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Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform

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Microtubule-associated protein tau is the major constituent of the paired helical filament, the main fibrous component of the neurofibrillary lesions of Alzheimer's disease. Tau is an axonal phosphoprotein in normal adult brain. In Alzheimer's disease brain tau is hyperphosphorylated and is found not only in axons, but also in cell bodies and dendrites of affected nerve cells. We report the production and analysis of transgenic mice that express the longest human brain tau isoform under the control of the human Thy-1 promoter. As in Alzheimer's disease, transgenic human tau protein was present in nerve cell bodies, axons and dendrites; moreover, it was phosphorylated at sites that are hyperphosphorylated in paired helical filaments. We conclude that transgenic human tau protein showed pre-tangle changes similar to those that precede the full neurofibrillary pathology in Alzheimer's disease.

Key words: Alzheimer's disease/microtubule-associated protein tau/nerve cells/neurofibrillary lesions/transgenic mice

Introduction

Abundant senile plaques and neurofibrillary lesions constitute the major neuropathological characteristics of Alzheimer's disease. Neurofibrillary lesions appear within the vast majority of nerve cells that degenerate during the course of the disease, where their presence is indicative of dementia. The paired helical filament (PHF), which is composed of the microtubule-associated protein tau, forms the principal fibrous component of the neurofibrillary lesions (neurofibrillary tangles, neuropil threads and senile plaque neurites; for reviews see Goedert, 1993; Trojanowski *et al.*, 1993). It is found in cell bodies, dendrites and axons of affected nerve cells.

By contrast, in normal brain, tau is an axonal protein whose known functions are to promote the assembly and stability of microtubules (Cleveland *et al.*, 1977; Binder *et al.*, 1985; Peng *et al.*, 1986). The pattern of tau expression is developmentally regulated; in fetal human brain there is a single isoform, but in adult brain there are six isoforms (Goedert *et al.*, 1989). The different isoforms are produced from a single gene by alternative mRNA splicing. The fetal isoform corresponds to the smallest of the adult isoforms. Tau is a phosphoprotein, and developmental regulation also extends to the level of phosphorylation in that fetal tau is phosphorylated at more sites than adult tau. A number of these sites have been identified by mass spectrometry and peptide sequencing, as well as by the use of phosphorylation-dependent anti-tau antibodies (Bramblett *et al.*, 1993; Brion *et al.*, 1993; Goedert *et al.*, 1993, 1994; Hasegawa *et al.*, 1993; Kenessey and Yen, 1993; Watanabe *et al.*, 1993). The vast majority of known sites are serine or threonine residues that are followed by a proline.

In Alzheimer's disease tau becomes hyperphosphorylated and this is believed to lead to its self-association, resulting in PHF formation. PHF-tau is unable to bind to microtubules, but will bind after dephosphorylation, indicating a consequence of hyperphosphorylation (Bramblett *et al.*, 1993; Yoshida and Ihara, 1993). By SDS–PAGE, PHF-tau migrates as three major bands, of apparent molecular masses 60, 64 and 68 kDa, that do not align with native or recombinant tau (Greenberg and Davies, 1990; Lee *et al.*, 1991; Goedert *et al.*, 1992a). Alignment is achieved after dephosphorylation, indicating that PHF-tau is hyperphosphorylated on all six brain isoforms (Goedert *et al.*, 1992a; Greenberg *et al.*, 1992; Liu *et al.*, 1993). Residues that are hyperphosphorylated in PHF-tau have been identified and many of these are serine/threonine–prolines (Lee *et al.*, 1991; Hasegawa *et al.*, 1992; Goedert *et al.*, 1993, 1994).

Protein kinases and/or protein phosphatases with a specificity for serine/threonine–prolines are therefore likely to be involved in the phosphorylation of tau in normal brain and its hyperphosphorylation in Alzheimer's disease. Accordingly, recent experiments have shown that recombinant tau can be phosphorylated *in vitro* at a number of sites that are hyperphosphorylated in PHFs by mitogen-activated protein (MAP) kinase (Drewes *et al.*, 1992; Goedert *et al.*, 1992b), glycogen synthase kinase-3 (GSK3; Hanger *et al.*, 1992; Mandelkow *et al.*, 1992; Ishiguro *et al.*, 1993) and cyclin-dependent kinase 5 (cdk5; Baumann *et al.*, 1993; Kobayashi *et al.*, 1993; Paudel *et al.*, 1993). The trimeric form of protein phosphatase 2A accounts for the majority of brain phosphatase activity towards tau phosphorylated by MAP kinase (Goedert *et al.*, 1992b). This work represents a first step towards an understanding of the mechanisms that lead to hyperphosphorylation of tau in Alzheimer's disease. However, it suffers from the inherent limitation that *in vitro* studies are not necessarily representative of the *in vivo* situation.

Therefore, to further our understanding of the role of tau in the pathogenesis of Alzheimer's disease, we have

expressed the longest human brain tau isoform (441 amino acid isoform) in transgenic mice under the control of the human Thy-1 promoter (Seki *et al.*, 1985). Approximately every second transgenic line expressed the human transgene at tau mRNA levels that were up to 5-fold higher than the endogenous murine tau mRNA levels. *In situ* hybridization on tissue sections from brain showed that human tau mRNA was expressed at high levels in nerve cells. Immunoblot analysis on whole murine brain did not reveal equally high tau protein levels, as transgenic human tau accounted for ~10% of the endogenous murine tau levels. However, immunohistochemical analysis showed that human tau was only detected in a small percentage of nerve cells, implying high levels of the transgene product in these cells. Interestingly, as in Alzheimer's disease, prominent somatodendritic staining was observed in addition to axonal staining. Moreover, tau protein was phosphorylated at some of the sites that are hyperphosphorylated in Alzheimer's disease.

Results

Comparison of different promoters

Three different promoters (rhombotin 1, Thy-1 and neuron-specific enolase) were tested so as to achieve high expression levels of transgenic human tau in nerve cells (Seki *et al.*, 1985; Sakimura *et al.*, 1987; Boehm *et al.*, 1990; Greenberg *et al.*, 1990). With construct ALZ3 (using the human rhombotin 1 promoter), eight founders were analysed, seven of which produced offspring harbouring the transgene. With the ALZ7 construct (using the human Thy-1 promoter), six founders were generated, all of which transmitted the transgene. With the ALZ11 construct (using the rat neuron-specific enolase promoter), five founders were generated, two of which were analysed directly for RNA expression; of the remaining three, the transgene was transmitted by two. The eight lines with the rhombotin 1 promoter showed either no detectable expression or only very low levels of expression, when comparing transgenic human with endogenous murine tau mRNA. Similarly, six lines and two founders with the neuron-specific enolase promoter linked to either human tau or an unrelated transgene, showed only low levels of transgenic mRNA. In addition, the murine neurofilament L promoter was tested for the expression of an unrelated cDNA. Of five lines analysed, none showed significant expression levels. By far the best results were obtained with the human Thy-1 promoter expression vector. Further analysis was therefore restricted to lines expressing the 441 amino acid isoform of tau from human brain driven by the human Thy-1 promoter (ALZ7 construct).

