment les constituants membranaires sont ségrégés dans l'un ou l'autre des compartiments pour assurer l'identité dendritique ou axonale? Toutes ces questions continuent encore à intriguer les biologistes cellulaires.

Comme mentionné précédemment, le cytosquelette neuronal est un déterminant intrinsèque majeur de l'architecture polaire des neurones (Ludin and Matus 1993; Mandell and Banker 1996; da Silva and Dotti 2002). Dans les prochaines sections nous allons présenter les différents composants du cytosquelette neuronal.

1.4. Le cytosquelette neuronal

Le cytosquelette est constitué de trois structures filamenteuses aux propriétés bien distinctes: les microfilaments d'actine, les filaments intermédiaires et les microtubules. Dans les neurones, ces structures permettent d'assurer à la fois la stabilité structurale nécessaire pour maintenir sa fonctionnalité et une certaine dynamique qui est indispensable aux changements morphologiques associés aux processus d'apprentissage et de mémoire.

1.4.1. Les microfilaments d'actine

Ces filaments polaires, de 5 à 8 nm de diamètre, polymérisent par l'addition de sous unités d'actine globulaire à leur pôle positif en présence d'ATP (Mitchison and Kirschner 1988). Il existe un grand nombre de protéines qui s'associent aux microfilaments d'actine (McGough 1998; Dos Remedios et al. 2003). Ces dernières peuvent assurer l'assemblage des monomères d'actine globulaire en actine filamenteuse, la réticulation des filaments en réseau, leur fasciculation, leur dépolymérisation et même leur liaison à la membrane plasmique (Hartwig and Kwiatkowski 1991). Dans la majorité des cellules, le réseau de microfilaments d'actine est cortical et se situe juste sous la membrane plasmique (Ludin and Matus 1993). Le réseau d'actine peut être hautement dynamique (Carlier 1993). En effet, la régulation de plusieurs protéines associées à l'actine, le plus souvent par un second messager, permet au réseau d'actine corticale de subir une restructuration locale pour répondre rapidement et efficacement à des signaux intracellulaires ou extracellulaires. Cette réorganisation peut permettre un changement morphologique ou une contraction de la cellule (Bray and White 1988; Dos Remedios et al. 2003).

Dans les cellules post-mitotiques, comme les neurones, les microfilaments d'actine ont un rôle très important dans l'établissement et le maintien de la morphologie neuronale. Au cours du développement neuronal, le réseau d'actine est impliqué dans le contrôle du guidage des cônes de croissance (Letourneau 1996; Dent et al. 2003). De plus, une forte concentration d'actine particulièrement dynamique est retrouvé aux épines dendritiques (Fischer et al. 1998; Micheva et al. 1998). Une réorganisation importante de l'actine au niveau de ces épines dendritiques se produit lors de

l'induction du phénomène de potentiation synaptique à long terme (LTP), forme élémentaire de mémoire au niveau cellulaire (Bliss and Collingridge 1993; Malenka and Nicoll 1999; Fukazawa et al. 2003) et l'inhibition de la polymérisation de l'actine altère le maintien de la LTP dans les neurones de l'hippocampe (Krucker et al. 2000; Fukazawa et al. 2003).

1.4.2. Les filaments intermédiaires

Les filaments intermédiaires sont caractéristiques du type cellulaire dans lesquels ils s'expriment. Ainsi, dans les cellules gliales, on retrouve la protéine fibrillaire acide des cellules gliales (GFAP) alors que dans tous les neurones on retrouve les neurofilaments (NF-L, NF-M et NF-H). En plus des neurofilaments (NFs), certains neurones peuvent exprimer une ou des protéines de filaments intermédiaires additionnelles telles que la périphérine, l'α-internexine et la nestine (Ho and Liem 1996).

Les NFs sont formés suite à la copolymérisation des trois sous unités NF-L, NF-M et NF-H. *In vitro*, la formation de dimères NF-L/NF-M ou NF-L/NF-H est nécessaire à l'assemblage des NFs (Ching and Liem 1993; Athlan and Mushynski 1997). L'assemblage antiparallèle et décalé de ces dimères donne des tétramères. Par la suite, huit tétramères s'assemblent pour produire un filament cylindrique de 10 nm (Julien and Mushynski 1998).

Les NFs joue un rôle important dans la stabilité axonale mais aussi dans le maintien du diamètre de l'axone (Hirokawa et al. 1984; Julien and Mushynski 1998). D'ailleurs, les NFs, plus nombreux dans l'axone que dans les dendrites, constituent un des principaux éléments du cytosquelette axonal (Hirokawa 1991).

Chez les patients atteints de sclérose latérale amyotrophique (ALS), on retrouve une accumulation anormale de NFs dans le corps cellulaire et les

axones des motoneurones (Hirano et al. 1984). Il a été proposé que des dérèglements dans les mécanismes d'assemblage et de transport des NFs contribueraient à la formation de ces agrégats dans cette maladie neurodégénérative (Julien and Mushynski 1998; Lariviere and Julien 2004).

1.4.3. Les microtubules

La tubuline est l'une des protéines les plus abondantes du système nerveux. En effet, elle représente 20% des protéines totales dans le cerveau adulte des mammifères. Les microtubules jouent un rôle essentiel dans le maintien de la structure des prolongements neuronaux ainsi que dans le transport intracellulaire (Laferriere et al. 1997). La quantité de tubuline dans le cerveau reflète l'importance des microtubules dans la morphologie des cellules nerveuses. En effet, le traitement des neurones en culture avec des drogues qui dépolymérisent les microtubules telles que la colchicine ou le nocodazole conduit à la rétraction des prolongements neuronaux (Matus 1988; Mandelkow and Mandelkow 1995).

Les microtubules sont formés de polymères de tubuline et de protéines associées aux microtubules (MAPs). Dans un premier temps, les isoformes de la tubuline α et β s'assemblent en dimères. Ces derniers polymérisent pour former un protofilament. Par la suite, 13 protofilaments s'assemblent en tubules de 25 nm de diamètre pour donner un microtubule (Laferriere et al. 1997). Pendant la division cellulaire, les microtubules sont dans un état d'équilibre hautement dynamique (instabilité dynamique), durant lequel il y a une alternance entre des périodes de polymérisation et de dépolymérisation (Laferriere et al. 1997). Dans les cellules post-mitotiques, les microtubules sont moins dynamiques que dans les cellules mitotiques. La plus grande stabilité des microtubules dans les neurones est attribuable à la

présence de protéines associées aux microtubules structurales (MAPs) (Laferriere et al. 1997). Ces protéines stabilisent les microtubules en diminuant leur instabilité dynamique (Drechsel et al. 1992; Takemura et al. 1992). Dans les neurones, on retrouve MAP1, MAP2 et Tau (Mandelkow and Mandelkow 1995) alors que dans les cellules en division expriment MAP4 (Chapin and Bulinski 1991).

Au cours des dernières années, plusieurs autres MAPs qui régulent la dynamique des microtubules ont été identifiées. Par exemple, la protéine «Stable Tubule Only Protein» (STOP) stabilise les microtubules en bloquant le recyclage de la tubuline et inhibant l'instabilité dynamique des microtubules (Bosc et al. 2003). D'autres protéines telle que LIS1 et doublecortine lient directement les microtubules et les stabilisent en diminuant leur instabilité dynamique (Horesh et al. 1999). Ces deux dernières sont aussi impliquées dans la migration cellulaire au cours du développement neuronal (Reiner 2000).

En contre partie, d'autres protéines s'associent aux microtubules et les déstabilisent. Ceci est le cas de la stathmine qui séquestre des sous unités de tubuline et provoque le désassemblage et la déstabilisation du réseau microtubulaire (Belmont and Mitchison 1996).

En plus de ces MAPs qui modulent la dynamique des microtubules, il existe deux autres classes de MAPs: les protéines motrices et les protéines qui médient la liaison entre les vésicules membranaires et les microtubules.

Les MAPs motrices sont capables de convertir l'énergie chimique, produite à la suite à l'hydrolyse de l'ATP, en une force mécanique leur permettant de se déplacer le long des microtubules. Ces protéines ont la capacité de détecter la polarité intrinsèque des microtubules (Vale et al. 1992). Cela se manifeste par l'existence de MAPs motrices qui assurent un

transport antérograde, les kinésines, et des MAPs motrices qui permettent le transport rétrograde, les dynéines (Hirokawa and Takemura 2005).

Récemment, plusieurs protéines impliquées dans la liaison entre les microtubules et différentes organelles membranaires ont été identifiées. Parmi ces protéines, on retrouve les «Cytoplasmic Linker Proteins» (CLIPs), les «CLIP-associating-proteins» (CLASPs) (McNally 2001; Mimori-Kiyosue and Tsukita 2003), les HOOKs (Walenta et al. 2001), GMAP-210 (Rios et al. 2004) et la SCG10 (Lutjens et al. 2000). Outre leur capacité de lier les organelles membranaires aux microtubules, certaines de ces protéines sont aussi capables de moduler la dynamique des microtubules. Par exemple, CLIP-115 et CLIP-170 stabilisent les microtubules (Komarova et al. 2002) alors que SCG10 et CLIPR-59 déstabilisent les microtubules (Gavet et al. 1998; Lallemand-Breitenbach et al. 2004).

Dans les prochaines sections, nous allons décrire les différents membres de la famille des MAPs structurales neuronales. L'expression et la distribution de ces dernières sont régulées de façon spatio-temporelle. Dans le cerveau humain, on distingue MAP 1a et 1b, MAP 2a, 2b, 2c et 2d ainsi que 6 isoformes de Tau (Hirokawa 1994; Mandelkow and Mandelkow 1995).

1.4.3.1 La protéine MAP1

Les deux isoformes de MAP1, MAP1a et MAP1b, sont transcrites à partir de deux gènes différents mais partagent une identité de séquence partielle (Schoenfeld et al. 1989). Au cours du développement neuronal, l'isoforme MAP1b serait la première MAP à être exprimée (Schoenfeld et al. 1989; Tucker 1990). L'expression de cette isoforme augmente pour atteindre son maximum au cours des premières phases du développement neuronal

(Schoenfeld et al. 1989; Diaz-Nido et al. 1990; Garner et al. 1990). Par la suite, l'expression de MAP1b diminue graduellement alors que celle de MAP1a augmente significativement (Matus 1988; Schoenfeld et al. 1989; Garner et al. 1990). L'apparition de la protéine MAP1a est post-natale et coïncide avec une phase de maturation de la circuiterie neuronale (Schoenfeld et al. 1989; Garner et al. 1990). Il est intéressant de souligner qu'à l'âge adulte, la protéine MAP1b est détectée uniquement dans les régions du cerveau reconnues pour avoir une activité continue de neurogenèse, comme l'hippocampe, le bulbe olfactif et la rétine (Schoenfeld et al. 1989; Diaz-Nido et al. 1990; Ludin and Matus 1993). Les protéines MAP1a et MAP1b sont exprimées de façon préférentielle dans les cellules du système nerveux et sont détectées dans tous les compartiments neuronaux (Gordon-Weeks et al. 1993; Ulloa et al. 1994; Gonzalez-Billault et al. 2004).

Le domaine de liaison aux microtubules de MAP1a et MAP1b, localisé dans la partie amino-terminale de la protéine, n'est pas homologue à celui de MAP2 et Tau (Noble et al. 1989). Ce domaine est constitué d'une région basique contenant un motif de 4 aa. répétés (Noble et al. 1989).

Plusieurs études ont montré le rôle de MAP1b lors de la différenciation neuronale *in vitro* et lors du développement du système nerveux murin. *In vitro*, MAP1b a la capacité de stabiliser les microtubules mais de façon moins efficace que MAP2 (Vandecandelaere et al. 1996). Dans des cellules non neuronales, la surexpression de MAP1b induit des effets modérés sur l'organisation des microtubules (Noble et al. 1989); par exemple elle n'induit pas la formation de faisceaux de microtubules (Takemura et al. 1992), contrairement aux effets dramatiques engendrés par la surexpression de Tau ou de MAP2. En outre, dans les cellules PC12 ou dans des cultures primaires de neurones, l'inhibition de l'expression de

MAP1b par l'ajout d'oligonucléotides anti-sens dans le milieu de culture diminue ou bloque la pousse neuritique (Brugg et al. 1993; DiTella et al. 1996). Quatre lignées de souris déficientes pour MAP1b ont été générées (Edelmann et al. 1996; Takei et al. 1997; Gonzalez-Billault and Avila 2000; Meixner et al. 2000). Bien que le degré de sévérité des phénotypes de ces différentes souris invalidées est discordant, toutes ces études montrent l'importance de MAP1b lors du développement du système nerveux murin (Gonzalez-Billault and Avila 2000).

1.4.3.2 La protéine MAP2

La protéine associée aux microtubules MAP2 compte parmi les protéines les plus abondantes dans les dendrites du système nerveux central mammifère adulte (Sanchez et al. 2000).

1.4.3.2.1. Structure primaire de MAP2

Il existe plusieurs isoformes de MAP2 générées par l'épissage alternatif d'un même transcrit primaire codé par un gène unique, localisé sur le chromosome 2 et contenant 20 exons (figure 2). Les isoformes de MAP2 sont classées, en deux groupes, selon leur poids moléculaire.On distingue MAP2 de haut poids moléculaire (HMWMAP2), représentées par MAP2a et MAP2b dont le poids moléculaire est respectivement de 280 et de 270 KDa ainsi que les isoformes de MAP2 de faible poids moléculaire (LMWMAP2), représentées par MAP2c et MAP2d dont le poids moléculaire est respectivement de 70 et 75 KDa (Tucker 1990; Sanchez et al. 2000).

MAP2 contient deux domaines fonctionnels: un domaine de liaison aux microtubules dans la partie carboxy-terminale et un domaine de projection dans la partie amino-terminale. À la jonction de ces deux domaines existe une région riche en proline (figure 2).

Structurellement, la différence majeure entre HMWMAP2 et LMWMAP2 est l'insertion d'une séquence additionnelle de 1372 aa. dans le domaine de projection. Les deux isoformes HMWMAP2, MAP2a et MAP2b, diffèrent par l'insertion d'une séquence de 83 acides aminés, dans le domaine de projection de MAP2a (Chung et al. 1996). Les isoformes MAP2a, MAP2b et MAP2c contiennent 3 séquences répétées de liaison aux microtubules alors que l'isoforme MAP2d en contient 4 (Ferhat et al. 1994). Ces séquences répétées contiennent chacune 18 aa. et sont séparées par des séquences de 13 ou 14 aa. (Sanchez et al. 2000). Les isoformes de MAP2 les plus étudiées et les mieux caractérisées sont MAP2b et MAP2c.

Au cours du développement du système nerveux, l'expression des isoformes de MAP2 est régulée de façon spatio-temporelle (Tucker 1990). Pendant le développement, l'expression de l'isoforme MAP2c, appelée aussi isoforme juvénile, atteint son pic à un moment qui coïncide avec la pousse neuritique (Tucker 1990; Sanchez et al. 2000). Par la suite, l'expression de MAP2c diminue graduellement pour disparaître à l'âge adulte, à l'exception de certaines régions du système nerveux adulte qui ont conservé leur capacité de se régénérer comme le bulbe olfactif et la rétine (Tucker 1990; Sanchez et al. 2000). Les isoformes HMWMAP2 sont spécifiquement distribuées dans le compartiment somato-dendritique, à l'âge adulte, alors que les isoformes LMWMAP2 peuvent se retrouver dans tous les compartiments neuronaux (Tucker 1990; Sanchez et al. 2000).

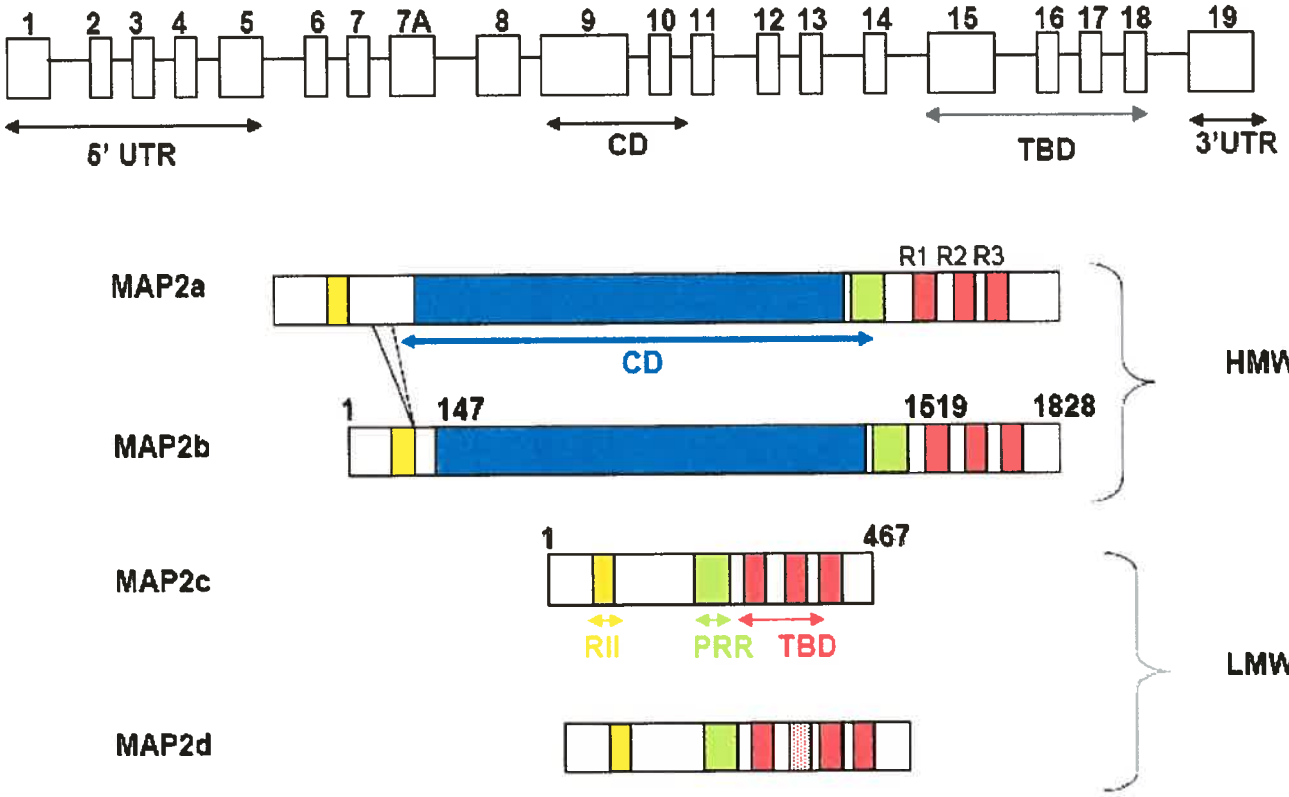
Figure 2 : Représentation schématique du gène et des différentes isoformes de la protéine MAP2. Les isoformes de MAP2 sont divisées en 2 groupes: MAP2 de haut poids moléculaire (MAP2a et MAP2b) ou HMWMAP2 et MAP2 de faible poids moléculaire ou LMWMAP2 (MAP2c et MAP2d). HMWMAP2 se distinguent de LMWMAP2 par l'insertion d'une séquence additionnelle de 1372 aa. dans la région amino-terminale (codée par les exons 9 à 11). Le domaine de liaison aux microtubules de MAP2 compte 3 ou 4 séquences répétées (3R ou 4R) de 18 aa. hautement conservées et espacées de 13 aa.. Toutes les isoformes de la protéine MAP2 contiennent une séquence de liaison à la sous unité RII de la PKA et une région riche en résidus proline (PRR). Adapté de (Sanchez et al. 2000)

TBD : domaine de liaison des microtubules.

RII : domaine de liaison de la sous-unité régulatrice RII de la PKA.

PRR : domaine riche en proline.

CD : domaine central de 1372 aa. qui différencient HMWMAP2 et LMWMAP2.


1 2 3 4 5 6 7 7A 8 9 10 11 12 13 14 15 16 17 18 19
5' UTR
CD
TBD
3'UTR
MAP2a
R1 R2 R3
CD
HMWMAP2
MAP2b
1
147
1519
1828
MAP2c
1
467
RII
PRR
TBD
LMWMAP2
MAP2d

1.4.3.2.2. Fonctions de MAP2

- ***Acquisition de la morphologie neuronale***

Les résultats de plusieurs études, *in vivo* et *in vitro*, ont montré l'implication de MAP2 dans la pousse neuritique et l'établissement de la polarité neuronale. Dans les neurones en culture, l'ajout d'oligonucléotides anti-sens qui suppriment l'expression de toutes les isoformes de MAP2, inhibe l'initiation de la pousse neuritique (Caceres et al. 1992; Sharma et al. 1994) alors que dans des cellules non neuronales, les cellules ovariennes d'insectes Sf9, la surexpression de MAP2 induit la formation de prolongements cytoplasmiques (Leclerc et al. 1993; Leclerc et al. 1996). Des souris déficientes pour la protéine MAP2 ont été générées. Phénotypiquement, mise à part une légère différence de poids de l'animal, ces souris, viables et fertiles, sont difficilement discernables des souris témoins (Sanchez et al. 2000). Toutefois, une analyse plus rigoureuse a montré que les souris déficientes pour MAP2 présentent une diminution de la densité des microtubules et de la longueur des dendrites (Harada et al. 2002). Ce phénotype, peu sévère, suggère la possibilité d'un mécanisme compensatoire entre les différentes MAPs. Cette hypothèse est appuyée par les résultats obtenus suite à l'analyse du phénotype des souris déficientes à la fois pour MAP2 et pour MAP1b. Ces animaux déficients doubles, qui ne survivent pas après leur naissance, présentent des altérations morphologiques multiples et majeures au niveau du système nerveux central et périphérique (Teng et al. 2001).

Récemment, une autre lignée de souris transgéniques MAP2 a été produite. Ces souris expriment une forme tronquée de MAP2 présentant une délétion des premiers 158 aa., commun à HMWMAP2 et LMWMAP2. Cette

séquence délétée contient le domaine de liaison de la sous unité RII de la PKA (Khuchua et al. 2003). Les dendrites des neurones de l'hippocampe de ces souris transgéniques sont plus courtes et largement désorganisées comparativement à celles des souris témoins (Khuchua et al. 2003).

- ***Organisation du cytosquelette neuronal:***

In vitro et *in vivo,* suite à l'association de la protéine MAP2 aux microtubules, ces derniers deviennent plus stables et plus rigides (Edson et al. 1993; Leclerc et al. 1993; Weisshaar and Matus 1993; Matus 1994). Bien que principalement liée aux microtubules MAP2 est capable d'interagir avec d'autres éléments du cytosquelette neuronal (Matus et al. 1981). Par exemple, dans les dendrites, au niveau des épines dendritiques, régions dépourvues de microtubules, MAP2 colocalise avec l'actine (Caceres et al. 1983; Morales and Fifkova 1989; Cunningham et al. 1997). *In vitro*, MAP2b et MAP2c sont capables de se lier à l'actine (Sattilaro 1986; Pedrotti et al. 1994; Cunningham et al. 1997). Toutefois, ces deux isoformes réorganisent différemment les microfilaments d'actine. Ainsi, MAP2b, est capable d'induire la formation de faisceaux parallèles d'actine alors que MAP2c réorganise les microfilaments d'actine en faisceaux à angle (Cunningham et al. 1997; Roger et al. 2004). La séquence de MAP2 responsable de sa liaison aux microfilaments d'actine a été identifiée. Cette dernière est localisée dans la deuxième séquence répétée du domaine de liaison aux microtubules (Correas et al. 1990). Cette donnée a été corroborée par une étude récente qui a montré que le domaine de liaison aux microtubules de MAP2 est nécessaire et suffisant pour la liaison et la fasciculation des microfilaments d'actine (Roger et al. 2004).

Par ailleurs, des études *in vitro* ont montré que la protéine MAP2 est aussi capable de lier la sous unité 70 KDa des neurofilaments (Leterrier et al. 1982; Heimann et al. 1985; Miyata et al. 1986). En effet, i*n vivo*, MAP2 fait partie des ponts qui relient les neurofilaments aux microtubules dans les dendrites des neurones moteurs de la moelle épinière (Hirokawa et al. 1988a).

Ainsi, en interagissant directement avec les trois éléments du cytosquelette neuronal, MAP2 peut jouer un rôle important dans l'organisation de ce dernier.

- ***Contribution à la distribution polarisée des protéines de signalisation***

La protéine MAP2 interagit également avec plusieurs autres protéines (tableau 1). L'interaction de MAP2 avec la PKA est la plus étudiée et la mieux caractérisée. Toutes les isoformes de MAP2 contiennent une séquence (de 31 aa.) de liaison à la sous unité régulatrice RII de la PKA (Obar et al. 1989; Rubino et al. 1989). Cette interaction permet à MAP2 de relocaliser et d'ancrer la PKA au cytosquelette neuronal à un compartiment spécifique où cette kinase pourrait accomplir sa fonction (Davare et al. 1999; Edwards and Scott 2000). D'ailleurs, l'analyse des souris mutantes nulles pour MAP2 a montré que MAP2 est essentielle pour la liaison de la PKA aux microtubules dans les neurones (Harada et al. 2002). En l'absence de MAP2, la quantité totale et l'activité de la PKA diminuent chez ces souris transgéniques (Harada et al. 2002). En contre partie, la PKA phosphoryle plusieurs sérines contenues dans le domaine de liaison aux microtubules et dans la région riche en proline de MAP2 (Sanchez et al. 2000; Harada et al. 2002; Khuchua et al. 2003). Chez des souris transgéniques exprimant une

forme tronquée de MAP2 (délétion des premiers 158 aa. contenant le site de liaison de la PKA) on observe une diminution de l'état de phosphorylation de MAP2 et une altération de la mémoire contextuelle reliée à la peur (Khuchua et al. 2003).

L'isoforme MAP2b peut interagir à la fois avec la PKA et la sous unité α-1 des canaux calciques de classe C - type L (Davare et al. 1999). Ces derniers, spécifiquement concentrés dans les épines dendritiques, sont d'importants régulateurs de la fonction neuronale. En effet, l'influx de calcium qui passe à travers les canaux calcique de type-L contrôle plusieurs phénomènes incluant la plasticité synaptique et l'excitabilité membranaire (Lipscombe et al. 2004). Suite à leur phosphorylation par la PKA, l'activité des canaux calciques de classe L est augmentée (Lipscombe et al. 2004). En interagissant directement et simultanément avec ces deux protéines, MAP2b faciliterait la phosphorylation rapide et efficace de ces canaux calciques par la PKA (Davare et al. 1999).

En outre, l'isoforme MAP2b peut aussi s'associer à la calmoduline. En effet, un site de liaison à la calmoduline dans la séquence additionnelle de 1372 aa de MAP2b a été identifié (Kotani et al. 1985; Kindler et al. 1990). Des études *in vitro* ont montré que l'interaction de MAP2 avec la calmoduline inhibe l'interaction de MAP2 avec l'actine (Kotani et al. 1985).

Par ailleurs, toutes les isoformes de MAP2 interagissent avec la protéine adaptatrice Grb2 et aussi avec les membres de la famille des protéines tyrosines kinases non récepteurs c-Src et fyn (Lim and Halpain 2000; Zamora-Leon et al. 2001; Zamora-Leon et al. 2005). *In vitro*, c-Src et Grb2 interagissent surtout avec une sous population de MAP2 soluble et non associée aux microtubules (Lim and Halpain 2000). Ces interactions MAP2/Grb2, MAP2/c-Src et MAP2/fyn sont médiées par les domaines SH3

de Grb2, de fyn et de c-Src et le domaine de liaison aux microtubules de MAP2 (Lim and Halpain 2000; Zamora-Leon et al. 2001).

- ***Régulation du transport des organelles membranaires***

Les microtubules jouent un rôle important dans le trafic intracellulaire (Goldstein and Yang 2000). Les MAPs motrices, telles que les kinésines et les dynéines, assurent le transport des organelles le long des microtubules (Hirokawa and Takemura 2005). Dans des essais *in vitro* et dans des cellules en culture, la présence de MAPs structurales (MAP2 et Tau) en excès sur les microtubules peut inhiber le transport assuré par les MAPs motrices (Heins et al. 1991; Hagiwara et al. 1994; Seitz et al. 2002). Une explication à ce phénomène serait que ces MAPs structurales rentrent en compétition avec les MAPs motrices pour un même site de liaison en C-terminal de la molécule de tubuline (Hagiwara et al. 1994; Sato-Harada et al. 1996; Terwel et al. 2002). Ainsi, en empêchant les MAPs motrices de se lier aux microtubules, la surexpression de MAPs structurales entraîne l'accumulation de certaines organelles membranaires au niveau du corps cellulaire (Sato-Harada et al. 1996; Ebneth et al. 1998; Seitz et al. 2002; Stamer et al. 2002).

Bien que ces résultats suggèrent l'implication des MAPs structurales dans la régulation du transport des organelles membranaires d'autres études sont nécessaires pour mieux comprendre ce phénomène.

Tableau 3 : Tableau résumant les interactions protéiques de MAP2 ainsi que les fonctions possibles de ces interactions.

Protéines	Fonctions possibles suite à l'interaction avec MAP2	Références
Microtubules	- Diminution de l'instabilité dynamique des microtubules - Augmentation de la rigidité des microtubules - Modulation de l'initiation de la pousse neuritique	(Caceres et al. 1992) (Sanchez et al. 2000) (Takemura et al. 1992)
Neurofilaments	- Liaison des microtubules et des neurofilaments *in situ*	(Hirokawa et al. 1988a)
Actine filamenteuse	- Liaison et réorganisation des microfilaments d'actine - Modulation de l'initiation la pousse neuritique - Liaison des microtubules aux microfilaments d'actine	(Cunningham et al. 1997; Roger et al. 2004) (Selden and Pollard 1986)
Calmoduline	- Modulation de l'interaction de MAP2 avec les microfilaments d'actine	(Kotani et al. 1985) (Kindler et al. 1990)
Mapmoduline	- Modulation de l'interaction de MAP2 avec les microtubules - Implication dans la régulation du transport membranaire	(Ulitzur et al. 1997) (Itin et al. 1999)
La sous unité RII de la PKA	- Localisation et ancrage de la PKA dans les dendrites - Modulation de l'initiation de la pousse neuritique	(Obar et al. 1989) (Harada et al. 2002)
Les canaux calciques de classe C- type L	- Liaison de la PKA aux canaux calciques	(Davare et al. 1999)
Les récepteurs NMDA	- Relocalisation et dégradation de MAP2 lors de l'ischémie cérébrale	(Buddle et al. 2003)
Porine (VDAC)	- Liaison des mitochondries au cytosquelette	(Linden and Karlsson 1996)
c-Src, Grb2 et Fyn	- Intégration et transduction du signal - Modulation de la phosphorylation de MAP2 par fyn	(Lim and Halpain 2000) (Zamora-Leon et al. 2005)
MARTA1 et 2 (MAP2-RNA Trans-Acting protein)	- Interaction entre MARTA1 et MARTA2 avec ARNm de MAP2 permet le ciblage de ce dernier dans les dendrites	{Rehbein, 2002 #1885; Rehbein, 2000 #1886}

1.4.3.2.3. Phosphorylation de MAP2

La phosphorylation est la modification post-traductionnelle la plus importante que la protéine MAP2 subit (Sanchez et al. 2000). En effet, *in vivo*, les différentes isoformes de MAP2 sont hautement phosphorylées (jusqu'à 46 moles de phosphate par mole de protéine) (Sanchez et al. 2000). Les sites de phosphorylation contenus dans la protéine MAP2 sont hautement conservés (autour de 90%) chez la souris, le rat et l'humain, ce qui témoigne bien de l'importance de la phosphorylation de MAP2 (Kindler et al. 1990).

Plusieurs protéines kinases sont impliquées dans la phosphorylation de MAP2. Parmi ces kinases, on retrouve les «Extracellular signaling-regulated Kinases» (ERKs), la glycogène synthétase 3β (Gsk3β), les protéines kinases dépendantes du cycle cellulaire (CDKs) et les «Microtubule-affinity-regulating kinases» (MARKs), la protéine kinase AMPc-dépendante (PKA), la protéine kinase Calcium/Calmoduline dépendante II (CamKII) ainsi que la protéine kinase calcium et phospholipides dépendante (PKC) (Sanchez et al. 2000). La majorité des sites d'action de ces kinases se retrouvent au niveau de la région riche en proline de MAP2 et du domaine de liaison aux microtubules (Sanchez et al. 2000).

Les différentes fonctions de MAP2 sont régulées selon l'état de phosphorylation de cette protéine (Riederer et al. 1995; Itoh et al. 1997; Ozer and Halpain 2000; Sanchez et al. 2000). Par exemple, la phosphorylation de MAP2 par la PKA, diminue la capacité de MAP2 de se lier à la tubuline et à l'actine et inhibe la protéolyse de MAP2 par les calpaines (Sanchez et al. 2000).

En outre, l'état de phosphorylation des différentes isoformes de MAP2 est régulé au cours du développement (Riederer et al. 1995). Dans les neurones de l'hippocampe en culture, une augmentation de l'état de phosphorylation de MAP2 coïncide avec l'augmentation de l'arborisation dendritique (Diez-Guerra and Avila 1993; Diez-Guerra and Avila 1995). De plus, l'ajout d'inhibiteurs de protéines kinases dans le milieu de culture, qui diminuent l'état de phosphorylation de MAP2, diminue l'arborisation dendritique (Audesirk et al. 1997). L'augmentation de l'état de phosphorylation de MAP2 a aussi été observée au cours du développement neuronal in vivo (Sanchez et al. 1995). Durant les premiers stades du développement neuronal, une forte activité des kinases, comparativement aux phosphatases, peut engendrer une phosphorylation accrue de MAP2 (Mawal-Dewan et al. 1994). Sachant que l'augmentation de l'état de phosphorylation de MAP2 peut inhiber son interaction avec les microtubules (Brugg and Matus 1991; Mandell and Banker 1996; Itoh et al. 1997), on peut envisager que cela permet une plus grande capacité de réorganisation du cytosquelette neuronal nécessaire pour l'élaboration de la morphologie neuronale (Sanchez et al. 2000).

Dans des neurones matures, des changements morphologiques peuvent avoir lieu suite à l'activité neuronale (Maletic-Savatic et al. 1999; Marrone and Petit 2002). Cette activité neuronale est accompagnée par des changements de l'état de phosphorylation de MAP2 (Halpain and Greengard 1990; Diaz-Nido et al. 1993; Quinlan and Halpain 1996). Ces résultats suggèrent qu'un changement de l'état de phosphorylation de MAP2 pourrait être à l'origine de la réorganisation du cytosquelette neuronal et du remodelage dendritique chez l'adulte (Quinlan and Halpain 1996).

1.4.3.3 La protéine Tau

1.4.3.3.1 historique

Tau a été décrite, pour la première fois, comme une protéine soluble co-purifiée avec les microtubules (Weingarten et al. 1975) et dont l'association avec ces derniers est régulée selon son état de phosphorylation (Cleveland et al. 1977). En effet, comme c'est le cas pour MAP2, lorsque Tau est dans un état moins phosphorylé, sa capacité à promouvoir l'assemblage et la stabilisation des microtubules est diminuée (Lindwall and Cole 1984). La recherche sur Tau a pris un essor considérable lorsqu'il a été montré qu'elle est l'élément principal des enchevêtrements neurofibrillaires ou tangles (NFTs= Neurofibrillary Tangles) retrouvés dans les cerveaux des personnes atteintes de la maladie d'Alzheimer (MA). Cette maladie neurodégénérative se traduit par une perte progressive et irréversible de la mémoire et des fonctions cognitives. La raison fondamentale de la neurodégénérescence des cellules nerveuses et des connections synaptiques qui conduit à la démence, dans la MA, reste encore inconnue. En plus de la mort neuronale, sur le plan histologique, deux types de lésions sont observées dans les cerveaux MA: les NFTs et les plaques séniles. Les plaques séniles sont extracellulaires et composées principalement du peptide β-amyloïde (Aβ) (St George-Hyslop et al. 1987; Tanzi et al. 1987). Ce dernier est généré par le clivage de la protéine précurseur du β-amyloïde (APP). C'est un peptide insoluble qui se dépose progressivement dans la totalité du cerveau et plus particulièrement dans le système limbique et les aires associatives du cortex (Dickson 1997). L'autre type de lésion, les NFTs, sont intracellulaires et reflètent un changement cytosquelettique important. En effet, dans les neurones affectés, Tau hyperphosphorylée se

retrouve sous forme de dépôts filamenteux insolubles qui ont un aspect, en microscopie électronique, de paires de filaments hélicaux (PHFs) ou de filaments droits (SFs) (Lee et al. 2001). Ces filaments forment les NFTs dans le compartiment somato-dendritique (Kosik et al. 1984; Grundke-Iqbal et al. 1986a; Grundke-Iqbal et al. 1986b). La formation des NFTs est précédée par un stade dit «pré-tangles» où la distribution de la protéine Tau hyperphosphorylée est plutôt diffuse dans le corps cellulaire (Braak et al. 1994).

Le rôle de Tau dans le processus de neurodégénérescence dans la maladie d'Alzheimer a été longtemps controversé. La découverte de mutations, dans le gène codant pour APP et associées aux cas familiaux de la MA, qui augmentent la production du Aβ (Balbin et al. 1992; Mullan et al. 1992; Felsenstein et al. 1994; Johnston et al. 1994) ainsi que les résultats démontrant la neurotoxicité du peptide Aβ (Davis and Van Nostrand 1996; Guo et al. 1999) ont grandement contribué à cette controverse.

Par ailleurs, la protéine Tau est également impliquée dans d'autres désordres neurodégénératifs (autres que la MA) communément appelés tauopathies (Tolnay and Probst 1999; Lee et al. 2001). Toutes les tauopathies sont caractérisées par l'accumulation intracellulaire de Tau hyperphosphorylée sous forme de dépôts filamenteux et insolubles (Lee et al. 2001). Des dépôts filamenteux de Tau sont présents dans les cerveaux des personnes atteintes de la MA, de démences fronto-temporales (FTDs), de la maladie de Pick et de deux désordres parkinsoniens: la paralysie supranucléaire progressive et la dégénérescence cortico-basale (Tolnay and Probst 1999; Lee et al. 2001). En 1997, lors d'une conférence consensus, le terme FTDP-17 (Frontotemporal dementia and parkinsonism linked to chromosome 17) a été introduit pour décrire des individus avec des

antécédants familiaux (autosomaux dominants) présentant à la fois des symptômes cliniques de FTDs et des troubles parkinsoniens (Foster et al. 1997).

