INTRODUCTION

The neurodegenerative process in Alzheimer disease (AD) is characterized by the progressive and irreversible deafferentation of the limbic system, association neocortex, and basal forebrain (1–6), accompanied by neurofibrillary pathology. This AD-related neurofibrillary pathology is characterized by accumulation of paired helical filaments (PHF) in neuronal cell bodies (tangles) (7–9), dendrites (neuropil threads) (10–12), and axons (plaque’s dystrophic neurites) (13). The PHF is mainly composed of hyperphosphorylated tau, a microtubule-associated protein predominantly expressed by neurons (14–18).

The mechanisms leading to neurofibrillary pathology in AD are currently under intense scrutiny. Among the explanations proposed, one possibility is that tau might be abnormally expressed or processed (19, 20). Supporting this possibility, recent studies have shown that frame-shift mutations in tau can be found in neurons of AD patients (21). In addition, mutations in the human tau gene cause fronto-temporal dementia and parkinsonism linked to chromosome 17 (22–24). Some mutations, including mutations in intron 10, induce increased levels of the functionally normal 4-repeat tau protein isoform, leading to neurodegeneration (25–27). Another possibility is that tau hyperphosphorylation might be a secondary neuronal response to amyloid toxicity and amyloid accumulation. In support of this, previous in vitro studies have shown that treatment of nerve cells with beta amyloid promotes tau hyperphosphorylation (28, 29). Similar results have been reported in vivo by intracerebral injection of beta amyloid (30, 31) and following brain injury (32). Recent studies have also investigated the possible occurrence of neurofibrillary pathology in amyloid precursor protein (APP) transgenic (tg) mice. Among them, some studies have reported the presence of phosphorylated tau epitopes (Alz50 immunoreactivity) in the neurons (33) and the accumulation of filaments in the plaques (34).

This latter study was performed in tg mice where a platelet-derived growth factor-B (PDGF-B) promoter drives a human APP (hAPP) minigene (PDAPP) (34, 35) encoding alternatively spliced hAPP (with a V—>F mutation in position 717). This mutation is associated with familial AD (36–38). The PDAPP tg mice showed an age- and brain region-dependent development of typical amyloid plaques, dystrophic neurites, loss of presynaptic terminals, astrocytosis, and microgliosis (34, 39). However, it is still unclear how early in the progression of the pathology the neurofibrillary alterations develop, and how these alterations compare with those observed in patients with AD. Previous studies have been focused in the analysis of patterns of tau immunoreactivity by light microscopy, but little or no information is available as to the subcellular distribution of these phosphorylated tau epitopes by electron microscopy in the brain of patients with AD or in PDAPP tg mice. Then, in order to more fully understand the characteristics of neurofibrillary pathology in...
PDAPP tg mice, ultrastructural and immunocytochemical studies were performed in 4- to 20-month-old tg mice and compared to AD cases. Studies were conducted using the AT8 antibody against phosphorylated tau (Ser-202 residue) because previous studies have shown that this is a highly sensitive marker capable of detecting early neurofibrillary alterations in AD (40, 41).

MATERIALS AND METHODS

Mouse and Human Brain Tissues

A total of 50 mice (4-, 8-, 12-, 16-, and 20-months-old) were included for the present study. Of them, 30 were heterozygous tg mice from a previously established PDAPP-109 line (n = 6 per each age group) (34), and 20 were age-matched non-tg littermates (n = 4 per each age group). Mice were anesthetized, perfused with cold saline, and fixed in 4% buffered paraformaldehyde. Brains were then serially sectioned at 40 μm with a Leica vibratome 2000 and cryoprotected sections were stored at −20°C, as previously described (42). The vibratomed sections were subsequently used for immunocytochemical and ultrastructural analysis as described below. In addition, for comparisons with the tg material, 40-μm-thick vibratome sections from the midfrontal cortex and hippocampus of 3 AD cases (mean PMT = 3.5 hours) from the Alzheimer Disease Research Center (ADRC) at the University of California San Diego were utilized.

