Neurobiology

Fragmentation of the Golgi Apparatus Induced by the Overexpression of Wild-Type and Mutant Human Tau Forms in Neurons

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Tau is a microtubule-associated protein enriched in the axonal compartment. In several neurodegenerative diseases including Alzheimer's disease, hyperphosphorylated tau accumulates in the somatodendritic compartment, self-aggregates, and forms neurofibrillary tangles. 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 green fluorescent protein (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 wild-type 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 neurofibrillary tangles. (*Am J Pathol* 2005, 166:1499–1514)

In normal brain, the microtubule-associated protein tau is involved in the formation and the stabilization of microtubules in the axon.¹ The expression of tau is developmentally regulated by alternative splicing.² Six isoforms are present in human brain.³ In pathological conditions, tau becomes hyperphosphorylated, detaches from microtubules, accumulates in the somato-dendritic compartment, and self-aggregates to form insoluble filaments.⁴ Alzheimer's disease (AD) is characterized by two neuropathological lesions, the amyloid plaques corresponding to extracellular aggregation of A β peptides and the neurofibrillary tangles (NFTs) formed of insoluble filaments containing hyperphosphorylated tau.⁵ Several other neurodegenerative diseases are characterized by prominent intracellular accumulations of filaments containing phosphorylated tau.⁶ 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).⁷ The FTDP-17 mutations were also found in individuals either presenting clinical and neuropathological phenotypes of corticobasal degeneration, Pick's disease, or progressive supranuclear palsy.⁶ At least 29 different mutations were identified.⁶ The majority of these mutations were located in the coding region or close to the splice donor site of intron 10.⁶ Most missense mutations seem to decrease the ability of tau to bind microtubules and increase its self-aggregation (ie, K250T, G272V, P301L, P301S, V337M, G389R, and R406W).⁶ The mutations that affect the exon 10 splicing lead to an imbalance of the tau isoform ratio (ie, S305N and S305S).⁶ The link

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between tau protein dysfunction and neurodegeneration was further confirmed in transgenic mice overexpressing the mutated forms of tau.^{8–10}

Microtubules contribute to the maintenance of neuronal architecture and also act as railways for the motor-based transport of membranous organelles.¹¹ 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.¹² The overexpression of tau in nonneuronal and neuronal cells leads to the accumulation of these organelles in the perinuclear region.^{13,14} 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.¹⁵ 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.^{4,16} Furthermore, an abnormal distribution and morphology of membranous organelles were reported in several neurodegenerative diseases including AD.^{17–20} In particular, a fragmentation of the Golgi apparatus (GA) was observed in neurodegenerating neurons of patients suffering from AD, amyotrophic lateral sclerosis, Creutzfeldt-Jakob disease, and multiple system atrophy.^{19,21,22}

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.¹¹ In a normal cell, the GA is composed of a series of flattened, parallel, interconnected cisternae organized around the microtubule-organizing center in the perinuclear region.²³ The fragmentation of the GA is characterized by its reorganization in small, round, disconnected, and dispersed elements.²³ A fragmentation of the GA occurs during mitosis in normal cells.^{24,25} This reorganization of the GA is also noted in apoptotic cells indicating that a fragmented GA can also be associated with cellular dysfunction.^{26,27} 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.²⁸ This fragmentation is reminiscent of the one observed in mitotic cells.²³

The fragmentation could have detrimental effects on the secretory activity of the GA.²⁹ 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.³⁰ In this case, the secretory activity of the GA is not perturbed.^{30–32} In degenerating neurons, the fragmentation of the GA is similar to that observed under microtubule depolymerizing conditions.³³ 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.³⁴ 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.⁸ These mice are characterized by motor and behavioral 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 endoplasmic reticulum 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 activity.