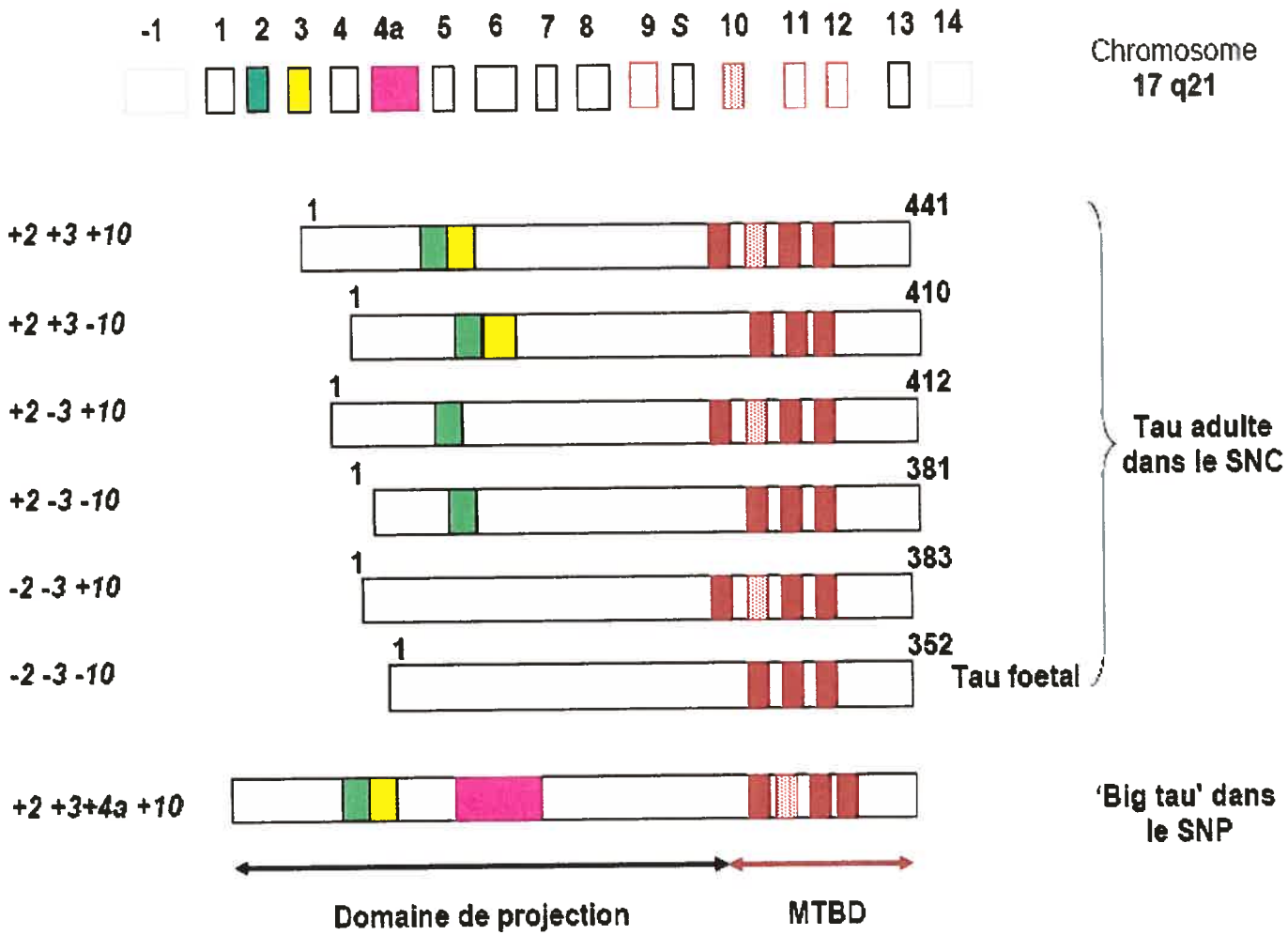
C'est la découverte de mutations dans le gène de Tau associées aux FTDP-17 qui a ressuscité l'intérêt pour Tau (Spillantini et al. 1998b). En effet, chez les patients atteints de FTDP-17, il a été observé uniquement des dépôts filamenteux de Tau hyperphosphorylée mais pas les plaques amyloïdes (Baker et al. 1997; Hutton et al. 1998; Poorkaj et al. 1998; Spillantini et al. 1998a; Spillantini and Goedert 1998). Ces mutations ont permis de montrer sans aucune ambiguïté que des anomalies qui affectent la protéine Tau sont suffisantes pour induire la neurodégénérescence et conduire à la démence (Spillantini et al. 1998b; Ingram and Spillantini 2002).

1.4.3.3.2 Organisation du gène de la protéine Tau humaine

Le gène humain de Tau est unique et est situé sur le chromosome 17 (17q21). Le transcrit primaire de Tau contient 16 exons (voir figure 3). Les ARNm contenant les exons 4a, 6 et 8 n'ont pas été détecté dans le cerveau (Lee et al. 2001). L'exon -1 et l'exon 14 sont transcrits mais non traduits. Les exons 1, 4, 5, 7, 9, 11, 12 et 13 sont des exons constitutifs alors que les exons 2, 3 et 10 sont alternativement épissés. Dans le système nerveux périphérique, l'insertion de l'exon 4a lors de l'épissage alternatif, donne une isoforme de haut poids moléculaire (~100 kDa) (Goedert et al. 1992). Tau est principalement neuronale mais certaines cellules non neuronales peuvent en contenir sous forme de traces (Buee et al. 2000). En conditions pathologiques, Tau peut être aussi exprimée dans les cellules gliales (Miyazono et al. 1993; Komori 1999; Ferrer et al. 2001; Yang et al. 2003).

L'expression de Tau est régulée de manière spatio-temporelle. Au cours de la vie fœtale, seule l'isoforme la plus courte de 352 aa. est présente (Lee et al. 2001). Après la naissance et au cours du développement neuronal, les autres isoformes commencent à être exprimées. Dans le cerveau humain adulte, six isoformes de Tau sont produites. Ces dernières diffèrent les unes des autres par la présence ou l'absence, à l'extrémité amino-terminale, d'un insert de 29 ou 58 aa. (codé par l'exon 3 et/ou l'exon 2) et d'une séquence répétée additionnelle de 31 aa. dans la partie carboxy-terminale (codée par l'exon 10). On parle alors de 3 isoformes Tau 3R et de 3 isoformes Tau 4R (figure 3). Ces 6 isoformes contiennent entre 352 et 441 acides aminés et leur poids moléculaires observés par SDS-PAGE varie entre 45 kDa et 65 kDa. Chez l'humain, l'épissage alternatif qui se produit au niveau de l'exon 10 est d'une grande importance. En effet, dans le cerveau adulte normal, les quantités des isoformes Tau 3R et Tau 4R sont équivalentes (Goedert et al. 1989). Si cet équilibre est perturbé, cela peut conduire à la neurodégénérescence (voir la section portant sur les mutations du gène codant pour Tau).

Figure 3: Représentation schématique des différentes isoformes de Tau. Six isoformes dans le système nerveux central (SNC) dont une seule qui s'exprime durant la vie fœtale. L'isoforme de 100 kDa est spécifique au système nerveux périphérique (SNP). L'expression des différents isoformes de la protéine Tau dans le SNC est régulée de façon spatio-temporelle. MTBD= domaine de liaison aux microtubules. PPR= région riche en proline. Entre les exons 9 et 10, un gène codant pour la saithoine (S). Cette protéine de 128 acides aminés serait impliquée dans la MA mais sa fonction exacte n'est pas encore été établie (Conrad et al. 2002).


-1 1 2 3 4 4a 5 6 7 8 9 S 10 11 12 13 14
Chromosome
17 q21
+2 +3 +10
1
441
+2 +3 -10
1
410
+2 -3 +10
1
412
+2 -3 -10
1
381
-2 -3 +10
1
383
-2 -3 -10
1
352
Tau foetal
Tau adulte
dans le SNC
+2 +3+4a +10
'Big tau' dans
le SNP
Domaine de projection
MTBD

1.4.3.3.3 Structure de la protéine Tau

Comme c'est le cas pour MAP2, la structure primaire de Tau comprend deux domaines: le domaine de liaison aux microtubules et le domaine de projection (Goedert et al. 1989; Himmler 1989). Ce dernier est situé en N-terminale et projette à la surface des microtubules ou il pourrait interagir avec d'autres éléments cellulaires. Le domaine de liaison aux microtubules, localisé en C-terminale, est une région très basique contenant, selon les isoformes, 3 ou 4 séquences répétées imparfaites (Goedert et al. 1989; Himmler 1989). Chacune des séquences répétées de Tau contient 18 aa. séparées par des séquences moins conservées de 13 à 14 aa. appelées inter-répétitions (Goedert et al. 1989; Himmler 1989). À la jonction de ces deux domaines, se retrouve une région centrale riche en résidus proline (PRR) dont le rôle est de réguler la fonction de Tau.

1.4.3.3.4 Fonctions de Tau

- ***Organisation du cytosquelette neuronal***

Plusieurs études *in vitro* ont montré que Tau permet la nucléation, la polymérisation et la stabilisation des microtubules (Maccioni et al. 1989; Bre and Karsenti 1990; Drechsel et al. 1992). C'est le domaine de liaison aux microtubules qui serait le principal responsable de l'interaction de Tau avec les microtubules (Drubin et al. 1986). Dans ce domaine, la séquence qui a la plus grande affinité avec les microtubules est celle qui recouvre les régions R1, IR 1-2 et R2 (Goode et al. 1997). Il en résulte que les isoformes Tau 4R sont capables de lier et de polymériser les microtubules plus efficacement que les isoformes Tau 3R (Lee et al. 1989; Goedert and Jakes 1990). Ceci serait très important au cours du développement neuronal où seule

l'isoforme la plus courte Tau 3R, est exprimée. Au cours de la vie fœtale, la plasticité neuronale est d'une importance primordiale pour l'établissement des prolongements neuronaux ainsi que des connexions entre les différents neurones. Dans le cerveau adulte, une plus grande stabilité des microtubules serait requise pour maintenir les prolongements et les connexions établies, on retrouve alors aussi bien les isoformes Tau 3R que Tau 4R.

Bien que la liaison de Tau aux microtubules soit principalement assurée par le domaine de liaison aux microtubules, des études ont rapporté l'implication du domaine de projection de Tau pour une liaison plus efficace aux microtubules (Kanai et al. 1992). En effet, une séquence de 7 aa., dans le domaine de projection de Tau, serait capable d'influencer la capacité de cette protéine à lier et à assembler les microtubules (Goode et al. 1997).

Tau semble pouvoir interagir avec les autres éléments du cytosquelette neuronal (microfilaments d'actine et neurofilaments) (Miyata et al. 1986; Cunningham et al. 1997). Certaines études, *in vivo* et *in vitro*, ont rapporté que Tau s'associe directement aux microfilaments d'actine (DiTella et al. 1994; Kempf et al. 1996; Cunningham et al. 1997). Il est toutefois important de souligner que toutes les études ne sont pas unanimes quant à la capacité de Tau de lier directement les microfilaments d'actine (Dehmelt and Halpain 2004; Roger et al. 2004). En effet, bien que les domaines de liaison de MAP2 et Tau aient une identité de séquence très importante, une récente étude a montré que seul le domaine de liaison aux microtubules de MAP2 serait capable de se lier aux microfilaments d'actine (Dehmelt and Halpain 2004; Roger et al. 2004).

Dans ces études, des essais *in vitro* ont clairement montré qu'une protéine Tau dont le domaine de liaison aux microtubules a été substitué par celui de la protéine MAP2 serait alors capable de lier les microfilaments

d'actine (Roger et al. 2004). Par ailleurs, la protéine Tau interagit avec la spectrine une protéine associée aux microfilaments d'actine, de sorte que l'interaction de Tau avec les microfilaments d'actine pourrait se faire indirectement via la spectrine (Carlier et al. 1984).

o *Formation de l'axone*

Plusieurs études dans différents systèmes cellulaires ont révélé le rôle de Tau dans l'établissement de la polarité neuronale. La suppression de l'expression de Tau par l'ajout d'oligonucléotides anti-sens dans des cultures primaires de neurones bloque la différenciation axonale (Caceres and Kosik 1990; Caceres et al. 1991). En contrepartie, lorsque Tau est microinjectée ou surexprimée dans des cellules non-neuronales, elle stabilise et favorise la formation de faisceaux de microtubules (Drubin et al. 1986; Drubin and Kirschner 1986; Kanai et al. 1989; Lewis et al. 1989; Lee and Rook 1992; Takemura et al. 1992; Barlow et al. 1994). De plus, dans les cellules ovariennes d'insecte Sf9 initialement sphériques, la surexpression de Tau induit la formation de longues extensions cytoplasmiques (Knops et al. 1991; Leclerc et al. 1993; Boucher et al. 1999). L'espacement entre les microtubules dans ces prolongements cytoplasmiques rappelle celui observé dans l'axone des neurones (Baas et al. 1991) (Chen et al. 1992).

Par ailleurs, le phénotype d'une lignée de souris mutantes nulles pour Tau ne semble pas se distinguer de celui des souris hétérozygotes ou des souris témoins (Harada et al. 1994). L'analyse histologique n'a pas montré d'anormalités majeures. D'ailleurs dans les axones de grand calibre, aucune différence n'a été décelée. Toutefois, dans les axones de petit calibre, une diminution de la densité des microtubules ainsi qu'une désorganisation du réseau des microtubules ont été observées (Harada et al. 1994).

Ce phénotype, peu sévère, pourrait être expliqué par une compensation par les autres MAPs en l'absence de Tau. D'ailleurs, chez les souris déficientes pour Tau, une augmentation de l'expression de MAP1A a été rapportée (Harada et al. 1994; Dawson et al. 2001). En outre, le phénotype des souris dépourvues à la fois de Tau et de MAP1B est beaucoup plus sévère que celui des souris dépourvues uniquement de Tau ou de MAP1B (Takei et al. 2000). Contrairement aux souris déficientes simples, les souris déficientes doubles (MAP1B et Tau) meurent durant les quatre premières semaines suivant leur naissance. La pousse neuritique est sévèrement compromise dans des cultures primaires de neurones prélevés de ces souris déficientes doubles (Takei et al. 2000). L'hypothèse de cette redondance fonctionnelle entre les différentes MAPs neuronales structurales a été corroborée par les résultats d'une autre étude. Des cultures primaires de neurones prélevés à partir d'une autre lignée de souris déficientes pour MAP1B ont été traitées avec des oligonucléotides anti-sens qui suppriment spécifiquement l'expression de Tau ou de MAP2 (Gonzalez-Billault et al. 2002). Les résultats de cette étude suggèrent le mode d'action synergique des trois MAPs structurales MAP1b, MAP2 et Tau lors de la pousse neuritique et de l'établissement de la polarité neuronale (Gonzalez-Billault et al. 2002).

- ***Intégration et transduction du signal***

Plusieurs études ont rapporté que Tau interagit avec la membrane plasmique (Brandt et al. 1995; Ekinci and Shea 2000; Maas et al. 2000). L'interaction de Tau avec la membrane plasmique se fait via son domaine de projection de Tau (Brandt et al. 1995) qui se lie au domaine SH3 de la tyrosine kinase de la famille des Src, la protéine Fyn (Lee et al. 1998; Lee et

al. 2004). Cette interaction serait dépendante de l'état de phosphorylation de Tau (Brandt et al. 1995; Ekinci and Shea 1999; Maas et al. 2000) et nécessaire pour l'induction de la pousse neuritique (Brandt et al. 1995; Klein et al. 2002).

Tau peut également se lier à certaines kinases telles que GSK3β (Sun et al. 2002; Agarwal-Mawal et al. 2003), CDK5 (Patrick et al. 1999) mais aussi des phosphatases telle que PP2A (Sontag et al. 1999) et PP1 (Liao et al. 1998). L'interaction de Tau avec ces kinases et ces phosphatases permettrait leur ciblage aux microtubules (Sun et al. 2002). En contrepartie, ces kinases permettraient la modulation de l'état de phosphorylation de Tau (voir section la phosphorylation de Tau).

- ***Régulation du transport membranaire***

Le rôle possible de Tau dans la régulation du transport le long des microtubules a été mis en évidence dans des essais *in vitro* (Heins et al. 1991; Jancsik et al. 1996; Seitz et al. 2002), dans les neurones en culture (Stamer et al. 2002) et dans plusieurs lignées cellulaires (Sato-Harada et al. 1996; Ebneth et al. 1998; Stamer et al. 2002). En effet, dans les cellules qui surexpriment la protéine Tau, on observe une accumulation des mitochondries, des peroxysomes, de vésicules golgiennes qui transportent le précurseur du Aβ (Ebneth et al. 1998; Stamer et al. 2002). Comme pour MAP2, l'effet inhibiteur de Tau sur le transport serait en grande partie due à une compétition entre les MAPs motrices et les MAPs structurales pour se lier aux microtubules (Hagiwara et al. 1994; Sato-Harada et al. 1996; Ebneth et al. 1998; Seitz et al. 2002; Stamer et al. 2002; Terwel et al. 2002). Dans les cellules de neuroblastome humain (N2a) surexprimant Tau, une accumulation des organelles membranaires au niveau du corps cellulaire a

aussi été observée. Ce phénotype est accompagné d'une plus grande vulnérabilité de ces cellules au stress oxydatif induit par l'ajout dans le milieu de culture de peroxyde d'hydrogène comparativement aux cellules témoins qui ont subit le même traitement. Ainsi, la surexpression de Tau peut contribuer à augmenter la vulnérabilité de ces cellules au stress oxydatif (Mandelkow et al. 2003).

Tableau 2: Tableau résumant les interactions protéiques de Tau et fonctions possibles de ces interactions.

Protéines	Fonctions possibles de l'interaction	Références
Microtubules	- Stabilisation des microtubules - Augmentation de la rigidité des microtubules - Module l'initiation de la pousse neuritique	(Caceres et al. 1991) (Leclerc et al. 1993) (Kosik and Caceres 1991)
NFs	- Lien entre les microtubules et les neurofilaments	(Miyata et al. 1986)
Actine	- Rôle possible lors de la pousse neuritique?	(Shahani and Brandt 2002)
Spectrine	- Régulation de l'interaction entre les microtubules et les microfilaments d'actine	(Carlier et al. 1984)
α-synuclein	- Modulation de la phosphorylation de Tau par la PKA	(Jensen et al. 1999)
Mapmoduline	- Modulation de l'interaction de Tau avec les microtubules	(Itin et al. 1999)
PP2A	- Liaison de PP2A aux microtubules - Modulation de la phosphorylation de Tau	(Sontag et al. 1999)
PP1	- Liaison de PP1 à aux microtubules - Modulation de la phosphorylation de Tau	(Liao et al. 1998)
Préseniline1	- Liaison de Tau à Gsk3β	(Takashima et al. 1998)
CDK5	- Liaison de CDK5 aux microtubules - Modulation de la phosphorylation Tau	(Sobue et al. 2000)
Gsk3β	- Liaison de Gsk3β aux microtubules - Modulation de la phosphorylation Tau	(Agarwal-Mawal et al. 2003)
14-3-3	- Liaison de Tau à CDK5 et à Gsk3β	(Agarwal-Mawal et al. 2003)
Fyn	- Modulation de la posphorylation de Tau - Intégration et transduction du signal	(Lee et al. 1998) (Lee et al. 2004)
Src	- Intégration et transduction du signal	(Lee et al. 1998)

1.4.3.3.5 Phosphorylation de Tau

La protéine Tau subit plusieurs modifications post-traductionnelles, telles que la phosphorylation, la O-glycosylation, l'ubiquitination, l'oxydation, la glycation, la désamination et le clivage. Toutefois, la phosphorylation constitue la modification post-traductionnelle la plus importante (Cleveland et al. 1977; Buee et al. 2000). À l'instar de MAP2, Tau contient de nombreux sites potentiellement phosphorylables. Dans le système nerveux central, l'isoforme la plus longue (441 aa.) contient 80 sites sérines ou thréonines et 5 sites tyrosines (Lee et al. 2001).

Un grand nombre de protéines kinases ont été impliquées dans la phosphorylation de Tau ce qui pourrait valoir à cette protéine le titre «d'accepteur universel de phosphate» (Stoothoff and Johnson 2005). Parmi les kinases qui peuvent phosphoryler Tau, on distingue: MAPKs, «Stress-Activated-Protein-Kinases», GSK3β, CDK5, CDC2, «Microtubules affinity Regulating Kinase» (p110-MARK), CamKII, PKA, PKC (Buee et al. 2000; Stoothoff and Johnson 2005). Toutes ces kinases ont pour cible des sites serines ou thréonines, mais Tau peut aussi être phosphorylée sur des résidus tyrosines par des tyrosines kinases telles que fyn (Williamson et al. 2002; Lee et al. 2004).

La phosphorylation par certaines kinases serait optimale lorsque Tau est préalablement phosphorylée par une autre kinase (Singh et al. 1996). Par exemple, une phosphorylation préalable de Tau par CDK5 favoriserait sa phosphorylation subséquente par Gsk3β (Sengupta et al. 1997). En outre, la kinase fyn, qui lie et phosphoryle directement Tau (Lee et al. 2004), est aussi capable de phosphoryler Gsk3β qui devient plus active. Par conséquent la liaison de fyn à Tau augmente non seulement la phosphorylation de cette dernière sur des résidus tyrosines mais facilite aussi la phosphorylation de Tau

par Gsk3β (Lesort et al. 1999). D'un autre côté, la déphosphorylation de Tau est assurée par un certain nombre de protéines phosphatases, telles que PP1, PP 2A et PP 2B (Buee et al. 2000).

La majorité des sites de phosphorylation de Tau sont localisées dans le domaine de liaison aux microtubules et de part et d'autre de ce dernier (Shahani and Brandt 2002). Comme nous l'avons mentionné plus haut, la phosphorylation de Tau régule négativement son interaction avec les microtubules (Drechsel et al. 1992; Biernat et al. 1993; Bramblett et al. 1993; Buee et al. 2000). Par exemple, *in vitro*, la phosphorylation seulement de la sérine 262, est suffisante pour diminuer dramatiquement l'affinité de liaison de Tau aux microtubules (Biernat et al. 1993; Drewes et al. 1995). Toutefois, la phosphorylation d'autres sites tels que la thréonine 205, la sérine 202, la sérine 214, la thréonine 231, la sérine 356 et la sérine 396 peuvent aussi affecter l'affinité de liaison de Tau aux microtubules (Bramblett et al. 1993; Sontag et al. 1996) (Biernat and Mandelkow 1999).

Le niveau de phosphorylation de Tau varie au cours du dévelopement. Chez le rat, des études ont montré que l'isoforme fœtale est plus phosphorylée à l'âge embryonnaire qu'à l'âge adulte (Kanemaru et al. 1992; Bramblett et al. 1993).

1.4.3.3.6 L'hyperphosphorylation de Tau et la neurodégénérescence

Des études ont rapporté que Tau isolée des PHFs contient en moyenne 6 à 8 moles de phosphate/moles de protéine (Kopke et al. 1993). Ce taux de phosphorylation est nettement et significativement plus élevé que celui de tau isolée à partir de cerveaux normaux qui est de 1.9 moles de phosphate/moles de protéine (Kenessey and Yen 1993; Kopke et al. 1993). Dans ce contexte, il est intéressant de rappeler que l'isoforme fœtale de Tau est aussi hautement

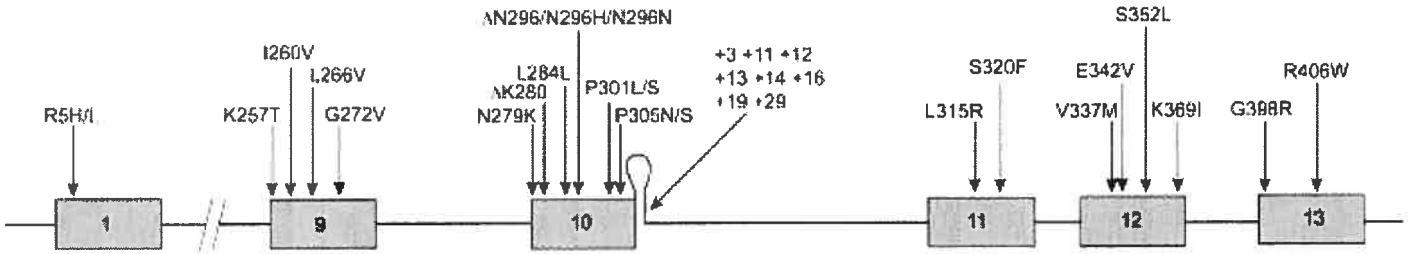
phosphorylée et présente même une immunoréactivité sur plusieurs résidus reconnus pour être hyperphosphorylés dans des conditions pathologiques (Yoshida and Ihara 1993; Bothwell and Giniger 2000).Toutefois, il n'existe aucune évidence que l'isoforme de Tau fœtale, hautement phosphorylée, s'autoagrège spontanément (Yoshida and Ihara 1993).

L'hyperphosphorylation de Tau peut entraîner des changements de conformation. Ces changements peuvent perturber l'association de Tau avec les microtubules ou d'autres partenaires cellulaires (Alonso et al. 1994; Ekinci and Shea 2000; Maas et al. 2000), altérer la distribution subcellulaire de Tau (Billingsley and Kincaid 1997) et augmenter la résistance de Tau à la protéolyse (Litersky and Johnson 1992; Litersky and Johnson 1995).

1.4.3.3.7 Les mutations de Tau et la neurodégénérescence

Jusqu'à ce jour 34 mutations ponctuelles et 17 polymorphismes codants associés aux FTDP-17 ont été identifiés dans le gène codant pour Tau (Rademakers et al. 2004). Les mutations peuvent être classées en deux grandes catégories : des mutations dans la séquence codante et des mutations introniques. Ces mutations engendrent la formation de filaments de Tau insolubles dont la morphologie, la composition en isoformes et la distribution sont différentes (voir Tableau 3 et les articles de revue (Lee et al. 2001; Rademakers et al. 2004; Brandt et al. 2005)). Par exemple, les mutations V337M et R406W induisent la formation de filaments droits ou des paires de filaments hélicoïdaux composés des 6 isoformes de Tau dans les neurones (Ingram and Spillantini 2002). La mutation P301L induit la formation de filaments tortueux contenant principalement des isoformes de Tau 4R dans les neurones et dans les cellules gliales (Ingram and Spillantini 2002).

Figure 4: Localisation des mutations associées aux FTDs au niveau de la séquence du gène codant pour la protéine Tau.



(Brandt et al. 2005)

Tauopathies/mutations	Localisation	isoformes insolubles	Distribution	Interaction avec les Mts	Épissage de E10
maladie d'Alzheimer		6 isoformes de Tau	Principalement neuronale	↓l'assemblage des Mts	Aucun
R5H	Exon 1	Principalement Tau 4R	Principalement gliales	↓l'assemblage des Mts	Aucun
R5L	Exon 1	Principalement Tau 4R	Neuronale et gliale	↓l'assemblage des Mts	Aucun
K257T	Exon 9 (R1)	Principalement Tau 3R	Principalement neuronale	↓l'assemblage des Mts	Aucun
I260V	Exon 9 (R1)	Principalement Tau 4R	N/D	↓l'assemblage des Mts	Aucun
L266V	Exon 9 (R1)	Les 6 isoformes de Tau	Neuronale et gliale	↓l'assemblage des Mts	Aucun
G272V	Exon 9 (R1)	Les 6 isoformes de Tau	Principalement neuronale	↓l'assemblage des Mts	Aucun
N279K	Exon 10 (IR 1-2)	Principalement Tau 4R	Neuronale et gliale	Aucun effet	↑l'épissage de E10
ΔK280	Exon 10 (IR 1-2)	N/D	N/D	↓ l'assemblage des Mts	↑ l'épissage de E10
L284L	Exon 10 (IR 1-2)	N/D	Neuronale et gliale	Aucun effet	↑ l'épissage de E10
N296N	Exon 10 (R2)	N/D	Neuronale et gliale	Aucun effet	↑ l'épissage de E10
ΔN296	Exon 10 (R2)	N/D	Neuronale et gliale	↓ l'assemblage des Mts	↑ l'épissage de E10
N296H	Exon 10 (R2)	Principalement Tau 4R	Principalement gliales	↓ l'assemblage des Mts	↑ l'épissage de E10
P301L	Exon 10 (R2)	Principalement Tau 4R	Neuronale et gliale	↓ l'assemblage des Mts	Aucun
P301S	Exon 10 (R2)	Principalement Tau 4R	Neuronale et gliale	↓ l'assemblage des Mts	Aucun
S305N	Exon 10 (IR 2-3)	N/D	Neuronale et gliale	Aucun effet	↑ l'épissage de E10
S305S	Exon 10 (IR 2-3)	N/D	Neuronale et gliale	Aucun effet	↑ l'épissage de E10
+3,+11,+12,+13,+14,+16	Intron 10	Principalement Tau 4R	Neuronale et gliale	Aucun effet	↑ l'épissage de E10
+19,+29	Intron 10	Principalement Tau 3R	N/D	↓ l'assemblage des Mts	↓ l'épissage de E10
L315R	E11	6 isoformes de Tau	Neuronale et gliale	↓ l'assemblage des Mts	Aucun
S320F	E11	6 isoformes de Tau	Neuronale et gliale	↓ l'assemblage des Mts	Aucun
V337M	E12 (IR 3-4)	6 isoformes de Tau	Principalement neuronale	↓ l'assemblage des Mts	Aucun
E342V	E12 (IR 3-4)	Principalement Tau 4R	Principalement neuronale	Aucun effet	↑ l'épissage de E10
S352L	E12 (IR 3-4)	N/D	N/D	N/D	N/D
K369I	E12 (R4)	6 isoformes de Tau	Principalement neuronale	↓ l'assemblage des Mts	Aucun
G389R	E13	6 isoformes de Tau	Principalement neuronale	↓ l'assemblage des Mts	Aucun
R406W	E13	6 isoformes de Tau	Principalement neuronale	↓ l'assemblage des Mts	Aucun

Les mécanismes moléculaires par lesquels la protéine Tau mutante conduit à la neurodégénérescence sont encore inconnus. Toutefois plusieurs études ont montré que les mutations peuvent affecter la protéine Tau à deux niveaux:

1) Certaines mutations peuvent modifier les propriétés biochimiques de Tau. L'analyse de l'affinité de liaison aux microtubules a montré que plusieurs mutations (ex. G272V, P301L, P301S, V337M et R406W) entraîneraient une réduction de la vitesse de polymérisation des microtubules et/ou une diminution d'affinité de la liaison aux microtubules (Hasegawa et al. 1998; Hong et al. 1998; Dayanandan et al. 1999; Rizzu et al. 1999). D'un autre côté, d'autres études soutiennent que l'effet de certaines mutations (ex. P301L, V337M et R406W) sur la liaison de Tau aux microtubules ne serait pas dramatique (DeTure et al. 2000). Dans les deux cas, la baisse d'affinité de Tau pour les microtubules serait équivalente à une perte partielle de sa fonction de stabilisation des microtubules.

2) D'autres mutations modifient l'épissage alternatif de l'exon 10 et provoquent un déséquilibre quantitatif entre les isoformes de Tau 3R et Tau 4R (en conditions normales, rapport =1, en conditions pathologiques il est de 2 à 3) (Hutton et al. 1998; Spillantini et al. 1998b). C'est le cas de la plupart des mutations introniques et également de certaines mutations codantes (ex. N279K, L284L, N296N, N296H, S305N, S305S et E342V) (Hutton et al. 1998; Spillantini et al. 1998b). Ce déséquilibre conduit à la formation de filaments insolubles contenant l'isoforme de Tau surproduite. Dans la majorité de ces mutations, c'est Tau 4R qui est surproduite (voir tableau 3). Puisque les isoformes Tau 4R ont une plus grande capacité de lier et de stabiliser les microtubules, que les isoformes Tau 3R, l'hypothèse de perte de fonction est invraisemblable. En l'occurrence, on pourrait imaginer que la surproduction de

Tau 4R provoquerait une saturation des sites de liaison disponibles sur les microtubules ce qui provoquerait une accumulation et une agrégation des isoformes surproduites dans le cytoplasme. Cette hypothèse reflète un gain de fonction toxique.

1.4.3.3.8 Les modèles animaux des tauopathies

Dans le but de mieux comprendre les mécanismes moléculaires, qui conduisent au dysfonctionnement et à l'accumulation des filaments insolubles lors du processus neurodégénératif, plusieurs modèles animaux surexprimant différentes isoformes humaines de Tau ont été générés (C. elegans et D. melanogaster, la lamproie et bien sur des souris transgéniques). En outre, suite à l'identification des mutations associées aux FTDP-17 d'autres modèles animaux ont été créés. Dans les sections suivantes nous allons décrire les modèles les plus pertinents (pour une revue complète voir Götz 2001; Brandt et al. 2005; Lee et al. 2005; Stoothoff and Johnson 2005).

- ***Modèles animaux chez les invertébrés***

Chez la drosophile, un homologue de Tau a été identifié, DMAP-85 (Cambiazo et al. 1995). Jusqu'à ce jour aucune mutation dans le gène codant pour la protéine DMAP-85 n'a été décrite. Des drosophiles transgéniques exprimant Tau humaine non mutée ou Tau humaine portant la mutation R406W ont été générées (Williams et al. 2000; Wittmann et al. 2001). Dans ces deux cas, les drosophiles transgéniques meurent prématurément et présentent une neurodégénérescence progressive et une accumulation de Tau anormalement phosphorylée. Toutefois, les drosophiles transgéniques exprimant Tau mutée ont un phénotype encore plus sévère (Wittmann et al. 2001). De plus, il est important de noter que dans les deux cas, la surexpression de Tau mutée ou

non, la mort neuronale survient sans la formation d'agrégats intraneuronaux (NFTs) (Wittmann et al. 2001). Ces résultats suggèrent que, dans ce modèle, la neurodégénérescence peut précéder, ou se produire indépendamment, de la formation de dépôts filamenteux de Tau.

- ***Souris exprimant Tau humaine non mutante***

Deux lignées de souris transgéniques exprimant l'isoforme de Tau humaine la plus courte (3R: -2,-3, -10) ou la plus longue (4R : +2+3+10) ont été générées (Gotz et al. 1995; Brion et al. 1999). Dans les deux cas, Tau est retrouvée à la fois dans les compartiments somato-dendritique et axonal (Gotz et al. 1995; Brion et al. 1999). Toutefois, chez ces souris transgéniques, aucune anomalie phénotypique ni neuropathologique n'a été observée (Gotz et al. 1995; Brion et al. 1999). Cette absence de phénotype pourrait être expliquée par le faible niveau d'expression du transgène par rapport à Tau endogène. Dans les deux cas, le transgène est sous le contrôle d'un promoteur qui induit un faible niveau d'expression (10 à 14% du niveau total de la protéine) (Gotz et al. 1995; Brion et al. 1999). Ainsi, d'autres souris transgéniques, surexprimant ces mêmes isoformes sous le contrôle d'un promoteur plus fort, ont été alors produites (Ishihara et al. 1999; Spittaels et al. 1999; Probst et al. 2000). Chez ces dernières, une hyperphosphorylation, une accumulation dans le compartiment somatodendritique de Tau et de la neurodégénérescence ont été observées (Ishihara et al. 1999; Spittaels et al. 1999; Probst et al. 2000). De plus, la dégénérescence axonale est accompagnée d'une accumulation, dans le corps cellulaire, des microtubules, des neurofilaments, des mitochondries, et de vésicules membranaires (Ishihara et al. 1999; Spittaels et al. 1999; Probst et al. 2000). Rappelons qu'un tel effet a été aussi observé suite à la surexpression de Tau dans des lignées cellulaires neuronales et non neuronales (Ebneth et al.

1998; Stamer et al. 2002). Ces observations suggèrent que la surexpression *in vivo* de Tau pourrait entraîner la neurodégénérescence et une perturbation du transport axonal.

Une autre approche a été employée pour permettre de produire des souris transgéniques exprimant simultanément les six isoformes de Tau humaines chez des souris mutantes nulles pour le gène Tau murin (Duff et al. 2000). Rappelons que dans le cerveau adulte murin, on retrouve seulement les trois isoformes de Tau 4R (les isoformes de Tau 3R ne seraient pas exprimées chez la souris adulte). Le laboratoire de Peter Davies a généré ces souris en utilisant l'ADN génomique humain de Tau (Andorfer et al. 2003). Les souris ainsi produites, en l'absence de Tau murine, représentent un modèle plus approprié à l'étude de la maladie d'Alzheimer. En effet, à partir de l'âge de trois mois, une redistribution de la protéine Tau du compartiment axonal vers le compartiment somato-dendritique a été observée. À six mois, des accumulations de Tau hyperphosphorylée sont détectées dans le compartiment somato-dendritique (ce qui correspondrait au stade pré-tangles). De plus, les auteurs ont décrit des agrégats de Tau et des PHFs chez des souris de 9 mois (Andorfer et al. 2003). Ainsi, cette approche élégante a permis de générer une lignée de souris qui développe une pathologie de Tau dont la distribution subcellulaire et la progression au cours du temps seraient comparables à celles qui ont lieu dans la maladie d'Alzheimer (Braak et al. 1994; Andorfer et al. 2003).

- ***Souris surexprimant la protéine Tau humaine portant des mutations associées aux FTDP-17***

Plusieurs lignées de souris transgéniques surexprimant la protéine Tau humaine portant des mutations associées aux FTDP-17 (G272V, P301L, P301S, V337M, R406W) ont été produites. Certaines lignées de souris développent seulement des agrégats insolubles de Tau hyperphosphorylée (P301S, R406W, G272V) alors que chez d'autres souris transgéniques (P301L, V337M) des NFTs ont aussi été observées. L'hyperphosphorylation de Tau décrite chez ces souris est similaire à celle décrite dans les tauopathies. De plus, l'hyperphosphorylation semble toujours précéder l'assemblage de filaments insolubles de Tau. Par ailleurs, dans certaines lignées de souris transgéniques (P301S et P301L) la mort neuronale ne serait pas attribuable à l'apoptose (Allen et al. 2002; Zehr et al. 2004) (voir aussi les articles de revue de (Götz 2001; Brandt et al. 2005; Lee et al. 2005)).