Immunocytochemical Analysis

In order to assess the overall patterns of tau and neurofilament immunoreactivity in anticipation of the ultrastructural analysis, vibratome sections were immunolabeled with mouse monoclonal antibodies against phosphorylated medium and high molecular weight neurofilaments (NFs) (SMI 312, 1:2000, Sternberger Monoclonals, Baltimore, MD) (43) or phosphorylation-dependent anti-tau (AT8, 1:200, Innogenetics, Ghent, Belgium), as previously described (42). Briefly, blind-coded vibratomed sections were incubated overnight at 4°C in primary antibodies, followed by incubation with the biotinylated antimouse secondary antibody (1:100, Vector, Burlingame, CA) and avidin-D-HRP (ABC Elite, Vector). Sections were then reacted with diaminobenzidine (DAB) (0.2 mg/ml) in 100 mM Tris-HCl (pH 7.4) with 0.001% hydrogen peroxide and analyzed by light microscopy.

Double-Immunofluorescent Labeling, Laser-Scanning Confocal Microscopy, and Quantitative Analysis of Plaques

In order to delineate the time course of cytoskeletal alterations in the tg mice, vibratomed sections from murine and human cases were incubated overnight at 4°C with a mixture of the rabbit polyclonal antibody against amyloid B-protein (R1280, 1:500, courtesy of Dr. Dennis Selkoe) (44) and either phosphorylated NF (SMI 312, 1:1000, Sternberger) (43) or phosphorylated tau (AT8, 1:100, Innogenetics) (40, 41), as previously described (43). Sections were then incubated with a mixture of FITC-conjugated anti-mouse IgG (1:75, Vector) and Texas red-conjugated anti-rabbit (1:75, Vector) secondary antibodies. The double-immunolabeled sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA) and mounted under glass coverslips with anti-fading media (VectaShield, Vector). All sections were processed simultaneously under the same conditions and experiments were performed twice in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached laser confocal scanning system MRC 1024 (BioRad, Watford, UK). For each case, 3 serial sections were analyzed. From each section, 5 fields within the frontal cortex and 5 fields within the hippocampal CA1 region were imaged. Serial optical z-sections (0.2-μm-thick) of the double-immunolabeled neuritic plaques were collected from each region using the dual channel imaging capability of the confocal microscope (10, 43). The Texas Red channel collected the R1280-immunolabeled amyloid deposits and the FITC channel collected the corresponding images of the SMI312- or AT8-immunolabeled elements in the plaques. Digitized confocal images from the hippocampal region were analyzed with the Image 1.43 system as previously described (10, 13) in order to estimate the number of plaques displaying AT8 or NF immunoreactivity. Briefly, confocal images from the hippocampus were reconstructed (cross section equivalent to 5 sq mm), and a threshold was set such that the total number of R1280, SMI312 and AT8 immunoreactive plaques per cross section was obtained. Total numbers were averaged among the 3 sections and calculations were performed to obtain the percentage of the R1280 immunoreactive plaques that displayed SM312 and AT8 immunoreactive dystrophic neurites.

Ultrastructural Analysis and Immunogold Labeling

Vibratome sections were embedded in epoxy and sectioned with the Reichert Ultracut-E ultramicrotome (Leica, Vienna, Austria), placed on 200-mesh copper grids, and stained in saturated ethanol/uranyl acetate and bismuth nitrate, as previously described (42). From each case, approximately 10 sections (80-nm-thick) were analyzed with the Zeiss EM10 Electron Microscope at 5, 10, 20, and 50 K magnifications in order to evaluate the ultrastructural characteristics of neurons, synapses, and plaques in tg mice and AD material. From each case, a total of 60 micrographs (15 at each of the 4 magnifications indicated) were prepared. In order to determine the relationship between neuritic processes, cellular elements and amyloid, additional series of 10 sections (80-nm-thick) were divided and transferred onto formvar carbon-coated slot grids. Serial numbered electron micrographs of selected areas containing neuritic plaques were obtained with a Zeiss EM10 electron microscope at a magnification of 3.3 K and 5 K for analysis as previously described (42).