Materials and Methods

Primary Hippocampal Cultures and Transfection

Primary hippocampal cultures were prepared as previously described.³⁵ Hippocampi from 18-day-old fetuses were treated with trypsin (0.25% at 37°C for 15 minutes) then washed in Hanks' 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 cocultured with a monolayer of glial cells in a serum-free medium supplemented with N2.³⁵ Forty-eight hours after plating, hippocampal neurons were transfected using a modified calcium phosphate transfection protocol.³⁶ Neurons were transferred in a six-well plate. To generate the calcium and DNA precipitate, 30 minutes before transfection 4 μ g of Qiagen-purified DNA in 60 μ l of 250 mmol/L CaCl₂ per well were mixed drop-wise with an equal volume of 2 $\times$ HBS (274 mmol/L NaCl, 10 mmol/L KCl, 1.4 mmol/L Na₂HPO₄, 15 mmol/L glucose, 42 mmol/L HEPES, pH 7.07). Cells were incubated with transfection precipitate for 30 minutes at 37°C and 5% CO₂. Then cells were washed three times with Hanks' balanced solution supplemented with 10 mmol/L Hepes and retransferred in the Petri dishes containing minimal essential medium/N₂ medium. The protein expression was allowed for 24 hours then the cells were fixed and processed for immunofluorescence. A GFP-tau4R con-

Figure 1. Schematic representation of tau protein. The tau isoform used in this study contains 4 repeats of 18 amino acids involved in tau binding to microtubules designated 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 substitutions 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.

struct 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 (Harvard University, Boston, MA).³⁷

Triton X-100 Extraction Procedure

Transfected neurons were extracted using a modified detergent-based extraction protocol.³⁸ Briefly, cells were rinsed in Hanks' balanced salt solution and extracted for 1 minute 30 seconds at 37°C in the extraction buffer (20 mmol/L PIPES, pH 6.9, 2 mmol/L MgCl₂, 2 mmol/L EGTA, 1 mmol/L phenylmethyl sulfonyl fluoride, and 0.1% Triton X-100). Then, the extracted neurons were fixed with cold methanol (−20°C) for 5 minutes, rehydrated in phosphate-buffered saline (PBS), and processed for immunostaining.

H₂O₂ Treatment to Induce Apoptosis in Neurons

Three days after plating, hippocampal neurons were treated with H₂O₂ (Fisher Scientific, Canada) to induce apoptosis. H₂O₂ was added to the medium at a final concentration of 400 μmol/L and then the neurons were incubated for 3 hours at 37°C and 5% CO₂. Neurons were then washed three times with PBS and fixed in 4% paraformaldehyde in PBS for 20 minutes.

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 minutes then cells were washed in PBS and immunolabeled as described previously.³⁹ 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, Indianapolis, IN) and used at a concentration of 1:100. Three Golgi markers were used: a polyclonal anti-MG-160 (kindly provided by Dr. Nicholas K. Gonatas, University of Pennsylvania, Philadelphia, PA) 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 Research Products, San Diego,

CA) 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, NY) was used. To label the microtubule-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, NY). Apoptotic cells were revealed by using a rabbit polyclonal antibody that recognizes the active form of caspase-3 (Chemicon International Inc., Temecula, CA) 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 fluorescein isothiocyanate (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 labeled cells were visualized with an axioplan Zeiss fluorescence microscope using either a ×63 or ×100 objective. For co-localization studies of the two Golgi markers, observations were made with a Leica TCS-SP1 confocal microscope using a ×100 objective and a ×4 zoom.

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, Mississauga, Ontario, Canada). Three sets of independent experiments were quantified. At least, 50 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 axioplan fluorescence microscope using a ×100 objective.

Perfusion of JNPL3 Mice

JNPL3 and age-matched control mice were purchased from Taconic Farms (Germantown, NY). The use of animals and all surgical procedures in this article were performed 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.

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 containing 1% bovine serum albu-

min and 2% normal donkey serum (Jackson ImmunoResearch Laboratories) 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, NY), recognizing hyperphosphorylated tau were used at a 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 labeled spinal cord sections were visualized with a Leica TCS-SP1 confocal microscope using either a $\times 63$ or $\times 100$ objective.

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 to their GA morphology. Images of the GA were captured by confocal microscopy with a $\times 63$ objective and a $\times 4$ zoom on the cell body.