Les souris transgéniques JNPL3 qui surexpriment l'isoforme Tau 4R portant la mutation exonique P301L sous le contrôle du promoteur de Prion, représentent un bon modèle d'étude (Lewis et al. 2000). La mutation P301L est la mutation la plus fréquente chez les patients atteints de FTDP-17 (Spillantini et al. 1998a). De plus, la lignée de souris transgéniques, produite par Lewis et al., récapitule bien le phénotype et les caractéristiques neuropathologiques retrouvées chez les patients portant la même mutation (Lewis et al. 2000).

- Le niveau d'expression du transgène est équivalent à celui de Tau endogène murine.
- Phénotypiquement, les souris JNPL3 montrent des changements de comportement (docilité, perte de vocalisation et négligence de l'hygiène) ainsi que des déficits moteurs (faiblesse et paralysie). De tels

changements reflètent les symptômes cliniques similaires à ceux des patients porteurs de la même mutation.

- Au niveau neuropathologique, ces souris présentent une gliose et une dégénérescence axonale (48% des neurones moteurs dégénèrent).
- Avec l'âge, ces souris développent des NFTs principalement au niveau de la moelle épinière alors que dans le cerveau les neurones affectés sont principalement au stade «pré-tangle».

La génération de ces modèles animaux a permis une meilleure compréhension des mécanismes qui conduisent au dysfonctionnement de Tau. Ainsi, une mutation ou une altération de l'état de phosphorylation de Tau peut être suffisante pour conduire à la mort neuronale mais la surexpression et/ou la mutation de Tau ne conduit pas toujours à la formation de NFTs.

1.5. Hypothèses et objectifs

Dans les neurones, les microtubules jouent un rôle clé lors de la formation et le maintien des prolongements neuronaux. Ces microtubules servent de support pour le transport intracellulaire assuré par les MAPs motrices. Au cours des dernières années, plusieurs études ont permis de mieux comprendre comment les kinésines et les dynéines médient le mouvement des organelles membranaires le long des microtubules (Hirokawa 1998; Allan and Schroer 1999; Karcher et al. 2002; Nakata and Hirokawa 2003). Cependant, l'inhibition de l'expression de ces MAPs motrices conduit seulement à la perte partielle de la distribution des organelles le long des microtubules (Feiguin et al. 1994; Harada et al. 1998). Ces résultats suggèrent que d'autres protéines, non motrices, seraient impliquées dans l'établissement de liens stables entre les microtubules et les organelles membranaires (Feiguin et al. 1994; Harada et al. 1998). Ainsi, les organelles membranaires peuvent s'associer avec les microtubules de manière dynamique ou de manière stable (Baumann and Walz 2001; Horton and Ehlers 2003a).

D'ailleurs, une nouvelle famille de MAPs qui établissement des liens stables entre les microtubules et les organelles membranaires ont été décrites (Rickard and Kreis 1996; Akhmanova et al. 2001; Mimori-Kiyosue and Tsukita 2003). Ainsi, il existe de plus en plus d'évidences que les microtubules, et leurs protéines associées, seraient aussi impliqués dans l'ancrage et la stabilisation des organelles membranaires dans les différents compartiments cellulaires.

La fonction de MAP2 et Tau la plus étudiée et la mieux caractérisée est leur capacité de lier et de stabiliser les microtubules (Cleveland et al. 1977; Kim et al. 1979). Toutefois, la désorganisation peu sévère au niveau du réseau microtubulaire chez les souris transgéniques déficientes pour Tau ou MAP2 (Harada et al. 1994; Takei et al. 2000; Dawson et al. 2001; Teng et al. 2001)

suggère que Tau et MAP2 pourrait avoir d'autres fonctions que celle de stabiliser les microtubules.

Dans cette thèse, nous avons voulu explorer la possible contribution possible de MAP2 et Tau dans la distribution polarisée des composants membranaires (particulièrement le RER et l'appareil de Golgi) au cours de la différenciation neuronale.

Dans un premier temps, nous avons montré que MAP2 est s'associée au RER *in vitro* et *in vivo*. Cette association semble impliquer p63, une protéine intégrale du RER, et le domaine de projection de MAP2. De plus, nos résultats indiquent que l'interaction de ces deux protéines permet d'effectuer un lien entre le RER et les microtubules *in vitro*.

D'autre part, dans une étude menée dans notre laboratoire, nous avons caractérisé une nouvelle association de Tau avec les membranes golgiennes (voir article en annexe). À la lumière de ces résultats, nous avons évalué l'effet de la surexpression de Tau humaine non mutante ou mutante (P301L, V337M et R406W) sur l'organisation de l'appareil de Golgi dans des cultures primaires de neurones. Nos résultats ont révélé qu'une surexpression de Tau peut altérer l'organisation de l'appareil de Golgi en le fragmentant. Cette fragmentation est d'autant plus importante si Tau porte les mutations associées aux FTDP-17. En outre, nous avons étudié l'organisation de l'appareil de Golgi chez les souris transgéniques JNPL3. Nos résultats montrent une fragmentation de l'appareil de Golgi chez les souris JNPL3 huit fois plus importante que celle observée chez les souris témoins. Cette altération semble être spécifique au Golgi (l'organisation du RE ne semble pas être affecté) et serait précoce dans le processus de neurodégénérescence puisque la fragmentation du Golgi peut avoir lieu avant même la formation des NFTs.

L'ensemble de nos résultats suggèrent que les protéines MAP2 et Tau pourraient jouer un rôle lors la polarisation des composants membranaires au cours du développement neuronal et a leur maintien à l'âge adulte.

2. premier article

"Interaction of microtubule-associated protein-2 and p63: a new link between microtubules and rough endoplasmic reticulum membranes in neurons

Interaction of microtubule-associated protein-2 and p63: a new link between microtubules and rough endoplasmic reticulum membranes in neurons

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2.1. SUMMARY

Neurons are polarized cells presenting two distinct compartments: dendrites and an axon. Dendrites can be distinguished from the axon by the presence of rough endoplasmic reticulum (RER). The mechanism by which the structure and distribution of the RER is maintained in these cells is poorly understood. In the present study, we investigated the role of the dendritic microtubule-associated protein, MAP2 in the RER membrane positioning by comparing their distribution in brain subcellular fractions as well as in primary hippocampal cells and by examining MAP2/microtubule interaction with RER membranes in vitro. Subcellular fractionation of rat brain revealed a high MAP2 content in a subfraction enriched with the ER markers, ribophorin and p63. Electron microscope morphometry confirmed the enrichment of this subfraction with RER membranes. In cultured hippocampal neurons, MAP2 and p63 were found to concomitantly compartmentalize to the dendritic processes during neuronal differentiation. Protein-blot overlays using purified MAP2c protein revealed its interaction with p63 and immunoprecipitation experiments performed in HeLa cells showed that this interaction involves the projection domain of MAP2. In an in vitro reconstitution assay, MAP2-containing microtubules were observed to bind to RER membranes in contrast to microtubules containing tau, the axonal MAP. This binding of MAP2c-microtubules was reduced when an anti-p63 antibody was added to the assay. The present results suggest that MAP2 is involved in the association of RER membranes with microtubules and thereby could participate in the differential distribution of RER membranes within a neuron.

2.2. INTRODUCTION

Neurons are polarized cells that present two distinct compartments, dendrites and an axon. These compartments can be distinguished by their morphology: dendrites are multiple and taper whereas the axon is unique and its diameter is uniform (Bartlett and Banker 1984; Dotti et al. 1988; Hillman 1988). Dendrites and axon also differ by their membranous organelle composition: RER is found in the somato-dendritic compartment but not in the axon and the number of free ribosomes is a lot higher in dendrites than in the axon (Bartlett and Banker 1984; Peters et al. 1991). The cytoskeletal elements are also distinctly distributed in these neuronal compartments: the number of microtubules is higher in dendrites than in the axon whereas the opposite is noted for neurofilaments (Bartlett and Banker 1984; Hirokawa 1991).

Microtubules are involved in the establishment of cell polarity. Notably, the microtubules of dendrites and axon contain different microtubule-associated proteins (MAPs): the microtubule-associated protein-2 (MAP2) is found in dendrites whereas tau is present only in the axon (Caceres et al. 1984; Hirokawa 1991; Ludin and Matus 1993). The contribution of these MAPs to the establishment of neuronal polarity has been well documented. Tau contributes to the axonal differentiation in primary neuronal cultures whereas MAP2 is involved in the differentiation of minor neurites, the neuronal processes that become dendrites, and in the maintenance of dendrites in adult neurons (Caceres and Kosik 1990; Caceres et al. 1991; Dinsmore and Solomon 1991; Caceres et al. 1992; Sharma et al. 1994; Sanchez et al. 2000). Despite the fact that MAP2 and tau are known to induce microtubule formation in neurons, their precise role in the elaboration of dendrites and axon remains elusive. Each of these proteins

presents different isoforms that are generated by alternative splicing. Splicing events occur in the microtubule-binding domain that is confined to three or four imperfect repeated domains of 18 amino acids located in the COOH-terminus and in the projection domain that extends at the surface of the microtubules (Buee et al. 2000; Sanchez et al. 2000). The latter domain, the projection domain, regulates the spacing between microtubules (Chen et al. 1992; Leclerc et al. 1996; Belanger et al. 2002). MAP2 and tau share sequence homology in the microtubule-binding domain and in the adjacent proline-rich region located between the projection domain and the microtubule-binding domain (Lewis et al. 1989; Felgner et al. 1997; Goode et al. 1997). The presence of a distinct class of MAPs in dendrites and axon suggests that these proteins may have another function than the stabilization of microtubules. Consistent with this hypothesis, microtubule formation by MAP2 and tau is not sufficient to induce process outgrowth in Sf9 cells (Knops et al. 1991; Leclerc et al. 1996; Belanger et al. 2002). Indeed, MAP2b promoted the formation of microtubules in these cells but only 7% of the MAP2b-expressing cells developed processes. Thus, the stabilization of microtubules does not seem to be the sole function of MAP2 in dendritic outgrowth. In recent years, our work and that of others showed that MAP2 could also interact with actin microfilaments and neurofilaments (Leterrier et al. 1982; Bloom and Vallee 1983; Heimann et al. 1985; Sattilaro 1986; Hirokawa et al. 1988; Correas et al. 1990; Cunningham et al. 1997). Thus, MAP2 could act as a cytoskeletal integrator in dendrites by linking together the three cytoskeletal elements. This role remains to be characterized.

Besides interacting with the three constituents of the neuronal cytoskeleton, MAP2 can also interact with signaling proteins. For example, MAP2 can interact with the regulatory subunit RII of the c-AMP dependent

protein kinase (PKA) (Rubino et al. 1989). In MAP2 knockout mice, there is a reduction of total PKA in dendrites and the rate of induction of phosphorylated CREB is reduced after forskolin stimulation (Harada et al. 2002). These events were accompanied by a decrease of dendritic elongation. From these data, one can conclude that MAP2 plays an important role in the polarized distribution of signaling proteins that regulate dendritic differentiation and plasticity.

In a recent study, we reported that MAP2 was found in a crude membrane preparation from mouse spinal cord homogenate suggesting that MAP2 could be associated with membranous organelles (Farah et al. 2003). In recent years, families of proteins called CLIPs and Hooks were shown to mediate the interaction between microtubules and membranous organelles (Pierre et al. 1992; De Zeeuw et al. 1997; Hoogenraad et al. 2000; Walenta et al. 2001). Interestingly, CLIP-115 was found to be responsible for the polarized distribution of a membranous organelle exclusively present in dendrites termed the dendritic lamellar bodies (DLB) (De Zeeuw et al. 1997). Until now, no microtubule-associated protein has been identified that contributes to the dendritic distribution of RER membranes. Our present data indicates that MAP2 could play such a role. Here, we report a novel association of MAP2 with RER membranes using subcellular fractionation, electron microscopy immunocytochemistry and electron microscopy in an in vitro reconstitution assay. Moreover, we showed that this association involves the interaction of MAP2 projection domain with a 63 kDa non-glycosylated type II integral RER membrane protein, termed p63, which was found to mediate the interaction between RER membranes and microtubules in a previous study (Schweizer et al. 1995; Klopfenstein et al. 1998; Klopfenstein et al. 2001). Taken together, our results suggest that the

interaction between MAP2 and p63 might contribute to the preferential distribution of RER membranes in the dendritic processes.

2.3. MATERIALS AND METHODS

2.3.1. Subcellular fractionation

Animals were purchased from Charles River (Charles River Laboratories Inc., Montreal, Quebec, Canada). The use of animals and all surgical procedures described in this article were carried out according to *The guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.* Brain was dissected from twenty adult rats Sprague-Dawley, the protocol previously described by Lavoie et al. was used to separate rough microsomes and Golgi elements from total microsomes (Lavoie et al. 1996). A schematic of the protocol is shown in Fig. 1A. Briefly, total microsomes were isolated by differential centrifugation and ER and Golgi elements were subsequently purified by ultracentrifugation in a sucrose step-gradient.

2.3.2. Immunoblot analysis

Protein assay was performed (Bio-Rad kit, Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Equal amounts of proteins were loaded in each lane and electrophoresed in a 7.5% polyacrylamide gel. Following separation, proteins were electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose strips were incubated with the primary antibodies during 90 min at room temperature. They were then washed with Phosphate Buffered Saline (PBS) and then incubated with the peroxidase-conjugated secondary antibodies. Membranes were again washed and then revealed by chemiluminescence (Amersham Pharmacia Biotech, Quebec, Quebec, Canada). The following primary antibodies were used: the monoclonal antibody anti-MAP2 (clone

HM2, Sigma, Oakville, ON), the monoclonal antibody anti-tau (clone Tau-5, Oncogene Research Products, San Diego, California), the monoclonal antibody anti-α-tubulin (clone DM 1A, Sigma, Oakville, ON), a polyclonal antibody against ribophorin II, a polyclonal and a monoclonal antibody against p63 (kindly provided by Dr. H.P. Hauri, University of Basel, Switzerland), a polyclonal antibody against Porin (Oncogene Research Products, San Diego, California), a polyclonal antibody against mannosidase II (kindly provided by Dr. M.G. Farquhar, University of California, San Diego) and a polyclonal against NaK-ATPase (kindly provided by Dr. D. Fambrough, The Johns Hopkins University, Maryland).

2.3.3. Electron microscopy

Microsomes isolated from brain were fixed using 2.5% glutaraldehyde, recovered onto Millipore membranes by the random filtration technique of Baudhuin et al. (Baudhuin et al. 1967) and processed for electron microscopy as previously described (Lavoie et al. 1996).

2.3.4. Pre-embedding electron microscope immuno-cytochemistry

Immunolocalization of HM2 was modified from that used previously by Dominguez et al. (Dominguez et al. 1991). 200 µg of the rough microsomal fraction (RM) were resuspended in 10% normal goat serum/saline (0.9% NaCl / 10 mM Tris-HCl pH 7.4) solution containing the primary antibody. The monoclonal HM2 antibody was used at a concentration of 1:200. The incubation was allowed to proceed overnight at 4°C. RM were then fixed for 30 min at 37°C in 0.05% glutaraldehyde solution and recovered onto Millipore membranes by the filtration technique of Baudhuin et al. (Baudhuin et al.

1967). Membranes were then washed with saline solution, blocked in 1% ovalbumine/PBS solution for 30 min at room temperature and incubated with the anti-mouse IgG-colloidal gold solution (Sigma, 1:10) diluted in 0.02% Polyethylene glycol/saline for 60min. Following the washing steps, membranes were fixed with 2.5% glutaraldehyde at 4°C overnight and processed for electron microscopy as described above.

2.3.5. Primary hippocampal cultures

Primary embryonic hippocampal cultures were prepared from18-day-old rat fetuses as previously described (Banker and Goslin 1998). After removal of meninges, hippocampi were treated with trypsin (0.25% at 37°C for 15 min.) then washed in Hank's balanced solution and dissociated by several passages through a constricted Pasteur pipette. The cells were then plated on glass coverslips coated with polylysine. Cells were plated at 200 000 cells per 60 mm Petri dishes. Then, after 4 h to allow the attachment of the cells to the substrate, the hippocampal cells were transferred either in a serum-free B-27 supplemented neurobasal medium or in N2 supplemented medium.

2.3.6. Immunofluorescence

Neurons were fixed in 4% paraformaldehyde/PBS for 45 minutes. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. The MAP2 protein was revealed using a monoclonal antibody directed against MAP2 (clone HM2, dilution 1:200) purchased from Sigma (Mississauga, Ontario, Canada) or the rabbit polyclonal anti-MAP2 (1:2000) (kindly provided by Dr. Richard Vallée, Columbia University, New York, USA). The endoplasmic reticulum was revealed using the A1/59 monoclonal anti-p63 (1:100) (kindly provided by Dr. H.P. Hauri, University of Basel, Switzerland).

To visualize tau protein, a rabbit polyclonal anti-tau antibody was used at a concentration of 1:750 (kindly provided by Dr. Virginia M-Y Lee, University of Pennsylvania, Philadelphia, USA). Microtubules were revealed using a rat polyclonal antibody directed against α-tubulin (Abcam, Cambridge, UK). We used the following secondary antibodies: a donkey anti-mouse conjugated to FITC (dilution 1:100), a donkey anti-rabbit conjugated to Rhodamine (1:500) an Alexa Fluor 647 anti-rat (1:400) or an AMCA anti-rat (1:300). All these secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada. These antibodies were diluted in 5% BSA/PBS. Incubations were carried out at room temperature for 1 h. After three washes in PBS, the coverslips were mounted in polyvinyl alcohol (Calbiochem, CA, USA). Fluorescently labeled cells were visualized with a Leica TCS-SP1 confocal microscope using 63x or 100x objectives or a Zeiss Axioplant fluorescence microscope using 20X or 40X objectives.

2.3.7. GFP- MAP2 fusion proteins

Rat MAP2c cDNA were inserted in the expression vector pEGFP-C1 (Clontech, CA, USA). The polylinker of this expression vector was modified, three cloning sites, BamHI, ScaI and NotI were inserted by annealing of the oligonucleotides on 5'TCGAGGGATCCAGTACTGCGGCCGCTTAATTAA-3' and 5'-CCCTAGGTCATGACGCCGGCGAATTAATTCTAG-3'. Then the annealed sequence was ligated into the vector pEGFP-C1 at the XhoI and BamHI restriction sites. Then the full length cDNA coding for MAP2c was excised, with BamHI and NotI restriction enzymes, from the BacPAK-HIS2 vector previously described(Belanger et al. 2002). The GFP tag was fused to the MAP2c sequence at its N-terminus. Deleted mutants corresponding to the projection domain of MAP2c (Proc: nucleotides 1-444) and to the microtubule-

binding domain (Mt: nucleotides 442-1551) were generated. These mutants were inserted in the vector pEGFP-C1. The pEGFP-C1 Tau4R plasmid was kindly provided by Dr. Ken Kosik(Lu and Kosik 2001).

2.3.8. Transfection of primary hippocampal neurons

After seven days in culture, hippocampal neurons were transfected using a modified calcium phosphate transfection protocol(Micheva et al. 2001). Briefly, neurons were transferred in a 6-well plate. The calcium and DNA precipitate was generated 30 min before transfection by mixing drop-wise 4µg of Qiagen-purified DNA in 60 µl of 250 mM $CaCl_2$ per well with an equal volume of 2X HBS (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2 HPO_4, 15 mM glucose, 42 mM HEPES, pH=7.07). Cells were incubated with transfection precipitate for 30 min at 37°C and 5% CO_2. Following incubation, cells were washed 3 times with Hank's balanced solution supplemented with 10 mM Hepes and retransferred in the original Petri dishes containing the glial momolayer and the N2 serum free medium. Protein expression was allowed to proceed for 24 h. The cells were then fixed and processed for immunofluorescence.

2.3.9. MAP2c and tau purification from Sf9 cells

Sf9 cells were purchased from the American Type Culture Collection (ATCC # CRL 1711; Rockville, MD). Cells were grown in Grace's medium (Gibco, BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT) as a monolayer at 27°C. For protein purification, Sf9 cells were grown as a suspension to obtain a final concentration of 1.5 x 10^6 cells/200ml total media volume and were then infected with MAP2c or tau viral stocks (Baas et al. 1994; Belanger et al.

2002). Infection was allowed to proceed for 48-72 h before the cells were centrifuged at 1000x g. The cell pellet was kept at -80°C until protein purification. Protein purification was performed using the boiling preparation method as previously described (Hernandez et al. 1986).

2.3.10. Overlay Assay

A fraction enriched in proteins of rough microsomes (RM) was prepared from rat liver as previously described(Lavoie et al. 1996). RM proteins from rat liver were separated on a 7.5% polyacrylamide gel. Following separation, proteins were electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membranes were then incubated for 1h at room temperature in the overlay buffer: 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 3% Non fat milk, 0.1% Tween. The membrane was then incubated, overnight at 4°C, in 500 µl of the overlay buffer plus 1mM DTT and 1mM PMSF containing 10 µg of the purified MAP2c. The following day, after two washes in TBS-Tween (25mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% tween 20), the membranes were incubated for 1h30 min in the overlay buffer containing 1mM DTT, 1mM PMSF with the monoclonal anti-MAP2 clone HM2 (1:1000) to detect the interactions of MAP2 with RM proteins. This was followed by an incubation a with a horseradish peroxidase-conjugated mouse antibody (Jackson Immunoresearch Laboratories). To reveal proteins, an ECL detection kit was used (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions.

2.3.11. Co-Immunoprecipitation

Adult or immature embryonic (E19) or neonatal (P0) Sprague-Dawley rat brain were dissected and homogenized in the immunoprecipitation (IP) buffer: 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 5mM EGTA, 5mM EDTA,

5mM Na_3VO_4, 50 mM NaF, 1mM DTT, 1% IGEPAL and a cocktail of protease inhibitors (Roche Diagnostics, Canada). The homogenate was sonicated for 5 sec at an amplitude of 6% and then centrifuged for 20 min at 13500 x g. The supernatant was used for the co-immunoprecipitation experiments. The monoclonal anti-MAP2 (clone HM2) or the polyclonal anti-p63 antibody was added to 1 ml of the supernatant and incubated overnight on a rocking platform at 4°C. 100 µl of protein A-Sepharose beads were then added to the immunoprecipitates and incubation was allowed to proceed for 1 h at 4°C. After a short spin at 14000 x g at 4°C, the supernatant was removed and the beads were washed 6 times with 1 ml cold IP buffer. Finally, the beads were resuspended in 50µl of loading buffer and boiled for 5 minutes. The protein A-sepharose was removed by centrifugation at 12000 x g at room temperature. The samples were analyzed by SDS-PAGE using 12% polyacrylamide gel.

2.3.12. Cell culture and protein expression

HeLa cells (ATCC # CRL CCL-2, Rockville, MD) were cultured in DMEM media (Gibco BRL, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% peniciline/streptomycine at 37°C in a humidified 5% CO_2 incubator. For transfection, HeLa cells were plated in 35 mm Petri dishes, and grown overnight to approximately 80% confluency. Cells were then transiently transfected using the polyfect transfection reagent (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Briefly, a mixture of 1,5 µg of plasmid DNA, 15 µl of polyfect reagent, and 100 µl of DMEM were incubated for 10 min at room temperature and 600 µl of cell culture media was then added to the complex. Cells were washed with PBS and subsequently incubated with the DNA-polyfect complex. The expression was allowed to proceed for 24h at 37°C. The

following day, after two washes with cold PBS, transfected HeLa cells were scrapped off into 1ml IP buffer and incubated on ice for 1h to lyse the cells. The lysate was sonicated for 5 sec at an amplitude of 6%, passed 10 times through a 25-gauge needle and then centrifuged at 14000 x g for 20 min at 4°C. The resulting supernatant was used to perform co-immunoprecipitation as described in the previous section. To immunoprecipitate the full length GFP-MAP2c fusion protein and the truncated form GFP-Proc, either the monoclonal anti-MAP2 clone HM2 (1:250) or the monoclonal anti-GFP antibody (Roche Diagnostics, Canada) was used. To immunoprecipitate GFP-Mt protein or the GFP-tau 4R protein the monoclonal anti-GFP antibody (Roche Diagnostics, Canada) was used at a concentration of 1:100.

2.3.13. Negative staining for electron microscopy

Tubulin (2mg/ml) was allowed to polymerize in the presence of MAP2c or tau protein (0.5 mg/ml) in the G-PEM buffer for 35 min at 37°C. Microtubules were then added to a freshly prepared nuclear fraction from rat liver and the incubation was allowed to proceed for 30 minutes. To monitor the presence of microtubules in this preparation, 5 µl of the supernatant were placed on a carbon-coated formvar-supported EM grid. After an incubation of 30 sec, the grid was rinsed with distilled water and stained with 1% (w/v) uranyl acetate for 30 sec. Samples were visualized with a Zeiss CM 902 transmission electron microscope.

2.3.14. Preparation of nuclear fractions

Nuclear fractions were prepared from rat liver homogenates (1:2, w/v) in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.5, 0.025 M KCl and 0.005 M $MgCl_2$ (0.25 M sucrose-TKM) using the procedure of Blobel and Potter (Blobel and

Potter 1966). The isolated nuclei were resuspended in cold 0.25M sucrose-TKM and centrifuged for 10 min at 1000x g. Nuclei from 10 ml of homogenate were resuspended by gentle stirring with a glass rod in 1.2 ml G-PEM buffer (80 mM PIPES, pH 6.9, 1 mM $MgCl_2$ and 1 mM EGTA to which 1 mM GTP was added prior to use). To detect p63 protein, the nuclear fraction was treated with Dnase according to a previously described protocol to get an enrichment of nuclear membranes (Dwyer and Blobel 1976).

2.3.15. In vitro microtubule membrane reconstitution

An in vitro microtubule-membrane reconstitution assay was modified from that previously described (Paiement 1981). Bovine brain tubulin protein was purchased from Cytoskeleton (Denver, CO). Tubulin (2 mg/ml) was allowed to polymerize in the presence of MAP2c or tau protein (0.5 mg/ml) in the G-PEM buffer for 35 min at 37°C. Freshly prepared nuclear fraction was resuspended in the G-PEM buffer to which 5 mM $MgCl_2$, 1 mM GTP, 2 mM ATP and a cocktail of protease inhibitors (Roche, Laval, Quebec, Canada) were added prior to use. 50 µl of this fraction were added to the polymerized microtubules and incubation was allowed to proceed for another 30 min at 37°C. The nuclei were then centrifuged at 1000 x g for 5 min at 37°C. The supernatant was discarded and the pellet was fixed using 2.5% glutaraldehyde and 1% sucrose in the G-PEM buffer. Fixation was performed at 37°C for 30 min and the samples were then processed for electron microscopy as described above. In two sets of experiments, the polyclonal anti-p63 antibody (1:50) was added to 50 µl of the nuclear preparation and incubated at room temperature for 30 min. Then, this preparation was incubated with the polymerized microtubules.

2.4. RESULTS

2.4.1. The distribution of MAP2 in subcellular fractions of rat brain

In a previous study, we reported that high molecular weight MAP2 proteins (HMW MAP2) were present in a crude membrane fraction isolated from mouse brain homogenates (Farah et al. 2003). In the present study, MAP2 distribution was studied using subcellular fractions from rat brain. MAP2 distribution was examined in adult rat brain subfractions prepared using a fractionation protocol designed to purify rat liver ER (Lavoie et al. 1996) and summarized in Fig. 1A. MAP2 distribution was compared to that of various markers for different cellular organelles: Na-K-ATPase, a plasma membrane marker, ribophorin and p63, markers of the endoplasmic reticulum, mannosidase II, a Golgi marker and Voltage-Dependent Anion Channel or VDAC, a mitochondrial marker (Fig. 1B). The cytoskeletal marker tubulin was also used to characterize the subfractions. HMW MAP2 was found in the total microsomal fraction (P) as shown in Fig. 1B. Furthermore, HMW MAP2 was found enriched in a subfraction containing p63 and ribophorin, two proteins mainly found in the rough ER (RER) compartment (Kelleher et al. 1992; Schweizer et al. 1993; Rajasekaran et al. 1995; Schweizer et al. 1995; Torre and Steward 1996). Based on the enrichment with these two markers, this subfraction was named RM for rough microsomes (Fig. 1B). This subfraction also contained mitochondrial membranes as revealed by the anti-VDAC antibody. MAP2 is known to interact with mitochondria (Jancsik et al. 1989; Linden et al. 1989; Jung et al. 1993). However, it seemed unlikely that MAP2 in the RM subfraction was associated only with mitochondria since: 1) the subfraction I3 presented an amount of VDAC similar to that of the RM subfraction but the amount of MAP2 in this subfraction was much lower than

that in the RM subfraction and 2) electron microscopy morphometry analysis confirmed that this subfraction contained very few mitochondria (see Fig. 2). Taken together, these data suggested that HMW MAP2 isoforms could be associated with RER membranes.

Since RER is mainly found in neuronal cell bodies and dendrites and MAP2 is a somato-dendritic MAP (Ludin and Matus 1993), it seems reasonable to consider an association between this MAP and RER. In this case, the RM subfraction was expected to contain little or no tau, the MAP found in the axon (Ludin and Matus 1993). Figure 1C illustrates that RM was highly enriched in MAP2 but in contrast contained very little tau.

Electron microscopy of the RM subfraction confirmed the presence of rough microsomes (Fig. 2A and B). Furthermore, morphometric analysis indicated that 51% of the vesicles in this subfraction presented ribosomes attached to the membrane. The microsomes in RM were heterogeneous in shape and size (Fig. 2A and B) and were often observed in association with membrane-free filaments with associated ribosomes (see ovals in Figs. 2A and B). A small amount of tubulin was detected by immunoblotting (Fig. 1B) but no microtubule was observed in the RM subfraction (Fig. 2A and B). Presumably, they were depolymerized at low temperature (4°C) during fractionation. Electron microscope immunocytochemistry was carried out to reveal the association of MAP2 with membranes in the RM subfraction (Fig. 2C and D). A quantitative analysis of the gold particles found on membranes was performed to demonstrate the preferential association of MAP2 with rough membranes (Table 1). Results from three different sets of experiments revealed that 88,6% of MAP2 immunogold labeling associated with membranes was present on rough membranes.

2.4.2. Somato-dendritic compartmentalization of p63 and MAP2 in primary hippocampal cultures

In primary hippocampal cultures, MAP2 is segregated to the somato-dendritic compartment during establishment of neuronal polarity (Caceres et al. 1984; Hirokawa 1991; Ludin and Matus 1993). Similarly, in mature neurons, RER is also found in the cell body and dendrites (Bartlett and Banker 1984). We examined whether MAP2 and RER concomitantly segregate to the somato-dendritic compartment in hippocampal neurons during their development. After one day in culture, the hippocampal neurons are polarized cells presenting 3 to 4 short minor neurites that will differentiate to become dendrites and a long thin neurite which develops into the axon (Bartlett and Banker 1984; Dotti et al. 1988). All these neurites terminate in a growth cone, a motile structure presenting a rich actin network at the periphery and bundles of microtubules at the center (Goslin et al. 1989). After ten days in culture, the dendrites and axon are differentiated and the synaptic contacts are established. MAP2 is found in both minor neurites and axon in the first days in hippocampal cultures and then becomes compartmentalized to the dendrites (Kempf et al. 1996). To examine whether the compartmentalization of MAP2 and RER occurs concomitantly in hippocampal neurons, the distribution of p63 was examined before (one-day old cultures) and after (seven-day old cultures) MAP2 becomes concentrated in the somato-dendritic compartment. To clearly demonstrate that RER membranes become enriched in the somato-dendritic compartment during neuronal differentiation, a polyclonal antibody directed against tau was used to distinghish dendritic and axonal processes. Thus, hippocampal neurons were double-labeled with a polyclonal antibody directed against tau and either with the anti-MAP2 antibody HM2 which recognizes both the high and low molecular weight isoforms of MAP2 expressed in these neurons or a

monoclonal antibody directed against p63 (Fig.3). Tubulin staining was used to visualize both dendritic and axonal processes (Fig.3). In one-day old primary hippocampal neurons, MAP2 and tau were present in all neuronal compartments as indicated by the tubulin staining. Similarly, p63 was also found in all neuronal compartments. In seven-day old hippocampal neurons, MAP2 and p63 were concentrated in the dendritic processes but barely evident in the axon as indicated by their poor co-localization with tau staining (Fig.3). Thus, as previously reported for the RER protein, ribophorin, p63 becomes enriched in the somato-dendritic compartment during neuronal differentiation (Torre and Steward 1996). These results show that MAP2 and the RER membranes are co-segregated in the somato-dendritic compartment during hippocampal cell differentiation.

We next determined the effects of overexpression of MAP2c on the distribution of microtubules and RER in primary hippocampal neurons. MAP2c overexpression causes the reorganization of microtubules into thin or thick bundles (Leclerc et al. 1996). Therefore, if RER is tightly associated with microtubules, it should undergo a reorganization similar to that of microtubules under MAP2c overexpression. Seven-day old neurons were transfected with a GFP vector containing cDNA of LMW MAP2, MAP2c. GFP-MAP2c was mostly found in dendrites in seven-day old control hippocampal neurons (Fig. 4A and A'). A similar distribution was noted for RER in these neurons. Seven-day old control neurons transfected with GFP vector revealed no discernable difference in RER distribution compared to control cells (Fig. 4B and B'). Overexpressing MAP2c produced no important changes in the structure of the dendrites and axon of seven-day old hippocampal neurons (data not shown) even though in some of the transfected cells, several thin extensions emerged from the cell body (Fig.4D). A similar phenotype has been previously described

in Sf9 cells expressing MAP2c (Leclerc et al. 1996; Belanger et al. 2002). However, transfection with GFP-MAP2c led to a reorganization of the microtubule network in the neuronal cytoplasm (Fig.4C'' and D''). In most of the MAP2c-transfected neurons, large microtubule bundles were noted in the cell body (Fig.4C''). In these cells, RER staining was found along these bundles (arrows in Fig.4C, C' and C"). In the MAP2c-transfected neurons harboring multiple thin extensions, very thin microtubule bundles were randomly distributed in the cell body and an important reorganization of RER staining was noted in the perikaryon (arrowhead in the inset of Fig.4D, D' and D") along these bundles (Fig.4D, D' and D''). In both types of MAP2c-transfected neurons, GFP-MAP2c and RER distribution were closely associated with the MAP2c-containing microtubule bundles (Fig.4C, C' and C" and D, D' and D''). The concomitant reorganization of both microtubules and RER observed in MAP2c-overexpressing neurons showed that RER and microtubules are intimately associated in hippocampal cells. However, the above results did not demonstrate whether MAP2c was directly involved in the association of RER with microtubules.

2.4.3. Interaction of MAP2 with the integral RER membrane protein p63

To identify the proteins mediating the association of MAP2 with ER membranes, we performed a western blot overlay experiment (Fig.5). The proteins contained in the RM subfraction isolated from adult rat liver were separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with MAP2c protein purified from Sf9 cells. The interaction of MAP2c with the proteins contained in the RM subfraction was revealed by using the anti-MAP2

antibody, HM2. As expected MAP2 staining was found in a doublet band corresponding to the molecular weight of tubulin. Moreover, a MAP2 immunoreactive band was also observed at an apparent molecular weight of 63 kDa. As shown in figure 5, this band reacted with an anti-p63 antibody indicating that p63 could be involved in the association of MAP2 with RER membranes. To further demonstrate this interaction, we tested whether p63 could co-immunoprecipitate with MAP2 in a homogenate prepared from adult rat brain (Fig.6A). The anti-MAP2 antibody, HM2 was used to immunoprecipitate MAP2. As shown in figure 6A, a band immunoreactive to the anti-p63 antibody was present in MAP2 immunoprecipitate. In the brain homogenate, two bands were revealed by the polyclonal anti-p63 antibody, one band at 63 kDa and one band of slightly higher molecular weight. The monoclonal anti-p63 antibody only revealed the lower band at 63 kDa. In the MAP2 immunoprecipitate, only the band at 63 kDa was revealed either using the monoclonal or the polyclonal antibody. We also tested whether the LMW MAP2 isoforms could interact with p63 in immature brain. Brain homogenate was prepared from embryonic (E19) rat or newborn (P0) rat (data not shown) and immunoprecipitation of either MAP2 or p63 was performed. As noted for HMW MAP2 isoforms, in the LMW MAP2 immunoprecipitate, p63 protein was also present (Fig. 6B). Most notably, MAP2c could be immunoprecipitated by using the anti-p63 antibody. From these results, one could conclude that the domain of MAP2 interacting with p63 was located in the peptide sequence common to HMW and LMW MAP2 isoforms.

MAP2 proteins have two main domains, the projection domain and the microtubule-binding domain located at the N- and C-terminal respectively. A construct corresponding to either the projection domain (Proc) or the microtubule-binding domain (Mt) of the LMW MAP2 isoform, MAP2c was

generated and fused to a GFP tag. To determine which of these domains interacted with p63, they were expressed in HeLa cells, a cell line expressing p63 but not MAP2 and their interaction with p63 was examined by co-immunoprecipitation. When MAP2 immunoprecipitation was performed on HeLa cells expressing full-length MAP2c, p63 was found in the immunoprecipitate (Fig.7). In Hela cells, one band of 63 kDa was revealed with both the monoclonal and polyclonal antibody directed against p63 as previously reported (Schweizer et al. 1995; Klopfenstein et al. 1998). However, when immunoprecipitation was performed on HeLa cells expressing full-length tau or the GFP alone, no p63 was detected in either tau or GFP immunoprecipitate. Most notably, when Proc was immunoprecipitated from HeLa cells with the anti-MAP2 antibody, HM2, p63 was found in the immunoprecipitate (Fig. 7). However, p63 was absent from the Mt immunoprecipitate indicating that the MAP2 interacting peptidic sequence with p63 is located within the first 150 a.a. of MAP2 isoforms.