For immunogold cytochemistry, as previously described (42), vibratome sections from non-tg and PDAPP tg mice and AD cases were fixed in 0.25% glutaraldehyde and 3% paraformaldehyde in 0.1M cacodylate buffer pH 7.4. Tissues were incubated in 100 mM glycine in sodium cacodylate buffer followed by brief fixation in 0.1% osmium tetroxide. Sections were dehydrated in a graded series of ethanol solutions. From each section, the frontal cortex and hippocampus were dissected and placed in gelatin capsules containing a polymerized layer of LR...
Fig. 1. Patterns of SMI312 and AT8 immunoreactivity in the mice by bright field light microscopy. All images are from the hippocampus of 12-month-old mice. While (A) SMI312 immunostained the axons in the non-tg mice, in (B) PDAPP tg mice SMI312 immunolabeled plaque dystrophic neurites. C: AT8 immunoreacted with the axons in the non-tg mice, while in (D) PDAPP tg mice AT8 immunostained the neuronal cell bodies. Only slight labeling of the neuronal cell bodies was observed with both antibodies (n). Scale bar = 30 μm.

white (medium grade, Ted Pella, Inc., Redding, CA). Fresh LR white was then added and polymerized for 24 hours in a 55°C oven. Blocks were sectioned on an Ultracut E and thin sections (6.0 nm–9.0 nm) were placed on nickel grids coated with a 0.5% paraflonian film. Grids were incubated at room temperature for 30 minutes with blocking buffer (TBS+0.8%BSA+0.1% gelatin+0.02% sodium azide) followed by a 12-hour incubation at room temperature with either the monoclonal antibody AT8 (40, 41), SMI312 or TA51 (45, 46). The latter antibody was raised to bovine-derived NF proteins and it recognizes a phosphorylation-dependent epitope in the carboxy terminus of the high molecular weight NF subunit in mice. This antibody is considered highly specific and does not cross-react with other cytoskeletal proteins. Incubation in primary antibody was followed by rabbit anti mouse-gold (5 nm, Zymed Laboratories, Inc., South San Francisco, CA). Grids were then fixed in 2% glutaraldehyde and postfixed with 2% uranyl acetate, followed by bismuth nitrate. Immunogold-labeled grids were examined with a Zeiss EM10 electron microscope. Control experiments consisted of incubating grids with secondary antibody-gold in absence of primary antibody.

Statistical Analysis

After the results were obtained, the code was broken and sets of data were assigned to their corresponding groups. Statistical analyses of the results were performed using the STAT VIEW II software package (Abacus Concepts, Piscataway, NJ) running on a Macintosh personal computer. Statistical comparisons among the groups were done by a 1-factor ANOVA with post-hoc Scheffe test. All values were expressed as mean ± SEM.

RESULTS

The antibodies against phosphorylated NF immunolabeled the neuronal cell bodies and the axons in the non-tg (Fig. 1A) and PDAPP tg mice (Fig. 1B). AT8 immunoreactivity was less commonly seen associated with the neuronal cell bodies, but was present in the axons of both non-tg (Fig. 1C) and tg mice (Fig. 1D). Consistent with previous studies (34), neuritic plaques were first observed in 8- to 10-month-old tg mice (Fig. 2). At this age the dystrophic neurites in the plaques...
displayed positive immunoreactivity with antibodies against phosphorylated NFs (Fig. 2), but not against phosphorylated tau (Fig. 2). Similar results were obtained in 12-month-old mice (Figs. 1B, 2A, 3A, C); however, in 16- to 20-month-old mice, the neuritic component of the plaques displayed positive immunoreactivity with antibodies against both phosphorylated NFs (Figs. 2A, 3B) and tau (Figs. 2A, 3D). Overall, a greater proportion of plaques in the hippocampus displayed phosphorylated NFs immunoreactivity compared to phosphorylated tau, however the percentage of plaques displaying positive dystrophic neurites increased with age for both cytoskeletal markers (Fig. 2B). In the PDAPPtg mice the neuronal cell bodies showed mild phosphorylated NF and tau immunoreactivity (Figs. 1D, 3C), but no neurofibrillary-like structures or neuropil threads were observed. Ultrastructural analysis was performed in order to gain more detailed information as to the subcellular alterations associated with phosphorylated NF and phosphorylated tau immunoreactivity.