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 $\mu\text{g}/\text{ml}$ of proteinase K in 10 mmol/L Tris, pH 8, for 30 minutes, then rinsed with Tris-buffered saline. As a positive control, the spinal cord sections from control mice were incubated with 1 $\mu\text{g}/\mu\text{l}$ of DNase I for 20 minutes and then rinsed in Tris-buffered saline and processed for TUNEL labeling according to the manufacturer'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 $\times 100$ objective.

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 analysis of variance to detect any interaction between the sets of experiments and/or the experimental groups (neurons expressing ei-

ther GFP alone, wild-type tau, or a mutated form of tau). Because 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 analysis of variance 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 analysis of variance to detect any interaction between the types of mice (control and transgenic) and/or between the different groups of age (3 to 5 months versus 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).

Results

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.³⁴ 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 Banker and Goslin.³⁵ After 1 day in culture, the hippocampal neurons are polarized cells presenting three to four short minor neurites that will differentiate to become dendrites and a long thin neurite that develops into the axon.^{40,41} In the subsequent days of culture, the minor neurites differentiate into dendrites. After 7 to 10 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-tau4R). The expression was allowed to proceed for 24 hours. Neurons were then fixed and processed for immunofluorescence 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 3-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.⁴² 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-tau4R retained its ability to bind to microtubules in this system. Transfected neurons were stained with an antibody directed against β -tubulin. A co-localization of GFP-tau4R and tubulin immunostaining was

Figure 2. Three-day-old hippocampal neurons expressing either GFP or GFP-tau4R. Neurons were fixed with 4% paraformaldehyde and 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 co-localization with microtubules. In neurons expressing wild-type tau4R, a co-localization of GFP-tau with microtubule bundles was revealed by the tubulin staining. **B:** Transfected neurons expressing GFP-tau4R were extracted with Triton X-100 and stained for MAP2, the dendritic microtubule-associated protein. GFP-tau4R and MAP2 remained attached to microtubules during the extraction procedure. **C:** GFP- and GFP-tau4R-expressing neurons were stained with an antibody directed against the Golgi marker, MG-160. In GFP-tau4R-expressing neurons, the GA was fragmented. **Inset:** $\times 2$ magnification of the GA. Scale bars: 4 μ m (A); 5 μ m (B); 10 μ m (C).

noted in the cell body and the neurites (Figure 2A). To further confirm the binding of GFP-tau4R to microtubules, transfected neurons were extracted in the microtubule-

Table 1. Morphological Analysis of the GA in Hippocampal Neurons Transfected Either with GFP, GFP-Tau 4R, GFP-P301L, GFP-R406W, or GFP-V337M

	Number of cells 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

The number of cells with a fragmented GA was significantly higher in neurons expressing wild-type human tau4R than in GFP-expressing neurons. The percentage of neurons expressing a mutant form of tau4R (P301L, V337M, or R406W) with a fragmentation of the GA was significantly higher than in wild type tau4R-expressing neurons. The statistical significance between the different groups was determined by using a one-way analysis of variance followed by the Tukey-Kramer multiple comparison post hoc test.

* $P < 0.001$.

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 labeled with an antibody directed against the dendritic microtubule-associated protein MAP2, which is known to remain attached to microtubules during the extraction procedure (Figure 2B).⁴³ To visualize the binding of GFP-tau4R to microtubules, neurons were stained with an antibody directed against GFP. As shown in Figure 2B, GFP-tau 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 tau4R-expressing neurons. To visualize the GA, an antibody directed against the conserved medial Golgi sialoglycoprotein MG-160 was used. In 3-day-old hippocampal neurons, expressing the GFP alone, the GA appeared as a juxtannuclear compact and clustered structure in the vicinity of the microtubule organizing center (Figure 2C). This distribution had previously been reported in differentiating neurons.⁴⁴ 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-immunolabeled Golgi membranes distributed around the nucleus.¹⁹ Neurons included in the quantification analysis presented no vacuole and had a normal morphological phenotype: three to four minor neurites of a length exceeding at least two to three times the diameter of the cell body and a long axon. According to these criteria, neurons having a very high expression level of GFP-tau4R were excluded because 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-tau4R-expressing neurons presenting a fragmented GA, transfected hippocampal neurons from three 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-tau4R and having a