2.4.4. In vitro reconstitution of the microtubule-MAP2-ER complexes

To show that MAP2 can act as a linker between the ER and microtubules, an in vitro membrane–microtubule reconstitution assay was developed. Briefly, ER membranes were incubated with microtubules containing MAP2 and the membrane-bound microtubules were co-sedimented by centrifugation. The association of microtubules with membranes was then examined by electron microscopy. Since both microtubules and ER membranes co-sediment at 100 000xg, we decided to use a nuclear preparation as a source of RER membranes. There are two main advantages to this preparation: 1) RER membranes are with nuclei (Gerace and Burke 1988) and therefore, the results

generated with nuclei are of relevance to the mechanism of interaction between MAP2-microtubules and RER and 2) nuclei sediment at low speed (1000 x g) in contrast to microtubules that sediment at high speed (100000x g). Thus, the only way microtubules can co-sediment with nuclei in this assay is through an association with the nuclei. A fraction of rat liver nuclei prepared using the Blobel and Potter procedure (Blobel and Potter 1966) was used because it is devoid of MAP2 and tau. Moreover, no tubulin was detected in this fraction by immunoblotting (Fig.8A). Finally, the presence of p63 in this nuclear preparation was confirmed by immunoblotting (Fig.8B).

The pre-polymerized microtubules used in the present assay were composed of pure bovine brain tubulin and MAP2c protein purified from Sf9 cells. As a negative control, microtubules were pre-polymerized using the axonal MAP, tau also purified from Sf9 cells. We tested the microtubule polymerizing activity of MAP2c and tau to eliminate the possibility that any difference in the number of microtubules bound to nuclei could be caused by a difference of tau and MAP2c capacity to form microtubules in our experimental conditions. To this purpose, MAP2c (0.5mg/ml) or tau (0.5mg/ml) was incubated with pure bovine brain tubulin (2mg/ml) in the G-PEM buffer. Following an incubation of 30 min at 37°C and a high speed centrifugation (100, 000 x g), the pellet and the supernatant were analyzed by western blotting for their content in tubulin, MAP2c and tau. As shown in Fig.8C, similar amounts of tubulin were found in the MAP2c and the tau pellets indicating that these proteins have a similar tubulin polymerizing activity in the present conditions. Lastly, microtubule formation by MAP2c and tau was examined at the electron microscopic level by negative staining. Individual and bundles of microtubules were observed in both MAP2c- and tau- microtubule preparations (Fig.8D and E). Nuclei were incubated with exogenous microtubules that were

pre-polymerized either in the presence of MAP2c or tau. Following the incubation, nuclei were pelleted at a centrifugation speed of 1000xg. Nuclei were then resuspended in fresh buffer and processed for electron microscopy. All incubations of nuclei with microtubules were performed at 37°C since microtubules depolymerize at lower temperature. No microtubule was observed in association with either the control nuclei or nuclei incubated in the presence of tau-prepolymerized microtubules (Fig.9A and B). To eliminate the possibility that the tau-containing microtubules depolymerized during the incubation with the nuclei, the presence of microtubules was monitored at the electron microscopic level by negative staining (Fig.8F). However, when nuclei were incubated with MAP2c-pre-polymerized microtubules, microtubules were observed as small cylinders with a constant diameter (~25 nm in diameter) sectioned in different orientations on their surface (see arrows in Fig.9C). Thus the above experiments showed that MAP2c- but not tau-pre-polymerized microtubules could associate with nuclei and this association was necessary for microtubules to co-sediment at 1000xg. These results were reproduced in five different series of experiments.

A quantitative analysis was carried out to evaluate the number of MAP2c-containing microtubules associated with the rat liver nuclei. Microtubules located within 25 nm of the outer nuclear membrane were included in the analysis since this is considered a physiologically relevant distance for microtubule interaction based on measurement of spacing between microtubules in bundles induced by MAP2c and tau in Sf9 cells (Leclerc et al. 1993; Belanger et al. 2002). Most of the microtubules (>80%) were organized in bundles of two or more microtubules. The number of microtubule bundles per nucleus was quantified on 19 nuclei from four different sets of experiments for a total of 76 nuclei. On average, 2.26 $\pm$ 0.25 microtubule bundles were

found associated with one nucleus. These results indicate that MAP2c might be involved in the interaction of RER membranes with microtubules *in vivo*. In the last two sets of experiments, an additional experimental condition was added to examine the contribution of p63 in the interaction of MAP2-containing microtubules with nuclei. To do this, the polyclonal anti-p63 antibody was added to the nuclei preparation 30 min before their incubation with the MAP2c-microtubules. In these two sets of experiments, anti-p63 antibodies were found to be able to reduce MAP2-microtubule binding to the nuclei. Quantification revealed 2 ± 0.26 bundles of MAP2c-microtubules associated with one nucleus in the control experiments whereas in presence of the anti-p63 antibody, 0.94 ± 0.14 bundle was attached to one nucleus. This indicated that p63 contributes to the association of MAP2c containing microtubules with nuclei.

2.5. DISCUSSION

In the present study, we describe a novel type of ER-microtubule interaction that is mediated by the dendritic MAP, MAP2. A subfraction enriched in rough ER microsomes (RM) was isolated from rat brain as indicated by its high content in the two RER proteins, ribophorin and p63, as well as by an electron microscope morphometric analysis using the ribosome as a morphological marker for RER. An important amount of MAP2 was detected in the RM subfraction by immunoblotting. In primary hippocampal neurons, MAP2 and p63 were co-segregated to the somato-dendritic compartment during neuronal differentiation. An overlay assay and co-immunoprecipitation experiments indicated that the association of MAP2 with RER membranes involved p63. The interacting domain of MAP2 with p63 was found to be located in the first 150 residues of MAP2 projection domain. Using an in vitro reconstitution assay, MAP2 was shown

to mediate the association between microtubules and RER membranes. Most notably, this association was significantly reduced in the presence of an anti-p63 antibody. Collectively, our results point to a role for MAP2 and p63 in the distribution and structural maintenance of RER in neurons.

Previous studies have shown that the ER membranes are associated with microtubules (Terasaki et al. 1986; Terasaki 1990; Terasaki and Reese 1994; Hirokawa 1998; Aihara et al. 2001). This association could be either dynamic for trafficking of ER membranes or stable for the positioning of these membranes within a cell (Terasaki 1990; Cole and Lippincott-Schwartz 1995; Hirokawa 1998). Depolymerization of microtubules using the drug nocodazole affects both the trafficking and positioning of ER membranes (Terasaki et al. 1986; Terasaki and Reese 1994; Waterman-Storer and Salmon 1998; Aihara et al. 2001). The ER movement along microtubules has been well documented (Terasaki 1990; Allan and Vale 1994; Waterman-Storer and Salmon 1998; Aihara et al. 2001). The sliding of ER membranes along microtubules seems to be driven by motor proteins such as kinesin and dynein (Le Bot et al. 1998; Lane and Allan 1999; Matanis et al. 2002; Bannai et al. 2004; Watson et al. 2005)

Moreover, it was shown that ER could be moved within a cell by its attachment to the microtubule-plus end (Waterman-Storer and Salmon 1998). However, when the expression of either dynein or kinesin is suppressed within a neuron, the association of membranous organelles including the ER with microtubules is not completely eliminated indicating that other proteins are involved in this association (Feiguin et al. 1994; Harada et al. 1998). These proteins would be involved in the positioning of membranous organelles along microtubules and thereby would allow the maintenance of the structure of these organelles. The maintenance of

membrane structure is particularly important in post-mitotic cells such as mature neurons where ER membranes are found along the length of dendrites and axon. Recently the relative proportion of dynamic and static ER was determined within hippocampal neurons. It was found that only a small ER subcompartment is dynamic in mature hippocampal neurons (Bannai et al. 2004). Proteins mediating a stable interaction between microtubules and membranous organelles could play a role in the maintenance of membrane structure and distribution.

Cytoplasmic linker proteins (CLIPs) for example, are known to establish a link between microtubules and membranous organelles. As such, CLIP-170 has been reported to mediate the interaction of endocytic carrier vesicles to microtubules (Pierre et al. 1992). Furthermore, CLIP-115 was shown to be responsible for the polarized distribution of the dendritic lamellar bodies (DLB) in neurons (De Zeeuw et al. 1997; Hoogenraad et al. 2000). Recently, a new class of proteins termed CLASPs (CLIP-associated proteins) was identified (Akhmanova et al. 2001). These proteins bind CLIPs and microtubules and have a microtubule-stabilizing effect. A family of proteins named Hooks also mediates the interaction between microtubules and membrane organelles. More specifically, Hook3 links the Golgi membranes to microtubules (Walenta et al. 2001). Futhermore, p63 was shown to be involved in the interaction of ER with microtubules in a non-neuronal cell line (Schweizer et al. 1995; Klopfenstein et al. 1998; Klopfenstein et al. 2001). Since p63 is an integral membrane protein, it was termed a CLIMP (cytoskeleton-linking membrane protein).

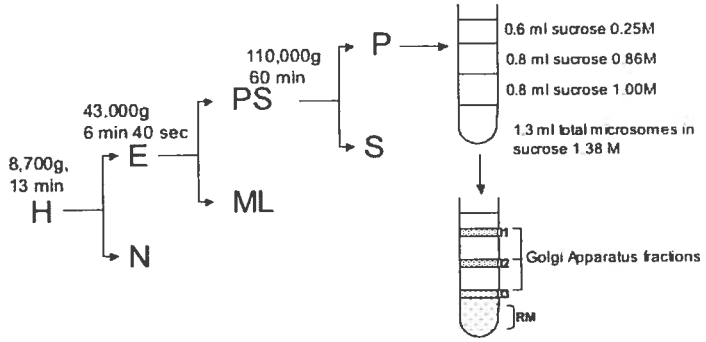
To our knowledge, no dendritic cytosolic linker protein has been identified so far that mediates the interaction between the ER and microtubules in neurons. Our results show that MAP2 plays such a role.

However, MAP2 is a cytosolic linker protein that has to be classified in a category of its own for two reasons: 1- the microtubule-binding domain of MAP2 has no sequence homology with that of CLIPs, CLASPs and Hooks 2- all the linker proteins identified so far bind to distal end of microtubules whereas MAP2 binds to microtubules along their length (Al-Bassam et al. 2002).

Our studies suggest that the association of MAP2 with RER membranes involves p63. The exact function of p63 remains to be clarified. However, convincing data were generated in COS cells showing its potential role in the positioning of the RER along microtubules. The overexpression of p63 in COS cells induced an important rearrangement of the ER from a punctate/reticular pattern to a tubular one (Klopfenstein et al. 1998). This was accompanied by the bundling of microtubules. The cytoplasmic domain of p63 was shown to be responsible for its microtubule bundling activity (Klopfenstein et al. 1998). In neurons, the highly polarized distribution of the RER in the somato-dendritic compartment might require more complex molecular interactions between RER membranes and microtubules than in non-polarized cells. In this context, the direct binding of p63 to microtubules would not be enough to determine the positioning of RER in neurons. Thus, the selective interaction of MAP2- but not tau-containing microtubules with p63 would contribute to the concentration of RER membranes in the somato-dendritic compartment.

Previous studies showed that microtubule motor proteins are involved in the positioning of ER membranes. The present results indicate that structural microtubule-associated proteins could also contribute to the distribution of ER membranes within a neuron. The activity of these proteins might vary to allow dendritic growth and remodeling.

Figure 1. Distribution of MAP2 in subcellular fractions prepared from adult rat brain A) Schematic representation of the subcellular fractionation procedure. A step-gradient of sucrose was used to separate both rough microsomes (RM) and Golgi derivatives (I1 and I2) from total membrane extract (P). **B)** Immunoblot analysis of the subcellular fractions obtained from adult rat brain. Fractions obtained following subcellular fractionation were electrophoresed on a 7.5% polyacrylamide gel (30 µg/lane) and transferred to a nitrocelllulose membrane as described in methods. Antibodies directed against MAP2 (HM2), the plasma membrane marker Na-K-ATPase, the endoplasmic reticulum markers, ribophorin and P63, the mitochondrial marker porin (VDAC), the Golgi marker mannosidase II and the cytoskeletal marker tubululin were used. **C)** Comparison of MAP2 and tau distribution in the RM subfraction. A monoclonal antibody directed against tau was used (clone tau5). E: cytoplasmic extract, N: nuclear fraction, P: total membrane extract, S: cytosolic fraction, I: interface, ML: mitochondria and lysosomes, PS: microsomes and cytosol and RM: rough microsomes.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I1
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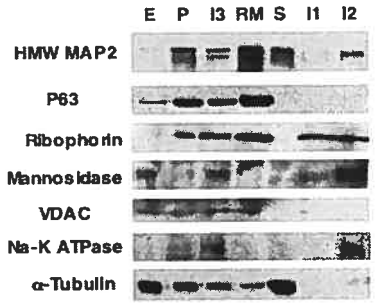
RM
Golgi Apparatus fractions

B)


Adult rat brain
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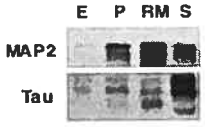
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MAP2
Tau

Figure 2. Electron microscopy of rat brain rough microsomes (RM).

A and B) Electron micrographs of the RM subfraction. In A, arrowheads point to ribosomes on the membranes of rough microsomes. The arrows point to smooth microsomes. The ovals in Figures A and B surround ribosomes associated with membrane-free filamentous structures. In B, rough microsomes reveal different morphologies: tubular (1), oval (2), dilated (3), cup-shaped (4) and with a spiral alignment of ribosomes (5).

C and D) Electron microscope immunocytochemistry of MAP2 in the RM subfraction. The monoclonal anti-MAP2 antibody HM2 (1:200) was revealed using a secondary anti-mouse antibody conjugated to 10nm colloidal gold particles. Immunogold labeling of MAP2 was found associated with smooth membranes (SM) as shown in C and with rough ER membranes (RM) shown in D. Scale bars Aand B=1μm, C=500nm and D=250nm.

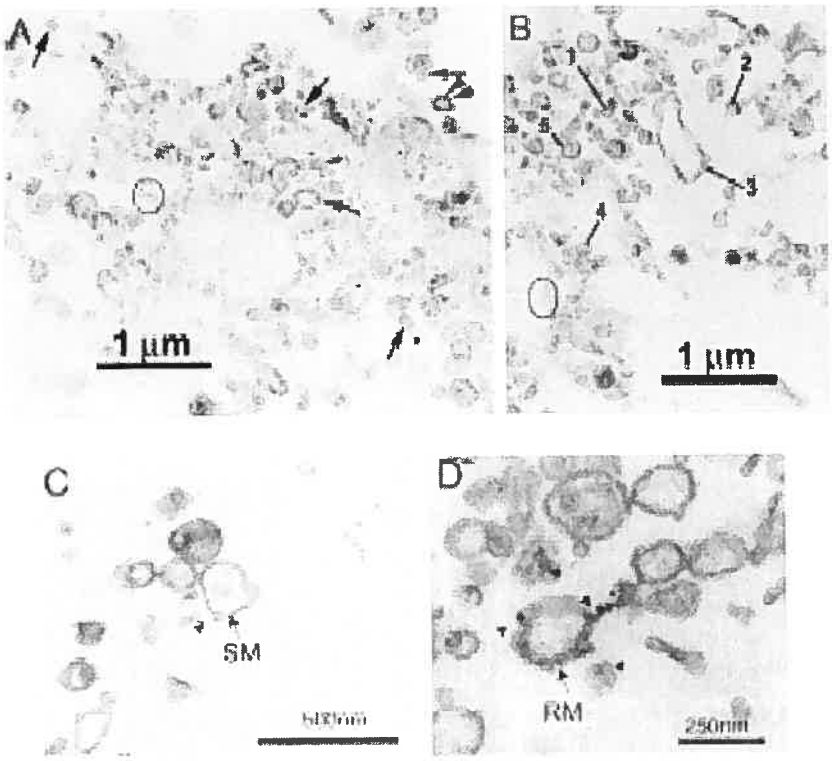

A
1 µm
B
1
2
3
4
1 µm
C
SM
D
RM
250nm

Figure 3. The somato-dendritic compartmentalization of MAP2 and p63 coincides in cultured hippocampal neurons. The distribution of MAP2, tau, p63 and tubulin is shown in one- and seven-day old cultured hippocampal neurons. The polyclonal anti-MAP2 antibody and the polyclonal anti-tau were revealed by using a donkey secondary anti-rabbit conjugated to FITC. The monoclonal anti-p63 antibody was revealed by using a donkey secondary anti-mouse antibody conjugated to rhodamine. The rat polyclonal anti-tubulin antibody was revealed by using a secondary anti-rat conjugated to AMCA. Tubulin staining was used to visualize both dendritic and axonal processes. In one-day old neuronal cultures, MAP2, p63 and tau were present in all processes whereas in seven-day old neuronal cutures MAP2 and p63 were enriched in dendrites and tau in the axon. Scale bar: 50µm.

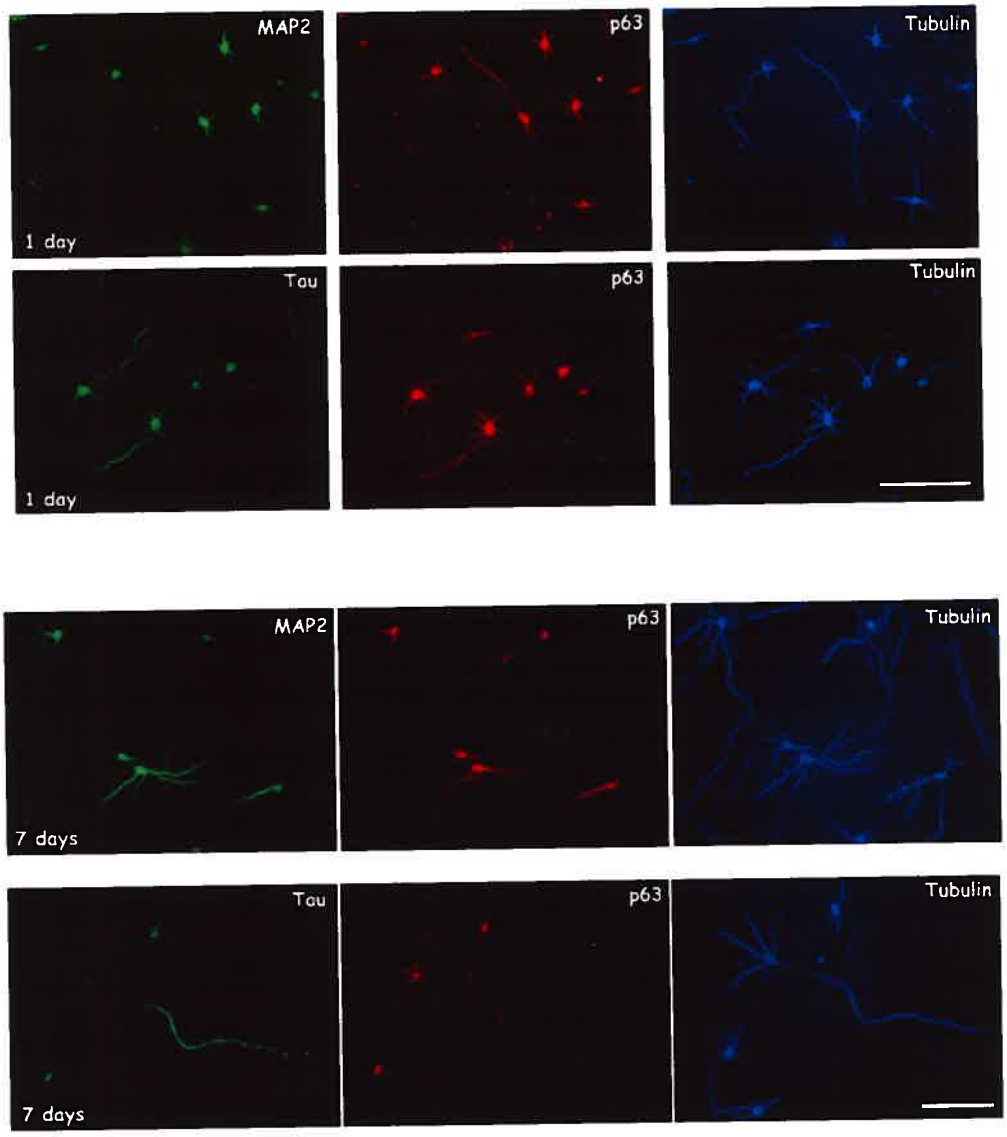

MAP2
p63
Tubulin
1 day
Tau
p63
Tubulin
1 day
MAP2
p63
Tubulin
7 days
Tau
p63
Tubulin
7 days

Figure 4. Overexpression of GFP-MAP2c fusion protein in hippocampal neurons. A and A' show the distribution of MAP2 (HM2 antibody) and the RER respectively in seven day-old cultured hippocampal neurons. The polyclonal antibody against ribophorin II was used to stain the RER. This antibody was revealed using a secondary anti-rabbit conjugated to rhodamine. Insets show a higher magnification of the cell body.

B) and B') Seven day-old neurons were transfected with the GFP protein alone as a control. Protein expression was allowed to proceed for 24hrs before the cells were fixed and processed for immunofluorescence. Insets show that the GFP protein does not induce a reorganization of the RER membranes.

C, C' and C" show the distribution of the GFP-MAP2c, ribophorin and tubulin staining in a transfected neuron presenting thick microtubule bundles. In these cells, RER staining is found on these bundles (arrows).

D, D' and D'' show the distribution of the GFP-MAP2c, ribophorin and tubulin staining in a transfected neuron presenting multiple thin extensions. In these cells, a reorganization of the RER was noted in the perikaryon (arrowhead in the inset) and along the thin bundles.

Scale bar for all figures except C, C' and C"= 20μm. Scale bar for C, C' and C"= 8μm. Scale bar for the insets= 4μm.

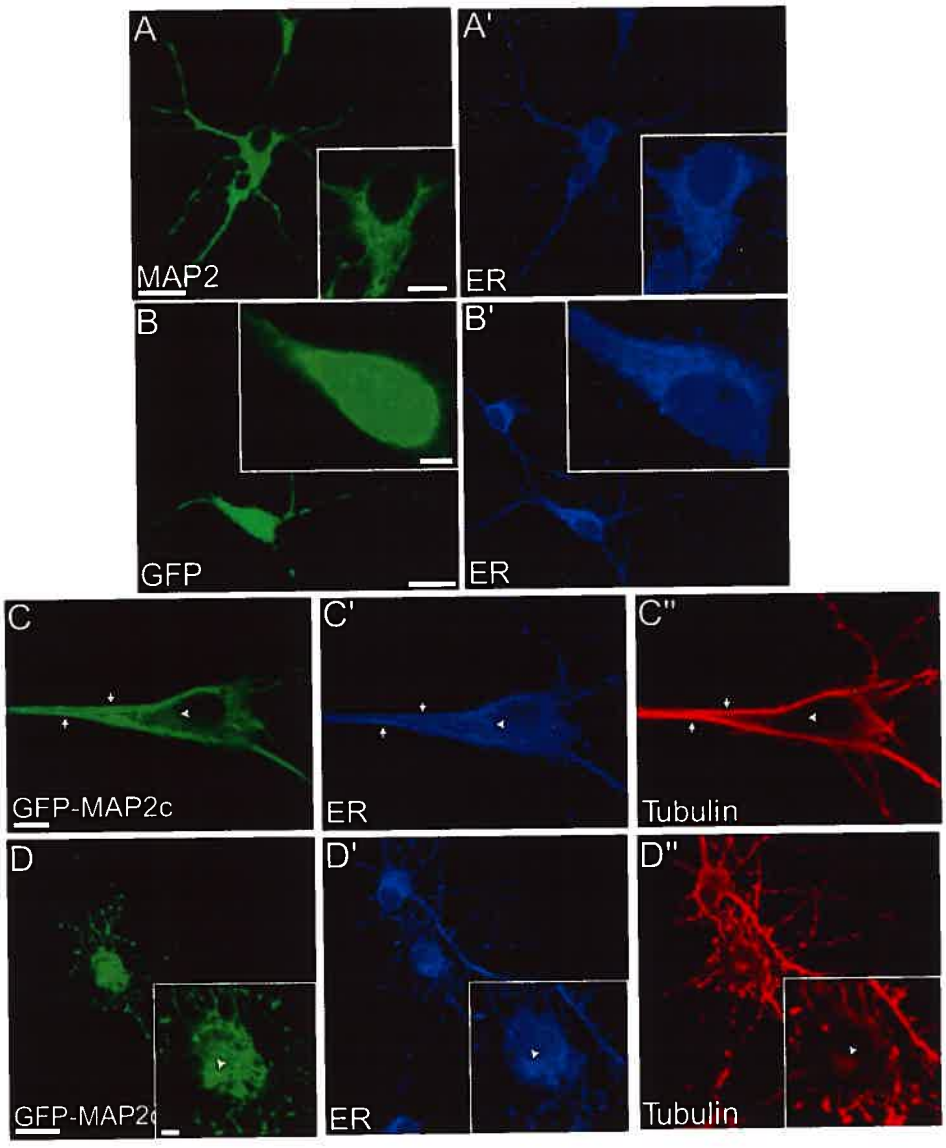

A
MAP2
A'
ER
B
GFP
B'
ER
C
GFP-MAP2c
C'
ER
C''
Tubulin
D
GFP-MAP2c
D'
ER
D''
Tubulin

Figure 5. The interaction of MAP2c and p63 in an overlay assay. An overlay assay using MAP2c purified from Sf9 cells was carried out to show the interaction of MAP2 with ER proteins from a rough microsomal fraction prepared from rat liver. The overlay experiment was performed as described in methods. In lane 1, no MAP2c protein was added to the overlay buffer. In lane 2, the membrane was incubated with purified MAP2c protein for 1h 30 min. In lane 3, the membrane of lane 2 was stained with an anti-p63 polyclonal antibody.

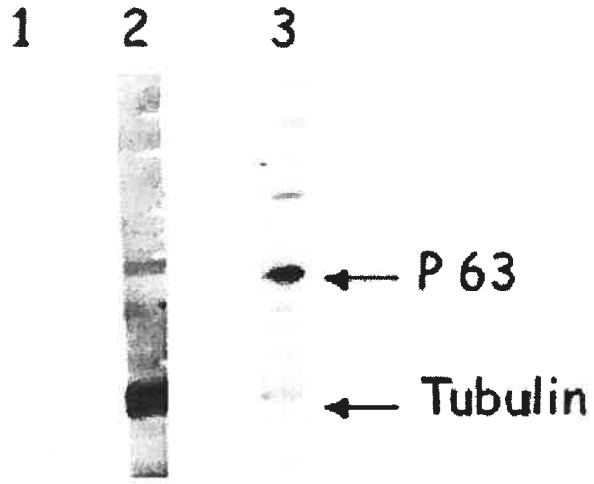
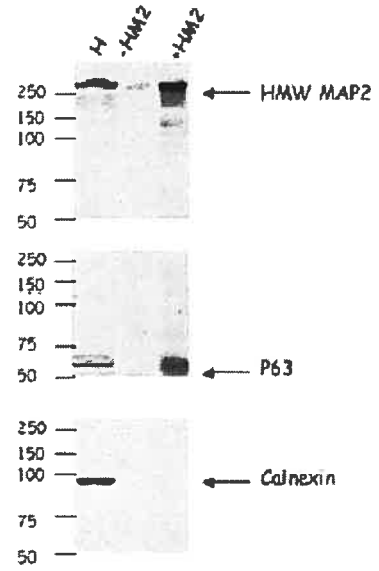

1
2
3
P 63
Tubulin

Figure 6. Co-immunoprecipitation of the MAP2/p63 complex from adult and embryonic (E19) rat brain homogenate. A) The interaction of MAP2 with p63 in adult rat brain was confirmed by co-immunoprecipitation. MAP2 was immunoprecipitated from an adult rat brain homogenate as described in Methods. The monoclonal anti-MAP2 HM2 (1:250) was used to immunoprecipitate the MAP2/p63 complex. The nitrocellulose membrane was then revealed with the monoclonal anti-MAP2 HM2 (1:1000) and then with the polyclonal anti-p63 (1:5000). The calnexin (CNX) was used as a negative control. Note that the polyclonal antibody anti-CNX recognizes the calnexin only in the adult rat brain homogenate. B) The interaction of MAP2 with p63 in embryonic rat brain was confirmed by co-immunoprecipitation. Either MAP2 or p63 was immunoprecipitated from a neonatal rat brain homogenate by using the monoclonal anti-MAP2 HM2 (1:250) or the polyclonal antibody directed against p63 (1:500). The western blot was then revealed with the monoclonal anti-MAP2 HM2 (1:1000) and then with the monoclonal anti-p63 (1:250). The precision plus protein standards from Bio-Rad were used for accurate protein sizes.

A Adult rat brain



B Embryonic E19 rat brain

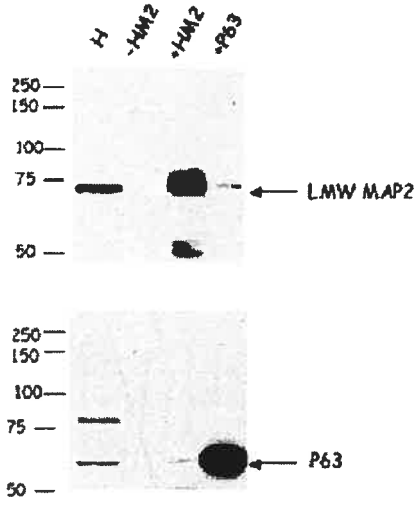
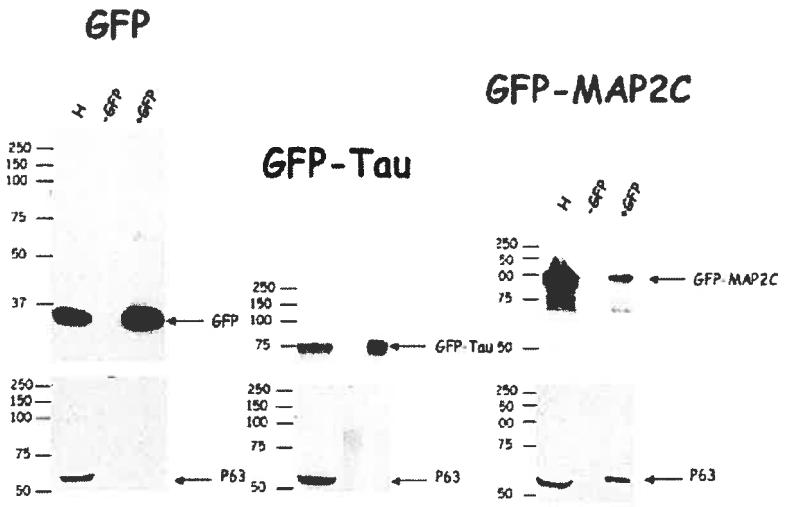


Figure 7. Interaction of the projection domain of MAP2 with p63. HeLa cells were transfected with an expression vector containing either GFP alone, GFP-tau, GFP-MAP2c, GFP-Proc, a deleted form of MAP2c corresponding to its projection domain or GFP-Mt, a deleted form of MAP2c corresponding to its microtubule domain. 24hrs after transfection, HeLa cells were lysed and co-immunoprecipitation experiments were carried out with an antibody directed against GFP. Our data showed that the binding peptidic sequence of MAP2 to p63 is located in the projection domain of MAP2c. Indeed, the anti-GFP antibody was able to co-immunoprecipitate either GFP-MAP2c and p63 or GFP-Proc and p63 but not GFP-Mt and p63. The GFP antibody was unable to co-immunoprecipitate either p63 and GFP-Tau or p63 and GFP alone. The precision plus protein standards from Bio-Rad were used for accurate protein sizes


GFP
GFP-MAP2C
GFP-Tau
H
-GFP
+GFP
250
150
100
75
50
37
GFP
GFP-Tau
GFP-MAP2C
P63

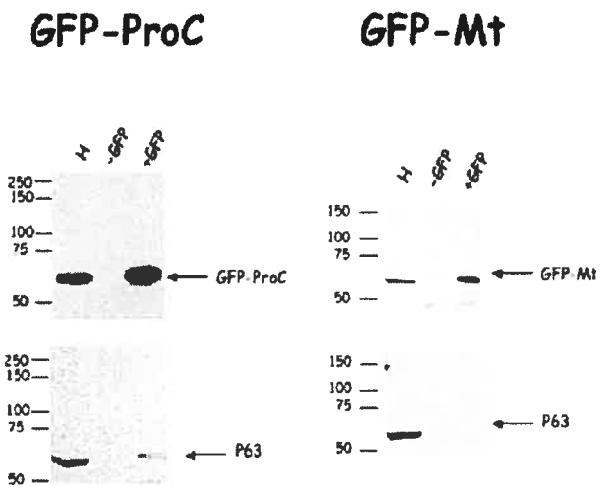

GFP-ProC
GFP-Mt
H
-GFP
+GFP
250
150
100
75
50
GFP-ProC
GFP-Mt
P63

Figure 8. In vitro reconstitution of the ER-MAP2-microtubule complexes. A) The nuclear fraction prepared was examined by western blotting for its content in tubulin. No endogenous tubulin was detected in this fraction. B) The presence of p63 in the nuclear fraction was confirmed by western blot by using the polyclonal-anti-p63 antibody. As noted in brain homogenate, in a subfraction enriched in rough membranes isolated from rat liver, the polyclonal antibody, anti-p63 revealed two bands. However, in a nuclear fraction isolated from rat liver and treated with Dnase to concentrate the membranes, only the band at 63 kDa was present. C) The microtubule polymerizing activity of MAP2c and tau was determined as described in Materials and Methods. Western blotting shows similar amounts of tubulin in the MAP2c and in the tau pellets indicating that these proteins have similar capacities to polymerize microtubules. D) Electron micrograph of MAP2c-pre-polymerized microtubules visualized by negative staining. E) Electron micrograph of tau-pre-polymerized microtubules visualized by negative staining. F) Electron micrograph illustrating that tau-pre-polymerized microtubules were intact after incubation with nuclei. Scale bar D, E and F= .25μm

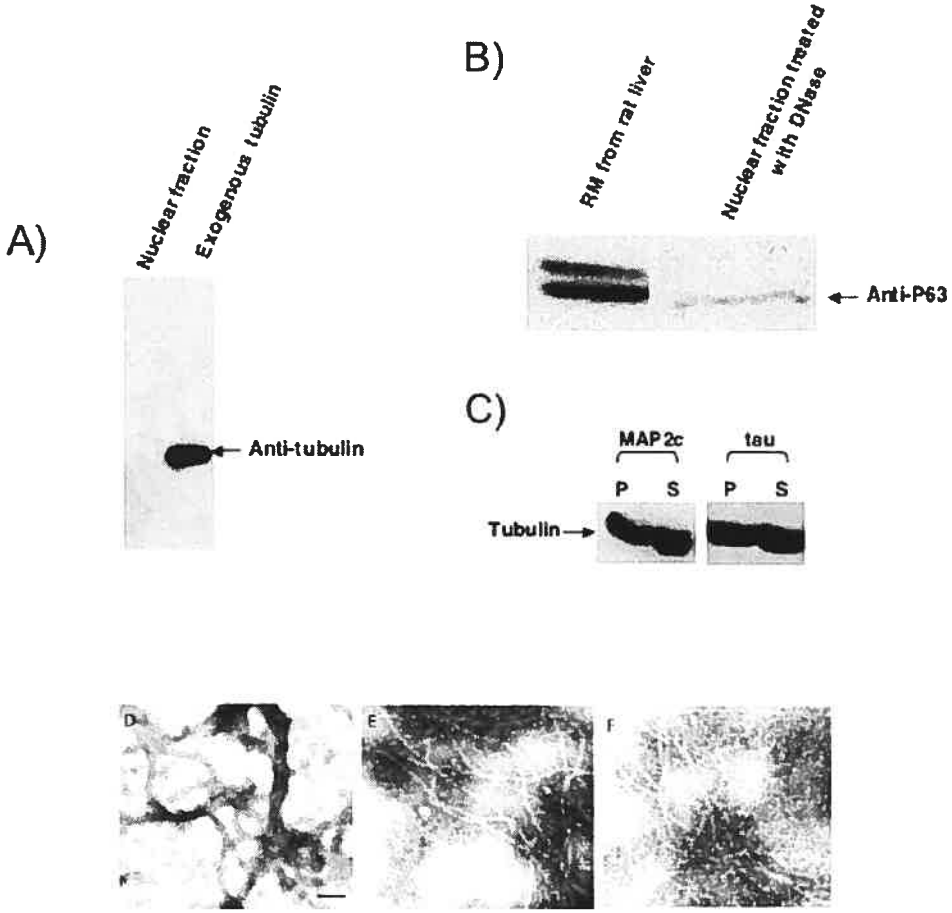
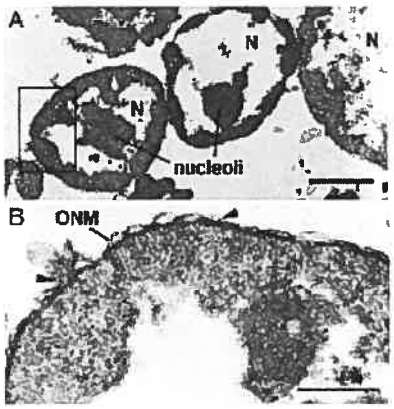

A)
Nuclear fraction
Exogenous tubulin
Anti-tubulin
B)
RM from rat liver
Nuclear fraction treated with DNase
Anti-P63
C)
MAP2c
tau
P
S
P
S
Tubulin
D
E
F

Figure 9. Electron micrographs of the in vitro reconstitution of the ER-MAP2-microtubule complexes. A) Nuclei (N) are shown after incubation in the presence of exogenously added tau-pre-polymerized microtubules. Under these conditions no microtubules were observed in association with the nuclei. A higher magnification of the region outlined by a rectangle in A is shown in B. Arrowheads point to ribosomes on the outer nuclear membrane (ONM). C) Micrographs schowing high power electron microscopy of the surface of nuclei after incubation in the presence of exogenously added MAP2c-pre-polymerized microtubules. Arrows point to cross and oblique sections of microtubules located within 25 nm of outer nuclear membrane. Scale bars: A= 2μm, B=1μm and C=500nm.