Electron microscopic analysis of sections stained with uranyl acetate and lead showed that in the PDAPP tg mice 12 months and older, there were cytoskeletal alterations characterized by accumulation of tubular/filamentous structures ranging in diameter from 12 nm to 15 nm (Fig. 4A, B) in the dystrophic neurites (Fig. 4) and neuronal cell bodies (Fig. 5). These structures were more commonly seen in the dystrophic neurites in association with distended or vacuolized membranous structures and multi-laminated bodies (Fig. 4C, D). In contrast, these cytoskeletal alterations were only occasionally seen in the neuronal cell bodies (Fig. 5) of older PDAPP tg mice. For example, in 20-month-old PDAPP tg mice, thick filamentous bundles in the cytoplasm of degenerating neurons (Fig. 5A, B) were observed in close proximity to laminated bodies (Fig. 5C) and ribosomes (Fig. 5D). Paired helical filaments were not identified in the PDAPP tg mice. To better understand the characteristics of these cytoskeletal alterations, additional immunogold analysis with antibodies against phosphorylated NF and phosphorylated tau were performed. Consistent with the light microscopic observations that showed moderate labeling of neurites in the mice (Fig. 1A, B), in both non-tg (Fig. 6A) and PDAPP tg mice (Fig. 6B) the antibody against phosphorylated NFs (SMI312) labeled filamentous structures along the axons. In the plaque dystrophic neurites and in some neuronal cell bodies, aggregates of these filamentous structures were also recognized by the SMI312 antibody in PDAPP tg mice (Fig. 6C, D). Similar results were obtained with the TA51 antibody (Fig. 7) against phosphorylated NFs, further confirming the specificity of the immunogold results. These filamentous structures immunolabeled by the NF antibodies were similar to those observed by TEM in the dystrophic neurites (Fig. 4A, B, D), but were different than the tubular structures associated with the vacuolized bodies (Fig. 4C). Overall, immunogold particles indicative of phosphorylated NFs were sparse and more often found in association with the dystrophic neurites (Fig. 6D, 7C, E) than with the neuronal cell bodies (Fig. 7D, F). Similar to the immunogold analysis, the double-labeling and confocal microscopy showed that phosphorylated NFs were mainly associated with dystrophic neurites in the plaques (Fig. 3A, B).

Consistent with the light microscopic observations that showed mild labeling of axons and neuronal cell bodies (Fig. 1C, D), in the non-tg mice, AT8 immunoreactivity was observed along the axoplasm as clusters associated with the microtubular network of myelinated axons (Fig. 8A). In PDAPP tg mice (Fig. 8B) and AD cases (Fig.
Fig. 3. Double labeling and laser scanning confocal microscopy of neuritic plaques in the hippocampus of PDAPP tg mice. Images collected by the FITC channel are in green (SMI312 or AT8) and those collected by the Texas red channel are in red (amyloid β protein). A: In 12-month-old and (B) 20-month-old PDAPP tg mice the plaques displayed abundant SMI312 immunoreactive neurites. C: In 12-month-old PDAPP tg mice the AT8 antibody labeled only the neuronal cell bodies but not the plaques. D: In 20-month-old PDAPP tg mice the AT8 antibody labeled the dystrophic neurites in the plaques. Scale bar = 10 μm.

