

Coexpression of Human cdk5 and Its Activator p35 with Human Protein Tau in Neurons in Brain of Triple Transgenic Mice

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The potential contribution of cyclin-dependent protein kinase 5 (cdk5) to hyperphosphorylate protein tau, as claimed in Alzheimer's disease, was investigated *in vivo*. We generated single, double, and triple transgenic mice that coexpress human cdk5 and its activator p35 as well as human protein tau in cerebral neurons. Whereas expression and increased cdk5-kinase activity was obtained, as measured *in vitro* and demonstrated *in vivo*, neither murine nor human protein tau was appreciably phosphorylated in the brain of double and triple transgenic mice. These mice behaved and reproduced normally. Silver impregnation and immunohistochemistry of brain sections demonstrated that neurofilament proteins became redistributed in apical dendrites of cortical neurons. This suggested a cytoskeletal effect, but no other relevant brain pathology became apparent. These observations indicate that cdk5/p35 is not a major protein tau kinase and that cdk5/p35 did not cause neurodegeneration in mouse brain, as opposed to cdk5/p25. © 2000 Academic Press

INTRODUCTION

Major lesions in the brain of patients suffering from Alzheimer's disease (AD) are the neurofibrillary tangles (NFT) composed of paired helical filaments (PHF). PHF consist of aggregated protein tau, a microtubule-associated protein, which is hyperphosphorylated relative to normal, cellular protein tau. PHF tau contains six to eight phosphate groups as opposed to an average of only about two per normal molecule of tau (Ksiezak-Reding *et al.*, 1992). At least 20 serine or threonine residues are potential phosphorylation sites in protein tau, and about half are of the serine/threonine-proline motif. Proline-dependent kinases were therefore proposed as putative protein tau kinases, including cyclin-dependent kinase 5 (cdk5) (Baumann *et al.*, 1993; Ishiguro *et al.*, 1992) among others like glycogen synthase kinases 3 α and β

(GSK-3 α and - β) (Ishiguro *et al.*, 1992), mitogen-activated protein kinase (MAP kinase), cell division cycle 2 kinase (cdc2 kinase) (Ledesma *et al.*, 1992), and cyclin-dependent kinase 2 (cdk2) (Baumann *et al.*, 1993; Ishiguro *et al.*, 1992). Although all have been demonstrated to phosphorylate protein tau *in vitro*, cdk5 and GSK-3 β in particular have attracted attention.

In mammalian brain, including human, cdk5 mRNA is actively transcribed (Hellmich *et al.*, 1992; Ino *et al.*, 1994; Lew *et al.*, 1994), predominantly in differentiated postmitotic neurons in brain, spinal cord, and peripheral ganglia (Ino *et al.*, 1994; Hellmich *et al.*, 1992; Gervasi and Szaro, 1995). The enzymatic activity of cdk5 is regulated by different activators, i.e., p39^{ncdk5ai} (Tang *et al.*, 1995), p67 (Shetty *et al.*, 1995), and p35 and its proteolytic derivatives (Lee *et al.*, 1996). p35 is exclusively expressed in neurons (Lew *et al.*, 1994; Tomizawa *et al.*, 1996), appears first in young migrating postmitotic neurons, and remains abundant only in areas of adult brain characterized by high neuronal plasticity (Delalle *et al.*, 1997). The evidence

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for the functional importance of cdk5 in brain development and maintenance is compelling. In mice lacking cdk5 (Gilmore *et al.*, 1998) or p35 (Chae *et al.*, 1997) the normal inside-out neurogenic gradient of the cerebral cortex is inverted. Increased cdk5/p35 activity in cortical neurons augmented neuritic outgrowth *in vivo* (Nikolic *et al.*, 1996).

Neurofilament proteins as well as microtubule-associated proteins (MAP) are substrates for cdk5, and cdk5-dependent phosphorylation may regulate their respective functions (Miyasaka *et al.*, 1993). In isolated systems, cdk5 phosphorylates the microtubule-associated protein tau on residues that are also phosphorylated in PHF tau (Ishiguro *et al.*, 1992; Paudel *et al.*, 1993; Baumann *et al.*, 1993). In transfected COS cells, cdk5-activator p23 increased phosphorylation of human protein tau (Michel *et al.*, 1998). Interestingly, cdk5 immunoreactivity was more prominent in pre-tangle neurons and in neurons bearing early stages of NFT in AD brain, indicating a role for cdk5 at a relative early stage (Pei *et al.*, 1998). A direct association of cdk5 with NFT (Yamaguchi *et al.*, 1996) and increased cdk-5 activity were described in brain of AD patients (Lee *et al.*, 1999) due to increased generation of p25 (Patrick *et al.*, 1999).

To investigate the effect of increased cdk5/p35 activity on phosphorylation of protein tau in neurons *in vivo*, we have generated double transgenic mice overexpressing cdk5 and p35 in the same neurons. The constructs were based on the engineered mouse thy-1 gene promoter to specifically direct expression of the transgene to neurons in the central nervous system (Moechars *et al.*, 1996, 1999; Spittaels *et al.*, 1999; Tesseur *et al.*, 2000). Since human protein tau is more extensively characterized than mouse protein tau, and in addition is expected to be the more relevant and suitable substrate, we have generated "triple" transgenic mice that coexpress human cdk5 and its activator p35, together with human protein tau in the same neurons. Despite this extensive effort, the evident increase in cdk5 kinase activity did not cause an appreciable hyperphosphorylation of murine or human protein tau. By silver impregnation and immunohistochemistry, neurofilaments and neurofilament proteins were redistributed in apical dendrites of cortical neurons, suggesting a mild cytoskeletal defect. The current observations do not, however, support a direct role for cdk5/p35 as a major protein tau kinase in transgenic mouse brain *in vivo*.