A
N
N
N
nucleoli
B
ONM

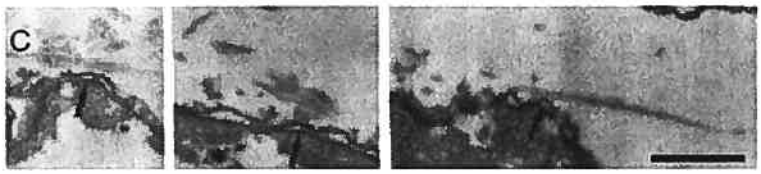

C

Table 1. Quantification of HMW MAP2 immunogold in the RM subfraction. The RM subfraction purified from rat brain was labeled by immunogold using anti-MAP2 antibody (clone HM2). The distribution of the gold particles was analyzed when HM2 antibody was omitted (-HM2) and when HM2 antibody was added (+HM2). The numbers represent the mean and the SEM of 3 sets of distinct experiments. An ANOVA (Tukey-Kramer Multiple comparisons Test) test was performed. *** means that the P value is less than 0,0001 which is considered extremely significant.

N=3	Number of gold particles -HM2 antibody	Number of gold particles + HM2 antibody
Smooth membranes	3,33±2,40	16,66±4,09
Rough membranes	9,33±0,66	129,66±9,90***

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3. deuxième article

"Fragmentation of the Golgi apparatus induced by the overexpression of wild type and mutant human Tau forms in neurons

Fragmentation of the Golgi apparatus induced by the overexpression of wild type and mutant human Tau forms in neurons

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Short running head : Fragmentation of the Golgi apparatus by tau

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3.1. ABSTRACT

Tau is a microtubule-associated protein enriched in the axonal compartment. In several neurodegenerative diseases including Alzheimer's disease, hyperphosphorylated tau accumulates in the somato-dendritic compartment, self-aggregates and forms neurofibrillary tangles (NFTs). A fragmentation of the neuronal Golgi apparatus (GA) was also observed in Alzheimer's disease. In the present study, we examined the effect of overexpressing human tau on the organization of the neuronal GA in rat hippocampal cultures and in JNPL3 mice expressing tau mutant P301L. GA fragmentation was noted in a significantly higher percentage of hippocampal neurons overexpressing wild-type human tau than in control neurons overexpressing GFP alone. Most importantly, in neurons overexpressing mutant forms of human tau (P301L, V337M or R406W), the percentage of neurons with a fragmented GA was 10% higher than that of neurons overexpressing wildtype human tau. In JNPL3 mice, a significantly higher percentage of motor neurons presented a fragmented GA compared to control mice. Interestingly, fragmentation of the GA was more frequent in neurons containing an accumulation and aggregation of hyperphosphorylated tau in the cell body than in neurons without these features. In both primary hippocampal neurons and JNPL3 mice, the tau-induced GA fragmentation was not caused by apoptosis. The present results implicate tau in GA fragmentation and show that this event occurs before the formation of NFTs.

3.2. INTRODUCTION

In normal brain, the microtubule-associated protein tau is involved in the formation and the stabilization of microtubules in the axon (Kosik and Caceres 1991). The expression of tau is developmentally regulated by alternative splicing (Himmler 1989). Six isoforms are present in human brain (Goedert et al. 1989). In pathological conditions, tau becomes hyperphosphorylated, detaches from microtubules, accumulates in the somato-dendritic compartment and self-aggregates to form insoluble filaments (Buee et al. 2000). Alzheimer's disease (AD) is characterized by two neuropathological lesions, the amyloid plaques corresponding to extracellular aggregation of Aβ peptides and the neurofibrillary tangles (NFTs) formed of insoluble filaments containing hyperphosphorylated tau (Morishima-Kawashima and Ihara 2002). Several other neurodegenerative diseases are characterized by prominent intracellular accumulations of filaments containing phosphorylated tau (Lee et al. 2001). These diseases are termed tauopathies. However, the implication of tau in neurodegeneration remained controversial until mutations in *tau* gene were identified and associated with fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Ingram and Spillantini 2002). The FTDP-17 mutations were also found in individuals either presenting clinical and neuropathologic phenotypes of corticobasal degeneration (CBD), Pick's disease (PiD) or progressive supranuclear palsy (PSP) (Lee et al. 2001). At least 29 different mutations were identified6. The majority of these mutations were located in the coding region or close to the splice donor site of intron 106. Most missense mutations seem to decrease the ability of tau to bind microtubules and increase its self-aggregation (ex. K250T, G272V, P301L, P301S, V337M, G389R and R406W) (Lee et al. 2001). The 4 mutations that affect the exon 10 splicing lead to an imbalance of the tau isoform ratio

(ex.S305N and S305S) (Lee et al. 2001). The link between tau protein dysfunction and neurodegeneration was further confirmed in transgenic mice overexpressing the mutated forms of tau (Lewis et al. 2000; Götz et al. 2001; Hutton et al. 2001). Microtubules contribute to the maintenance of neuronal architecture and also act as railways for the motor based transport of membranous organelles (Van Vliet et al. 2003). In recent years, other than stabilizing microtubules, tau was shown to be involved in the trafficking of membranous organelles including mitochondria, peroxisomes, endoplasmic reticulum and Golgi vesicles (Mandelkow et al. 2003). The overexpression of tau in non-neuronal and neuronal cells lead to the accumulation of these organelles in the perinuclear region (Ebneth et al. 1998; Stamer et al. 2002). Tau would affect vesicle trafficking by inhibiting the binding of motor proteins such as kinesins to microtubules as suggested by an in vitro competition assay (Seitz et al. 2002). In a neuron, the transport of membranous vesicles in dendrites and the axon is essential for the maintenance of synapse integrity. Consistently, a loss of synapses is observed in AD brain (Callahan and Coleman 1995; Buee et al. 2000). Futhermore, an abnormal distribution and morphology of membranous organelles were reported in several neurodegenerative diseases including AD (Gonatas et al. 1992; Stieber et al. 1996; Gonatas et al. 1998; Hirai et al. 2001). In particular, a fragmentation of the GA was observed in neurodegenerating neurons of patients suffering from AD, Amyotrophic Lateral Sclerosis (ALS), Creutzfeldt-Jakob disease (CJD) and multiple system atrophy (MSA) (Gonatas et al. 1998; Sakurai et al. 2000; Sakurai et al. 2002). The GA is involved in several important cellular functions including transport, processing and targeting of all proteins synthesized in the rough endoplasmic reticulum and destined for the secretory pathways (Van Vliet et al. 2003). In a normal cell, the GA is composed of a series of flattened,

parallel, interconnected cisterna organized around the microtubule organizing center (MTOC) in the perinuclear region (Cole and Lippincott-Schwartz 1995). The fragmentation of the GA is characterized by its reorganization in small, round, disconnected and dispersed elements (Cole and Lippincott-Schwartz 1995). A fragmentation of the GA occurs during mitosis in normal cells (Lucocq et al. 1987; Lucocq and Warren 1987). This reorganization of the GA is also noted in apoptotic cells indicating that a fragmented GA can also be associated with cellular dysfunction (Chiu et al. 2002; Nozawa et al. 2002). Furthermore, a fragmentation of the GA can be experimentally induced by the depolymerization of microtubules and by an alteration of the trafficking of vesicles between the endoplasmic reticulum and the GA (Presley et al. 1998). This fragmentation is reminiscent of the one observed in mitotic cells (Cole and Lippincott-Schwartz 1995). The fragmentation could have detrimental effects on the secretory activity of the GA (Waschulewski et al. 1996). This was observed in apoptotic cells where fragmentation of the GA is characterized by a spatial dissociation of the Trans Golgi Network (TGN) and the Golgi stacks. However, when a fragmentation of the GA is induced by depolymerization of microtubules, TGN membranes remain associated with the Golgi fragments (Cole et al. 1996). In this case, the secretory activity of the GA is not perturbed (Boyd et al. 1982; Rogalski et al. 1984; Cole et al. 1996). In degenerating neurons, the fragmentation of the GA is similar to that observed under microtubule depolymerizing conditions (Stieber et al. 1998). However, it is still unknown whether GA activity is perturbed by this reorganization in neurons. The cellular events leading to the fragmentation of the GA are not well characterized in neurons. A fragmentation of the GA was recently reported in primary cultures of astrocytes overexpressing wild type human tau protein (Yoshiyama et al. 2003). This implies that tau could have similar effects on the

morphology of the GA in neurons. Here, we investigated this point by overexpressing wild-type and mutated human forms of tau in primary hippocampal neurons. We also examined the morphology of the GA in JNPL3 transgenic mice that overexpress the mutated human form of tau, P301L(Lewis et al. 2000). These mice are characterized by motor and behavioural deficits. Motor deficits are associated with the presence of NFTs in motor neurons and their consequent death. Thus, JNPL3 mice represent a good model to study the sequential cascade of events that lead to neuronal dysfunction and death by tau. In the present study, we report that the overexpression of either wild-type human Tau4R or the mutant forms of Tau4R P301L, R406W and V337M induces a fragmentation of the GA in hippocampal neurons. Furthermore, a fragmentation of the GA was observed in motor neurons of JNPL3 mice. Most notably, this fragmentation occurred before the formation of NFTs indicating that it is an early event in the pathogenesis of tau. Moreover, the tau-induced fragmentation of the GA was not associated with apoptosis both in primary hippocampal neurons and in JNPL3 mice. A qualitative morphological analysis revealed no obvious change of the ER organization in hippocampal neurons either overexpressing wild-type or mutated tau forms and in JNPL3 mice. Collectively, our data indicate that a fragmentation of the GA induced by tau dysfunction might contribute to the alteration of neuronal activit

3.3. MATERIALS AND METHODS

3.3.1. Primary hippocampal cultures and transfection

Primary hippocampal cultures were prepared as previously described(Banker and Goslin 1998). Hippocampi from 18-day-old fetuses were treated with trypsin (0.25% at 37°C for 15 min) then washed in Hank's balanced solution and dissociated by several passages through a constricted Pasteur pipette. The cells were then plated on glass coverslips coated with polylysine. Hippocampal neurons were co-cultured with a monolayer of glial cells in a serum-free medium supplemented with N235. Fourty-eight hours after plating, hippocampal neurons were transfected using a modified calcium phosphate transfection protocol(Micheva et al. 2001). Neurons were transferred in a 6-well plate. To generate the calcium and DNA precipitate, 30 min before transfection, 4∝g of Qiagen-purified DNA in 60 µl of 250 mM CaCl2 per well were mixed drop-wise with an equal volume of 2X HBS (274 mM NaCl, 10 mM KCl, 1.4 mM Na2 HPO4, 15 mM glucose, 42 mM HEPES, pH=7.07). Cells were incubated with transfection precipitate for 30 min at 37°C and 5% CO2. Then cells were washed 3 times with Hank's balanced solution supplemented with 10 mM Hepes and retransferred in the petri dishes containing MEM/N2 medium. The protein expression was allowed for 24 hours then the cells were fixed and processed for immunofluorescence. A GFP-tau4R construct and three GFP- tau4R mutants, GFP-P301L, GFP-V337M and GFP-R406W were used (Figure 1). These GFP tau constructs were kindly provided by Dr. Ken Kosik(Lu and Kosik 2001).

3.3.2. Triton X-100 extraction procedure

Transfected neurons were extracted using a modified detergent based extraction protocol(Mandell and Banker 1996). Briefly, cells were rinsed in HBSS and extracted for 1 min 30 sec, at 37°C in the extraction buffer (20 mM PIPES, pH=6.9, 2 mM MgCl2, 2 mM EGTA, 1 mM PMSF and 0.1% Triton X-100). Then, the extracted neurons were fixed with cold methanol (-20°C) for 5 min, rehydrated in PBS and processed for immunostainning.

3.3.3. H_2O_2 treatment to induce apoptosis in neurons

Three days after plating, hippocampal neurons were treated with H_2O_2 (Fisher scientific, Canada) in order to induce apoptosis. H_2O_2 was added to the medium at a final concentration of 400 ∝M and then the neurons were incubated for 3 hours at 37°C and 5% CO_2. Neurons were then washed 3 times with PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes.

3.3.4. Immunofluorescence on primary hippocampal neurons

Twenty-four hours after transfection, neurons were fixed in 4% paraformaldehyde in PBS for 20 minutes. Then, after several washes in PBS, cells were permeabilized using a solution of 0.2% Triton X-100 in PBS for 5 min then cells were washed in PBS and immunolabelled as described previously(Belanger et al. 2002). To visualize the microtubules, a monoclonal antibody directed against β-tubulin (DSHB, University of Iowa, Iowa City, IA) was used. The anti-GFP mouse monoclonal antibody was purchased from Roche (Roche Molecular Biochemicals, IN, USA) and used at a concentration of 1:100. Three Golgi markers were employed: a polyclonal anti-MG-160 (kindly provided by Dr. Nicholas K. Gonatas, University of Pennsylvania, Philadelphia) at a concentration of 1:500, a rat polyclonal anti-TGN38 (Serotech Inc. distributed by Cedarlane laboratories, Hornby, Canada) at a concentration of 1:100 and MG-130 (Oncogene, USA) at a concentration of 1:500. To stain the endoplasmic reticulum, an anti-ribophorin II kindly provided by Dr. G. Kreibich (New York

University, New York, USA) was used. To label the microtuble-associated-protein-2, we used a rabbit polyclonal anti-MAP2 at a concentration of 1:2000 (kindly provided by Dr. Richard Vallee, Columbia University, New York). Apoptotic cells were revealed by using a rabbit polyclonal antibody that recognizes the active form of caspase-3 (Chemicon International Inc.) at a concentration of 1:50. The following secondary antibodies were used: a donkey anti-mouse conjugated to Rhodamine (1:500), a donkey anti-rabbit conjugated to Rhodamine (1:500), a donkey anti-mouse conjugated to FITC (dilution 1:100). All these secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada. We also used an Alexa Fluor 647 anti-rat (1:400) and an Alexa Fluor 647 anti-sheep (1:400) (Molecular Probes, Eugene, OR). Fluorescently labelled cells were visualized with an axioplant Zeiss fluorescence microscope using either a 63X or 100X objective. For colocalization studies of the two Golgi markers, observations were made with a Leica TCS-SP1 confocal microscope using a 100X objective and a 4X zoom.

3.3.5. Quantitative analysis of the percentage of tau transfected hippocampal neurons with a fragmented GA

A fragmented GA was defined as disconnected, small and round Golgi elements dispersed in the cell body and immunoreactive to MG-160. The quantitative analysis was performed using the image analysis software Northern eclipse (Empix Imaging, Mississauuga, Ontario, Canada). Three sets of independent experiments were quantified. At least, fifty transfected neurons per set of experiment were analyzed. Two pictures for each transfected neuron were captured: one of the GFP signal and one of the GA staining. These images were obtained with a Zeiss axoplant fluorescence microscope using a 100X objective.

3.3.6. Perfusion of JNPL3 mice

JNPL3 and age matched control mice were purchased from Taconic (Germantown, NY, USA). The use of animals and all surgical procedures in this article were carried out according to the guide to the care and use of experimental animals of the Canadian council on animal care. JNPL3 and age matched control mice were perfused using 0.9% saline and 4% paraformaldehyde. The spinal cord was dissected and incubated for 12 hours in 4% paraformaldehyde at 4°C.

3.3.7. Immunofluorescence on spinal cord sections of JNPL3 mice

Fifty µm sections of the spinal cord were made using a vibratome. The spinal cord sections were then incubated in a blocking solution (BS) containing 1% BSA and 2% Normal Donkey Serum (Jackson Immunoresearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada) in PBS. Sections were permeabilized using 0.5% Triton X-100 for 2 hours and then incubated in primary antibodies diluted in the PBS for 24 hours at room temperature. Two monoclonal antibodies, PHF-1 and CP-13 (kindly provided by Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, New York), recognizing hyperphosphorylated tau were used at concentration of (1:10) on the spinal cord sections of control and transgenic mice. GA was visualized using an anti-MG-160 antibody and an anti-TGN38 antibody described above. After several washes with PBS, the spinal cord sections were incubated with the appropriate secondary antibodies diluted in PBS containing 0.5 % Triton X-100 for 2 hours at room temperature. Fluorescently labelled spinal cord sections were visualized with a Leica TCS-SP1 confocal microscope using either a 63X or 100X objectives.

3.3.8. Quantitative analysis of the percentage of motor neurons presenting a fragmented GA in JNPL3 mice

More than 150 neurons per animal were analyzed and classified with regard of their GA morphology. Images of the GA were captured by confocal microscopy with a 63X objective and a 4X zoom on the cell body.

3.3.9. TUNEL staining on spinal cord sections of JNPL3 mice

To detect apoptotic cells, the terminal dUTP nick-end labeling assay (TUNEL) was performed using the Fluorescein-FragEL DNA fragmentation detection Kit (Oncogene Research Products). Briefly, sections of the spinal cord of JNPL3 transgenic mice were incubated with 20∝g/ml proteinase K in 10 mM Tris pH 8, for 30 min, then rinsed with TBS. As a positive control, the spinal cord sections from control mice were incubated with µg/µl DNase I for 20 min then rinsed in TBS and processed for TUNEL labelling according to the manufacture's recommendations as were spinal cord sections from JNPL3 mice. Then the spinal cord sections were labeled using the CP-13 antibody to detect hyperphosphorylated tau protein and an antibody directed against MG-160 to stain the GA. The samples were then observed with a Leica confocal microscope using a 100X objective.

3.3.10. Statistical analysis

The Instat Graphpad software was used to perform the statistical analysis. The percentage of hippocampal neurons overexpressing either GFP alone, wild-type tau or a mutated form of tau and presenting a fragmented GA was examined in three sets of experiments. These percentages were subjected to a Two-way ANOVA to detect any interaction between the sets of experiments and/or the experimental groups (neurons expressing either GFP alone, wild-type tau or a mutated form of tau). Since no interaction was found between the sets of experiments ($p=0.1712$), they were combined. However, an interaction was found between the experimental groups ($p<0.0001$). The differences of the percentage of hippocampal neurons presenting a fragmented GA between the different experimental groups were analyzed by a One-way ANOVA followed by the Tukey-Kramer Multiple Comparisons Post-hoc test. The counts of motor neurons with a fragmented GA in spinal cord sections were subjected to a Two-way ANOVA to detect any interaction between the types of mice (control and transgenic) and/or between the different groups of age (3 to 5 months vs. 10 to 12 months). The statistical analysis revealed an interaction between the types of mice and the groups of age. Then, for each group of age, a T-test was performed to detect differences between the types of mice (control and transgenic).

3.4. RESULTS

3.4.1. Fragmentation of the GA by the overexpression of wild-type human Tau4R in primary hippocampal neurons

Recently, it was reported that the overexpression of wild-type human tau in astrocytes induces a fragmentation of the GA (Yoshiyama et al. 2003). In the present study, we investigated whether the neuronal GA was also affected by the overexpression of wild-type human tau 4R. To explore this point, we examined the effect of tau overexpression on the morphology of the GA in primary hippocampal

cultures. These cultures were prepared according to the protocol established by Goslin and Banker(Banker and Goslin 1998). After one day in culture, the hippocampal neurons are polarized cells presenting 3 to 4 short minor neurites that will differentiate to become dendrites and a long thin neurite which develops into the axon(Bartlett and Banker 1984; Dotti et al. 1988). In the subsequent days of culture, the minor neurites differentiate into dendrites. After seven to ten days in culture, the dendrites and axon are fully developed and the synaptic contacts are established. Two-day old neurons were transfected with an expression vector containing either GFP alone or GFP fused to wild-type human tau 4R (GFP-tau 4R). The expression was allowed to proceed for 24 hours. Neurons were then fixed and processed for immunoflurescence to reveal the organization of the GA. The GFP protein alone was present in the cell body, dendrites and the axon in transfected hippocampal neurons (Figure 2A). In three-day old hippocampal cultures, endogenous tau proteins are not compartmentalized to the axon as noted in vivo but are also found in the cell body and dendrites(Dotti et al. 1987). In our cultures, GFP-tau 4R presented a distribution similar to that of endogenous tau and was present in all neuronal compartments (Figure 2A). We also examined whether GFP-tau 4R retained its ability to bind to microtubules in this system. Transfected neurons were stained with an antibody directed against β-tubulin. A colocalization of GFP-tau 4R and tubulin immunostaining was noted in the cell body and the neurites (Figure 2A). To further confirm the binding of GFP-tau 4R to microtubules, transfected neurons were extracted in the microtubule-stabilizing buffer containing Triton X-100 before fixation. During the extraction procedure, the unbound cytosolic proteins are removed and only the cytoskeleton-bound proteins remain. To monitor the extraction procedure, neurons were labelled with an antibody directed against the dendritic microtubule-associated protein MAP2, which is known to remain attached to microtubules during the extraction procedure (Kempf et al. 1996) (Figure 2B). To visualize the binding of GFP-tau 4R to microtubules, neurons

were stained with an antibody directed against GFP. As shown in figure 2B, GFP-tau 4R staining was still found along microtubules after the extraction indicating that transfected human tau could bind to microtubules in rat hippocampal neurons.

The morphology of the GA was examined in GFP-tau 4R-expressing neurons. To visualize the GA, an antibody directed against the conserved medial Golgi sialoglycoprotein MG-160 was used. In three-day old hippocampal neurons, expressing the GFP alone, the GA appeared as a juxtanuclear compact and clustered structure in the vicinity of the MTOC (Figure 2C). This distribution had previously been reported in differentiating neurons (Lowenstein et al. 1994). Sometimes, the neuronal GA extended into the proximal region of the dendrites as a compact structure. A fragmented GA was defined as numerous, small, round and disconnected MG-160 immunobabelled Golgi membranes distributed around the nucleus (Gonatas et al. 1998). Neurons included in the quantification analysis presented no vacuole and had a normal morphological phenotype: 3 to 4 minor neurites of a length exceeding at least 2 to 3 times the diameter of the cell body and a long axon. According to these criteria, neurons having a very high expression level of GFP-tau 4R were excluded since their morphological phenotype was modified. Indeed, these neurons developed multiple fine neurites emerging from the cell body. To determine the percentage of GFP and GFP-tau 4R-expressing neurons presenting a fragmented GA, transfected hippocampal neurons from 3 sets of experiments were analyzed (for details on the total number of cells analyzed for each construct see Table 1). The percentage of neurons expressing GFP-tau 4R and having a fragmented GA (30.89% $\pm$ 1.7) was significantly higher than that of neurons expressing GFP alone (6.5% $\pm$ 0.4).

3.4.2. Fragmentation of the GA is more frequent in primary hippocampal neurons overexpressing the mutant human tau forms than in neurons overexpressing wild-type human tau

Mutations in tau gene are known to decrease its microtubule binding affinity (Nagiec et al. 2001) and to increase its tendency to self-aggregate (Nacharaju et al. 1999; Vogelsberg-Ragaglia et al. 2000). Here, we investigated whether these mutations would induce a fragmentation of the GA in primary hippocampal neurons. Two-day old hippocampal neurons were transfected with an expression vector containing either the mutant human form of tau, P301L, V337M or R406W fused to a GFP tag (Figure 3) (Lu and Kosik 2001). Twenty-four hours following transfection, neurons were fixed and stained either with anti-tubulin antibody to reveal the microtubular network or with the anti-MG-160 antibody to reveal the organization of the GA. As noted for wild-type human tau, the mutant human forms of tau were found to co-localize with microtubule bundles as revealed by tubulin immunostaining (Figure 3). Furthermore, the detergent based extraction procedure described above was used to show the association of the mutated human forms of tau with microtubules (Figure 4). In order to determine the percentage of cells presenting a fragmented GA, three different experiments were performed (Table 1). In each set of experiment, control neurons were transfected either with the expression vector containing GFP alone or wild-type human tau 4R. For the quantitative analysis, the transfected neurons were selected according to the morphological criteria described in the previous section. Fragmentation of the GA was noted in 39.01% , 42.68% and 41.04% neurons expressing the mutant forms of tau P301L, V337M and R406W respectively (Figure 5).

Thus, the percentage of neurons having a fragmented GA was significantly higher (10%) following the expression of a mutant form of tau compared to that of

wild-type tau (Table 1). The expression level of GFP fusion proteins did not seem to make a difference with regard to GA fragmentation since this phenomenon was noted in neurons presenting a low, moderate and high protein level of tau. For example, in most neurons included in the quantitative analysis, the protein level of the mutated form of tau, P301L was lower than both that of the other mutated forms tested and that of wild-type tau (Figure 5B). However, the percentage of neurons expressing P301L tau and presenting a fragmented GA was similar to that of the other mutated forms of tau (Table 1). The present data show that, in primary hippocampal cultures, the mutant human forms of tau give rise to a significantly higher number of neurons with a fragmented GA than wild-type human tau.

3.4.3. Fragmentation of the GA in JNPL3 mice overexpressing the mutant human tau P301L

In the JNPL3 transgenic mice expressing the mutant human tau form P301L, tau becomes hyperphosphorylated, accumulates in the somato-dendritic compartment and forms NFTs in motor neurons of the spinal cord(Lewis et al. 2000). We used this mouse model to verify whether the mutated form of tau P301L induces a fragmentation of the GA in vivo and whether this fragmentation preceeds or is concomitant with the formation of NFTs. Two groups of animals were examined: 3- to 5-month old mice that did not have any behavioural or motor disturbances and 10- to 12-month old mice that presented mild symptoms such as apathy, reduction of grooming and delayed righting reflexes. Fragmentation of the GA was examined in JNPL3 and control mice with the same genetic background using the anti-MG-160 antibody. Large motor neurons at the thoracic and lumbar levels were included in the present study. In 10-month old control mice, the GA appeared as perinuclear granular and tubular profiles often extending into apical dendrites (Figure 6A). In 10-month old JNPL3 mice, motor neurons with a normal

GA as well as motor neurons with a fragmented GA were observed (Figure 6B and C). In the last case, the GA was reorganized in several small, round, disconnected and dispersed elements (Figure 6C). The percentage of cells presenting a fragmented GA was significally higher in JNPL3 mice (8.5%± 1.4) compared to age-matched control mice (0.67%± 0.04). In 3- to 5-month old control and transgenic mice, a similar percentage of neurons had a fragmented GA. These results are presented in Table 2 and illustrated in Figure 7A.

To determine whether GA fragmentation occurred before or concomitantly with the accumulation of hyperphosphorylated tau in the somato-dendritic compartment, the morphology of the GA was examined in neurons without and with an accumulation of hyperphosphorylated tau in the somato-dendritic compartment in JNPL3 mice. Hyperphosphorylated tau was detected with the antibodies PHF-1 and CP-13. PHF-1 recognizes tau when serine 396 and serine 404 are phosphorylated48 and CP-13 when the serine 202 is phosphorylated (Ishizawa et al. 2003). Hyperphosphorylated tau was barely detectable in the cell body of motor neurons in control mice as shown by the staining of the tau phosphodependent antibody CP-13 (Figure 6A). In 3 to 5-month old and 10-month old JNPL3 mice, the staining of spinal cord sections with the CP-13 antibody revealed that in some motor neurons, an accumulation of hyperphosphorylated tau was observed in the somato-dendritic compartment.

In some motor neurons with hyperphosphorylated tau, the morphology of the GA was similar to that of neurons without hyperphosphorylated tau (Figure 6B). However, some motor neurons with hyperphosphorylated tau presented a fragmented GA. The percentage of neurons containing hyperphosphorylated tau and displaying a GA fragmentation was evaluated in 3- to 5-month old and in 10- to 12-month old transgenic mice. For the quantitative analysis, 50 motor neurons of each category were analyzed per animal. Fragmentation of the GA was more frequent in motor neurons with an accumulation of hyperphosphorylated tau in the somato-dendritic

compartment than in neurons which did not show this accumulation. Indeed, in 3- to 5-month old transgenic mice, only 1.5% of motor neurons without accumulation of tau had a fragmented GA compared to 4% of neurons with an accumulation. However, this difference was not statistically significant. In 10- to 12-month old transgenic mice, the percentage of neurons with an accumulation of hyperphosphorylated tau in the somato-dendritic compartment and a fragmented GA was significantly higher (16%) than the percentage of neurons without accumulation (1.3%) (Figure 7B).

Finally, to determine whether the fragmentation of the GA preceded or was concomitant with the formation of tau aggregates, two classes of motor neurons were analyzed in JNPL3 mice: one having hyperphosphorylated tau in the somato-dendritic compartment but no aggregate and one having tau aggregates but no NFTs. The number of motor neurons having tau aggregates and NFTs was significantly higher in 10-12 month old mice than in 3- to 5-month old mice as reported in a previous study. A motor neuron without tau aggregation and one with tau aggregation are illustrated in Figure 6C and D respectively. The phospho-dependent anti-tau antibody, CP-13 was used to detect the motor neurons with and without aggregation of hyperphosphorylated tau. For the quantitative analysis, the GA morphology of fifty motor neurons from 10-12 month old JNPL3 mice was examined. Our data showed that 64% of the neurons containing tau aggregates presented a fragmented GA. Taken together, the present results indicate that the fragmentation of the GA is an early event in tau pathogenesis that occurs before the formation of NFTs.

3.4.4. Association of the TGN membranes with Golgi fragments in neurons overexpressing wild-type and mutated human tau

In a normal cell, the TGN is located on the trans side of the GA and a colocalization of TGN (TGN38) and Golgi (GM-130 or MG-160) markers is noted.

This colocalization is associated with a normal secretory activity of GA. Apoptotic cells present a fragmented GA (Chiu et al. 2002; Nozawa et al. 2002). In these cells, the co-localization between TGN and Golgi markers is lost (Lane et al. 2002) and this correlates with a decrease of the secretory activity of the GA (Lowe et al. 2004). Here, we examined whether fragmented GA induced by wild-type and mutated forms of tau was similar to that found in apoptotic cells. This was examined both in hippocampal cultures and JNPL3 mice. Transfected hippocampal neurons expressing either GFP alone, Tau 4R or one of the three mutant forms of tau 4R (P301L, V337M or R406W) were double immunostained for TGN38, a TGN marker and MG-160, a medial Golgi marker. Colocalization of these two markers in transfected neurons presenting a fragmented GA was examined by confocal microscopy. Thirty neurons with a fragmented GA were analyzed per tau construct. In control hippocampal neurons, TGN (TGN38) and Golgi (MG-160) markers co-localized as shown by immunofluorescence (Figure 8). This colocalization was preserved in neurons transfected with all constructs (Figure 8). However, when primary hippocampal neurons were treated with H_2O_2 to induce apoptosis, TGN38 and MG-160 staining did not co-localize as previously reported in nonneuronal cell (Figure 9). An antibody directed against activated caspase-3 was used to detect the apoptotic neurons. The same analysis was conducted on spinal cord motor neurons from 10-month old JNPL3 mice. Ten motor neurons per animal were examined. As noted in transfected hippocampal neurons, a co-localization was observed between the TGN and Golgi markers (data not shown). Moreover, to detect apoptotic neurons, spinal cord sections were examined by TUNEL staining (Figure 10). As a positive control, spinal cord sections from control mice were treated with DNAse I and processed for TUNEL staining. Motor neurons containing hyperphosphorylated tau and having a fragmented GA were not TUNEL-positive in JNPL3 spinal cord sections. In a recent study, it was also shown that in JNPL3 mice, oligodendrocytes but not neurons undergo apoptosis

(Zehr et al. 2004). Taken together, the above observations indicate that the fragmentation of the GA induced by tau protein or by mutant FTDP-17 related forms of tau is different from that observed in apoptotic cells.

Finally, no obvious change was noted in the distribution of endoplasmic reticulum markers in both JNPL3 mice (data not shown) and in hippocampal neurons overexpressing either wild-type or a mutated form of tau indicating that this organelle was not significantly affected (Figure 11).

3.5. DISCUSSION:

The present data showed that the overexpression of human tau protein resulted in a fragmentation of the GA in primary hippocampal neurons. Furthermore, the percentage of neurons presenting a fragmented GA was higher in neurons expressing tau mutants P301L, V337M and R406W than in neurons expressing wild-type human tau 4R. A fragmentation of the GA was also noted in JNPL3 mice expressing the mutated form of tau P301L. In these mice, the fragmentation of the GA preceded the formation of NFTs indicating that it is an early event in tau pathogenesis. Finally, our data showed that the TGN membranes were associated to the Golgi fragments in tau overexpressing neurons suggesting that the fragmentation of the GA was not induced by apoptosis.

Recently, an ultrastructural study on JNPL3 transgenic mice revealed that the cytoplasmic organelles were dispersed throughout the perikarya in motor neurons. Moreover, the Golgi complex was fragmented but no morphological change was observed for mitochondria or other membranous organelles indicating that the expression of mutated tau exerted a specific effect on the GA (Lin et al. 2003). This was shown in two types of neurons: neurons presenting NFTs and ballooned neurons containing vacuoles. In the present study, we showed that the GA fragmentation occurs at an early stage of tau pathogenesis. Indeed, the GA was fragmented and dipersed in asymptomatic JNPL3 mice. Beside slight apathy, the transgenic mice examined in the present study did not show any major motor or behavioural deficits. Moreover, our immunocytochemical analysis revealed that neurons containing hyperphosphorylated tau and tau aggregates but no NFTs had a fragmented GA. However, the fragmentation of the GA was more frequent in neurons containing aggregates of hyperphosphorylated tau. The above observations indicate that tau dysfunction can lead to the fragmentation of the GA which occurs early in the process

of tau pathogenesis before the formation of NFTs. The present results corroborate earlier data showing that fragmentation of the GA was found in neurons without NFTs in Alzheimer's brain tissue (Stieber et al. 1996). Fragmentation of the GA was reported in human brain tissue from patients suffering from AD (Gonatas et al. 1998), ALS (Gonatas et al. 1992), CJG (Sakurai et al. 2000) and MSA(Sakurai et al. 2002). It has also been observed in motor neurons of transgenic mice expressing the mutation G93A of the human Cu/Zn superoxide dismutase 1 (SOD1) associated with familial ALS cases (Mourelatos et al. 1996; Stieber et al. 2000). Most importantly, this fragmentation of the GA was noted at an early stage of the neurodegenerative process in these mice as reported here in JNPL3 mice. Interestingly, in transgenic mice overexpressing the human NF-H gene that develop a motor neuronopathy resembling ALS, motor neurons did not present a fragmented GA (Stieber et al. 2000). This suggests that the fragmentation of the GA is not a secondary cellular response to cell death. Many neurological disorders, including PD, dementia with Lewy bodies and MSA present fibrillar agregates of α-synuclein such as Lewy bodies and Lewy neurites (Hardy and Gwinn-Hardy 1998) (Trojanowski et al. 1998; Gosavi et al. 2002). In COS cells, a fragmentation of the GA was induced by the addition of α-synuclein non-fibrillar oligomers in the medium (Gosavi et al. 2002). Thus, GA fragmentation seems to occur at an early stage of α-synuclein dysfunction before the formation of fibrillar aggregates. Taken together, the above observations indicate that the fragmentation of the GA is an early event in the process of neurodegeneration. Most of the neurodegenerative diseases that present a fragmentation of the GA were associated with deficient axonal transport (Sheetz et al. 1998; Williamson and Cleveland 1999; LaMonte et al. 2002). This is supported by the fact that these diseases are characterized by the accumulation and formation of protein aggregates in the cell body (Hardy and Gwinn-Hardy 1998; Buee et al. 2000; Rao and Nixon 2003).

Interestingly, GA fragmentation can be experimentally induced by altering the transport of vesicles (Fujiwara et al. 1988) (Siddhanta et al. 2000).

Cytoskeletal abnormalities including an alteration of the microtubule network were noted in neurodegenerative diseases (Brandt 2001). The morphology and the localization of the GA is intimately linked to microtubules. Indeed, the perinuclear localization and the clustered morphology of the GA is perturbed by the depolymerization of microtubules during mitosis (Lucocq et al. 1987; Lucocq and Warren 1987). Moreover, the drugs which depolymerise microtubules such as nocodazole and those which stabilize them such as taxol, induce a fragmentation of the GA in neuronal and non-neuronal cells (Rogalski and Singer 1984). In the present study, we can not exclude the possibility that the fragmentation of the GA was induced by the effect of tau on the organization of the microtubules. Indeed, in tau transfected neurons, a higher number of microtubule bundles was observed. Overexpression of tau could alter the molecular links between Golgi membranes and microtubules. For example, motor proteins such as kinesins are known to be involved in the trafficking of vesicles between the endoplasmic reticulum and the GA (Lippincott-Schwartz et al. 1995). Any perturbation of this trafficking could result in fragmentation of the GA. Thus, tau could compete with kinesin for binding to microtubules (Ebneth et al. 1998; Stamer et al. 2002; Mandelkow et al. 2003) and thereby impair the transport of vesicles from the ER to the GA. However, in astrocytes, the overexpression of tau affected vesicle transport by altering microtubule dynamics due to a specific reduction of stable detyronisated microtubules and by decreasing kinesin levels. These alterations resulted in a disruption of the kinesin-dependent transport and a GA fragmentation. A similar cascade of events could lead to GA fragmentation caused by tau dysfunction in a neuron. The fact that a fragmentation of the GA was more frequent in neurons expressing a mutant form of

tau than in neurons expressing wild-type tau might indicate that these mutant differently interact with microtubules and more severely impair their function.

Overexpression of tau could perturb the signaling pathways involved in the maintenance of the morphological phenotype of the GA. In JNPL3 mice, tau is hyperphosphorylated as observed in AD and FTD brain indicating an imbalance of kinase and phosphatase activity (Buee et al. 2000). During mitosis, the fragmentation of the GA depends on the sequential activation of the kinases MEK1 and cdc2-kinase (Colanzi et al. 2003). Moreover, phosphorylation of spectrin by a reduction of phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P2) synthesis induced its dissociation from Golgi membranes and a fragmentation of the GA (Siddhanta et al. 2003).

Fragmentation of the GA can also be induced by brefeldin A, a Golgi toxin that inactivates Arf1, a small GTPase associated with the GA membranes (Fujiwara et al. 1988) (Donaldson and Jackson 2000). Recently, in neurons, it was shown by using two golgi toxins Brefeldin A (BFA) and nordihydroguaiaretic acid (NDGA) that the fragmentation of GA was concomittant to a transient increase of glycogen synthetase kinase 3β (GSK3-β) activity and tau phosphorylation (Elyaman et al. 2002). Several studies have highlighted the pivotal role of GSK-3β in tau hyperphosphorylation in neurodegenerating neurons (Bhat and Budd 2002). According to the above observations, one could imagine that the imbalance of kinases and phosphatases leading to tau dysfunction could also affect the organization of the GA. This imbalance seems to take place earlier in transgenic mice overexpressing mutant human tau than in mice expressing wild-type tau as revealed by the hyperphosphorylation of tau at an early stage in these mice (Lee et al. 2001). This could contribute to a higher percentage of neurons with a fragmented GA in neurons expressing mutant human forms of tau than in neurons expressing wild-type tau.