Figure 3. Three-day-old hippocampal neurons expressing either GFP-P301L, GFP-V337M, or GFP-R406W. The neurons were fixed with 4% paraformaldehyde and 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 μ m.

fragmented GA ($30.89 \pm 1.7\%$) was significantly higher than that of neurons expressing GFP alone ($6.5 \pm 0.4\%$).

Fragmentation of the GA Is More Frequent in Primary Hippocampal Neurons Overexpressing the Mutant Human Tau Forms P301L, R406W, and V337M than in Neurons Overexpressing Wild-Type Human Tau

Mutations in tau gene are known to decrease its microtubule binding affinity⁴⁵ and to increase its tendency to self-aggregate.^{46,47} 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).³⁷ Twenty-four hours after 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). To determine the percentage of cells presenting a fragmented GA, three

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, and 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 μ m.

different experiments were performed (Table 1). In each set of experiments, 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%) after 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 because 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

Figure 5. Fragmentation of the GA in 3-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 juxtannuclear, 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-immunolabeled membranes around the nucleus. **Inset:** $\times 2$ 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 .

number of neurons with a fragmented GA than wild-type human tau.

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.⁸ 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 precedes or is concomitant with the formation of NFTs. Two groups of animals were examined: 3- to 5-month-old mice that did not

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 morphology 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 bars: 4 μm (**A**, **B**, **D**); 8 μm (**C**).

have any behavioral 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

Table 2. Number of Motor Neurons with a Fragmented GA in Control and JNPL3 Mice

Number of animals	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 to 5 months control mice			
1	1	97	1.03
12	2	185	1.08
13	0	131	0.00
14	2	99	2.02
3 to 5 months transgenic mice			
11	2	186	1.08
12	1	178	0.56
13	3	127	2.36
14	1	167	0.60
10 to 12 months control mice			
11	1	147	0.68
12	1	170	0.59
13	1	135	0.74
10 to 12 months transgenic mice			
11	11	154	7.14
12	11	158	6.96
13	10	88	11.36

observed (Figure 6, B 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 significantly higher in JNPL3 mice ($8.5 \pm 1.4\%$) compared to age-matched control mice ($0.67 \pm 0.04\%$). 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.⁴⁹ Hyperphosphorylated tau was barely detectable in the cell body of motor neurons in control mice as shown by the staining of the tau phospho-dependent 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

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.

in motor neurons with an accumulation of hyperphosphorylated tau in the somato-dendritic compartment than in neurons that 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- to 12-month-old mice than in 3- to 5-month-old mice as reported in a previous study.⁸ A motor neuron without tau aggregation and one with tau aggregation are illustrated in Figure 6, C 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 50 motor neurons from 10- to 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.

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 co-localization of TGN (TGN38) and Golgi (GM-130 or MG-160) markers is noted. This co-localization is associated with a normal secretory activity of GA. Apoptotic cells present a fragmented GA.^{26,27} In these cells, the co-localization between TGN and Golgi markers is lost⁵⁰ and this correlates with a decrease of the secretory activity of the GA.⁵¹ 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, Tau4R, or one of the three mutant forms of tau4R (P301L, V337M, or R406W) were double immunostained for TGN38, a TGN marker and MG-160, a medial Golgi marker. Co-localization 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 co-localization was preserved in

neurons transfected with all constructs (Figure 8). However, when primary hippocampal neurons were treated with H₂O₂ to induce apoptosis, TGN38 and MG-160 staining did not co-localize as previously reported in nonneuronal cells (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.⁵² 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).

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 tau4R. 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.⁵³ 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 dispersed in asymptomatic JNPL3 mice. Beside slight apathy, the transgenic mice examined in the present study did not show any major

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-tau4R, GFP-P301L, GFP-V337M, or GFP-R406W were stained for MG-160 and TGN38. These markers co-localized in transfected neurons with a fragmented GA indicating that the polarity of the GA was preserved. Scale bar, 3 μ m.