RNA blot analysis and in situ hybridization

Five lines with the ALZ7 construct were analysed at 3 months of age and three lines were found to express high levels of transgenic tau mRNA in brain. The transgenic lines expressed a tau band of 2.2 kb in addition to the major murine tau band of ~6 kb, reflecting the expression of mRNA from the ALZ7 construct (Figure 1). The relative levels of transgenic tau mRNA were up to 5-fold higher than those of endogenous murine tau mRNA (Figure 1). RNA blot analysis of brain, kidney, lung, liver, spleen and heart showed that the transgene was only

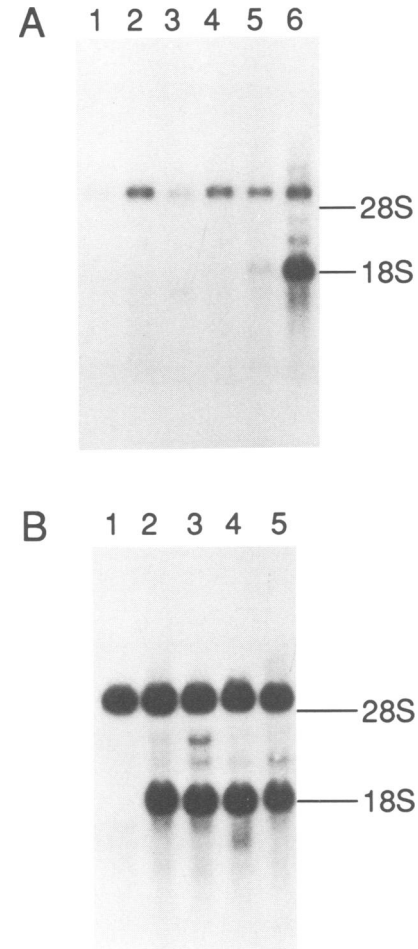


Fig. 1. RNA blot analysis of ALZ7 lines. Total RNA from brain and peripheral tissues of ALZ7 transgenic and control (C57BL/6) mice was analysed by RNA blotting using a human tau probe. The different lanes were as follows. (**A**) Lane 1, C57BL/6 kidney; lane 2, C57BL/6 brain; lane 3, ALZ7–7–47 kidney; lane 4, ALZ7–7–47 brain; lane 5, ALZ7–10–38 brain; lane 6, ALZ7–4–30 brain. (**B**) Lane 1, C57BL/6 brain; lane 2, ALZ7–20–23 brain; lane 3, ALZ7–5–53 brain; lane 4, ALZ7–4–32 cerebellum; lane 5, ALZ7–4–32 cerebrum. (In the numbering of the organs, the first number refers to the transgene, the second to the founder animal of the particular line and the third to the F1 animal derived from the particular founder animal.) The positions of the 28S and 18S rRNA bands are shown.

expressed in brain, with no detectable human tau mRNA in any of the other tissues analysed (Figure 1).

The cellular localization of transgenic tau mRNA was investigated by *in situ* hybridization using an oligonucleotide probe that is specific for human tau mRNA (Figure 2). In hippocampus from transgenic animals, specific hybridization was observed over granule cells of the dentate gyrus and pyramidal cells of the cornu ammonis (CA) layers when the probe in the anti-mRNA sense orientation was used (Figure 2a). No specific hybridization was observed with the probe in the mRNA sense orientation (Figure 2b). Moreover, only background hybridization was observed with the anti-mRNA sense probe in hippocampus and other brain regions from a control mouse (Figure 2c). Specific hybridization was observed throughout the brain of transgenic animals, with some variation in intensity between brain regions and between nerve cells within a given brain region. Representative examples are