The fragmentation of the GA is also observed in apoptotic cells. Additional morphological criteria such as cell shrinkage, nuclear condensation, and formation of apoptotic bodies are characteristic of apoptotic cells (Strasser et al. 2000). The fragmentation of the GA in apoptotic cells is similar to that observed during mitosis (Sesso et al. 1999). However, in these cells, there is a loss of the GA polarity as revealed by the lack of co-localization between TGN membranes and the membranes of the medial Golgi compartment. Interestingly, in hippocampal neurons overexpressing either wild-type human tau or FTD related human mutants of tau, TGN membrane and medial Golgi membrane markers co-localized. This colocalization was also observed in JNPL3 mice indicating that the fragmentation of the GA induced by tau is not related to an apoptotic process. Indeed, no apoptosis was noted in hippocampal neurons overexpressing human tau and in motor neurons of the JNPL3 mice (Zehr et al. 2004).

Futhermore, the hippocampal neurons and the motor neurons in JNPL3 mice that presented a fragmented GA did not show morphological changes related to apoptosis. Consistently, a nonapoptotic neurodegenerative process was reported in transgenic mice overexpressing either the human mutated form of tau P301S (Allen et al. 2002) or V337M (Tanemura et al. 2002).

Fragmentation of the GA occurs at an early stage of several neurodegenerative diseases. However, its functional impact on the GA activity remains to be determined. GA is involved in several cellular functions including transport, processing and targeting of all proteins synthesized in the rough endoplasmic reticulum and destined to the secretory pathways, the plasma membrane or lysosomes (Van Vliet et al. 2003). In polarized cells such as neurons, transport and targeting of proteins to the dendrites and the axon is crucial to maintain cell polarity. Therefore, one can imagine that an alteration of the GA could impair the highly regulated process of protein sorting in neuronal compartments.

Such functional alteration of the GA was recently demonstrated in CHO cells overexpressing either wild-type or mutant SOD1. In these cells, a fragmentation of the GA and a dysfunction of the secretory pathway were noted (Stieber et al. 2004). Interestingly, the perturbation of microtubule network could affect the secretion capacity of the GA (Parczyk et al. 1989; De Almeida and Stow 1991). Thus, by perturbing the microtubule network, tau could alter the secretory function of the GA. Futher studies are needed to address this point.

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Figure 1: Schematic representation of tau protein. The tau isoform used in this study contains 4 repeats of 18 amino acid involved in tau binding to microtubules esignated R1 to R4 (black bars) and lacks the exon 2, 3, 4a and 6 in the amino-terminal. The arrows represent the positions of the amino acid substitions in FTDP-17 related tau mutants: P301L, V337M and R406W mutations. P301L mutation is located in the second repeat, V337M in the third interrepeat and R406W in the C-terminal domain outside the microtubule binding domain of tau protein.

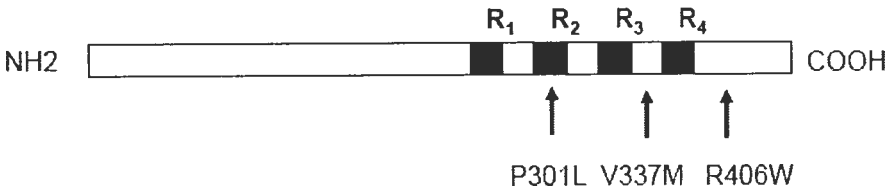

R1
R2
R3
R4
NH2
COOH
P301L
V337M
R406W

Figure 2: Three-day old hippocampal neurons expressing either GFP or GFP-tau 4R. Neurons were fixed with 4% paraformaldehyde then immunostained with an anti β-tubulin antibody.

A) In control cells transfected with GFP alone, the overexpressed protein was diffusely distributed in the cytoplasm and did not show any colocalization with microtubules. In neurons expressing wild-type tau 4R, a co-localization of GFP-tau with microtubule bundles was revealed by the tubulin staining. B) Transfected neurons expressing GFP-tau 4R were extracted with Triton X-100 and stained for MAP2, the dendritic microtubule-associated protein. GFP-tau 4R and MAP2 remained attached to microtubules during the extraction procedure. C) GFP and GFP-tau 4R expressing neurons were stained with an antibody directed against the Golgi marker, MG-160. In GFP-tau 4R–expressing neurons, the GA was fragmented. Inset: 2 times magnification of the GA. Scale bar: A= 4 μm, B= 5 μm and C= 10μm.

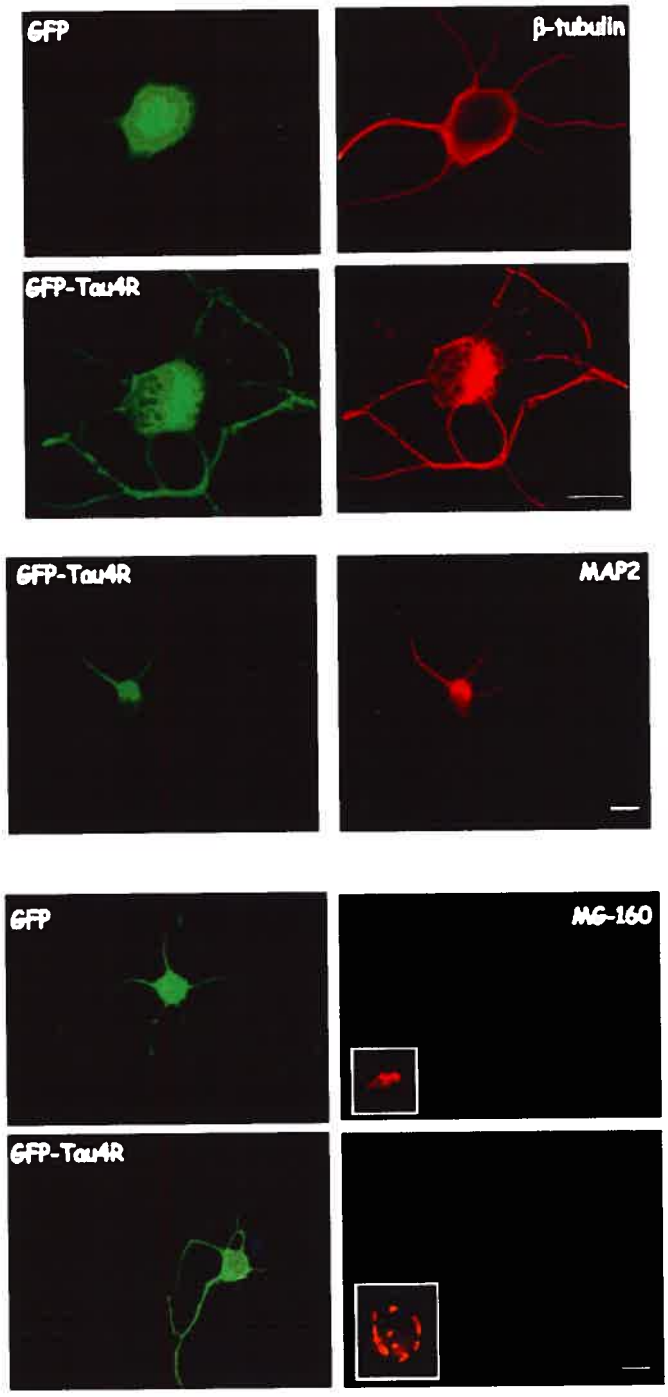

GFP
β-tubulin
GFP-Tau4R
GFP-Tau4R
MAP2
GFP
MG-160
GFP-Tau4R

Figure 3: Three-day old hippocampal neurons expressing either GFP-P301L, GFP-V337M or GFP-R406W. The neurons were fixed with 4% paraformaldehyde then immunostained with an anti β-tubulin antibody. In neurons expressing GFP-P301L, -V337M and -R406W, tubulin staining revealed a co-localization of GFP-tau mutants with microtubule bundles. Scale bar= 4 μm.

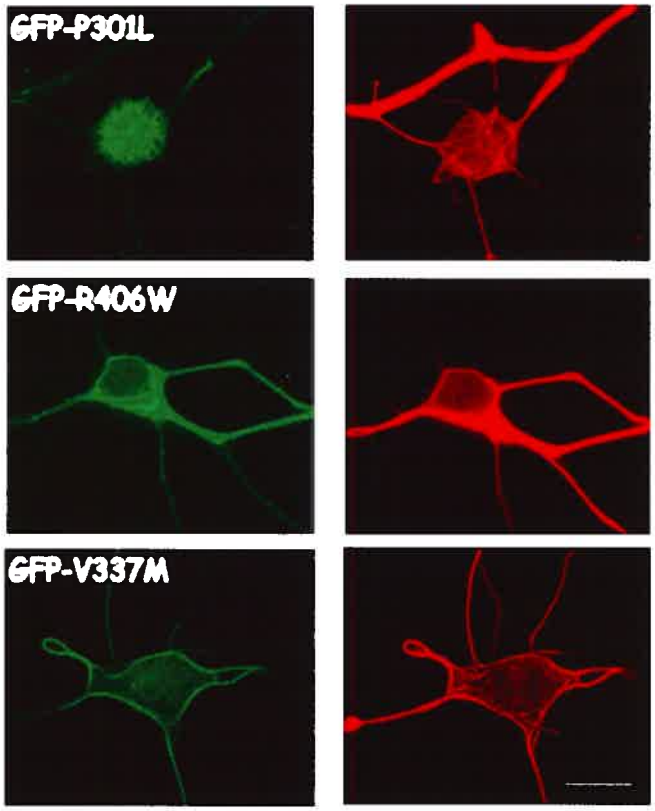

GFP-P301L
GFP-R406W
GFP-V337M

Figure 4: Three-day old hippocampal neurons expressing either GFP-P301L, GFP-V337M or GFP-R406W and extracted with Triton X-100. The neurons were extracted using a Triton X-100 based protocol, fixed with cold methanol then double-immunostained with an anti-GFP antibody and a polyclonal anti-MAP2 antibody. In control cells transfected with GFP alone, the overexpressed protein was totally extracted (data not shown). In neurons expressing any of the three FTD related mutant forms of tau, the GFP-tau remained attached to the microtubules during the extraction procedure. Microtubule-bound tau was found in the cell body, minor neurites and the axon. On the other hand, the dendritic MAP, MAP2 was only attached to the microtubules located in minor neurites and the cell body.

Scale bar=5 μm.

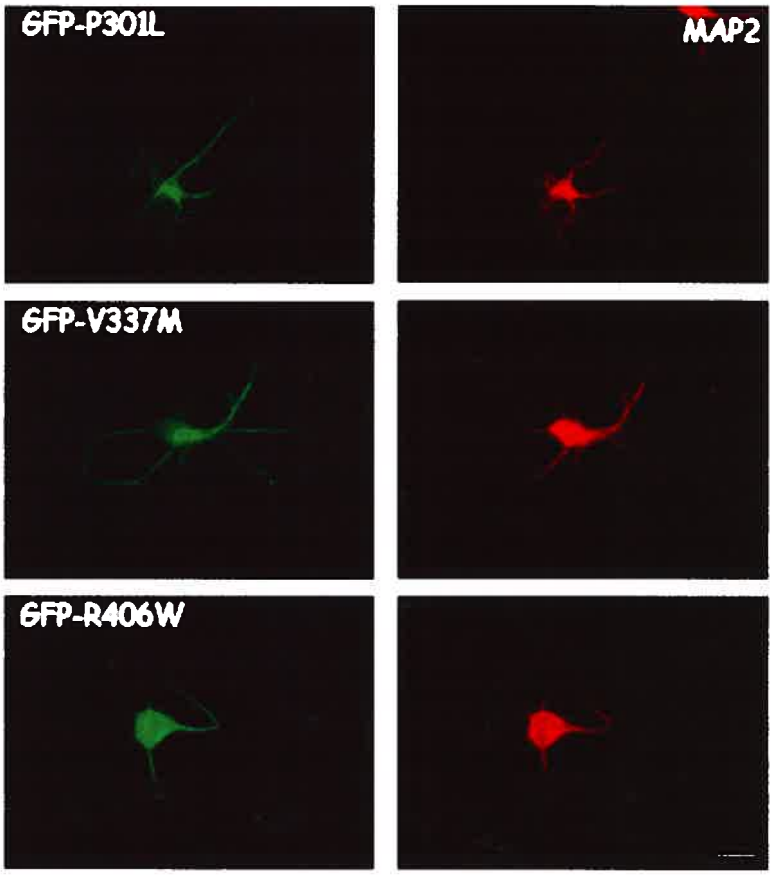
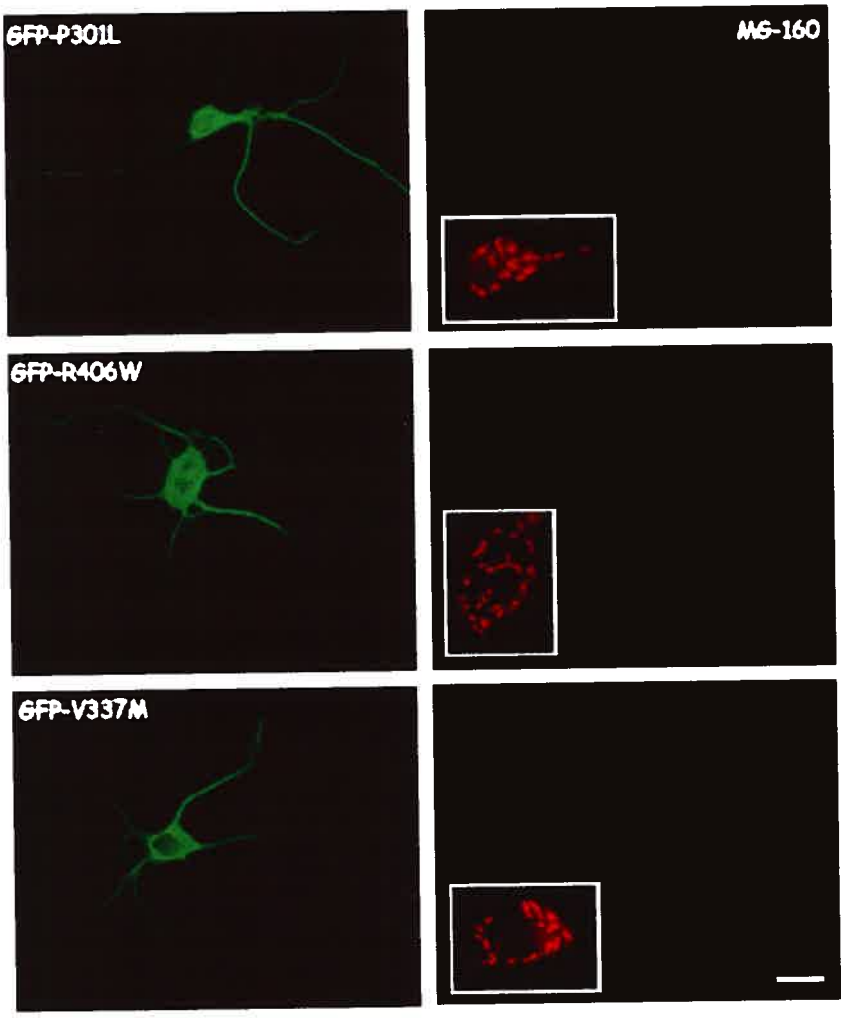

GFP-P301L
MAP2
GFP-V337M
GFP-R406W

Figure 5: Fragmentation of the GA in three-day old hippocampal neurons transfected with either GFP-P301L, GFP-V337M or GFP-R406W.

A) Neurons were fixed with 4% paraformaldehyde and stained with a polyclonal antibody directed against the Golgi protein, MG-160. In contrast to control cells where the GA appeared as a juxtanulear compact and clustered structure, in neurons expressing any of the three FTD related mutant forms of tau, the GA was fragmented. A fragmentation of the GA was defined as numerous, small, round and disconnected MG-160 immunolabelled membranes around the nucleus. Inset: two times magnification of the GA

B) A transfected neuron presenting a low protein level of the tau mutant P301L but having a fragmented GA. Scale bar = 10 μm.


GFP-P301L
MG-160
GFP-R406W
GFP-V337M

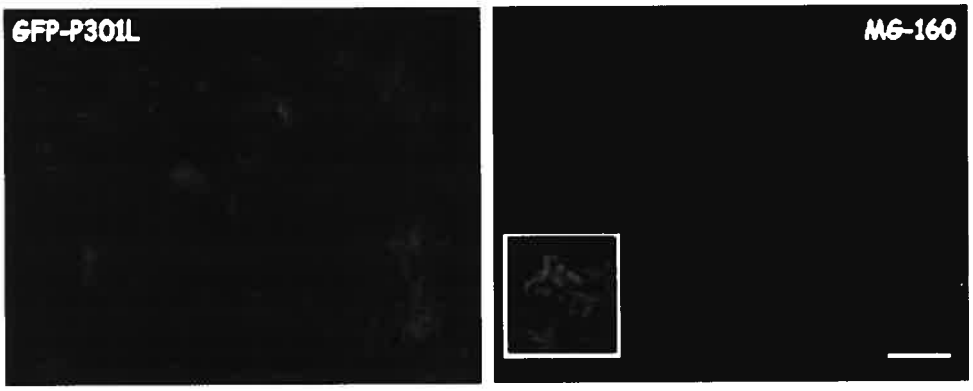

GFP-P301L
MG-160

Figure 6: Motor neurons from spinal cord sections of a 10-month old control and JNPL3 mouse. Sections were double immunostained with the phospho-dependent tau antibody, CP-13 and an antibody directed against the Golgi protein, MG-160. A and A'), a motor neuron from a 10-month old control mouse that did not show any staining for CP-13 antibody. The GA presented a normal distribution.

B and B') Two motor neurons from a 10-month old JNPL3 transgenic mouse. The motor neuron on the left but not the one on the right presented an accumulation of hyperphosphorylated tau in the cell body as revealed by the CP-13 staining. In these two motor neurons, the morpholgy of the GA appeared normal.

C and C') A motor neuron from a 10-month old transgenic mouse with an accumulation of hyperphosphorylated tau protein in the cell body and with a fragmented GA.

D and D') A motor neuron from a 10-month old transgenic mouse that presented an accumulation and aggregation of hyperphosphorylated tau in the cell body. In this motor neuron, the fragmented GA was displaced on one side of the cell body by the tau aggregate. Scale bar: A, B, D= 4 μm and C= 8 μm.

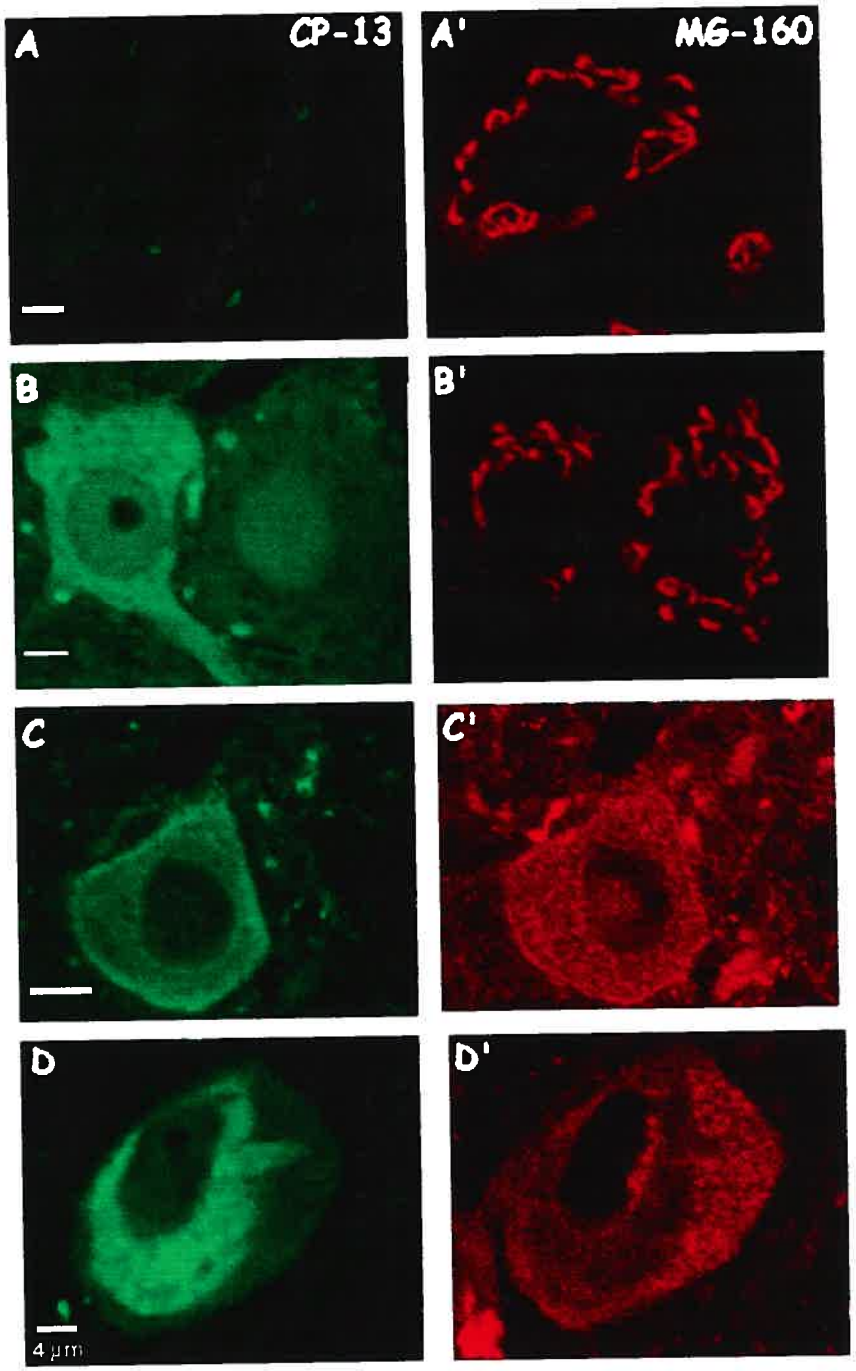

A
CP-13
A'
MG-160
B
B'
C
C'
D
D'
4 μm

Figure 7: Histograms showing the percentage of motor neurons with a fragmented GA in control and JNPL3 mice. Four 3- to 5-month old JNPL3 mice and four age matched control mice were analyzed. Three 10- to 12-month old transgenic mice and three age matched control mice were included in the present study

A) ~150 neurons per mouse were examined

B) 150 neurons per mouse with and without an accumulation of hyperphosphorylated tau were analyzed.

Figure 7A

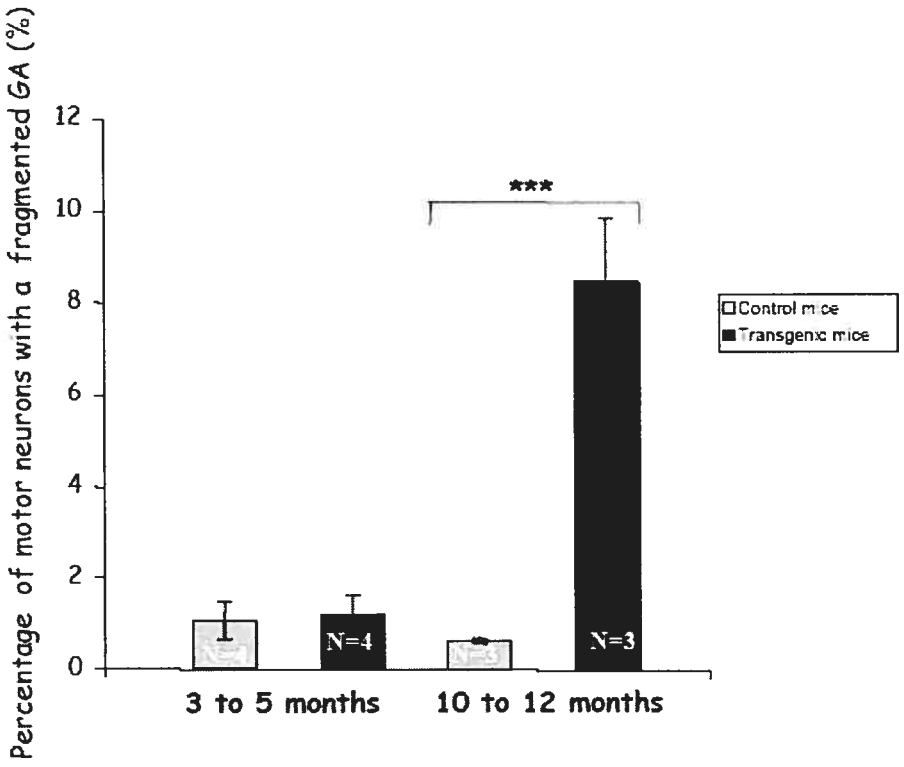

Percentage of motor neurons with a fragmented GA (%)
12
10
8
6
4
2
0
***
N=4
N=3
3 to 5 months
10 to 12 months
Control mice
Transgenic mice

Figure 7B

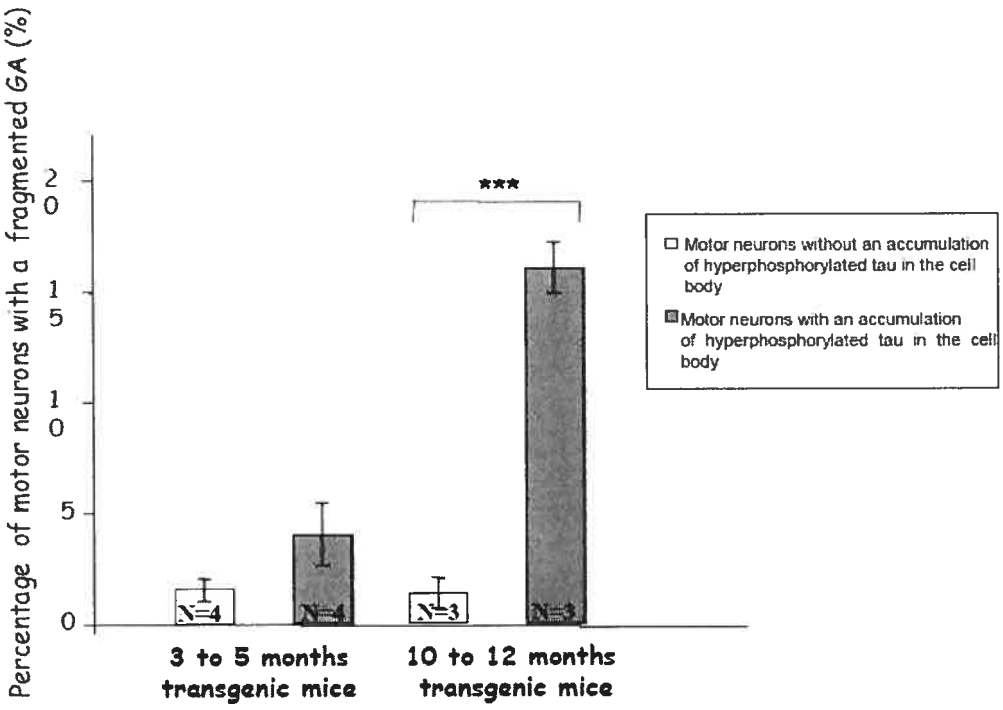

Percentage of motor neurons with a fragmented GA (%)
2 0
1 5
1 0
5
0
***
N=4
N=4
N=3
N=3
3 to 5 months transgenic mice
10 to 12 months transgenic mice
Motor neurons without an accumulation of hyperphosphorylated tau in the cell body
Motor neurons with an accumulation of hyperphosphorylated tau in the cell body

Figure 8: Confocal microscopy analysis of the distribution of TGN38 and the Golgi membrane marker MG-160 in transfected hippocampal neurons with a fragmented GA. Transfected neurons expressing either GFP, GFP-tau 4R, GFP-P301L, GFP-V337M or GFP-R406W were stained for MG-160 and TGN38. These markers colocalized in transfected neurons with a fragmented GA indicating that the polarity of the GA was preserved. Scale bar= 3 μm.

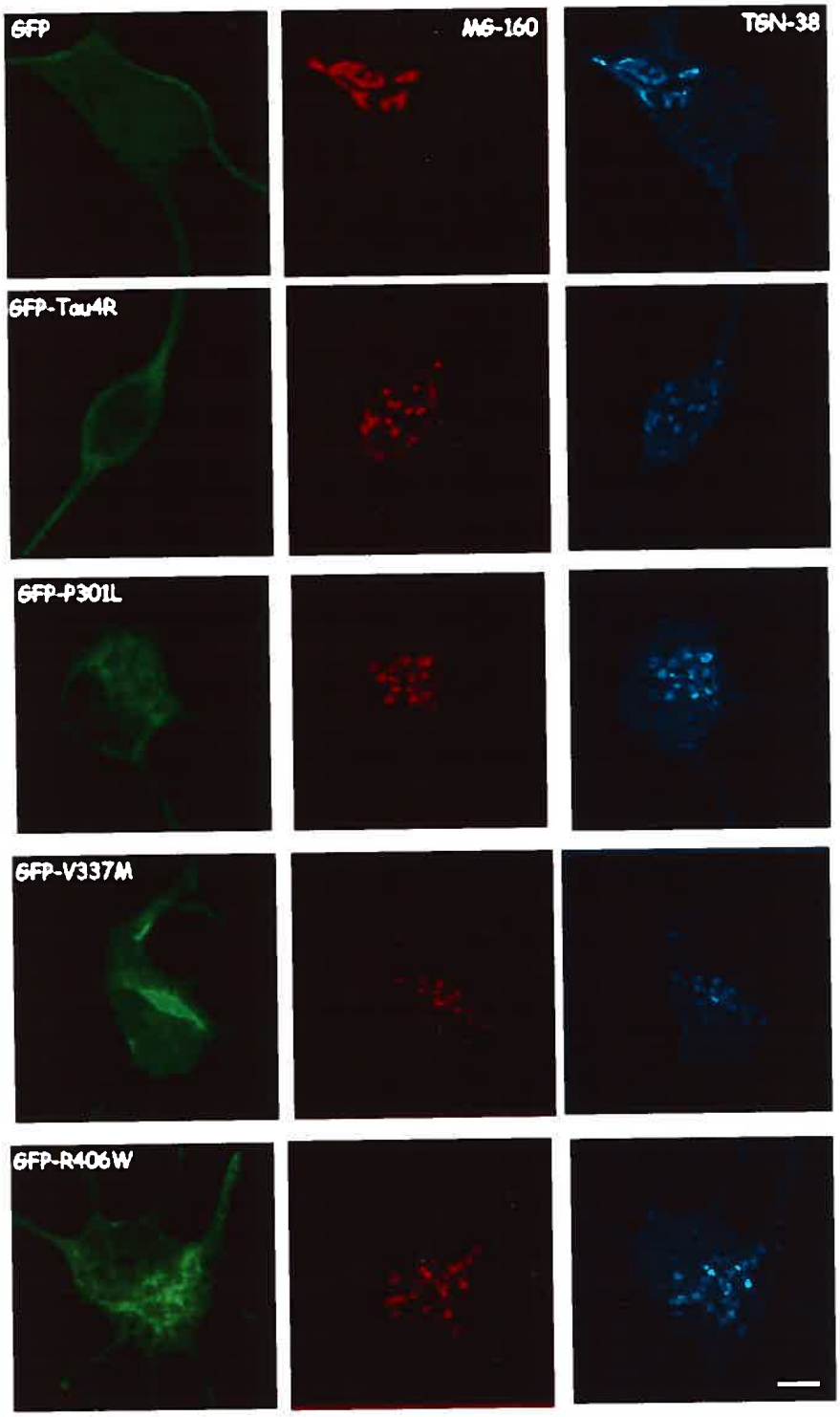

GFP
M6-160
TGN-38
GFP-Tau4R
GFP-P301L
GFP-V337M
GFP-R406W

Figure 9: Confocal microscopy analysis of the distribution TGN38 and the Golgi marker GM-130 in hippocampal neurons. These two Golgi markers co-localized in control hippocampal neurons as shown by immunofluorescence. In H_2O_2 treated hippocampal neurons, TGN38 and GM-130 staining did show a complete co-localization. The arrows indicate the Golgi structures that did not contain both markers. The staining of activated caspase-3 in the nucleus indicated that the neuron was apoptotic. Scale bar= 3 μm.

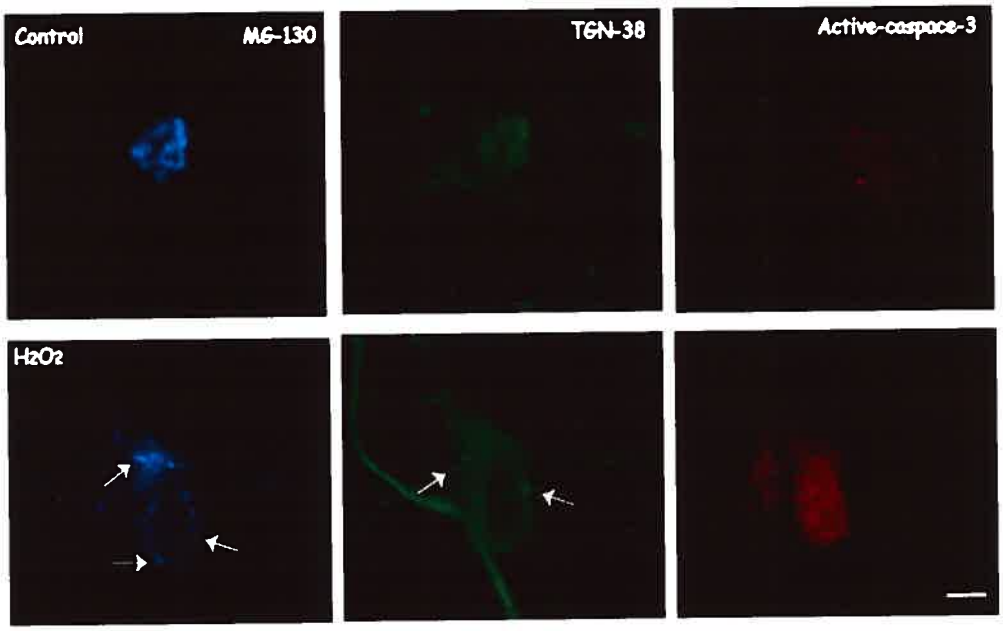

Control
MG-130
TGN-38
Active-caspace-3
H2O2

Figure 10: Apoptotic motor neurons were analyzed by using the terminal dUTP nick-end labeling assay (TUNEL) on sections of the spinal cord from JNPL3 transgenic mice. As a positive control, spinal cord sections from control mice were treated with DNase I. In 40 control spinal cord sections treated with DNase I (A, B and C), TUNEL-positive neurons were detected as indicated by the nuclear staining (C). The DNAse I treated sections were also stained with the CP-13 antibody (A) and with the antibody directed against MG-160 (B). No staining to CP-13 was detected in control motor neurons. As shown in D,E and F, in JNPL3 mice spinal cord sections, a motor neuron immunoreactive to CP-13 (D) and presenting a fragmented GA (E) as revealed by the anti-MG-160 antibody was not TUNEL-positive (F). Scale bar: 8 μm

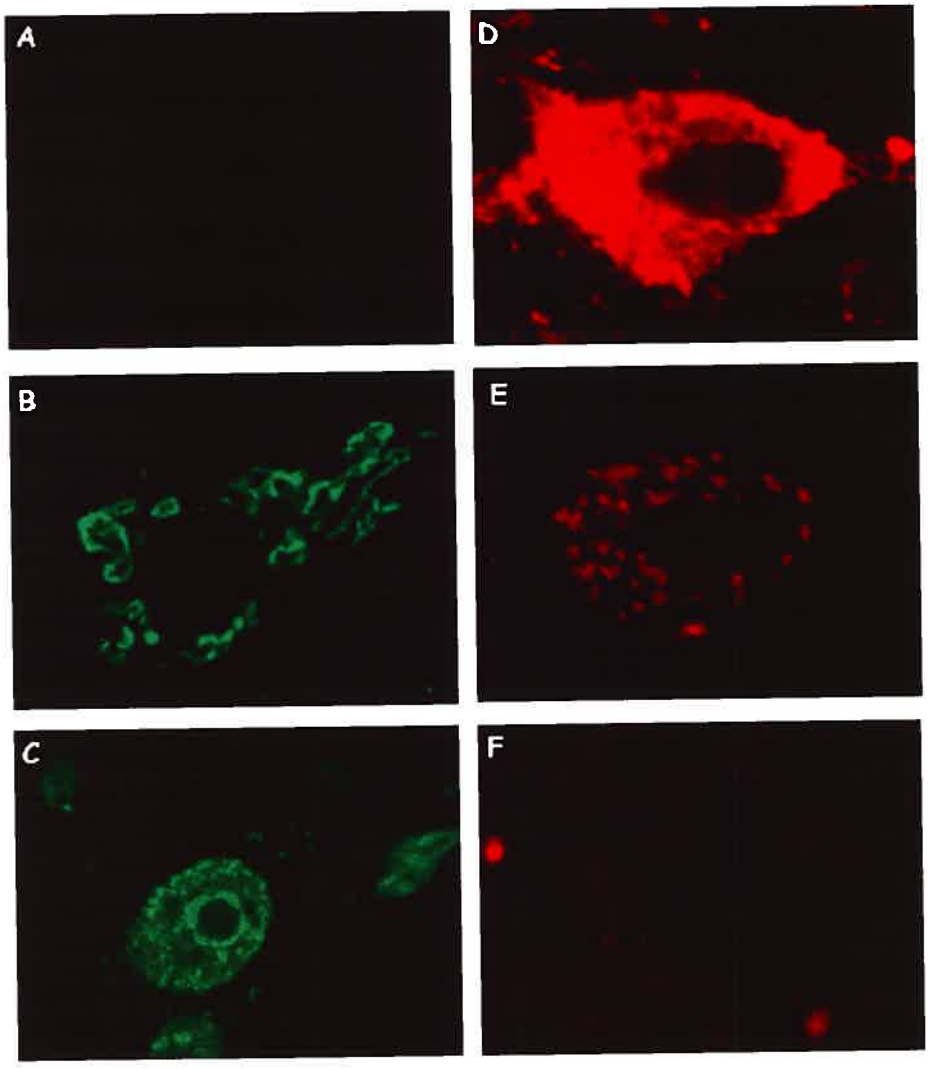

A
D
B
E
C
F

Figure 11: Confocal microscopy analysis of the distribution of the ER in transfected hippocampal neurons presenting a fragmented GA. Transfected neurons expressing either GFP, GFP-tau 4R, GFP-P301L, GFP-V337M or GFP-R406W were stained for Ribophorin II, a marker of the rough ER. No major change in the ER morphology was detected in transfected neurons presenting a fragmented GA. Scale bar= 3 µm.

