Tau Filament Formation in Transgenic Mice Expressing P301L Tau*

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Mutations in the microtubule-associated protein tau, including P301L, are genetically coupled to hereditary frontotemporal dementia with parkinsonism linked to chromosome 17. To determine whether P301L is associated with fibril formation in mice, we expressed the longest human tau isoform, human tau40, with this mutation in transgenic mice by using the neuron-specific mouse Thy1.2 promoter. We obtained mice with high expression of human P301L tau in cortical and hippocampal neurons. Accumulated tau was hyperphosphorylated and translocated from axonal to somatodendritic compartments and was accompanied by astrocytosis and neuronal apoptosis indicated by terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling. Moreover, P301L tau formed abnormal filaments. Electron microscopy of sarcosyl-insoluble protein extracts established that the filaments had a straight or twisted structure of variable length and were ~15 nm wide. Immunoelectron microscopy showed that the tau filaments were phosphorylated at the TG3, AT100, AT8, and AD198 epitopes in vivo. In cortex, brain stem, and spinal cord, neurofibrillary tangles were also identified by thioflavin-S fluorescent microscopy and Gallyas silver stains. Together, our results show that expression of the P301L mutation in mice causes neuronal lesions that are similar to those seen in human tauopathies.

Hereditary frontotemporal dementia with parkinsonism linked to chromosome 17 is a group of neurodegenerative diseases characterized by early behavioral changes accompanied by subsequent cognitive and motor disturbances. More than a dozen families were identified, with diverse but overlapping clinical features. Pathological changes include selective frontotemporal atrophy, neuronal loss, gliosis, and spongiosis in several brain areas in addition to abundant filamentous inclusions composed of hyperphosphorylated tau protein in neurons and, to some extent, in glial cells (1).

Tau is an axonal, microtubule-associated phosphoprotein in normal adult brain (2). Tau has tubulin-polymerizing activities to some extent, in glial cells (1).

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* The abbreviations used are: 4R, four-repeat; 3R, AD, Alzheimer’s disease; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; S1, somatosensory cortex 1; M1, motor cortex 1; CA, cornu ammonis.

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to detect tau phosphorylated at serine 199; MC1 (Dr. Peter Davies; diluted 1:10) was used to detect the conformational ALZ50 epitope; and rabbit anti-glial fibrillary acidic protein IgG (Sigma, catalog no. G-9269; diluted 1:400) was used to detect activated astrocytes. For peroxidase and diaminobenzidine stainings, secondary antibodies were obtained from Vector Laboratories (Vectorstain ABC kits PK-6101 and PK-6102). For immunofluorescence, secondary antibodies were obtained from Molecular Probes (ALEXA-FLUOR series).

**Immunoblot Analysis—** Brains from transgenic and control mice (aged 3 weeks to 8 months) were weighed and Dounce homogenized in 2.5% (v/v) perchloric acid in phosphate-buffered saline (PBS), allowed to stand on ice for 30 min, and centrifuged for 10 min at 10,000 × g. The supernatants were dialyzed against 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride and used for immunoblot analysis as described, using equal amounts except for ALZ17, for which only half the amount of extract has been used (10). Ponceau staining of the membranes was included to confirm loading of comparable amounts of protein.

Sarcoyol extractions were done as described (10). In brief, brain tissue of 8-month-old pR5 transgenic and control mice were homogenized in 10 volumes of buffer consisting of 10 mM Tris-HCl (pH 7.4), 0.8 mM NaCl, 1 mM EDTA, and 10% sucrose. An Alzheimer’s disease (AD) brain sample was included for comparison. The homogenate was spun at 20,000 × g for 20 min, 0.200 × g. The supernatant was brought to 1% N-lauroylsarcosine (Sigma, 100 mM, pH 7.4), and incubated 1 h at room temperature while shaking. After a 1-h spin at 100,000 × g, the sarcosyl-insoluble pellets were resuspended in 50 mM Tris-HCl (pH 7.4) and stored at 4 °C.