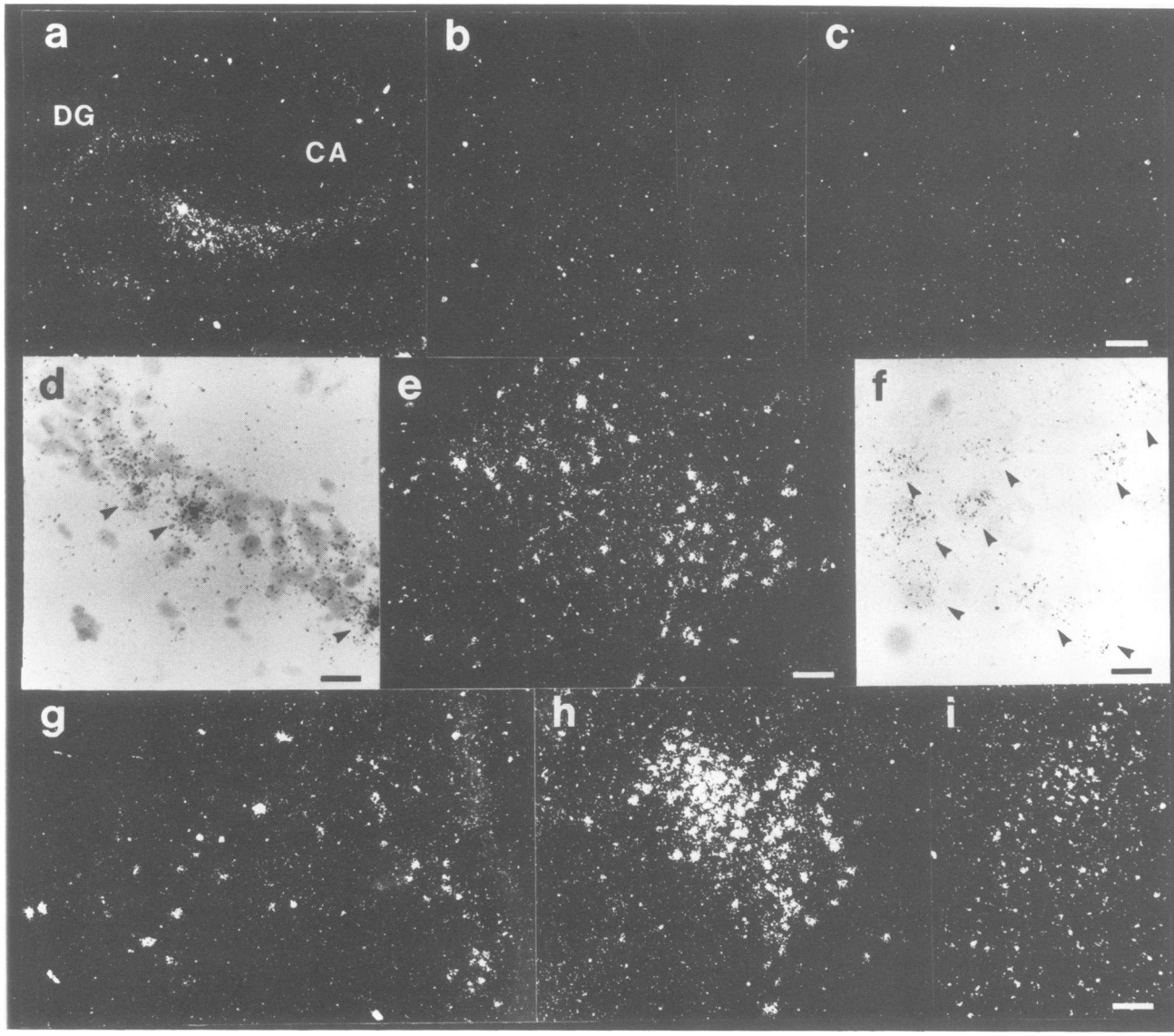


Fig. 2. Cellular localization of human tau mRNA in murine brain from the ALZ7-4 line. (**a** and **b**) Dark-field photomicrographs of hippocampal formation from transgenic murine brain following hybridization with the oligonucleotide probe in the anti-mRNA sense (a) or mRNA sense (b) orientation. (**c**) Dark-field photomicrograph of hippocampal formation from control murine brain following hybridization with the oligonucleotide probe in the anti-mRNA sense orientation. (**d**) Light-field photomicrograph of dentate gyrus from transgenic murine brain following hybridization with the oligonucleotide probe in the anti-mRNA sense orientation. (**e** and **f**) Dark-field (e) and light-field (f) photomicrographs of pontine raphe magnus nucleus from transgenic murine brain following hybridization with the oligonucleotide probe in the anti-mRNA sense orientation. (**g**–**i**) Dark-field photomicrographs of deep cerebellar nuclei (g), hypoglossal nucleus (h) and subiculum (i) from transgenic murine brain with the oligonucleotide probe in the anti-mRNA sense orientation. Arrows indicate strongly hybridizing granule cells of the dentate gyrus in (d) and hybridization-positive nerve cells in the pontine raphe magnus nucleus in (f). DG, dentate gyrus; CA, cornu ammonis. Scale bars: 360 μm in (c) (for a–c); 75 μm in (d); 135 μm in (e) (for e, g and h); 30 μm in (f); 200 μm in (i).

shown in Figure 2d–i. No specific hybridization was seen in white matter.

Immunoblot analysis

Brains from 3 month-old transgenic and control mice were homogenized in 2.5% perchloric acid to determine the relative tau levels by immunoblot analysis. Since native tau protein runs as a smear as a consequence of heterogenous phosphorylation, the different tau isoforms are better visualized as discrete bands following dephosphorylation. Brains from three ALZ7 lines were selected on the basis of high expression of transgenic tau mRNA. They were analysed for tau protein with antibody 134. All three lines showed similar expression levels. The representative immunoblot is displayed in Figure 3. Each extract is shown before and after treatment with alkaline phosphatase. Following alkaline phosphatase treatment, four discrete bands were seen with tau from control brain, presumably reflecting the presence of four tau isoforms in adult murine brain (Figure 3, lane 2). The same four bands were present in tau from transgenic mice following alkaline phosphatase treatment, but another tau band was also seen which ran with a slightly higher apparent molecular mass than the largest tau band from control murine brain (Figure 3, lanes 4, 6, 8 and 10). This additional band corresponds to transgenic human tau. It ran at the same position as the recombinant 441 amino acid isoform of human tau and was the only band immunoreactive with Tau14 (Kosik

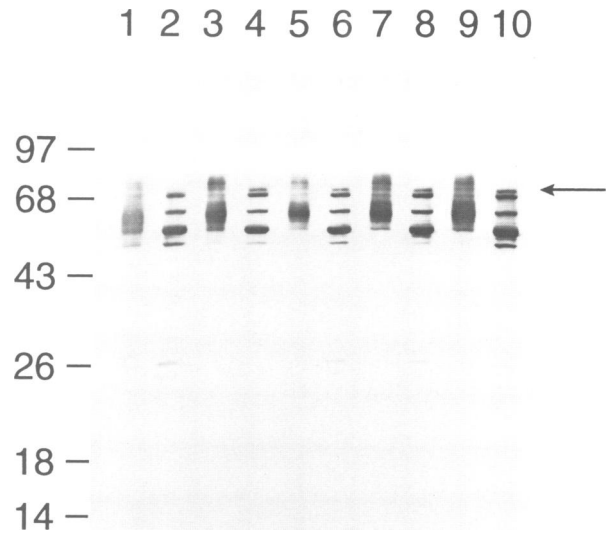


Fig. 3. Immunoblot analysis of ALZ7 lines. Tau protein was extracted from brain of ALZ7 transgenic and control (C57BL/6) mice and analysed by immunoblotting using the tau antiserum 134. Each sample was analysed before and after dephosphorylation with alkaline phosphatase. The different lanes were: lanes 1 and 2, C57BL/6 before and after alkaline phosphatase treatment; lanes 3 and 4, ALZ7–4–102 before and after alkaline phosphatase treatment; lanes 5 and 6, ALZ7–4–31 before and after alkaline phosphatase treatment; lanes 7 and 8, ALZ7–5–53 before and after alkaline phosphatase treatment; lanes 9 and 10, ALZ7–20–123 before and after alkaline phosphatase treatment. (In the above numbering the first number refers to the transgene, the second to the founder animal of the particular line and the third to the F1 animal derived from the particular founder animal.) The $M_r \times 10^{-3}$ are shown to the left.

et al., 1988; Trojanowski *et al.*, 1989), an antibody that distinguishes between human and murine tau (data not shown). The level of human tau relative to murine tau was ~10%.

Immunohistochemistry

The cellular distribution of tau protein was investigated in brain from 3 month-old control and transgenic mice by using phosphorylation-independent (Figures 4 and 5) and phosphorylation-dependent (Figure 6) anti-tau antibodies. In paraffin-embedded horizontal sections from transgenic murine brain, strong staining of the nerve cells and their processes was observed with antibodies N-Tau5 (Figure 4A) and 133. In contrast, N-Tau5 (Figure 4B) and 133 gave only weak neuropil staining in paraffin-embedded horizontal sections from control murine brain.