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 not 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 .

motor or behavioral 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 that 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.¹⁷

Fragmentation of the GA was reported in human brain tissue from patients suffering from AD,¹⁹ amyotrophic lateral sclerosis,¹⁸ CJD,²¹ and multiple system atrophy.²² 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 amyotrophic lateral sclerosis cases.^{54,55} 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 amyotrophic lateral sclerosis, motor neurons did not present a fragmented GA.⁵⁶ This suggests that the fragmentation of the GA is not a secondary cellular response to cell death. Many neurological disorders, including Parkinson's disease, dementia with

Lewy bodies, and multiple system atrophy present fibrillar aggregates of α -synuclein such as Lewy bodies and Lewy neurites.^{57,58} In COS cells, a fragmentation of the GA was induced by the addition of α -synuclein nonfibrillar oligomers in the medium.⁵⁹ 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.^{60–62} This is supported by the fact that these diseases are characterized by the accumulation and formation of protein aggregates in the cell body.^{4,57,63} Interestingly, GA fragmentation can be experimentally induced by altering the transport of vesicles.^{64,65}

Cytoskeletal abnormalities including an alteration of the microtubule network were noted in neurodegenerative diseases.⁶⁶ 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.^{24,25} Moreover, the drugs that depolymerize microtubules such as nocodazole and those that stabilize them such as taxol, induce a fragmentation of the GA in neuronal and nonneuronal cells.⁶⁷ In the present study, we cannot exclude the

Figure 10. Apoptotic motor neurons were analyzed by using the 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 control spinal cord sections treated with DNase I (**A–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** to **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 μ m.

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.⁶⁸ Any perturbation of this trafficking could result in fragmentation of the GA. Thus, tau could compete with kinesin for binding to microtubules^{12–14} and thereby impair the transport of vesicles from the endoplasmic reticulum to the GA. However, in astrocytes, the overexpression of tau affected vesicle transport by altering microtubule dynamics due to a specific reduction of stable deetyroninated microtubules and by decreasing kinesin levels.³⁴ 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 mutants 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.⁴ During mitosis, the fragmentation of the GA depends on the sequential activation of the kinases MEK1 and cdc2-kinase.⁶⁹ Moreover, phosphorylation of spectrin by a reduction of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] synthesis induced its dissociation from Golgi membranes and a fragmentation of the GA.⁷⁰ 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.^{64,71} Recently, in neurons, it was shown by using two Golgi toxins Brefeldin A and nordihydroguaiaretic acid that the fragmentation of GA was concomitant to a transient increase of glycogen synthetase kinase 3 β (GSK3- β) activity and tau phosphorylation.⁷² Several studies have highlighted the pivotal role of GSK-3 β in tau hyperphosphorylation in neurodegenerating neurons.⁷³ 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.⁶ 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.⁷⁴ The fragmentation of the GA in apoptotic cells is similar to that observed during mitosis.⁷⁵ 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.⁵⁰ 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 co-localization 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.⁵² Furthermore, 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 P301S⁷⁶ or V337M.⁷⁷

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 reticu-

Figure 11. Confocal microscopy analysis of the distribution of the endoplasmic reticulum in transfected hippocampal neurons presenting a fragmented GA. Transfected neurons expressing either GFP, GFP-tau4R, GFP-P301L, GFP-V337M, or GFP-R406W were stained for Ribophorin II, a marker of the rough endoplasmic reticulum. No major change in the endoplasmic reticulum morphology was detected in transfected neurons presenting a fragmented GA. Scale bar, 3 μ m.

lum and destined to the secretory pathways, the plasma membrane, or lysosomes.¹¹ 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.⁷⁸ Interestingly, the perturbation of microtubule network could affect the secretion capacity of the GA.^{79,80} Thus, by perturbing the microtubule network, tau could alter the secretory function of the GA. Further studies are needed to address this point.

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