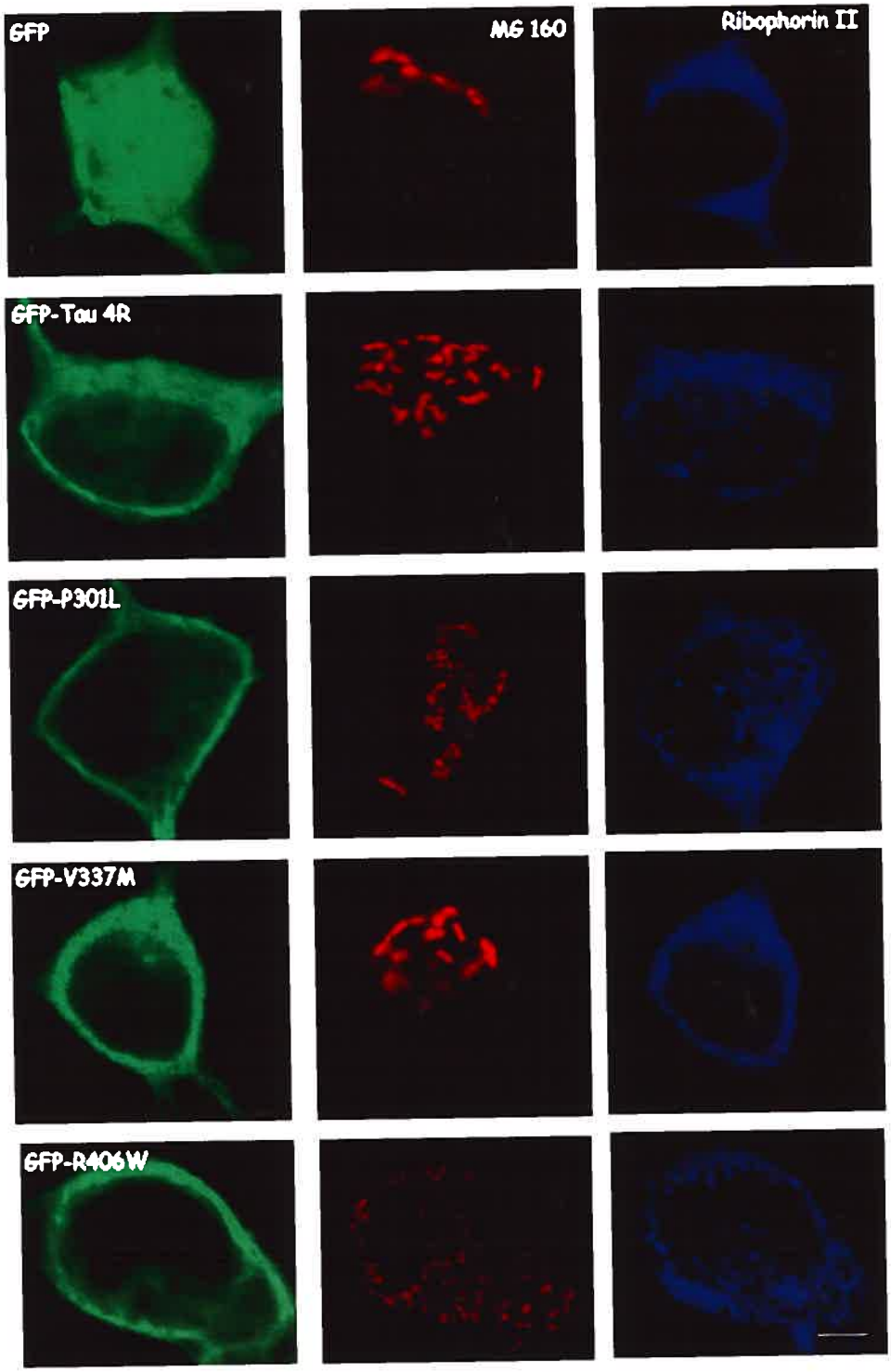

GFP
MG 160
Ribophorin II
GFP-Tau 4R
GFP-P301L
GFP-V337M
GFP-R406W

Table 1: Morphological analysis of the GA in hippocampal neurons transfected either with GFP, GFP-Tau 4R, GFP-P301L, GFP-R406W or GFPV337M.

The number of cells with a fragmented GA was significantly higher in neurons expressing wild-type human tau 4R than in GFP expressing neurons. The percentage of neurons expressing a mutant form of tau 4R (P301L, V337M or R406W) with a fragmentation of the GA was significantly higher than in wild type tau 4R-expressing neurons. The statistical significance between the different groups was determined by using a one-way ANOVA followed by the Tukey-Kramer Multiple Comparison Post-hoc test. The three asterisks indicate that the p value is <0,001.

	Number of cell examined	Percentage of cells with a fragmented GA	SEM
GFP	167	6,5%	0,4
GFP-Tau 4R	162	30,89%***	1,7
GFP-P301L	166	39,01%***	2,3
GFP-R406W	168	41,04%***	0,52
GFP-V337M	164	42,68%***	0,72

Table 2: Number of motor neurons with a fragmented GA in control and JNPL3 mice

	Number of motor neurons with a fragmented GA	Total number of motor neurons examined per animal	Percentage of motor neurons with a fragmented GA
3-5 months control mice			
1	1	97	1.03%
2	2	185	1.08%
3	0	131	0.00%
4	2	99	2.02%
3-5 months transgenic mice			
1	2	186	1.08%
2	1	178	0.56%
3	3	127	2.36%
4	1	167	0.60%
10-12 months control mice			
1	1	147	0.68%
2	1	170	0.59%
3	1	135	0.74%
10-12 months transgenic mice			
1	11	154	7.14%
2	11	158	6.96%
3	10	88	11.36%

3.6. REFERNCES

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4. Discussion générale

Nos travaux visaient à déterminer la possible implication des MAPs structurales dans la ségrégation des organelles membranaires au cours du développement neuronal.

4.1. Interaction de MAP2 avec le RER:

Dans la cellule, les microtubules et le réticulum endoplasmique sont deux structures intimement liées (Terasaki et al. 1986; Terasaki and Reese 1994). Les mécanismes qui permettent le déplacement du RE le long des microtubules ont été bien décrits (Waterman-Storer and Salmon 1998). Cependant, dans les neurones, une grande partie du RE, ne serait pas dynamique mais plutôt stable (Bannai et al. 2004). Pourtant les mécanismes moléculaires qui permettent l'établissement de liens stables entre les microtubules et le RE, dans les neurones, sont encore mal connus.

Sachant que dans les neurones matures, la protéine MAP2 de haut poids moléculaire et le RER sont compartimentalisés dans les dendrites (Bartlett and Banker 1984b; Peters et al. 1991; Torre and Steward 1996) nous avons étudié la contribution possible de MAP2 dans la ségrégation du RER. Dans le cadre de cette étude, nous avons effectué des expériences de fractionnement subcellulaire à partir de cerveau de rat adulte. Les analyses biochimiques et l'immunocytochimie en microscopie électronique des fractions ainsi obtenues a permis de révéler que dans les neurones MAP2 s'associe avec les membranes du RER. L'étude de la différenciation des neurones de l'hippocampe in vitro a permis de montrer que MAP2 et le RER co-ségrégent dans le compartiment somato-dendritique. De plus, des expériences de recouvrement par immunobuvardage et de co-immunoprécipitation ont montré que MAP2 interagit avec la protéine p63 via le domaine de projection de MAP2. En outre, un essai de reconstitution *in vitro* a montré que MAP2, contrairement à Tau,

peut effectuer un lien entre les microtubules et le RE. Ainsi, MAP2 serait la première protéine dendritique identifiée à effectuer le lien entre les microtubules et le RER.

Au cours des dernières années, le rôle joué par plusieurs autres protéines associées aux microtubules non motrices dans l'assemblage et l'organisation des organelles membranaires a été bien documenté (Pierre et al. 1992; De Zeeuw et al. 1997; Perez et al. 2002; Pernet-Gallay et al. 2002; Howard and Hyman 2003; Mimori-Kiyosue and Tsukita 2003; Lallemand-Breitenbach et al. 2004; Rios et al. 2004). Par exemple, dans les dendrites, CLIP-115 est associée à la fois aux microtubules et à des structures membranaires très spécialisées appelées corps lamellaires dendritiques (CLDs). Ces CLDs seraient impliqués dans la formation et la stabilité des jonctions communicantes dans les dendrites (De Zeeuw et al. 1995). La structure et la localisation subcellulaire de CLIP-115 suggèrent que cette protéine contrôle la formation et la translocation des CLDs (De Zeeuw et al. 1997).

Dans les cellules mitotiques, deux protéines ubiquistes jouent un rôle dans la liaison des microtubules et le RE. La première protéine est la p63 (que nous avons identifié comme partenaire de MAP2 dans les neurones) et la deuxième et la p22. La p63 (ou CLIMP-63 «cytoskeleton-linking membrane protein-63») est une protéine intégrale de type II du réticulum (Schweizer et al. 1993). Dans les cellules non neuronales, une étude a rapporté que le domaine cytoplasmique de p63 lie les microtubules (Klopfenstein et al. 1998). Cependant, lors de leur essai in vitro, Klopfenstein et al. ont utilisé des microtubules purifiés à partir du cerveau. Sachant que ces microtubules contiennent inévitablement des MAPs neuronales (entre autre MAP2), il n'était pas clair si la p63 se liait directement ou indirectement à la tubuline (Klopfenstein et al. 1998). Nos résultats suggèrent que la p63 se lie

indirectement à la tubuline via MAP2 dans les neurones. Dans les cellules non-neuronales, MAP4 pourrait assurer le lien entre la p63 et la tubuline. D'où la nécessité d'autres études pour clarifier ce point. Dans les cellules non neuronales, la protéine p22 s'associe préférentiellement avec les membranes du RE et joue un rôle dans la dynamique et l'organisation des microtubules et du RE (Andrade et al. 2004). Des études antérieures ont montré que l'association de p22 aux microtubules est indirecte et se ferait via un facteur cytosolique encore non identifié (Timm et al. 1999). La p22 est exprimée dans les neurones (Barroso et al. 1996) et il serait intéressant de vérifier si la liaison aux microtubules de p22 se fait via MAP2. Dans ce contexte, l'essai de recouvrement par immunobuvardage a révélé que MAP2 s'associe à au moins 5 protéines (incluant la p63 et la tubuline ainsi que 3 protéines de faible poids moléculaire). D'autres expériences seront nécessaires pour identifier les autres partenaires de MAP2 au niveau du RE.

4.1.1. Fonctions possibles de l'interaction de MAP2 avec le RE:

Mise à part l'établissement de liens stables entre les microtubules et le RER, est ce que l'association entre MAP2 et le RE pourrait assurer d'autres fonctions?

Le réticulum endoplasmique assure plusieurs fonctions dans la cellule. Le RE est le siège de synthèse et de maturation des protéines transmembranaires ainsi que celles destinées à la voie de sécrétion (Hegde and Lingappa 1999). De plus, le RE représente la principale réserve dynamique de calcium intracellulaire (Verkhratsky 2005).

En effet, le calcium est un messager intracellulaire important qui gouverne plusieurs fonctions incluant la différenciation cellulaire, la croissance,

la polarité, l'excitabilité membranaire, l'exocytose et l'activité synaptique (Berridge et al. 2000; Verkhratsky 2005). Dans la cellule, l'homéostasie du calcium est étroitement régulée par des mécanismes de transport actif mais aussi par des mécanismes de stockage (Berridge 1998; Berridge et al. 2000). Au moins deux groupes principaux de canaux permettent l'entrée du calcium dans la cellule: les canaux calciques voltage dépendants et les canaux calciques messagers dépendants tels que les canaux- récepteurs du glutamate de type NMDA (Berridge et al. 2000; Verkhratsky 2005). D'un autre côté, comme mentionné plus haut, dans la cellule le RE joue un rôle primordial dans la régulation de la concentration spatio-temporelle du calcium intracellulaire nécessaire pour une activité neuronale coordonnée (Rose and Konnerth 2001). Dans ce contexte, il est intéressant de souligner que la protéine MAP2 interagit non seulement avec les sous unités 2A et 2B des récepteurs NMDA (Husi et al. 2000; Buddle et al. 2003) mais aussi avec la sous unité α-1 des canaux calciques voltage dépendants de type-L et de classe-C (Davare et al. 1999). En outre, et à la lumière de nos résultats, MAP2 interagit avec le RE, principale réserve intracellulaire de calcium. Ainsi, grâce à ses interactions, MAP2 pourrait représenter un effecteur central dans les voies de signalisation régulée par le calcium.

4.2. Interaction de Tau avec les membranes golgiennes:

Nous avons récemment décrit une nouvelle interaction entre les membranes Golgiennes et la protéine Tau. Cette interaction a été révélée suite à la caractérisation biochimique et en microscopie électronique des fractions subcellulaires préparées à partir de cerveaux de rat adulte. De plus, un marquage immunocytochimique en microscopie électronique sur les motoneurones de la moelle épinière de souris a montré la présence de la

protéine Tau au niveau de l'appareil de Golgi. La quantification de la distribution du marquage à l'or colloïdal suggère que la protéine Tau s'associe préférentiellement avec le sous compartiments médial et trans du Golgi (voir article en annexe). Cette distribution polarisée laisse présager une fonction spécifique de la protéine Tau au niveau de l'appareil de Golgi.

4.2.1. Fonctions possibles de l'interaction de Tau avec l'appareil de Golgi:

4.2.1.1. Établissement de liens stables entre les microtubules et l'appareil de Golgi:

Les microtubules jouent un rôle primordial dans le maintien de la structure et la fonction de l'appareil de Golgi (Thyberg and Moskalewski 1999; Palmer et al. 2005). En s'associant à la fois avec l'appareil de Golgi et les microtubules Tau pourrait permettre l'établissement de liens stables entre les membranes golgiennes et les microtubules.

D'autres protéines associées aux microtubules interagissent avec les membranes golgiennes. Parmi ces dernières, on distingue la FTCD (p58) (Bashour and Bloom 1998; Hennig et al. 1998), GMAP-210 (Pernet-Gallay et al. 2002; Rios et al. 2004), Hook3 (Walenta et al. 2001), CLIPR-59 (Perez et al. 2002; Lallemand-Breitenbach et al. 2004) et la SCG10 (Gavet et al. 1998; Lutjens et al. 2000). Les protéines Hook3, FTCD et GMAP-210 sont concentrées dans le sous compartiment cis du Golgi (Walenta et al. 2001; Pernet-Gallay et al. 2002) alors que SCG10 et CLIPR-59 sont enrichies dans le sous compartiment trans du Golgi (Lutjens et al. 2000; Perez et al. 2002). Les résultats de plusieurs études laissent présager que ces protéines jouent un rôle important dans le positionnement de l'appareil de Golgi et l'établissement de liens stables entre les microtubules et l'appareil de Golgi. Cependant, la

fonction exacte de l'association de toutes ces protéines avec l'appareil de Golgi n'est pas encore bien connue (sauf GMAP-210). Dans une étude récente, Rios et al. ont montré que la protéine GMAP-210 recrute la tubuline-γ au niveau du compartiment cis du Golgi ce qui permet d'assembler et de positionner l'appareil de Golgi au niveau des centrosomes (Rios et al. 2004). En outre, la déplétion ou la surexpression de GMAP-210 induit la fragmentation de l'appareil de Golgi (Pernet-Gallay et al. 2002; Rios et al. 2004). La protéine Hook3 joue également un rôle important dans le maintien de l'architecture de l'appareil de Golgi puisque la surexpression de cette dernière induit la fragmentation de l'appareil de Golgi (Walenta et al. 2001).

4.2.1.2. Ancrage de certaines protéines kinases et phosphatases aux membranes à l'appareil de Golgi:

Plusieurs protéines kinases et phosphatases sont localisées au niveau de l'appareil de Golgi. L'activité de ces protéines régulent non seulement la structure mais aussi la fonction de l'appareil de Golgi (Austin and Shields 1996; Preisinger and Barr 2005). Au niveau des membranes golgiennes, on retrouve, entre autres, la PKA (Nigg et al. 1985a; Nigg et al. 1985b), la CDK5 (Paglini et al. 2001), la PKC (Schultz et al. 2004), la Gsk3β (Piché 2004) et la protéine phosphatase PP2A (Hancock et al. 2005). Il est intéressant de souligner que toutes ces protéines kinases et phosphatases interagissent avec la protéine Tau (Sontag et al. 1999; Sobue et al. 2000; Sun et al. 2002; Agarwal-Mawal et al. 2003). Par conséquent un des rôles possibles de l'interaction de Tau avec les membranes golgiennes serait la localisation et l'ancrage de ces protéines kinases et phosphatases à la surface de l'appareil de Golgi. Une altération dans la distribution de ces protéines kinases et phosphatases (suite à une mutation ou

une hyperphosphorylation de la protéine Tau) pourrait avoir pour conséquence une perturbation de l'organisation et/ou de la fonction du Golgi.

4.2.1.3. Ciblage des vésicules golgiennes au compartiment axonal:

Lors du développement neuronal, l'apport de protéines membranaires par la voie de sécrétion est un processus fondamental qui accompagne la pousse neuritique et la polarité neuronale (Horton and Ehlers 2003b). Dans les neurones, les mécanismes moléculaires qui permettent le ciblage et l'accumulation des protéines membranaires dans l'un ou l'autre des compartiments sont encore peu connus (Winckler and Mellman 1999; Winckler 2004). Une fonction possible à l'association de Tau avec les membranes golgiennes serait le ciblage des vésicules golgiennes destinées à l'axone. En effet, l'enrichissement de Tau au niveau de l'axone et son association préférentielle au compartiment médial et trans du Golgi fait de cette protéine un bon candidat pour le ciblage et la ségrégation de certaines vésicules golgiennes dans l'axone. Dans ce contexte, il est intéressant de rappeler que la surexpression de Tau inhibe le transport des vésicules golgiennes qui transportent le précurseur du Aβ (APP) (Stamer et al. 2002) et peut également interférer avec la fonction sécrétoire du Golgi (Zhao et al. 2005). Toutefois cette hypothèse reste encore spéculative et plusieurs expériences sont nécessaires pour la vérifier.

4.2.2. Altération de l'organisation de l'appareil de Golgi suite à la surexpression de Tau:

Nos résultats indiquent que la surexpression de Tau induit la fragmentation de l'appareil de Golgi. Nous avons exprimé des formes humaines de Tau dans des cultures primaires de neurones isolés de l'hippocampe d'embryons de rats. L'étude qualitative et quantitative a montré que l'expression de la forme humaine normale de la protéine Tau induit la fragmentation du Golgi dans 30% des neurones transfectés en comparaison à 6% dans les neurones témoins. Dans le cas des neurones exprimant l'une des formes humaines mutantes de Tau, l'appareil de Golgi est fragmenté dans 40% des neurones transfectées. En outre, un phénotype similaire a été observé chez les souris transgéniques JNPL3. Le nombre de motoneurones qui présentent une fragmentation de l'appareil de Golgi est huit fois plus élevé chez les souris JNPL3 que chez les souris témoins. Dans ce modèle animal, la fragmentation de l'appareil de Golgi serait un événement précoce au cours du processus de neurodégénérescence puisque nous avons été en mesure d'observer des motoneurones avec un Golgi fragmenté chez des souris transgéniques asymptomatiques et avant même l'apparition de NFTs. De plus, ce changement morphologique serait spécifique au Golgi (pas de changement morphologique majeur de la structure du RE n'a été observé) et indépendant de l'apoptose.

D'autres études ont rapporté la fragmentation de l'appareil de Golgi suite à la surexpression de Tau dans des cultures primaires d'astrocytes (Yoshiyama et al. 2003). De plus, chez des souris transgéniques qui surexpriment certaines formes humaines mutantes de Tau (V337M et P301L) des études en microscopie électronique ont révélé une fragmentation de l'appareil de Golgi (Tanemura et al. 2002; Lin et al. 2003).

Les mécanismes moléculaires exacts conduisant à la fragmentation de l'appareil de Golgi suite à la surexpression de la protéine Tau humaine mutante ou non sont encore inconnus. Nous ne pouvons pas non plus prétendre que la fragmentation observée suite à la surexpression de Tau serait une conséquence de l'altération des liens entre Tau et l'appareil de Golgi. Toutefois nous pouvons émettre quelques hypothèses pour expliquer ce phénomène.

La fragmentation de l'appareil de Golgi peut avoir lieu suite à la perturbation ou l'altération des microtubules. En effet, dans des cellules en culture l'ajout de drogues, tels que le nocodozole ou le taxol, induit une fragmentation de l'appareil de Golgi (Hoshino et al. 1997; Minin 1997). Par conséquent la fragmentation du Golgi dans notre étude pourrait être due à la perturbation de l'organisation des microtubules suite à la surexpression de la protéine Tau.

La fragmentation de l'appareil de Golgi peut également être induite suite à la perturbation des protéines impliquées dans le transport entre le RE et l'appareil de Golgi (Harada et al. 1998; Tang et al. 2001; Kauppi et al. 2002; Xu et al. 2002). L'utilisation de toxines telles que la Bréfeldine-A (BFA) ou l'acide nordihydroguaiarétique (NDGA) qui bloquent le transport entre le RE et de l'appareil de Golgi provoquent également la fragmentation de l'appareil de Golgi (Fujiwara et al. 1988; Elyaman et al. 2002). En effet, l'utilisation de BFA et NDGA dans les cultures primaires de neurones induit non seulement la fragmentation de l'appareil de Golgi mais aussi une augmentation de l'activité de la kinase GsK3β et de l'état de phosphorylation de la protéine Tau (Elyaman et al. 2002). Le transport kinésine dépendant qui assure le transport des vésicules entre l'appareil de Golgi et le RE est altéré suite à la surexpression de Tau (Ebneth et al. 1998; Stamer et al. 2002; Mandelkow et al. 2003). Ainsi la fragmentation du Golgi pourrait être due à la perturbation du transport entre le

Golgi et le RE induite par la présence de Tau en excès dans les neurones de l'hippocampe et les souris JNPL3.

Durant la mitose, la structure périnucléaire et compacte du Golgi subit également une série de transformations morphologiques dramatiques qui conduit à la fragmentation de l'appareil de Golgi et sa ségrégation aux cellules filles (Lucocq et al. 1987; Lucocq and Warren 1987). La dissection de ce processus au niveau moléculaire a permis d'identifier, comme agent de fragmentation, principalement deux kinases MEK1 et cdc2. Des expériences in vitro et in vivo ont montré que l'activation séquentielle de MEK1 et de CDC2 est nécessaire pour la fragmentation de l'appareil de Golgi durant la mitose (Lowe et al. 1998; Colanzi et al. 2000; Kano et al. 2000). Rappelons que l'activité de ces deux kinases est associée à la phosphorylation aberrante de la protéine Tau dans les conditions pathologiques telles que la maladie d'Alzheimer (Tsujioka et al. 1999; Husseman et al. 2000; Pei et al. 2002). D'ailleurs, la neurodégénérescence chez des patients atteints de la maladie d'Alzheimer ainsi qu'un modèle de souris transgénique de Tau a été associée avec la réexpression de certaines protéines impliquées dans la régulation du cycle cellulaire (entre autres cdc2) et dans l'initiation de la synthèse d'ADN (Herrup and Arendt 2002; Andorfer et al. 2005; Webber et al. 2005). Ainsi une activité aberrante de certaines protéines kinases pourrait induire l'hyperphosphorylation de Tau et aussi la fragmentation de l'appareil de Golgi.

Enfin, la fragmentation de l'appareil de Golgi se produit lors de l'apoptose (Nozawa et al. 2002). Toutefois nos résultats ainsi que les résultats d'une autre étude nous indiquent que dans les neurones moteurs de la moelle épinière des souris transgéniques JNPL3 et les cultures primaires de neurones de l'hippocampe la fragmentation de l'appareil de Golgi ne serait pas due à l'apoptose (Zehr et al. 2004; Liazoghli et al. 2005).

4.2.3. Altération de la distribution subcellulaire de la protéine Tau dans la maladie d'Alzheimer:

Des données préliminaires, générées dans notre laboratoire, indiquent qu'il y aurait une altération de la distribution subcellulaire de Tau dans le cerveau des patients atteints de la maladie d'Alzheimer. En effet, une analyse quantitative de la distribution subcellulaire de Tau montre la présence de Tau dans une fraction enrichie en membranes du RE dans des cerveaux de patients atteints de la maladie d'Alzheimer alors que dans des cerveaux témoins Tau est enrichie dans une fraction qui contient des marqueurs golgiens. Ces résultats concordent avec d'autres observations rapportées dans la littérature. Des études en microscopie électronique ont montré la localisation périnucléaire des filaments de Tau qui sont attachés aux membranes du RE chez des sujets atteints de la maladie d'Alzheimer (Gray et al. 1987; Okamoto 1991; Mena et al. 1996; Bondareff et al. 1998).

L'altération de la distribution de Tau, dans ces conditions pathologiques, pourrait non seulement perturber la fonction de Tau au niveau de l'appareil de Golgi mais aussi altérer la fonction du RE (exemple: contrôle de qualité des protéines nouvellement synthétisée). D'ailleurs, les résultats de plusieurs études suggèrent que la mort neuronale dans la maladie d'Alzheimer serait due à l'induction du stress du RE (O'Neill et al. 2001; Paschen 2003; Katayama et al. 2004).

4.3. Conclusions et perspectives:

La cellule contient une panoplie de protéines capables d'interagir à la fois avec les organelles membranaires et les microtubules. Certaines de ces protéines ancrent ou entravent le mouvement des organelles membranaires alors que d'autres les libèrent ou les transportent. La distribution d'une organelle

membranaire résulte de la combinaison de ces différents phénomènes. Nos résultats suggèrent que Tau et MAP2 pouraient être impliquées dans l'ancrage du Golgi et du RER aux microtubules dans les neurones. Cependant ces découvertes soulèvent plusieurs interrogations.

- Quels sont les autres partenaires de MAP2 au niveau du RER?
- Quel (s) est (sont) la (les) fonction (s) de la protéine MAP2 associée au RER?
- Est-ce que Tau fait partie d'un complexe protéique au niveau de l'appareil de Golgi? Si c'est le cas quelles sont les protéines golgiennes qui interagissent avec Tau?
- Quel (s) est (sont) la (les) fonction (s) de Tau associée à l'appareil de Golgi?
- Quelles sont les conséquences fonctionnelles de la fragmentation de l'appareil de Golgi suite à la surexpression de Tau (exemple une altération de la ségrégation des vésicules membranaires ou la modification de capacité sécrétoire de l'appareil de Golgi)?
- Est-ce que l'altération de la distribution subcellulaire de Tau observée dans les conditions pathologiques chez les sujets atteints de la maladie d'Alzheimer (des membranes golgiennes vers les membranes du RE) perturbe les fonctions du RE?

L'ensemble de nos travaux ouvrent de nouvelles perspectives de recherche pour une meilleure compréhension des mécanismes moléculaires qui régissent l'établissement de la polarité neuronale au cours du développement normal et sa perte au cours du processus neurodégénératif.

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ANNEXES

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"Association of tau with the Golgi membranes: a new key in tau function"

Association of tau with the Golgi membranes: a new key in tau function

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ABSTRACT

Tau, a microtubule-associated protein enriched in the axon, is known to stabilize and promote the formation of microtubules during axonal formation. Several studies have reported that tau was associated to membranes (Papasozomenos and Binder 1987; Jung et al. 1993; Brandt et al. 1995). However, many of these interactions are still controversial. In the present study, we further characterized the interaction of tau with membranous elements by examining the distribution of tau in subfractions enriched in either Golgi or endoplasmic reticulum (ER) membranes isolated from rat brain. A subfraction enriched in markers of the medial Golgi compartment, MG160 and mannosidase II, presented a high tau content indicating that tau could be associated to Golgi membranes. Electron microscope morphometry confirmed the enrichment of this subfraction in Golgi membranes. Futhermore, double-immunogold labeling experiments were conducted, on this subfraction, to show the association of tau with Golgi vesicles. Tau-immunogold was observed on vesicles labeled with either an antibody directed against MG160, the medial Golgi marker or TGN38, a marker of the Trans Golgi network. The association of tau with the medial Golgi membranes was confirmed by co-immunoprecipitation of a Golgi protein complex containing MG160 with an anti-tau antibody. Futhermore, confocal microscope analysis of the distribution of tau and MG160, in cultured hippocampal neurons, revealed that these two proteins partially co-localized at the periphery of the Golgi membranes. Electron microscope immunogold was employed to confirm the presence of tau on the Golgi membranes in intact neurons in the spinal cord. Most notably, this technique revealed a gradient of association of tau with Golgi membranes. Tau was found in all Golgi compartments but increased toward the trans side of the

Golgi. Finally, the pool of tau present in the Golgi subfraction was found to be phosphorylated at the sites recognized by the phosphorylation-sensitive antibody PHF-1.

INTRODUCTION

Tau is a neuronal microtubule-associated protein (MAP) that is enriched in the axonal compartment (Buee et al. 2000). In vitro studies have shown that tau can stabilize microtubules by decreasing their dynamic instability (Garcia and Cleveland 2001). Consistently, the suppression of tau expression results in a decrease of a pool of stable microtubules in primary neuronal cultures (Caceres et al. 1991). Moreover, the suppression of tau expression impairs the differentiation of the axonal process in these cultures (Caceres et al. 1991; Dawson et al. 2001). Recently, tau was shown to be involved in the transport of membranous organelles. Overexpression of tau in both neuronal and nonneuronal cells resulted in the accumulation of mitochondria, Golgi membranes and peroxisomes in the perinuclear region and their loss at the cell periphery (Ebneth et al. 1998; Stamer et al. 2002). This redistribution of membranous organelles might be caused by the impairment of the motor protein binding to microtubules by tau. Because of the increased association of tau with microtubules, this may cause a steric hindrance along microtubules preventing microtubule-motor interaction. Indeed, in vitro studies showed that kinesin binding to microtubules was decreased in the presence of tau (Heins et al. 1991). Tau and kinesin binds to α-tubulin and β-tubulin respectively (Santarella et al. 2004). Thus, the decrease of kinesin binding to microtubules by tau can not be explained by their competition to bind to tubulin. More recently, it was reported that the overexpression of tau in astrocytes also induced an accumulation of membranous organelles in the perinuclear region (Yoshiyama et al. 2003). This was accompanied by a reduction of detyrosinated tubulin and a decrease of kinesin protein level. Thus, tau might impair the transport of organelles by affecting diverse aspects of it.

In addition to its interaction with microtubules, tau was also found to be associated with the plasma membrane (Brandt et al. 1995). This interaction was shown to be necessary to induce axonal-like processes in PC12 cells. A proline rich sequence located in the amino-terminal of tau is involved in this interaction by mediating the association of tau to the SH3 domains of Fyn and src non-receptor tyrosine kinases (Lee et al. 1998). The localization of tau at the plasma membrane suggests that tau might establish a link between external signals and the microtubule network. Futhermore, other membranous associations were reported for tau such as its association with the outer membrane of mitochondria (Jung et al. 1993). However, these interactions remain controversial.

The function of tau is tightly regulated by its phosphorylation state. For example, an increase of tau phosphorylation reduces its binding affinity for microtubules (Buee et al. 2000). As noted for its binding to microtubules, the association of tau to the plasma membrane is also regulated by its phosphorylation state. Indeed, an increase of tau phosphorylation impairs its association with the plasma membrane (Maas et al. 2000). In Alzheimer's disease (AD), tau becomes hyperphosphorylated and aggregates in insoluble filaments called paired helical filaments (PHFs) (Lee et al. 2001). It is believed that hyperphosphorylated tau contributes to neurodegeneration by detaching from microtubules and destabilizing the axonal microtubule network. However, since tau interaction with the plasma membrane is also abolished by an increase of its phosphorylation, it indicates that in neurodegenerative conditions, the interaction of tau with the plasma membrane could also be compromised and contribute to neuronal dysfunction.

Microtubules are known to contribute to the cellular distribution of membranous organelles and to the maintenance of their morphology (Lucocq et al. 1987; Lucocq and Warren 1987). This occurs through numerous proteins able to link both microtubules and membranous organelles (Bloom and Brashear 1989; Lutjens et al. 2000; Walenta et al. 2001; Lallemand-Breitenbach et al. 2004; Rios et al. 2004). Most importantly, these proteins can contribute to the segregation of membranous organelles within a polarized cell such as a neuron. Indeed, CLIP-115 by establishing a link between microtubules and a membranous organelle termed lamellar bodies, contributes to its the polarized distribution in the somato-dendritic compartment in neurons (De Zeeuw et al. 1997). Here, we describe the association of tau with a subpopulation of Golgi membranes by using subcellular fractionation, co-immunoprecipitation and electron microscope immunocytochemistry in rat brain. The present results indicate that tau could be involved in linking together Golgi membranes and microtubules.

MATERIALS AND METHODS

Subcellular fractionation

Adult Sprague-Dawley rats were purchased at Charles River (Charles River Laboratories Inc., Montreal, Quebec, Canada). The use of animals and all surgical procedures described in this article were carried out according to *The guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.* Brain was dissected from twenty adult Sprague-Dawley rats. Subcellular fractions were generated by using the protocol previously described by Lavoie et al. (Lavoie et al. 1996). A schematic of the protocol is shown in Fig. 1. Briefly, total microsomes were isolated by differential centrifugation and ER and Golgi elements were subsequently separated by ultracentrifugation in a sucrose step-gradient.

Immunoblot analysis

Protein assay was performed (Bio-Rad kit, Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Equal amounts of proteins were loaded in each lane and electrophoresed in a 7.5% polyacrylamide gel. Following separation, proteins were electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose strips were incubated with the primary antibodies during 90 min at room temperature. They were then washed with Phosphate Buffered Saline (PBS) and then incubated with the peroxidase-conjugated secondary antibodies. Membranes were again washed and then revealed by chemiluminescence (Amersham Pharmacia Biotech, Quebec, Quebec, Canada). The following primary antibodies were used: the monoclonal antibody anti-MAP2 (clone HM2, Sigma, Oakville, ON), the monoclonal antibodies anti-tau, Tau-1 directed against unphosphorylated tau (Oncogene Research Products, San Diego, California) and 49.2 (kindly provided by Virginia M-Y Lee, University of Pennsylvania, Philadelphia, USA), a polyclonal antibody anti-tau (kindly provided by Virginia M-Y Lee, University of Pennsylvania, Philadelphia, USA), the monoclonal antibodies AT8

(Pierce Endogen, USA), CP9 and PHF-1 (kindly provided by Dr. Peter Davies, Albert Einsten, NY, USA) directed against phosphorylated tau, the monoclonal antibody anti-α-tubulin (clone DM 1A, Sigma, Oakville, ON), a polyclonal antibody against ribophorin (kindly provided by Dr. G. Kreibich, New York University School of Medicine, New York, New York), a polyclonal antibody against calnexin (Stressgen Biotechnologies, Victoria, BC, Canada), a polyclonal antibody against mannosidase II (kindly provided by Dr. M.G. Farquhar, University of California, San Diego), a polyclonal against NaK-ATPase (kindly provided by Dr. D. Fambrough, The Johns Hopkins University, Maryland), a monoclonal directed against MG160 (kindly provided by Dr. N. Gonatas, University of Pennsylvania, Philadelphia), GM130 (Oncogene, USA) and TGN38 (Serotec, UK).

Co-immunoprecipitation

Total microsomes prepared from adult rat brain were resuspended in a buffer containing 50 mM Tris-HCl, 150 mM NaCl and .2% Triton X-100, pH 7.4. The microsomes were sonicated for 5 sec at amplitude 6% and then were centrifuged for 5 min at 12000 rpm to sediment protein aggregates. The supernatant was incubated with a primary antibody directed against tau (polyclonal antibody) overnight at 4°C. The following day the sepharose beads were added to the solution for two hours. The beads were washed several times. The immunoprecipitate was analyzed by western blot.

Electron microscopy on the Golgi subfraction

Microsomes isolated from brain were fixed using 2.5% glutaraldehyde, recovered onto Millipore membranes by the random filtration technique of Baudhuin et al. (Baudhuin et al. 1967) and processed for electron microscopy as previously described (Lavoie et al. 1996).

Cell cultures

Primary embryonic hippocampal cultures were prepared as previously described(Banker and Goslin 1998). Hippocampi from 18-day-old fetuses were treated with trypsin (0,25% at 37°C for 15 min) then washed in Hank's balanced solution and dissociated by several passages through a constricted Pasteur pipette. The cells were then plated on glass coverslips coated with polylysine. Then, after 4 h to allow the attachment of the cells to the substrate, the hippocampal cells were inverted to face a monolayer of glial cells in a serum-free medium.

Immunofluorescence

Neurons were fixed in 4% paraformaldehyde/PBS for 30 minutes. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. The tau protein was revealed either using the monoclonal antibody 49.2 or a polyclonal antibody. The Golgi apparatus was revealed using either a monoclonal antibody directed against GM130, MG160 or a polyclonal antibody directed against TGN38. To visualize the microtubules, either a polyclonal antibody directed against tubulin (Abcam, Cambridge, UK) was used (1:100) or a monoclonal antibody directed against β-tubulin (DSHB, University of Iowa, Iowa City, IA). We used the following secondary antibodies: a donkey anti-rat conjugated to FITC (dilution 1:100), a donkey anti-rabbit conjugated to Rhodamine (1:500) (Jackson Immunoresearch Laboratories, Bio/Cam, Mississauga, Ontario, Canada), an Alexa Fluor 647 anti-mouse (1:400) and an Alexa Fluor 488 anti-rabbit (1:200) (Molecular Probes, Eugene, OR). All these antibodies were diluted in 5% BSA/PBS. Incubations were carried out at room temperature for 1 hour. After three washes in PBS, the coverslips were mounted in polyvinyl alcohol (Calbiochem, CA, USA). Fluorescently labelled cells were visualized with a Leica TCS-SP1 confocal microscope using 63x or 100x objectives.