Strongly labelled nerve cells were observed in most brain regions; however, their numbers were relatively small, accounting for only a few per cent of the total nerve cell population. In cerebral cortex and hippocampus nerve cell bodies, axons and dendrites of individual pyramidal cells were strongly labelled (Figure 5A and C). Other nerve cells showed lower staining intensities, and still others were unlabelled (Figure 5A and C). The

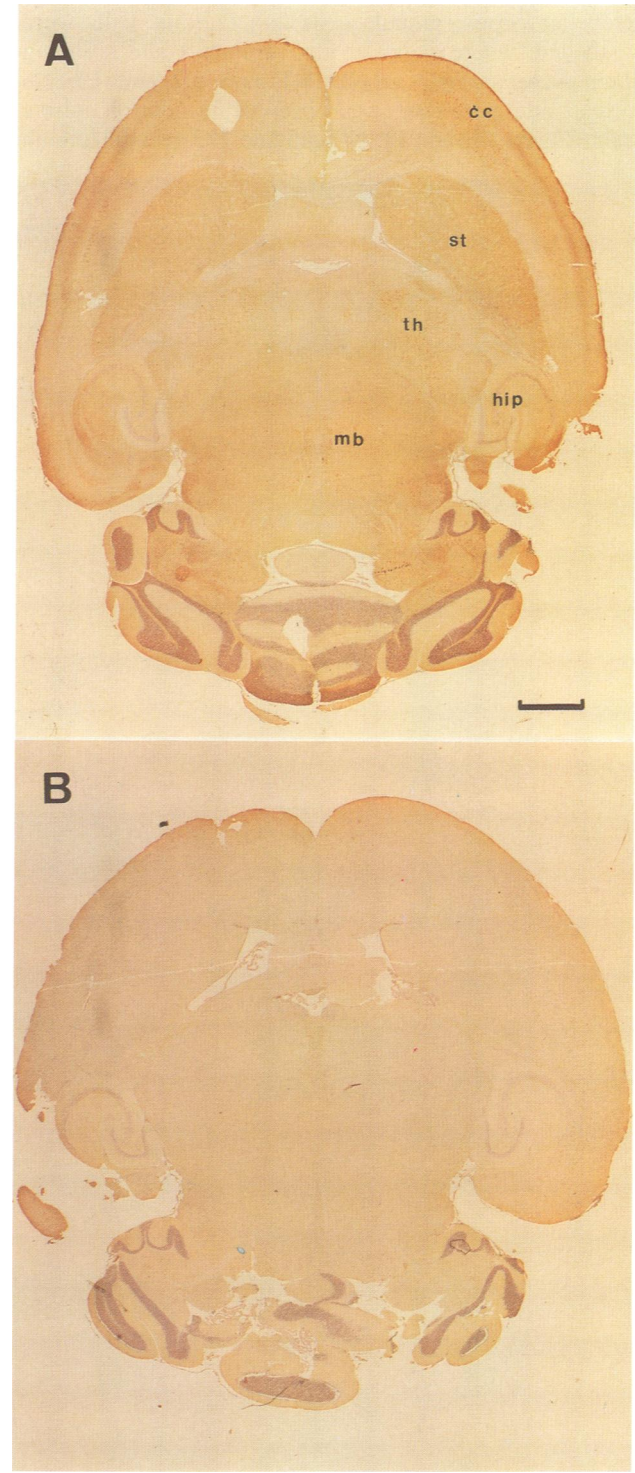
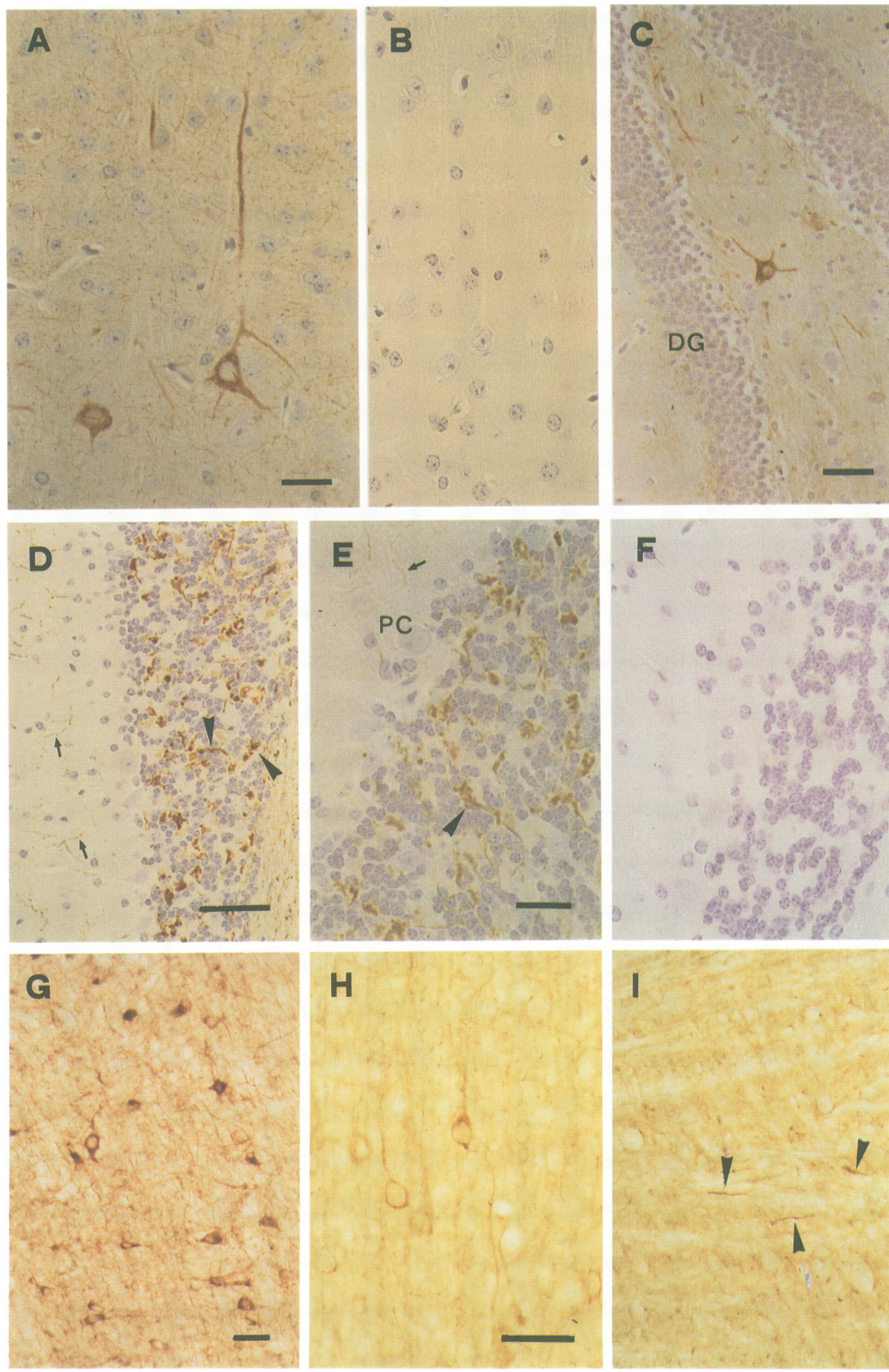