**RESULTS**

**Overexpression of Human Tau—** The neuron-specific elements of the mouse Thy1.2 promoter were used to express the longest human four-repeat tau isoform containing the two amino-terminal exons 2 and 3 (2’-3’4’R), along with human pathogenic mutation P301L (pR5 construct). Ten founders were obtained, four of which were used for further analysis on the basis of their expression levels: By immunoblot analysis of similar amounts of perchloric acid-soluble protein extracts, as judged by Ponceau stainings, a strong immunoreactive 66-kDa protein that corresponded to the transgenic tau band was identified in animals of some pR5 lines; it reacted with the anti-tau antibody Tau-1 that recognizes both human and murine tau (Fig. 1A), as well as with antibody HT7 against human, but not murine, tau (Fig. 1B). Soluble tau brain tissue levels for line pR5-183 were ~70% of endogenous tau (Fig. 1A, lane 4, upper transgenic band representing human tau compared with lower bands representing the endogenous murine tau isoforms). As expected, transgenic tau was absent from the wild-type brain protein extracts (Fig. 1B, lane 8). Amounts of sarcosyl-insoluble tau (Fig. 1C) were generally higher in the pR5-183 (Fig. 1C, lane 1) than in our transgenic mice that express human tau without the mutation (ALZ17 line; Ref. 11; Fig. 1C, lane 3). Protein extracts from pR5-183 mice were therefore subsequently used for electron microscopy. The transgenic mutant tau in pR5-183 mice was hyperphosphorylated, as indicated by several phosphorylation-dependent anti-tau antibodies (data not shown).

To determine the distribution and localization of tau in brain, a 3-month-old mouse of the pR5-183 line was analyzed by immunohistochemistry using antibody HT7. Expression levels of human tau were high in hippocampus, fornix fimbriae, amygdala, spinal cord, and cortex (Fig. 2A), weaker in brainstem and striatum, and not detectable in olfactory bulb and cerebellum. A sagittal section of the hippocampus showed numerous HT7-positive pyramidal neurons in the CA1 region but not in CA3. The mossy fiber projections of the hippocampus were strongly stained, whereas staining intensity was somewhat weaker in dentate gyrus granule cells (Fig. 2A and C). In CA1 pyramidal neurons human tau was present in axons but accumulated also in cell bodies and apical dendrites (Fig. 2B). In brain stem, HT7-positive cells were identified, in addition to axonal spheroids, and cells that expressed tau were strongly stained, with granular accumulation of tau (Fig. 2D). In addition, a subset of pyramidal cells in the cerebral cortex was HT7...
immunoreactive. As for CA1 pyramidal cells, we found granular immunostaining of cell bodies and dendrites (Fig. 2E). In comparison with wild-type human tau transgenic ALZ17 mice, staining of cell bodies and dendrites of the dentate gyrus granule cells was less pronounced in pR5-183 mice, as determined with antiserum AD199 (Fig. 2F and G) or antibody HT7 (data not shown). In other brain areas, staining patterns were comparable. Neurofibrillary tangles were identified in spinal cord, brain stem, and cortical layers 5 and 6 by thioflavin-S fluorescent microscopy (Fig. 2H) and Gallyas silver stains (Fig. 2I).

Abnormal Phosphorylation and Conformation of tau—To determine the phosphorylation status of tau in 3-month-old pR5 mice, we used a panel of phosphorylation- and conformation-dependent anti-tau antibodies. Antibody TG3, a phosphorylation- and conformation-dependent antibody, stained pyramidal neurons of the CA1 region of the hippocampus of transgenic mice (Fig. 3A) but not controls (Fig. 3B). TG3 stained numerous pyramidal neurons in cortices of transgenic (Fig. 3C) but not control (Fig. 3D) mice. The conformation-dependent antibody MC1 that detects the ALZ50 epitope of AD tau stained pyramidal neurons of the CA1 region and the cortex of transgenic (Fig. 3, E and G) but not control (Fig. 3, F and H) mice. The same staining pattern was obtained with antibody AT180, which detects tau phosphorylated at threonine 231 and serine 235 (Fig. 3, I–M). Antibody AD199 directed against phosphorylated serine 199 stained hippocampal and cortical neurons moderately, antibody AT8 directed against serines 202 and 205 stained only weakly and was mainly restricted to cortical neurons, whereas antibodies AD2 and PHF1 directed against serines 396 and 404 did not detect any neurons. Staining with antibodies 12E8 and AT100 revealed similar patterns in transgenic and control mice (data not shown).

Astrocytosis and Apoptosis—To detect activated astrocytes, we used an antibody against glial fibrillary acidic protein. This

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**FIG. 1.** Immunoblot analysis of tau protein in brains from mice of pR5 lines. A, tau protein extracted with perchloric acid from brains of different 3-month-old mice of pR5 lines (lanes 1–7; lane 4, line pR5-183) and a wild-type control mouse (lane 8) was analyzed by immunoblotting using anti-tau antibody Tau-1. This antibody recognizes murine and human tau. B, the blot was stripped and reprobed with HT7, a human tau-specific antibody. The arrow points to the human tau band. C, sarcosyl-insoluble tau from the brains of 8-month-old mice of line pR5-183 (lane 1), a wild-type control mouse (lane 2), and a wild-type human tau transgenic ALZ17 mouse (lane 3) analyzed by immunoblotting using antibody HT7. The arrow points to the human tau band.