8C), AT8 immunolabeling was also observed in the axons as clusters associated with the cytoskeleton. In the plaque dystrophic neurites of PDAPP tg mice, immunogold particles indicative of AT8 immunoreactivity were observed as clusters in the midst of the tubule-filamentous aggregates (Fig. 8E). These clusters of gold particles were associated with electrodense structures distributed in the midst of the filamentous aggregates (Fig. 8E). Overall, the tubulo-filamentous aggregates labeled by the antibodies against phosphorylated NFs and tau in the PDAPP tg mice were similar with the difference that phosphorylated NFs were diffusely distributed and phosphorylated tau immunoreactive was clustered. A similar pattern for NF (not shown) and AT8 immunogold labeling was observed in the PHF containing tangles in AD cases (Fig. 8F). These filamentous structures immunolabeled by AT8 antibodies were similar to those observed by TEM in the dystrophic neurites (Fig. 4A, B, D), but were different than the tubular structures associated with the vacuolated bodies (Fig. 4C). Overall, immunogold particles indicative of phosphorylated tau were more abundant than phosphorylated NFs and more often found in association with axons and the dystrophic neurites (Fig. 8) than with the neuronal cell bodies. This was consistent with the double-labeling and confocal microscopy (Fig. 3C, D).

DISCUSSION

The present study showed that in PDAPP tg mice, phosphorylated NF (SMI312) immunoreactivity preceded the appearance of phosphorylated tau (AT8) immunoreactivity in the dystrophic neurites in the plaques, indicating that the development of phosphorylated tau epitopes in the neuritic plaques is a late event. Consistent with these findings, immunocytochemical studies in AD patients have shown that in cases with milder AD, the neuritic plaques display predominantly anti-APP, anti-chromogranin, anti-synaptophysin and NF immunoreactivity, while in severe AD cases, phosphorylated tau immunoreactivity is more abundant (13, 47–52). This suggests that the appearance of phospho-tau immunoreactivity in the plaque neurites is related to the development of neurofibrillary tangles (NFTs) in AD patients. In support of this possibility, previous studies using Golgi impregnation methods as well as confocal microscopy have shown that PHF containing neurites in the plaques.
are derived from the neuronal process of NFTs (12). Although there is some controversy as to the relationship between AD severity (determined by the Braak system) and stage of the disease (determined clinically), most evidence appears to support the possibility that tangles increase with the progression of the disease and that they are rather uncommon at earlier stages (53–55). In contrast, plaques appear early and reach a plateau at later disease stages (56), suggesting that NFTs might appear in very old tg mice. In support of this possibility, we observed mild NF and tau pathology in 20-month-old tg mice. These alterations are similar to those observed in early stages of AD patients (57). However, since no PHF were observed in the oldest PDAPP tg mice, it is possible that other factors such as accelerated aging and/or increased longevity of the mice might be needed to obtain more overt tangle pathology.

The relationship between plaque and tangle formation in AD is a subject of controversy. While some studies suggest that tangles seed the formation of plaques, others propose that plaque and NFT formation might be independent (58). Since amyloid deposition precedes the appearance of dystrophic neurites, it is possible that the neurotoxic effects of Aβ might be partially responsible for the cytoskeletal alterations observed in the PDAPP tg
Fig. 5. Ultrastructural analysis of the cytoskeletal alterations in the hippocampal neuronal cell bodies of PDAPP tg mice. In older (20-month-old) PDAPP tg mice, thick bundles of filamentous structures (arrows) were found in the cytoplasm of degenerating neurons (A, B) in close proximity to laminated bodies (C) and ribosomes (D). Scale bars: A and B = 5 μm; C and D = 1 μm.

mice. In support of this possibility, studies have shown that intracerebral injections of Aβ result in increased phosphorylated tau immunoreactivity in the adjacent neurons, without the formation of PHF (30, 31). Similar effects on tau phosphorylation and NF aggregation have been described in other APP tg mice (33, 59) and in experimental models of neuronal injury (32, 60). However, this is the first study to describe the subcellular distribution of AT8 and phosphorylated NF epitopes in APP tg mice. Taken together, these data suggest that increased Aβ deposition might contribute to neurofibrillary pathology in AD, but that other factors may also play a role.