Fig. 4. Low-power view of horizontal brain sections from a 3 month-old transgenic mouse of the ALZ7-4 line (**A**) and an age-matched control mouse (**B**) stained with antibody N-Tau5. The paraffin-embedded sections (4 μm) were counterstained with haematoxylin. At this magnification, diffuse tau immunostaining was present throughout the transgenic murine brain, but not the control brain. Strong labelling is seen in (A) in cerebral cortex (cc), hippocampus (hip), striatum (st), thalamus (th), midbrain (mb) and cerebellum. Scale bar: 1 mm in (A) (for A and B).

staining appeared homogenous or granular, but not fibrillar. In some regions of grey matter only nerve cell axons were strongly labelled for transgenic human tau. Thus, in

cerebellar cortex, granule cells and Purkinje cells were unlabelled, whereas mossy fibre terminals and climbing fibres were strongly labelled (Figure 5D and E). No corresponding staining was observed in control murine brain (Figure 5B and F). N-Tau5 and 133 recognize both murine and human tau. However, an identical staining pattern was observed in transgenic murine brain with antibody Tau14 which only recognizes human tau (Figure 5G), indicating that the observed staining was due to transgenic human tau. In vibratome sections from trans-



genic murine brain that had been processed free-floating strong labelling of nerve cell bodies, dendrites and axons was observed with N-Tau5 and 133 (Figure 5H). In control murine brain, staining was only seen in nerve fibre tracts, reflecting an axonal localization of tau protein (Figure 5I). No staining of nerve cell bodies or dendrites was observed in control murine brain.

When the phosphorylation-dependent anti-tau antibodies PHF1 and AT8 were used on paraffin sections from transgenic murine brain, individual nerve cell bodies and their processes were stained (Figure 6A, C and E). No staining was observed in brain from control mice (Figure 6B, D and F). Fewer nerve cells were stained with PHF1 and AT8 than with N-Tau5, 133 or Tau14. The staining intensity with PHF1 and AT8 was always less than that with anti-tau antibodies that are not phosphorylation-dependent. However, the reactivities with PHF1 and AT8 indicate that a significant fraction of tau from transgenic murine brain was phosphorylated at Ser202 and Thr205, as well as at Ser396 and Ser404. Silver impregnation of brain sections from transgenic mice according to Gallyas (1971) and Holmes (1943) failed to give a specific reaction product. No positive reaction was observed with thioflavin S.

Discussion

An understanding of the pathogenesis of Alzheimer's disease requires the development of experimental animal models which reproduce the neuropathological characteristics of the disease, namely abundant neuritic plaques, neurofibrillary lesions and neuronal cell death within specific brain regions. Such animal models are also required for the testing of compounds that might prevent, arrest or reverse the pathological process. Attempts have been made to reach this goal through the generation of transgenic animals, using constructs that lead to expression of the whole or part of the amyloid precursor protein (Quon *et al.*, 1991; Lamb *et al.*, 1993; Higgins *et al.*, 1994).

The development of neurofibrillary tangles, neuropil threads and dystrophic neurites associated with some amyloid plaques is another major pathological hallmark of Alzheimer's disease. Over recent years it has become clear that microtubule-associated protein tau in a hyperphosphorylated state is the major component of the neurofibrillary lesions (for reviews see Goedert, 1993; Trojanowski *et al.*, 1993). Moreover, candidate protein kinases and protein phosphatases, whose abnormal activity may lead to the hyperphosphorylation of tau, have been identified. However, to date, the development of neurofibrillary lesions has not been specifically addressed in a transgenic animal model. We have taken the first step in that direction by expressing human tau protein in the brain of transgenic mice. In adult human brain tau exists as six isoforms which differ from each other by the presence or absence of three inserts (Goedert *et al.*, 1989). As a transgene, the longest human brain tau isoform, with two inserts in the N-terminal half and four tandem repeats in the C-terminal half, was chosen (cDNA clone hTau40; Goedert *et al.*, 1989). We used the human Thy-1 promoter as the promoter, and the β-globin p(A)/splice cassette as the 3′ element. By RNA blot analysis, expression of human tau mRNA was found in brain but not in a number of peripheral tissues. By *in situ* hybridization, human tau mRNA was found to be present in nerve cells throughout the brain of transgenic mice, including the brain regions that are affected by the neurofibrillary pathology of Alzheimer's disease. However, the mRNA levels were variable between brain regions; moreover, within a given brain region, some nerve cells expressed higher levels of human tau mRNA than others. These differences in expression were present in different lines and probably reflect the activity of the human Thy-1 promoter in mouse.

The levels of human tau protein were analysed in whole brain from transgenic mice by using antiserum 134 which recognizes the C-terminus of tau (Goedert *et al.*, 1989), a sequence identical in all murine and human tau protein isoforms. Following alkaline phosphatase treatment, four discrete tau bands were observed in control murine brain. In brain from transgenic mice, an additional tau band was observed which corresponded to the longest human brain tau isoform. The human tau protein band accounted for ~10% of total tau in transgenic murine brain; similar levels of human tau protein were observed in different transgenic lines. The relative levels of human tau mRNA were always higher than the expected protein levels, implying that more human tau mRNA was being transcribed than translated.

By immunohistochemistry, tau protein staining was not ubiquitously distributed in transgenic murine brain. We have used three different anti-tau antibodies that are not phosphorylation-dependent, two of which (N-Tau5 and 133) recognize both murine and human tau, and one (Tau14) which recognizes only human tau. N-Tau5 and 133 gave strong staining of a small percentage of nerve cells throughout the brain of transgenic mice, with a