**FIG. 2.** Tau immunoreactivity in brain from a 3-month-old mouse of the pR5 line reveals high expression of human tau. A, sagittal section of the hippocampus shows HT7-positive pyramidal neurons in CA1. Staining is less intense for dentate gyrus (dg) granule cells. Also note strong immunostaining of the mossy fiber (mf) projection in sector CA3 of the hippocampus. B, CA1 pyramidal neurons accumulate human tau in cell bodies and apical dendrites. C, the mossy fiber network is intensely stained, revealing transport of human tau into the axon. D, in brain stem, HT7-positive cells are found with granular accumulation of tau, in addition to axonal spheroids. E, in cerebral cortex, a subset of pyramidal cells is HT7-immunoreactive. Note the intense, granular immunostaining of cell bodies and dendrites. No immunoreactivity was observed in control mice. F and G, staining of cell bodies and dendrites of dentate gyrus granule cells with antiserum AD199 is less pronounced in pR5-183 mice (F) compared with wild-type human tau transgenic ALZ17 mice (G). H and I, neurofibrillary tangles are identified in several brain areas including spinal cord by thioflavin-S fluorescent microscopy (H) and Gallyas silver stains (I). Scale bars: A, F, and G, 100 μm; B–E, 20 μm.
antibody revealed activated astrocytes in the amygdala and in cortical areas of transgenic mice that contained numerous tau-positive neurons (Fig. 4A). No such activated astrocytes were observed in control amygdala and cortex (Fig. 4B). Also, no astrocyte activation was found in the hippocampus of transgenic mice (data not shown). To identify cells that underwent apoptosis, we used TUNEL stains and found many TUNEL-positive neurons in the somatosensory cortex that contained numerous tau-positive neurons (Fig. 5A). By contrast, only very few TUNEL-positive cells were present in wild-type mice, and staining was faint compared with transgenic mice (Fig. 5B). As negative control, we omitted the POD convert solution (Fig. 5C), and, as a positive control, we pretreated sections with DNase I (Fig. 5D). No TUNEL-positive neurons were identified in the hippocampus of transgenic mice (data not shown). We correlated numbers of HT7-positive neurons with glial fibrillary acidic protein-positive astrocytes and TUNEL reactivity in the somatosensory cortex and counted per visual field 50 (±14) HT7-positive neurons, 19 (±6) activated astrocytes, and 7 (±3) dark TUNEL-positive cells in transgenic brain compared with no HT7-positive neurons, 5 (±2) activated astrocytes, and 3 (±3) faint TUNEL-positive cells in the wild-type control (Fig. 4C). To correlate astrocytosis and tau expression in different brain areas, we compared in transgenic brain motor cortex M1 and somatosensory cortex S1 and counted per visual field 14 (±4) HT7-positive neurons and 5 (±1) activated astrocytes in the M1 cortex compared with 50 (±14) HT7-positive astrocytes and 19 (±6) activated astrocytes in the S1 cortex (Fig. 4D).

Tau Filaments—We prepared sarcosyl protein extracts for electron microscopy analyses in parallel from brain tissue of an AD patient, 8-month-old pR5 transgenic mice, and control mice and found tau filaments in both AD and transgenic mouse brains. The tau filaments in AD brain had a width of ~20 nm (Fig. 6, A and B), and those in transgenic mice were ~15 nm wide and significantly shorter (Fig. 6C). No such filaments were present in any of the control mice (data not shown). To identify phosphoepitopes of tau, we incubated the filaments with a panel of phosphorylation-dependent antibodies. Immunogold electron microscopy of extracts obtained from AD (Fig. 6, D and F) and pR5 transgenic (Fig. 6, E and G) mice brains using TG3 (Fig. 6, D and E) or AT8 (Fig. 6, F and G) identified several 6-nm, gold-decorated filaments. These filaments were also stained by the antibodies AT100 and AD199 (data not shown).