The present study also showed that while phosphorylated NF-immunoreactive gold particles were diffusely distributed along the filaments observed in the DN and axons, phospho-tau immunoreactivity was observed as clusters. This suggests that tau is abnormally compartmentalized in the axons in the tg mice and associates with NF rather than with MT, indicating that phosphorylated NF and tau are differentially compartmentalized, and that tau serves as an anchor site for filaments in the axons. Very few immuno-electron-microscopic studies in experimental models of NFT formation are currently available, and none have been published describing the subcellular distribution of AT8 epitopes in AD. Among the few studies published, the ultrastructural analysis in the aged sheep is of special interest (61). This study showed that Alz50 and AT8 immunoreactivity was associated with granular deposits in the cytoplasm of NFTs and at their dendritic
Fig. 6. Ultrastructural patterns of SMI312 immunogold labeling in the hippocampus of 12-month-old mice. In both non-tg (A) and PDAPP tg mice (B), the antibody against phosphorylated neurofilaments diffusely labeled filamentous structures along the axons. In the neuronal cell body (C) and in the plaque dystrophic neurites (D), gold particles were observed in association with aggregates of these filamentous structures in PDAPP tg mice. Scale bar = 1 μm.

branch points. For the most part, immunostaining was associated with ribosomes and rough endoplasmic reticulum. Similar to patients with AD, in aged sheep brains, Alz50 and AT8 immunoreactivity was associated with PHFs. However, consistent with our observations in the PDAPP tg mice, studies in AD have shown that abnormal phosphorylation of tau appears to occur first at Ser-202 (AT8 epitope) in dystrophic neurites, then Ser-202 in the soma, and finally at Ser-396 in dystrophic neurites and tangles (40, 41). Although demonstration of insoluble hyperphosphorylated tau by immunoblot will be needed to further confirm the results in the PDAPP mice, taken together, these results suggest that abnormal phosphorylation of tau at Ser-202 in dystrophic neurites represent an early neuropathological event.

The modeling of neurofibrillary pathology in animals other than the sheep has proven to be a difficult task. Previous studies have shown that tg overexpression of low molecular weight tau under the regulatory control of the HMGCR promoter (62), or high molecular weight tau under the control of the human Thy1 promoter (63), results in somatodendritic distribution and hyperphosphorylation of tau protein, similar to the pretangle changes that precede the neurofibrillary pathology in AD. However, neither neuronal dysfunction nor behavioral alterations were observed. To overcome these problems, new tg mouse models overexpressing wildtype or mutant fetal or adult forms of tau at high levels have been generated. In the model developed by Spittaels et al (64), overexpression of the high molecular weight tau under the
Fig. 7. Ultrastructural patterns of TA51 immunogold labeling in the hippocampus of 12-month-old mice. In both non-tg (A) and PDAPP tg mice (B), the antibody against phosphorylated NFs (TA51) diffusely labeled filamentous structures along the axons. In the plaque dystrophic neurites (C, E) and in the neuronal cell body (D, F), gold particles were observed in association with aggregates of these filamentous structures (arrows) in the PDAPP tg mice. N = nucleus. Electron micrographs in panels (C, D) are low power (×5000) images of panels E, F (×25,000). Scale bar = 1 μm.
regulatory control of the mouse (Mo) Thy1 promoter resulted in axonal degeneration in the forebrain and spinal cord accompanied by accumulation of NFs and sensorimotor deficits. Similarly, the model developed by Ishihara et al (65) where low molecular weight tau was overexpressed under the control of the MoPrP gene resulted in motoneuron disease and formation of dystrophic neurites with abundant NFs. No PHF of NFT were observed in the 2 models. The alterations in these 2 models were interpreted to be similar to tauopathies such as FTD and Guam/ALS (64, 65).

In summary, the present study showed that in the PDAPP tg model, most of the cytoskeletal alterations are associated with dystrophic neurites in the plaques, and to a lesser extent with the neuronal cell bodies. The progression and compartmentalization of the phosphorylated NFs and tau epitopes in the PDAPP tg mice recapitulates some of the alterations observed in early stages of AD.

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