Fig. 5. (**A**–**F**) Brain sections from an ALZ7-4 transgenic mouse (A and C–E) and an age-matched control mouse (B and F) stained with antibodies N-Tau5 (A–D) and 133 (E and F). The paraffin-embedded sections (4 μm) were counterstained with haematoxylin. (A) Cerebral cortex: two immunopositive pyramidal neurons. Note the intense and homogenous staining of nerve cell bodies and dendrites; many tau-positive fibres, probably axonal in nature, are seen in the neuropil background. (B) No corresponding staining was observed in cerebral cortex from a control mouse. (C) Hippocampus: pyramidal neuron of ammonian field CA4 showing intensely stained soma and dendrites. N-Tau5 immunoreactivity was also observed in pyramidal nerve cells in other fields of the cornu ammonis, especially CA3. Granule cells in the dentate gyrus (DG) remained unstained. (D and E) Cerebellar cortex: numerous mossy fibre terminals in the granule cell layer (some of them are labelled by an arrowhead) and climbing fibres (arrows) in the molecular layer are labelled with N-Tau5 (D) and 133 (E). Note that granule cells and Purkinje cells (PC) are unlabelled. (F) No corresponding staining was observed in cerebellar cortex from a control mouse. (**G**–**I**) Vibratome brain sections (40 μm) from an ALZ7 transgenic mouse (G and H) and an age-matched control mouse (I) stained with antibodies Tau14 (G) and N-Tau5 (H and I). (G) Nucleus centralis oralis pontis from an ALZ7 mouse. Strong labelling of many nerve cell bodies, dendrites and axons was observed throughout the brain with antibody Tau14 which recognizes human tau but fails to recognize murine tau. The staining pattern with Tau14 was indistinguishable from that observed with N-Tau5 or 133. Only background staining was observed in control murine brain stained with Tau14 (data not shown). (H) Cerebral cortex from an ALZ7 murine brain, showing strong labelling of individual neuronal cell bodies and dendrites with N-Tau5. (I) Subcortical white matter from a control murine brain. Labelling of axons (arrows) with N-Tau5. Nerve cell bodies and dendrites were not stained. Scale bars: 25 μm in (A) (for A and B); 50 μm in (C); 50 μm in (D); 25 μm in (E) (for E and F); 50 μm in (G); 50 μm in (H) (for H and I).

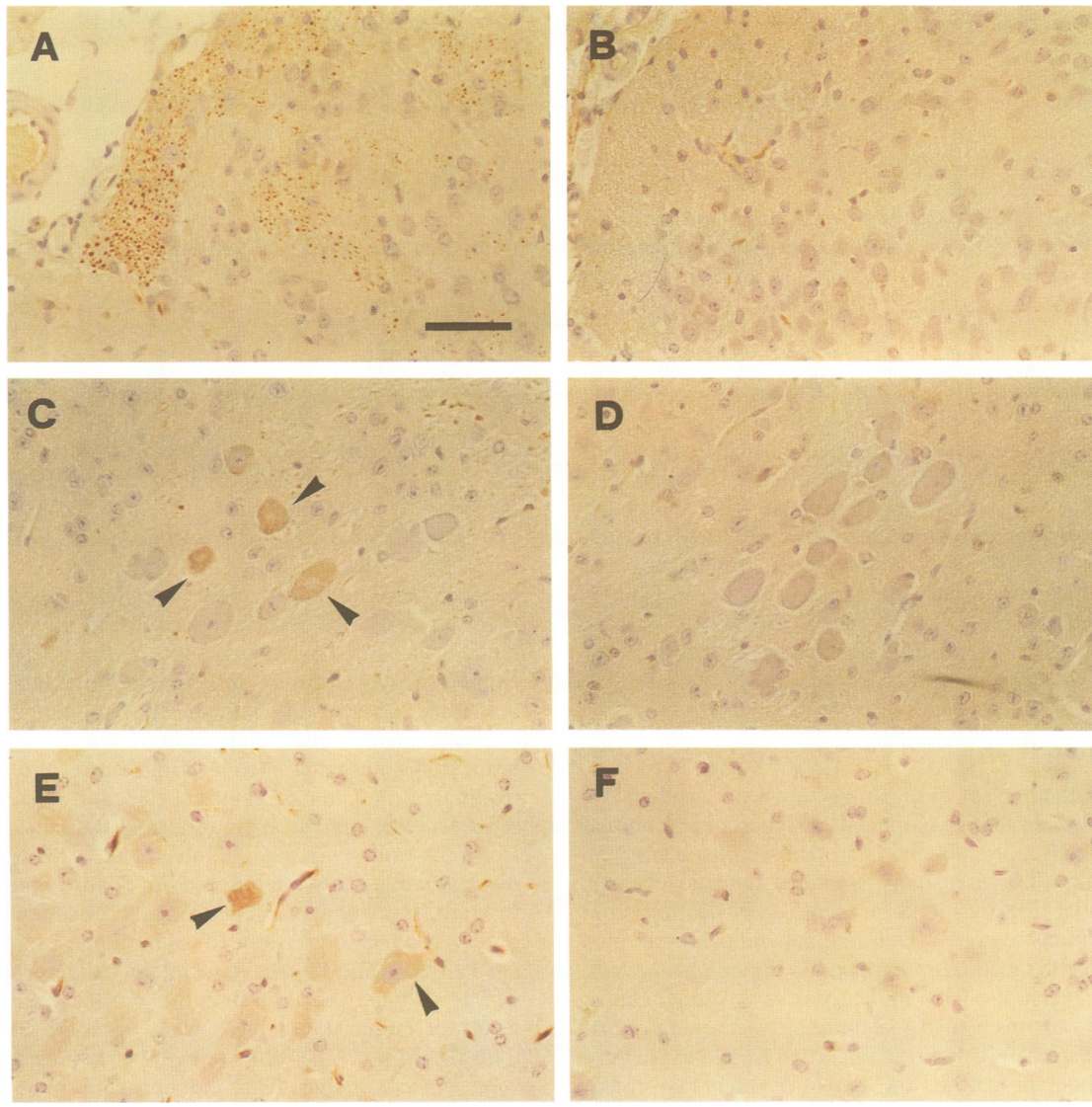


Fig. 6. (**A**–**F**) Brain sections from an ALZ7-4 transgenic mouse (A, C and E) and an age-matched control mouse (B, D and F) stained with phosphorylation-dependent anti-tau antibodies PHF1 (A–D) and AT8 (E and F). The paraffin-embedded sections (4 μm) were counterstained with haematoxylin. Both antibodies stained individual neurons or nerve cell groups only in transgenic murine brain, but in smaller numbers and with lesser intensity than N-Tau5, 133 or Tau14. (A) Nuclei pontis from an ALZ7 murine brain. Intensely PHF1-positive cross-sectioned nerve fibres, probably axons. (B) Lack of PHF1 staining in nuclei pontis from control murine brain. (C) Nucleus of the mesencephalic root of the trigeminal nerve showing some PHF1-positive nerve cell bodies (arrowheads) among unstained neurons. (D) Lack of PHF1 staining in the nucleus of the mesencephalic root of the trigeminal nerve from a control murine brain. (E) Nucleus centralis oralis pontis from an ALZ7 murine brain. Some neurons (arrowheads) show moderate cell body labelling with AT8. (F) No AT8 staining was observed in nucleus centralis oralis pontis from a control mouse. Scale bar: 50 μm in (A) (for A–F).

somewhat larger number of positive nerve cells in subcortical brain structures. However, immunopositive nerve cells were observed in cerebral cortex and hippocampus, two brain regions that are prominently affected by neurofibrillary lesions in Alzheimer's disease. A similar staining pattern was observed in all the mice from two different transgenic lines that were investigated in detail by immunohistochemistry.