DISCUSSION

The results of this study show that transgenic expression of human P301L mutant tau under control of the murine Thy1.2 genomic expression vector leads to the formation of sacrosyl-insoluble, 15-nm-wide tau filaments. By comparison, tau filaments in frontotemporal dementia with parkinsonism linked to chromosome 17 patients with the P301L mutation (Dutch family 1) consist of 15-nm-wide, slender, twisted filaments with variable periodicity, in addition to a few straight filaments (1).
and the tau filaments obtained from AD brains consist of 20-nm-wide, paired helical or straight filaments. The filaments in our P301L tau transgenic mice have the same width as those in the human disease associated with the P301L mutation (Dutch family 1). They were, however, shorter than filaments enriched from AD brains, which we included as positive control because of the unavailability of a brain sample from Dutch family 1. One likely explanation for the prevalence of short filaments in our mice is that the longest tau isoform expressed in our mouse contained two calpain recognition motifs for proteolytic degradation that may favor the formation of shorter filaments. In addition to the P301L mutation, the filament formation in our mice may also be related to high expression levels achieved by using the murine Thy1.2 expression vector, as shown by immunoblot analyses. A possible contribution of high expression levels to tau filament formation in our mouse lines is supported by the concentration dependence of filament assembly in vitro that resembles a nucleation-dependent process (18, 19). Together, the failure of previous attempts to model tau filament formation in transgenic mice may be related to either the absence of disease-causing mutations in the expression constructs or low expression levels (11, 20–25) or both.

In our mice, the murine Thy1.2 promoter directed expression of tau mainly to hippocampal and cortical neurons. Brain regions, which in human disease are spared from tau pathology, such as the cerebellum, did not express detectable levels of tau, as determined by immunofluorescence. As in human tauopathies (26), tau accumulated not only in axons but also in cell bodies and dendrites. To establish phosphoepitopes that are related to tau aggregation and filament formation, we used a panel of phosphorylation-dependent antibodies. In brains from patients of Dutch family 1, phosphorylation-dependent antibodies AT8, AT100, AT190, AT270, PHF1, and 12E8 stained numerous deposits in several brain regions, including the cortex, the dentate gyrus, and the CA1 region of the hippocampus. These deposits were mainly of the pre-tangle type and located in the perinuclear region and cell body and sometimes extended to the apical dendrites of neurons (1). In our model, AT100 and 12E8 did not discriminate wild-type from transgenic mice. AT8 staining was weak in transgenic mice and mainly restricted to a subset of cortical neurons, where it was found not only in cell bodies but also in dendrites. AT100 and PHF1 did not stain any neuron at up to 6 months of age. Age may account for this apparent difference between human disease and the mouse model, especially because filaments obtained from 8-month-old transgenic mice were AT100- and AT8-positive. AT180 and also the conformation-dependent antibodies MC1 and TG3, which were not included in the human study, revealed strong somatodendritic staining in both cortical and hippocampal pyramidal neurons of transgenic mice. Antibodies MC1 and TG3 recognize a distinct pathological conformation of the tau molecule in AD. In normal autopsy-derived brain tissue, tau is not stained by these antibodies. Our data indicate that transgenic tau underwent a conformational change favoring filament formation. Indeed, by immunogold electron microscopy of sarcosyl extracts, the AT8, AD199, AT100, and TG3 epitopes were identified on tau filaments in our P301L transgenic mice. For comparison, in the human study only one antibody, AT8, was used that labeled tau filaments (1). The distance of the gold particles from the core of the filament is similar to that reported for AD filaments (10). The space between the filaments and the gold particles corresponds to the known fuzzy coat of the filaments (10, 16). Taken together, tau in the nonfilamentous and filamentous states is hyperphosphorylated at several sites, which are also hyperphosphorylated in human tauopathies.

In Dutch family 1, neurological examination of brains revealed gliosis and severe neuronal loss in frontal and temporal...
cortex and variable loss in the parietal cortex, whereas the hippocampus showed only mild to moderate neuronal loss and gliosis (1). These data are consistent with our findings. Despite comparable levels and phosphorylation of human tau in hippocampus and cortex of transgenic mice, we found evidence for apoptosis and astrogliosis in cortical areas and in the amygdala but not in the hippocampus. Astrogliosis was positively correlated with levels of human tau expression, as shown for the M1 and S1 cortices. Therefore, we cannot exclude the possibility that this pathology progresses and will include the hippocampus with advanced aging in older mice.

Together, our data show that the P301L mutation in combination with high expression levels can cause the formation of abnormal tau formations in neurons in mice. Filament formation was also reported recently in mice expressing 2'3'-4R tau, along with the human pathogenic mutation P301L, under control of the mouse prion protein promoter. These mice show an advanced neurological phenotype, likely reflecting differences between 2'3'-4R and 2'3'-4R tau (27). In our P301L mice, filaments were phosphorylated at distinct epitopes, and their formation was accompanied by astrogliosis and apoptosis. These data indicate that P301L is a key pathogenic factor, and they underscore its pathophysiological role in frontotemporal dementia with parkinsonism linked to chromosome 17. Moreover, our results suggest the use of these mice, either alone or in combination with a pathogenic APP mutation, for the study of both the pathophysiology and the prevention of tau filament formation in neurodegenerative diseases.

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