Electron microscope immunocytochemistry on spinal cord sections

Adult mice were deeply anesthetized with sodium pentobarbital (somnotol, 65 mg/kg i.p.). They were perfused through the ascending aorta with 20 ml of 0.1M cacodylate buffer (CB, pH 7.4; room temperature), followed by 100 ml freshly prepared fixative containing 2.5% glutaraldehyde (J.B.EM, Pointe Claire, Dorval, Québec, Canada) and 1% paraformaldehyde (Fisher, NJ, USA) in CB. The spinal cords were removed and kept in the same fixative for 1h at room temperature after which they were washed in CB. The spinal cords were cut in sections of 100µm with a vibratome. The sections were postfixed in 1% osmium tetroxide (J.B.EM, Pointe Claire, Dorval, Québec, Canada) and 1.5% potassium ferrocyanide (Sigma) in CB for 30 min at 4°C. After an extensive wash in buffer, the sections were dehydrated in a graded series of alcohols at 4°C, then washed twice for 10 min in 100% ethanol at room temperature and embedded in LR white resin (J.B.EM). The resin was polymerized under anaerobic conditions for 48 h at 56°C. Ultrathin sections (approximately 60 nm) were cut with an ultramicrotome and placed on single slot nickel grids coated with Pioloform. These sections were then processed for postembedding immunocytochemistry with an antibody directed against tau (polyclonal antibody) as described in our previous study (Micheva et al. 1998).

Statistical analysis

The statistical significance of the number of gold particles in the Golgi compartments on spinal cord sections was determined using an ANOVA one-way test followed by Tukey-Kramer multiple comparison test. Statistical significance was accepted if $p<0.05$.

RESULTS

The distribution of tau in subcellular fractions prepared from adult rat brain

The present study aimed to further characterize the interaction of tau with membranous elements. To do this, subfractions enriched in ER and Golgi membranes were purified from adult rat brain using the fractionation protocol summarized in Figure 1A (Lavoie et al. 1996). These subfractions were characterized by using different membranous markers: ribophorin and calnexin as ER markers, mannosidase II, MG160, GM130 and TGN38 as Golgi markers and Na-K-ATPase as a marker of the plasma membrane. A shown in Figure 1B, the ER markers were concentrated in the RM fraction. The Golgi markers were enriched in the subfractions termed I2 and I3 each of these fractions presenting an enrichment of Golgi proteins belonging to distinct compartment. Based on staining intensity, GM130, a marker of the cis-Golgi was concentrated in the I2 subfraction and MG160 and mannosidase II, two markers of the medial Golgi compartment were enriched in the I3 subfraction. TGN38 was enriched in both I2 and I3 subfractions. The plasma membrane marker, Na-K-ATPase was also present in the I2 and I3 subfractions. Tau was enriched in the cytosolic fraction as noted for tubulin. Surprisingly, a significant amount of tau was present in the I2 subfraction containing plasma membrane and Golgi membrane markers (Figure 1B). Previous studies reported that tau was associated to the plasma membrane (Brandt et al. 1995). However, in the present subcellular fractionation conditions, the distribution of tau and the plasma membrane marker, Na-K-ATPase did not correspond since tau was barely detectable in the I3 subfraction. Interestingly, the distribution of tau was similar to that of two markers of the medial Golgi compartment, MG160 and mannosidase II (Velasco et al. 1993; Torre and Steward 1996; Gonatas et al. 1998). This suggested a direct association of tau with the membranes of the medial Golgi compartment. Very little tau was found in the I3 subfraction enriched in the Golgi cis compartment marker, GM130.

Similarly, a small amount of tau was found in the RM fraction enriched in ER markers. In contrast, MAP2, the dendritic MAP that shares sequence homology with tau in the microtubule-binding domain was highly concentrated in the RM fraction. This specific interaction of MAP2 with ER membranes was characterized elsewhere (Farah et al. 2005). Taken together, the above data suggested that tau could be associated to Golgi membranes of the medial compartment in the I2 subfraction. However, the plasma membrane marker Na-K-ATPase was also noted in this subfraction and therefore the association of tau with these membranes could not be ruled out.

Localization of tau in the I2 subfraction

To confirm the association of tau with the Golgi membranes, electron microscope immunocytochemistry of tau was performed on the I2 subfraction. Both vesicles and tubules with associated fenestrations reminiscent of the Golgi membranes were found in this subfraction (Figure 2). Quantification of the gold particle distribution from three different sets of experiments revealed that 82.1% of tau staining was associated with membranes in the I2 subfraction (see Table 1). To confirm the association of tau with the Golgi membranes, double electron microscope immunocytochemistry was carried out using either TGN38 or MG160 as Golgi markers and an anti-tau antibody (Figure 3). Tau immunolabeling was found on membranes labeled either with MG160 or TGN38. A quantitative analysis revealed on average 32% (n=2) and 54% (n=2) of the membranous labeling of tau on membranes containing MG160 and TGN38 respectively. The rest of tau staining was found on non-labeled membranes or associated with the background. Thus, from these data one could conclude that tau was associated to membranes of the medial Golgi compartment and the TGN. The double labeling studies suggest that tau protein detected by western blot in the I2 membrane subfraction was found on bone fide Golgi membranes. The association of tau with the Golgi membranes was independent of microtubules since none were observed in this subfraction.

Co-immunoprecipitation of a Golgi protein complex and tau in adult rat brains

The association of tau with the Golgi membranes was further demonstrated by co-immunoprecipitation experiments. Total microsomes from adult rat brains were prepared by a standard method (Farah et al. 2003). An anti-tau antibody was used to immunoprecipitate tau and the presence of Golgi proteins in tau immunoprecipitate was analyzed by western blotting. As shown in Figure 4A, the protein MG160 was present in tau immunoprecipitate indicating that tau was associated to membranes of the medial Golgi compartment. However, the presence of the cis Golgi compartment marker, GM130 was not or barely detectable in tau immunoprecipitate. This confirmed the above data from the subcellular fractionation that showed very little tau in the I3 subfraction immunoreactive to GM130. The ER marker, calnexin was detected at background level in tau immunoprecipitate showing that, in the present experimental conditions, tau was predominantly associated to the Golgi membranes.

To verify whether the association of tau with the Golgi membranes was peripheral, the I2 subfraction was treated with sodium carbonate buffer at pH 11.0, a procedure known to dissociate the peripheral proteins from the membranes (Figure 4B) (Wiedenmann et al. 1985). Under this treatment, tau mostly detached from the membranes indicating that it is a peripheral membrane protein in the I2 rat brain Golgi subfraction.

Enrichment of tau in the perinuclear region containing the Golgi apparatus in primary hippocampal neurons

We examined the co-localization of tau with the Golgi membranes in primary hippocampal cells. As previously described, after one day in culture, the hippocampal neurons are polarized cells presenting 3 to 4 short minor neurites that will differentiate to become dendrites and a long thin neurite which develops into the axon (Bartlett and Banker 1984; Dotti et al. 1988). All these neurites terminate in a growth cone, a motile structure presenting a rich actin network at the periphery and bundles of microtubules at

the center (Goslin et al. 1989). In the subsequent days of culture, the minor neurites differentiate into dendrites. After seven to ten days in culture, the dendrites and axon are fully developped and the synaptic contacts are established. To study the distribution of tau in primary hippocampal neurons, cells were labeled with either the monoclonal antibody, 49.2 or a polyclonal antibody (kindly provided by Dr. V.-W. Lee, University of Pennsylvania, Philadelphia, USA) that recognize all tau isoforms expressed in these neurons. The Golgi apparatus (GA) was stained with an antibody directed against either GM130, MG160 or TGN38. In seven-day old primary hippocampal neurons cultured in N2 medium, tau does not compartmentalize to the axonal process but remains in all neuronal compartments(Figure 5) (Kempf et al. 1996). The distribution of the GA in seven-day old hippocampal neurons examined using any antibody recognizing the Golgi proteins enumerated above, was mostly restricted to the cell body in the perinuclear region (Figure 5). However, some staining was also present along the neurites (Figure 5). In neurons that were double labeled for tau and MG160, an enrichment of tau staining was found in the perinuclear region where the GA is located. In a merged image of MG160 (red) and tau (green) staining, a yellow signal was observed at the periphery of the GA tubules indicating a coincidence of the two staining in this region. Futhermore, tau and the Golgi markers presented similar enrichments along the neurites (Figure 5). Collectively, the above observations indicated a possible association between tau and the Golgi membranes in hippocampal neurons.

Localization of tau at the Golgi apparatus by electron microscope immunocytochemistry on motoneurons

The localization of tau at the GA was examined in motoneurons of mouse spinal cord by electron microscope immunocytochemistry. These neurons have the advantage of presenting a large GA with multiple stacks of Golgi saccules which appear discontinuous around the nucleus (Peters et al. 1976). No gold particles were found when the primary antibody directed against tau was omitted as well as when this primary

antibody was preabsorbed with pure tau protein. As expected, tau staining was found on the axonal microtubules (Figure 6). Some staining was also present in the cytoplasm not associated to any structure. Remarkably, tau staining was observed on the Golgi membranes and appeared to be more concentrated to one side of the GA (Figure 7). To confirm this, a quantitative analysis of the number of gold particles per μm^2 of the different Golgi compartments, cis, medial and trans was carried out. Fenestration was used to identify the cis side of the GA and clathrin-coated vesicles to identify the trans Golgi side (Peters et al. 1976; Rambourg and Clermont 1990; Marsh et al. 2001). For the quantitative analysis, a total of 35 GA that presented a morphology allowing to distinguish the cis and trans side were selected from two animals. The surface of each Golgi compartment was directly measured on enlarged electron negatives using the image analysis system Northern Eclipse. Then, the number of gold particles found in these compartments was counted (gold particles/μm^2). The quantitative analysis revealed that 14.3 ± 2.1, 22.6 ± 2.4 and 36.7 ± 3.5 gold particles/μm^2 were found on the cis, medial and trans Golgi compartments respectively (Figure 8). The number of gold particles per μm^2 of Golgi membranes was significantly higher on the trans Golgi compartment than that on the cis and medial compartments. This data was consistent with the immunoblot data and suggested that tau was preferentially associated with membranes of the medial and trans Golgi.

Phosphorylated tau associated with Golgi membranes

It was previously shown that the pool of tau found at the plasma membrane was not phosphorylated at the sites recognized by the phosphorylation-sensitive antibodies PHF-1, AT-8 and AT-180 and was dephosphorylated at the Tau-1 antibody site in PC12 cells (Maas et al. 2000). The presence of phosphorylated tau at the sites recognized by the phosphorylation-sensitive antibodies PHF-1, AT-8 and CP9 was shown in normal adult rat brain by western blot (Jicha et al. 1999). To verify whether phosphorylated tau was found on the Golgi membranes in adult rat brain, we investigated the

phosphorylation state of tau in the I2 Golgi subfraction. In contrast to the pool of tau found at the plasma membrane, tau present in the I2 subfraction was phosphorylated at the sites recognized by the antibody PHF-1 (Ser396/Ser404) (Figure 9). However, very little immunoreactivity to the AT-8 antibody was noted in this fraction indicating that Ser199, Ser202 and Thr205 were not highly phosphorylated. Consistent with this result, tau found in the I2 subfraction was immunoreactive for the antibody Tau-1 that recognizes tau dephosphorylated at the region extending from 187-205 residues (Buee et al. 2000). Moreover, tau present in the I2 subfraction did not react with the phosphorylation-sensitive antibody CP9 directed against tau phosphorylated on Thr231. Phosphorylated tau at PHF-1 is most likely associated to Golgi membranes in the I2 subfraction. This fraction also contains membranes originating from the plasma membrane and the ER. According to a previous study, tau phosphorylated at the sites of the PHF-1 antibody was not found at the plasma membrane and in the present study, we showed that very little tau phosphorylated at the PHF-1 sites is found in the RM fraction. Taken together, these results strongly indicate that a pool of tau phosphorylated at Ser396 and Ser404 is associated to the Golgi membranes.

By using the phosphorylation-sensitive antibodies described above, we also detected distinct pools of phosphorylated tau in the I3 and RM subfractions. Tau found in the I3 subfraction was mainly immunoreactive to the AT-8 antibody. Moreover, no immunoreactivity to Tau-1 antibody was detected in this subfraction. On the other hand, tau found in the RM fraction was weakly immunoreactive to AT-8, PHF-1 and CP-9. From these results, one can conclude that different pools of phosphorylated tau exist within a neuron and these pools are most likely associated to different membranous compartments.

DISCUSSION

In the present study, we demonstrated that tau was associated to the Golgi membranes. Tau was preferentially found in a Golgi subcellular fraction enriched in membranes originating from the medial Golgi compartment and TGN. Electron microscope immunocytochemistry using double-labeling confirmed that tau was associated with membranes labeled with the Golgi markers GM160 and TGN38. The interaction of tau with the medial Golgi membranes was further confirmed by co-immunoprecipitation of a Golgi protein complex that contained the medial Golgi protein, MG-160 with an anti-tau antibody. Electron microscope immunocytochemistry revealed that tau association with the Golgi membranes was found in all Golgi compartments but increased toward the trans side. Moreover, the pool of tau present in the Golgi fraction was phosphorylated at the sites recognized by the phosphorylation-sensitive antibody PHF-1.

The most obvious function of tau on the Golgi membranes would be to mediate the interaction between these membranes and microtubules. In this context, tau is not the sole microtubule-binding protein that could play such a role. Indeed, other microtubule-associated proteins including FTCD, SCG10, GMAP-210, CLIPR-59 and Hook3, are also associated with the Golgi membranes (Bloom and Brashear 1989; Lutjens et al. 2000; Walenta et al. 2001; Lallemand-Breitenbach et al. 2004; Rios et al. 2004). As noted for tau in the present study, their association with the GA does not depend on microtubules. However, these proteins differ from each other and from tau on several aspects. First, they do not share any sequence homology in their binding domain to either Golgi membranes or microtubules. Second, they distinctly interact with microtubules and exert diverse effects on microtubule polymerization. CLIPR-59 and GMAP-210 bind to the plus and minus ends of microtubules respectively (Lallemand-Breitenbach et al. 2004; Rios et al. 2004). CLIPR-59 exerts a depolymerizing effect on microtubules. The binding location of FTCD and Hook3 on microtubules has not been

identified yet. These proteins were shown to induce tubulin polymerization but to a lower extent than tau (Bloom and Brashear 1989; Rios et al. 2004). SCG10 belongs to a family of proteins including the ubiquitous cytosolic protein stathmin that destabilize microtubules by mainly sequestrating free tubulin (Lutjens et al. 2000). Third, FTCD, SCG10, GMAP-210, CLIPR-59 and Hook3 arbor a distinct distribution on the Golgi membranes. FTCD, Hook3 and GMAP-120 are located on the cis-Golgi side whereas CLIPR-59 and SCG10 are found on the trans side. In the case of tau, our results indicate that tau concentration on the Golgi membranes increases towards the trans side of the Golgi complex. Although it is believed that FTCD, SCG10, GMAP-210, CLIPR-59 and Hook3 mediate the association of the Golgi membranes with microtubules and contribute to the positioning and morphology of the GA within a cell, the respective function of these proteins on the Golgi membranes remains to be elucidated except for GMAP-210. GMAP-210 is found on cis-Golgi membranes and binds the minus end of microtubules located at the centrosomes. In a recent study, it was shown that GMAP-210 recruits γ-tubulin complexes to the cis-Golgi membranes and this event is necessary for the pericentriolar position of the GA (Rios et al. 2004). Alpha- and beta-tubulin were also found to be attached to the Golgi membranes (Yamaguchi and Fukada 1995). The proteins involved in recruiting these tubulin isoforms to the Golgi membranes have not been identified yet. Tau could play such a role within a neuron.

A recent study showed that the overexpression of tau in astrocytes induced a fragmentation of the GA (Yoshiyama et al. 2003). We have also observed a fragmentation of the GA in primary hippocampal neurons transfected with human tau forms and in the JNPL3 mice expressing the mutant human form of tau P301L (Liazoghli et al. 2005). The mechanisms involved in this fragmentation of the GA by tau remain elusive. However, in previous studies, a fragmentation of the GA was consistently observed when the link between microtubules and the GA was perturbed (Lucocq et al. 1987; Lucocq and Warren 1987; Cole et al. 1996). For example,

depolymerization of microtubules by nocodazole or colchicine resulted in a fragmentation of the GA (Boyd et al. 1982). Overexpression of Hook3 or GMAP-210, two proteins that mediate the interaction between microtubules and the Golgi membranes, also induced a dispersion and fragmentation of the GA (Walenta et al. 2001; Pernet-Gallay et al. 2002). Taken together, the above observations suggest that tau could be involved in the link between the Golgi membranes and microtubules and thereby could contribute to the maintenance of the GA morphology within a neuron.

Most notably, tau was not uniformly distributed on the Golgi membranes but an increase of its concentration was noted towards the trans side of the GA. The microtubule-destabilizing protein, SCG10 which is neuronal specific is also enriched on the trans side of the GA (Lutjens et al. 2000). Interestingly, the association of SCG10 to the Golgi membranes was shown to be necessary for its targetting to the growth cones. Thus, the association of tau to the GA might be involved in its axonal sorting. On the other hand, tau might serve as a tag on vesicles emerging from the GA destined to the axonal process. Consistent with this possibibility, in our subcellular fraction enriched in Golgi membranes, an important percentage of tau immunogold labeling was found on small vesicles. The trafficking pathways of the biosynthesis of integral membrane proteins is well characterized. Their synthesis occurs in the rough endoplasmic reticulum and then they go through the Golgi complex where they are packaged into carrier vesicles that will be transported either to distinct cellular compartments to deliver their contents to the plasma membrane (Van Vliet et al. 2003). Sorting of vesicles destined to different cellular compartments occurs at the TGN (Gleeson et al. 2004; Sytnyk et al. 2004). Two mechanisms seem to regulate the targeting of integral membrane proteins to the axon (Sampo et al. 2003). Some proteins are targeted both to the somato-dendritic and axonal compartment but are only retained at the plasma membrane of the axon whereas other proteins are selectively delivered to the axonal plasma membrane. In this latter case, tau could act as a axonal localization signal for the delivery of the cargo

proteins to the axonal plasma membrane. Moreover, tau could remain associated with the cargo proteins after their integration to the plasma membrane since tau was found associated with this membrane.

As noted for the binding of tau to microtubules, the association of tau with membranes seems to be regulated by its phosphorylation state (Sampo et al. 2003). Indeed, the phosphorylation of tau abolished its binding to the plasma membrane in PC12 cells. The pool of tau found in the Golgi enriched subcellular fraction was phosphorylated at the sites recognized by the phospho-dependent antibody PHF-1 whereas the pool of tau found at the plasma membrane was not (Sampo et al. 2003). A gradient of tau phosphorylation was observed along the axon in primary hippocampal cultures (Mandell and Banker 1996). From these data, it appears that different pools of phosphorylated tau exist along the axon which could preferentially interact with either the Golgi membranes or the plasma membrane. In degenerating neurons, tau becomes hyperphosphorylated (Lee et al. 2001). Therefore, an aberrant phosphorylation of tau might result in an alteration of its association with membranes and contribute to its dysfunction.

The detection of tau on the GA by immunogold labeling showed that tau is present in the neuronal cell body. Although most of the studies state that tau is an axonal MAP, several observations suggest that tau is also located in the cell body and dendrites. In previous studies, tau was found in the somato-dendritic compartment in adult rat brain (Papasozomenos and Binder 1987). However, its phosphorylation state differed from that in the axon. Similar results were reported in primary hippocampal neurons (Mandell and Banker 1996). In Drosophila, tau is also distributed in all neuronal compartments in the adult retina (Heidary and Fortini 2001). The above observations indicate that different pools of tau exist within a neuron that present a different state of phosphorylation and subcellular distribution. The function of each of these pools remain

to be elucidated. This is a crucial step to better understand the implication of tau in neuronal function and degeneration.

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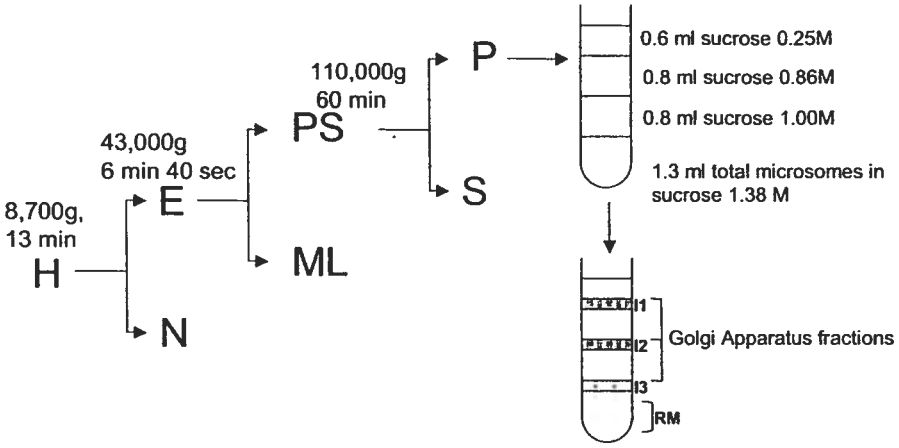
Figure 1. Immunoblot analysis of adult rat brain subfractions. A) Summary on the subcellular fractionation procedure. B) Fractions obtained following subcellular fractionation of adult rat brains were electrophoresed on a 7.5% polyacrylamide gel (30 µg/lane) and transferred to a nitrocelllulose membrane as described in methods. A monoclonal antibody directed against tau was used (clone tau5) as well as antibodies directed against the Golgi markers mannosidase, MG160, GM130 and TGN38, the plasma membrane marker Na-K-ATPase, the endoplasmic reticulum markers, ribophorin and calnexin, tubulin and MAP2 (HM2). E: cytoplasmic extract, P: total membrane extract, S: cytosolic fraction, I: interface, RM: rough microsomes.


8,700g,
13 min
H
43,000g
6 min 40 sec
E
N
110,000g
60 min
PS
ML
P
S
0.6 ml sucrose 0.25M
0.8 ml sucrose 0.86M
0.8 ml sucrose 1.00M
1.3 ml total microsomes in
sucrose 1.38 M
I1
I2
I3
RM
Golgi Apparatus fractions

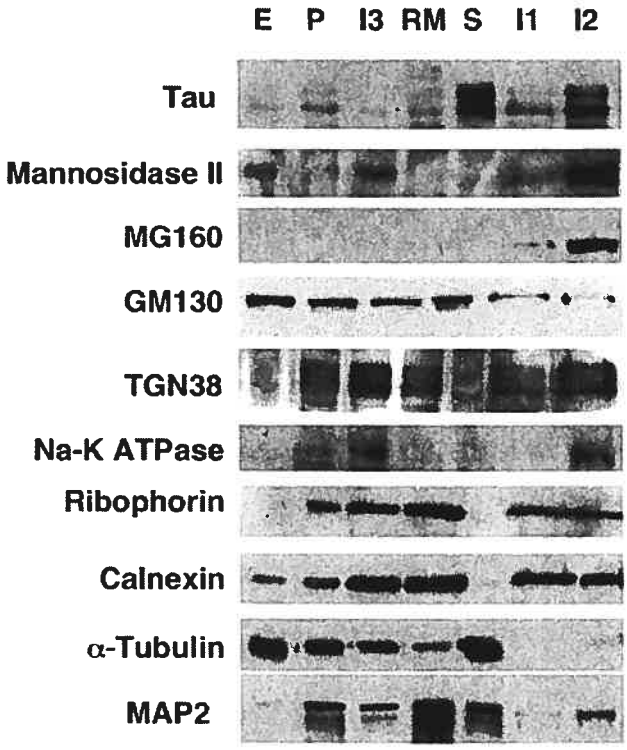

E
P
I3
RM
S
I1
I2
Tau
Mannosidase II
MG160
GM130
TGN38
Na-K ATPase
Ribophorin
Calnexin
α-Tubulin
MAP2

Figure 2. Electron microscopy of the I2 subfraction. Electron micrographs of the I2 fraction. Arrowheads point to short flattened membranes which are typical of Golgi membranes. The arrows point to small vesicles (less than 100nm diameter). Scale bar: 500nm

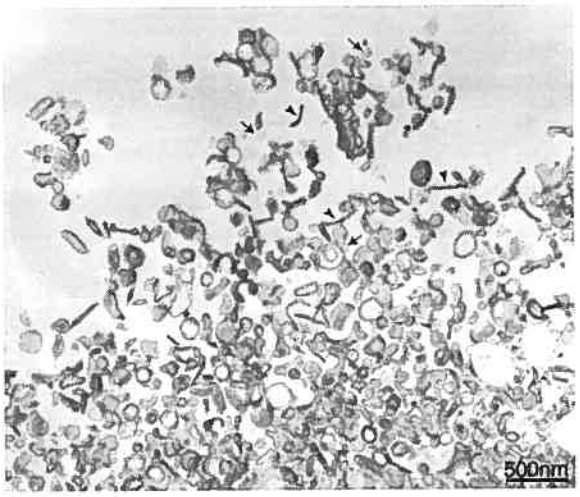

500nm

Figure 3. Electron microscope immunocytochemistry of tau in the I2 subfraction. A, B and C) A monoclonal anti-tau antibody clone 49.2 (1:200) and a polyclonal antibody directed against TGN38 were revealed using a secondary anti-mouse antibody conjugated to 10nm colloidal gold particles and a secondary anti-sheep antibody conjugated to 5nm colloidal gold particles respectively. Tau and TGN38 were found on the same short flattened membranes and vesicles. D, E and F) A monoclonal anti-tau antibody clone 49.2 (1:200) and a polyclonal antibody directed against MG160 were revealed using a secondary anti-mouse antibody conjugated to 10nm colloidal gold particles and a secondary anti-rabbit antibody conjugated to 5nm colloidal gold particles respectively. Tau and MG160 were found on the same short flattened membranes and vesicles. Scale bar: A, B, D and E= 150nm C and F=100nm

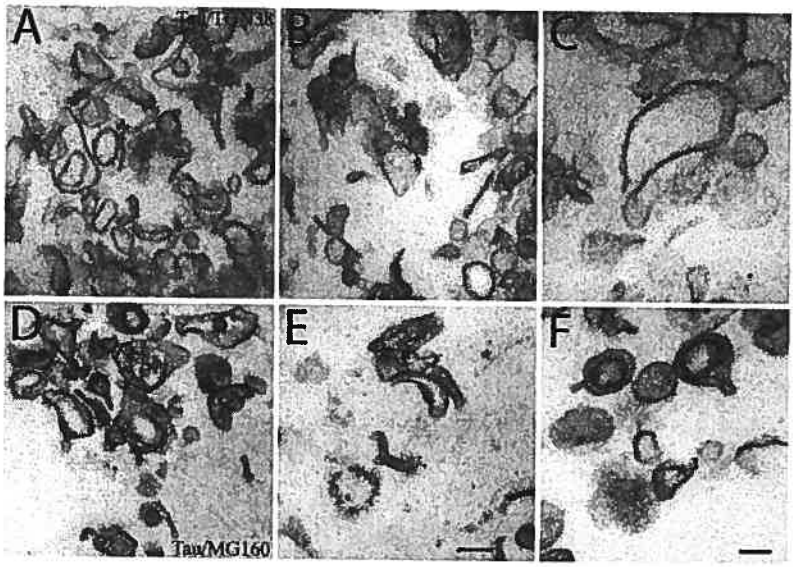
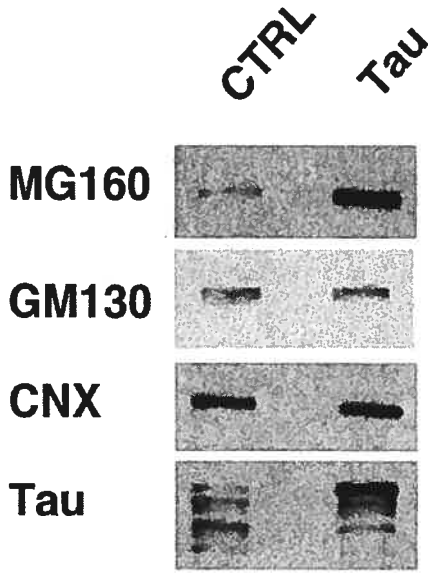


Figure 3

Figure 4. Co-immunoprecipitation of a Golgi protein complex containing MG160 with an anti-tau antibody. A) MG160 was found in tau immunoprecipitate. On the other hand, GM130 and calnexin were only detectable at the background level. B) Western blot analysis of the I2 fraction before and following washing with sodium carbonate pH11.0 as described in methods. A little amount of tau was found in the I2 fraction following the washing step suggesting that the association of tau with the golgi apparatus is peripheral.


CTRL
Tau
MG160
GM130
CNX
Tau

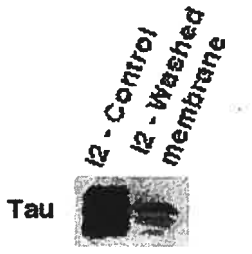

I2 - Control
I2 - Washed membrane
Tau

Figure 5. A partial co-localization of tau and MG160 in primary hippocampal neurons. Primary hippocampal neurons were double-stained with an anti-tau (green) and an anti-MG160 (red) antibody. Some overlap (yellow) of the two staining was noted at the periphery of the Golgi membranes and along the neurites (overlay). Scale bar= 20μm

Figure 6. Electron microscope immunocytochemistry on the sections of the spinal cord showing tau staining on microtubules. Tau immunogold was found along the microtubules in motor neurons. Scale bar=100 nm

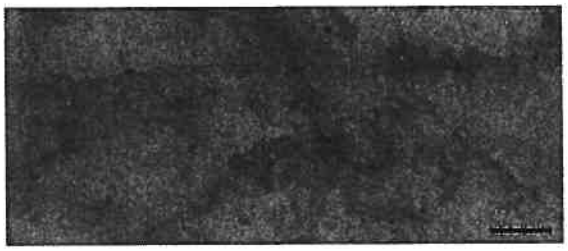


Figure 7. Electron microscope immunocytochemistry on a section of a mouse spinal cord. Tau immunogold was observed on the Golgi membranes and appeared to be more concentrated at the trans side of GA. Fenestration was used to identify the cis side of the GA and clathrin-coated vesicles to identify the trans side. Scale bar=500nm

Figure 8. Quantitative analysis of tau immunogold on the GA membranes. A quantitative analysis of the number of gold particles per μm^2 of membrane of the different Golgi compartments, cis, medial and trans revealed that tau was more concentrated on the trans side of the GA than on the cis side.

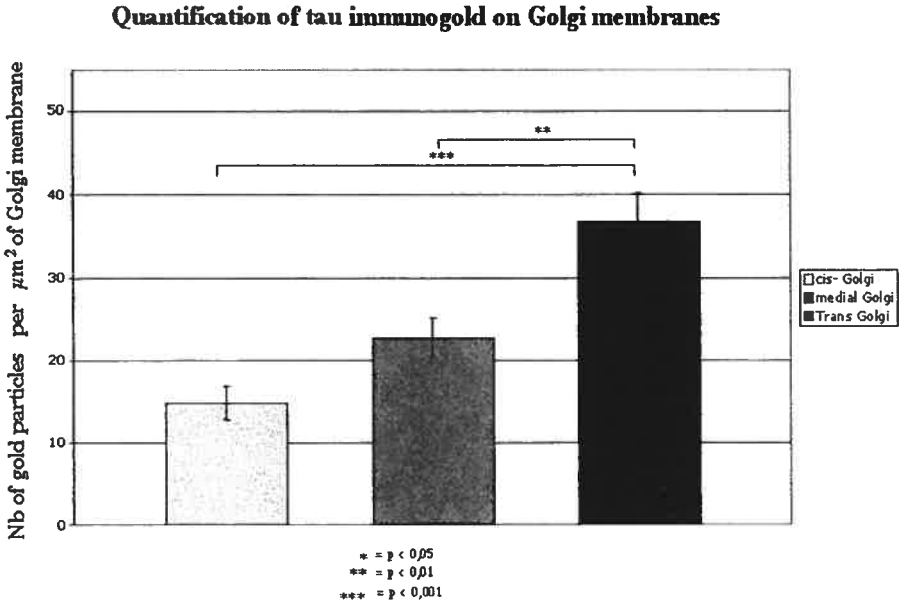

Quantification of tau immunogold on Golgi membranes
Nb of gold particles per µm² of Golgi membrane
50
40
30
20
10
0
**
***
cis- Golgi
medial Golgi
Trans Golgi
* = p < 0,05
** = p < 0,01
*** = p < 0,001

Figure 8

Figure 9. Immunoblot analysis of the phosphorylation state of tau in the I2 subfraction. Fractions obtained following subcellular fractionation of adult rat brains were electrophoresed on a 7.5% polyacrylamide gel (30 µg/lane) and transferred to a nitrocelllulose membrane as described in methods. The subcellular fractions were analysed by using the tau phosphorylation-sensitive antibodies PHF-1, AT-8 and CP9 and the antibody Tau-1 directed against unphosphorylated tau. A polyclonal antibody directed against tau was used to reveal total tau. E: cytoplasmic extract, P: total membrane extract, S: cytosolic fraction, I: interface, RM: rough microsomes.

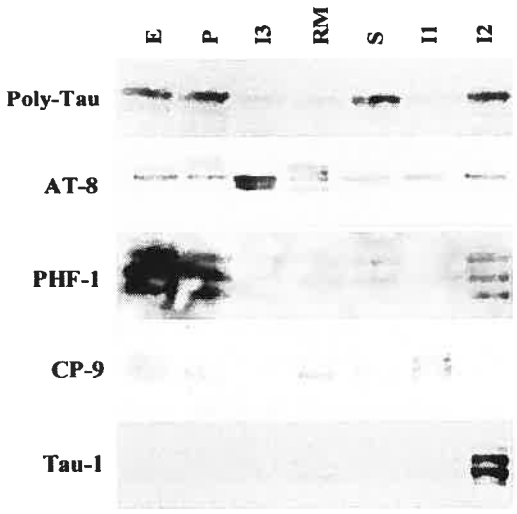

E
P
I3
RM
S
I1
I2
Poly-Tau
AT-8
PHF-1
CP-9
Tau-1

Table 1: Quantification of the number of gold particles associated with membranes in the I2 subfraction

N=3	Number of gold particles on membranes
Control (anti-tau antibody omitted)	**103**
Anti-Tau-antibody (clone 49.2)	**833**

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ANNEXE II
CONTRIBUTIONS AUX ARTICLES

Article 1:

J'ai contribué à toutes les étapes de cet article. Ceci inclut les résultats préliminaires (préparation de membranes crues à partir de cultures primaires de neurones corticales et à partir de cellules Cos-7 surexprimant les différentes protéines de fusion GFP-MAP2). Ces résultats préliminaires, ne sont pas présentés dans le cadre de cet article mais présentés sous forme d'affiche dans divers congrès et journées scientifiques, ont mené à l'identification de l'interaction de MAP2 et du RER. Dans le premier article, Carole Abi Farah a effectué le fractionnement subcellulaire à partir du cerveau de rat adulte ainsi que sa caractérisation biochimique et en microscopie électronique (les figures 1 et 2). Je suis responsable de l'étude de la distribution de MAP2 et de la p63 au cours du développement neuronal *in vitro* (figure 3). J'ai effectué les transfections des neurones de l'hippocampe et les marquages immunocytochimiques. L'observation et la prise de photos en microscopie confocale des neurones transfectés ont été effectuées en collaboration de Sébastien Perrault et Carole Abi Farah (Figure 4). Je suis responsable des expériences de recouvrement par immunobuvardage ainsi que de l'identification de l'interaction de MAP2c et de la p63 (figure 5). J'ai également effectué toutes les expériences de co-immunoprécipitation *in vivo* (E19, P0 et rats adultes) et *in vitro* dans les cellules HeLa exprimant les différentes protéines de fusion GFP-MAP2 (les figures 6 et 7). L'essai de reconstitution in vitro a été initié par Carole Abi Farah. Après le départ de Carole, j'ai reproduit ses résultats et rajouté des contrôles supplémentaires

importants (l'anticorps anti-p63, anti-MAP2...). Ces essais ont été effectués en collaboration avec Angela Anton qui m'a aidé lors de la préparation des noyaux à partir du foie de rats adultes et Michel Lauzon qui m'a aidé en microscopie électronique. Dans cette étude, les cultures primaires de neurones ont été effectuées par Mylène Desjardins, j'ai fait les cultures primaires des cellules gliales. L'article a été rédigé en collaboration avec Carole Abi Farh, Dr. Jacques Paiement et Dre. Nicole Leclerc. Pour cette étude, deux spécialistes du RE étaient parmi nos collaborateurs: Dr. Kreibich (New York University) et Dr. Paiement (Université de Montréal).

Article 2 :

Je suis responsable de toutes les expériences effectuées dans cet article sauf une partie des résultats des souris transgéniques JNPL3 qui a été effectuée par Sébastien Perrreault (les figures 6 et 7). J'ai effectué les cultures primaires de cellules gliales et Mylène Desjardins a effectué les cultures primaires des neurones de l'hippocampe. J'ai fait toutes les autres figures et rédigé le manuscrit avec Docteure Nicole Leclerc. Docteure Christina Micheva (Stanford University) a été notre collaboratrice dans cette étude. Docteure Micheva nous a donné des conseils pratiques ainsi que les protocoles pour réussir la transfection des neurones de l'hippocampe. De plus, Docteure Micheva s'est beaucoup investit dans le cadre d'une autre étude. Dans cette dernière, nous avons exploré l'effet des MAPs neuronales sur le transport des organelles membranaires: mitochondries, peroxisomes, réticulum endoplasmique et l'appareil de Golgi (résultats non présentés dans cette thèse).