At the single-cell level, intense antibody staining was observed not only in axons, but also in nerve cell bodies and dendrites. This contrasts with the predominantly axonal localization of tau in normal adult brain (Binder *et al.*, 1985; Peng *et al.*, 1986). Thus, in vibratome sections from control murine brain we only observed the staining of nerve fibre tracts with N-Tau5 and 133, reflecting an axonal localization of tau. In transgenic murine brain, additional somatodendritic labelling was observed. With antibody Tau14, strong axonal and somatodendritic staining was observed in transgenic murine brain, with only background staining in control murine brain. This demonstrates that the cellular localization of tau observed in transgenic murine brain with N-Tau5, 133 and Tau14 resulted from the staining of transgenic human tau, with no significant contribution by endogenous murine tau. This pattern resembles that observed in Alzheimer's disease, where tau is found in the form of neurofibrillary lesions in cell bodies, axons and dendrites of affected nerve cells. Moreover, it has been suggested that pre-tangle neurons can be distinguished from both normal nerve cells and tangle-bearing nerve cells by virtue of the

fact that they show a somatodendritic localization of tau (Bancher *et al.*, 1989). The staining pattern observed in positive nerve cells from transgenic mice was very similar to that of pre-tangle neurons. It was homogenous or granular, but non-fibrillar. No positive reaction was observed either by silver staining according to Gallyas (1971) or Holmes (1943) or with thioflavin S, indicating that tau was not in the form of PHFs.

The reason for the strong somatodendritic tau staining in some nerve cells from transgenic murine brain is unknown. The most likely explanation is that it resulted from the overproduction of tau. By immunoblotting on whole transgenic murine brain, human tau accounted for ~10% of murine tau; by immunohistochemistry, only a fraction of nerve cells was stained for human tau, implying that in a given tau-positive nerve cell the level of transgenic human tau far exceeded that of endogenous murine tau. The mechanisms that underlie the axonal localization of tau in normal brain are not understood. However, it is conceivable that the overproduction of tau could override these mechanisms and result in its accumulation in the somatodendritic compartment. Although there is no evidence to suggest that tau is being overproduced in the end stages of Alzheimer's disease (Goedert *et al.*, 1989), such a mechanism might operate within neuronal subpopulations in the early stages of the disease. Interestingly, it has been shown that tau protein mRNA levels are elevated in cerebral cortex from Down's syndrome individuals (Oyama *et al.*, 1994). It is well established that most individuals with Down's syndrome develop the neuropathology of Alzheimer's disease.

An additional difference between normal tau and PHF-tau is that the latter is hyperphosphorylated. It is widely believed that hyperphosphorylation of tau precedes PHF assembly and that it is both necessary and sufficient for PHF assembly. We have therefore used the phosphorylation-dependent anti-tau antibodies PHF1 (Greenberg *et al.*, 1992; Otvos *et al.*, 1994) and AT8 (Mercken *et al.*, 1992; Goedert *et al.*, 1993, 1995) on brain sections from control and transgenic mice. Whereas no specific staining was observed in control mice with either antibody, both PHF1 and AT8 labelled a substantial number of nerve cells in transgenic murine brain. This indicates phosphorylation at Ser202 and Thr205, and at Ser396 and Ser404, four of the sites that are hyperphosphorylated in PHF-tau (Lee *et al.*, 1991; Goedert *et al.*, 1993, 1995; Otvos *et al.*, 1994). The labelling with PHF1 and AT8 in transgenic mice was similar to that observed in Alzheimer's disease, where both antibodies stain neurofibrillary lesions (Mercken *et al.*, 1992; Goedert *et al.*, 1993; Schmidt *et al.*, 1994), and was distinct from that seen in control brain, where they give no specific staining.

Nerve cell bodies, axons and dendrites were labelled with PHF1 and AT8 in transgenic murine brain. They represented a subset of the nerve cells stained with N-Tau5, 133 and Tau14, indicating that hyperphosphorylation may have been the consequence rather than the cause of the aberrant localization of tau within immunoreactive nerve cells. The temporal connection between the hyperphosphorylation and somatodendritic localization of tau in the development of neurofibrillary lesions is unknown. A recent study has shown that in nerve cells of human transentorhinal cortex, intense nerve cell staining with AT8 precedes the formation of neurofibrillary lesions, indicating that tau in pre-tangle neurons is hyperphosphorylated (Braak *et al.*, 1994).

Taken together, our results indicate that transgenic mice that express the longest human brain tau isoform under the control of the human Thy-1 promoter show early changes that are associated with the development of the neurofibrillary lesions of Alzheimer's disease. They represent a first step towards the development of an authentic experimental animal model for the intraneuronal pathology of Alzheimer's disease.

Materials and methods

DNA constructs

The cDNA encoding the longest human brain tau isoform (clone hTau40; Goedert *et al.*, 1989) was subcloned into three different expression vectors using the human rhombotin 1, the human Thy-1 and the rat neuron-specific enolase promoters, respectively. A fourth expression vector using the human neurofilament L promoter, together with 7 kb of endogenous sequences (Beaudet *et al.*, 1992), failed to give high transcript levels in an unrelated transgene. It was therefore omitted from our study. The human rhombotin 1 expression vector was generated by subcloning hTau40 cDNA downstream of an 8 kb *Xho*I–*Nru*I fragment promoter element derived from clone pA27 (Boehm *et al.*, 1990) and upstream of a 900 bp simian virus (SV) 40 early region pA/splice cassette as the 3′ control region (ALZ3 transgene construct, according to our nomenclature). The human Thy-1 promoter expression vector was produced by subcloning hTau40 cDNA downstream of a 3.6 kb *Eco*RI fragment promoter element. A Kozak consensus sequence (5′-ACCGCCGCC-3′) had been introduced upstream of the initiator codon of tau (Kozak, 1987). The promoter fragment of human Thy-1 was obtained after the introduction by site-directed mutagenesis of an *Eco*RI site in the context of the initiator codon in an 8.2 kb genomic fragment (Seki *et al.*, 1985). As a 3′ control element, a 1.6 kb *Pst*I–*Bam*HI β-globin pA/splice cassette (Pircher *et al.*, 1989) was used (ALZ7 transgene construct, according to our nomenclature). For the neuron-specific enolase expression vector, hTau40 cDNA was subcloned downstream of a 2.3 kb fragment of the rat neuron-specific enolase promoter (Sakimura *et al.*, 1987) and upstream of a 900 bp SV40 pA/splice cassette used for the human Thy-1 expression construct (ALZ11 transgene construct, according to our nomenclature). All constructs were freed of vector sequences prior to microinjection.

Generation of transgenic mice

Transgenic mice were produced by pronuclear microinjection of B6D2F1×B6D2F1 embryos, as described previously (Hogan *et al.*, 1986). Founders were identified by PCR analysis of lysates from tail biopsies using two different primer pairs. An initial subset of transgenic founders was subsequently analysed by Southern blot analyses. As the Southern blots gave the same results as the PCR, the progeny and additional founders were identified solely by PCR. Founder animals were intercrossed with C57BL/6 animals to establish lines.

RNA blot analysis

Total RNA was isolated from brain and peripheral tissues by the guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1986). The RNA was electrophoresed on a 0.9% formaldehyde gel with 20 μg total RNA per lane. After separation, the RNA was stained with ethidium bromide to visualize the 28S and 18S rRNA bands for normalization, followed by its transfer to a Hybond N^+ nylon membrane. Prehybridization was performed for 1 h at 65°C in 0.2 M NaP buffer, pH 7.2, 35% formamide, 1% bovine serum albumin, 1 mM EDTA and 7% SDS. The randomly primed ^{32}P-labelled, heat-denatured plasmid probe was added to the prehybridization solution and hybridization was performed overnight at 65°C. Blots were washed in 30 mM NaP, 0.1% SDS at 65°C.

In situ hybridization

Two 45mer oligonucleotides in the antisense or the sense orientation were used. The design of the oligonucleotides took advantage of the presence of a stretch of 11 amino acids in human tau (residues 16–26) that is not found in murine tau. The oligonucleotide sequences were:

antisense, 5′-GCCCCCCTGATCTTTCCTGTCCCCCAACCCGTACG-TCCCAGCGTG-3′; and sense, 5′-CACGCTGGGACGTACGGGTTGG-GGGACAGGAAAGATCAGGGGGGC-3′.

The oligonucleotides were tailed with [α-^{35}S]dATP (1300 Ci/mmol; New England Nuclear) and used at 500 000 c.p.m./slide. The brain sections were cut at 15 μm, transferred onto gelatinized glass slides, fixed in 4% paraformaldehyde for 10 min, washed in PBS and kept in 95% ethanol until use. The sections were prehybridized at room temperature for 2 h in 50% formamide hybridization mixture, followed by overnight hybridization at 42°C. Washes were performed in 2× SSC (1× SSC = 150 mM sodium chloride, 15 mM sodium citrate), 0.1% SDS at room temperature for 2 h, with two changes of washing solution, followed by an overnight wash at 42°C in 1× SSC, 0.1% SDS. The slides were dehydrated in 70% ethanol, dried and dipped in photographic emulsion. They were developed after 4 weeks at 4°C.

Immunoblot analysis

Brains from transgenic mice and control mice were dounce homogenized in 2.5% (v/v) perchloric acid (0.5 ml/g), allowed to stand on ice for 20 min and centrifuged for 10 min at 10 000 *g*. The supernatants were dialysed against 50 mM Tris−HCl, pH 7.4, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and used for immunoblot analysis, as described (Goedert and Jakes, 1990). Dephosphorylation of tau with *Escherichia coli* alkaline phosphatase was performed as described (Goedert *et al.*, 1994).

Several anti-tau antibodies were used. Antiserum 134 is directed against the C-terminus of tau (Goedert *et al.*, 1989) and recognizes all six human brain and all four murine brain tau isoform bands. Tau14 is a monoclonal antibody that recognizes residues 141–149 of human tau (in the numbering of the longest human brain tau isoform; Kosik *et al.*, 1988; Trojanowski *et al.*, 1989); it recognizes all human tau isoforms, but fails to recognize murine tau. PHF1 is a monoclonal antibody that recognizes tau in a phosphorylation-dependent manner (Greenberg *et al.*, 1992; Lang *et al.*, 1992); it recognizes human tau isoforms when phosphorylated at Ser residues 396 and 404 (Otvos *et al.*, 1994). AT8 is a monoclonal antibody that recognizes tau in a phosphorylation-dependent manner (Mercken *et al.*, 1992; Goedert *et al.*, 1993); it recognizes human tau isoforms when phosphorylated at Ser202 and Thr205 (Goedert *et al.*, 1995).

Histochemistry and immunohistochemistry

Progeny from two founder lines generated with the ALZ7-4 construct (2–3 months of age) and an equal number of age-matched control mice were analysed histologically. Immunohistochemistry was performed using anti-tau antibodies Tau14, AT8 and PHF1 (see above). In addition, we used antisera 133 and N-Tau5. Antiserum 133 is not phosphorylation-dependent and was raised against N-terminal residues 1–16 of human tau (Goedert *et al.*, 1989). It recognizes all known tau isoforms. Antiserum N-Tau5 is a novel antiserum that was also raised against amino acids 1–16 of human tau (P.Frey, unpublished results). N-Tau5 has characteristics indistinguishable from those of antiserum 133.

Primary antibodies were applied to 4 μm parasaggital, coronal and horizontal sections of formaldehyde immersion-fixed and paraffin-embedded brains of ALZ7 transgenic mice and age-matched controls. In some experiments, brain tissue was fixed in Bouin's solution, followed by paraffin-embedding. Some animals were perfused transcardially with a mixture of 4% paraformaldehyde in saline/sodium potassium phosphate buffer, pH 7.4. Brains of perfused animals were cut sagitally on a vibratome without prior embedding and the 40 μm sections were processed free-floating. Paraffin-embedded sections (4 μm) of the medial temporal lobe, including the hippocampus, from a normal human brain (male, 60 years old) and from a patient with a histologically confirmed diagnosis of Alzheimer's disease (male, 78 years old) were immunostained in parallel with the murine brain sections. Sections were treated with normal goat serum (prior to using polyclonal antibodies) or normal horse serum (prior to using monoclonal antibodies) to block non-specific sites. This was followed by an overnight incubation with the primary antibody at 4°C. Staining was revealed using the ABC method, with the appropriate Vectastain kit (Vector Laboratories, Peterborough, UK). Paraffin-embedded sections were weakly counterstained with haematoxylin. Specificity of staining was confirmed by concurrent processing of tissue sections where the primary antibody had been omitted or preadsorption of the primary antibodies with recombinant human tau protein. To enhance staining, some sections were treated with citrate buffer at 97°C (Catoretti *et al.*, 1993). Parasaggital paraffin-embedded tissue sections were also used for silver impregnation according to Gallyas (1971), which specifically stains the neurofibrillary lesions of Alzheimer's disease, and Holmes (1943), which is also used for identifying neurofibrillary lesions. Tissue sections were also stained with thioflavin S.

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