MATERIALS AND METHODS

Constructs and Transgenic Mice

Human cdk5 cDNA (gift from I. Hoffmann, Heidelberg, Germany) and p35 cDNA (gift from L. H. Tsai, Boston, MA) were ligated into the engineered mouse thy-1 gene construct, essentially as described for similar constructs (Moechars *et al.*, 1996, 1999; Spittaels *et al.*, 1999; Tesseur *et al.*, 2000). The linearized *PvuI*-*NotI* restriction fragment of the construct was isolated and microinjected into 0.5-day-old mouse pre-nuclear embryos from the FVB/N strain as described (Hogan *et al.*, 1994; Moechars *et al.*, 1996, 1999; Spittaels *et al.*, 1999; Tesseur *et al.*, 2000). Southern blotting of *StuI*-digested mouse tail biopt DNA identified transgenic founders. The probes used were obtained by PCR: for cdk5 the forward primer was 5'-CCCCACCACAGAATCCA-3' and the reverse primer was 5'-TAACAGCGGACGGGAATC-3'; for p35 the forward primer was 5'-CCCCACCACAGAATCCA-3' and the reverse primer was 5'-CAAGGTCCCCGTTTCTCC-3'. The cDNA probes were radioactively labeled with [α -³²P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA). The nylon membranes were prehybridized, hybridized, washed, and exposed to autoradiographic film as described (Umans *et al.*, 1994). Human tau40 transgenic mice were generated in a similar manner, as described (Spittaels *et al.*, 1999). Routine genotyping of offspring of founders carrying human cdk5, p35, and tau40 was performed on tail-biopt DNA by PCR.

Northern Blotting of Mouse Brain RNA Extracts

Total RNA was isolated from brain from wild-type and transgenic mice (TRIzol reagent) according to the manufacturer's instructions (Life Technologies, Rockville, MD). Twenty micrograms of total RNA was fractionated by electrophoresis in a 1% formaldehyde agarose gel and transferred to Hybond-N membranes (Amersham, UK). Blots were hybridized with the appropriate probes as described (Lorent *et al.*, 1995). The human tau40 probe was generated by PCR with forward primer 5'-ACCCCATCCCTACCAACA-3' and reverse primer 5'-GCAGGCGGCTCTTACTAG-3'. The cdk5 and p35 cDNA probes were described above.

Western Blotting of Mouse Brain Protein Extracts

Brain tissue from wild-type and cdk5 transgenic mice was homogenized in ice-cold, freshly made

buffer containing 10 mM Tris (pH 7.4), 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 200 μ M Na_3VO_4 , 0.5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 0.7 μ g/ml pepstatin, 1 mM PMSF, 1% Triton-X-100 (Van Lint *et al.*, 1993). The homogenate was centrifuged at 100,000g for 35 min at 4°C and the supernatant was stored at -70°C after protein concentration was determined (Bio-Rad detergent compatible protein assay, Hercules, CA). Aliquots corresponding to 20 μ g of protein were denatured, reduced, separated on 4–20% Tris-glycine-buffered polyacrylamide gels (Novex, Frankfurt, Germany), and transferred to nitrocellulose membrane (Hybond ECL, Amersham) as described (Spittaels *et al.*, 1999; Tesseur *et al.*, 2000). The membranes were incubated with a polyclonal antibody to cdk5 (H-291 at 67 ng/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) and further developed for ECL detection after incubation with horseradish peroxidase-conjugated secondary antibody, as described (Spittaels *et al.*, 1999).

Extracts from prefrontal cortex of human brain and whole brain from wild-type and p35 transgenic mice were homogenized in ice-cold buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin, 5 mM NaF, 5 mM Na_3VO_4 , and 100 μ g/ml PMSF (Patrick *et al.*, 1999). The homogenates were cleared by centrifugation at 13,000g for 30 min at 4°C and supernatants were stored at -70°C. Aliquots equivalent to 25 μ g of protein were denatured, reduced, separated on 4–20% Tris-glycine-buffered polyacrylamide gel, and transferred to nitrocellulose membrane. Membranes were treated and incubated as described, with a polyclonal antibody to p35 (C-19 at 400 ng/ml) (Santa Cruz Biotechnology) as indicated.

For Western blotting of protein tau, mouse brain was homogenized in ice-cold buffer containing detergents, a cocktail of proteinase inhibitors as well as phosphatase inhibitors, i.e., 0.1 M MES (pH 6.4), 0.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, 200 μ M PMSF, 20 mM NaF, 200 μ M Na_3VO_4 , 1 μ M okadaic acid, 5 μ g/ml soybean trypsin inhibitor, 1% Triton X-100, 1% sodium desoxycholate, and 0.1% SDS. After centrifugation at 100,000g for 30 min at 4°C, supernatants were stored at -70°C. Prior to electrophoresis, supernatants were precleared by incubation with immobilized protein G (Pierce, Rockford, IL) for 2.5 h at 4°C to eliminate endogenous immunoglobulins present in the brain extracts. After centrifugation, aliquots of the supernatants were denatured, reduced, separated on 8%

Tris-glycine-buffered polyacrylamide gels, and transferred to nitrocellulose membranes. These were incubated with one of the following monoclonal antibodies: TAU-5 (500 ng/ml) (Pharmingen, San Diego, CA), AT-8 (2 μ g/ml) (Mercken *et al.*, 1992; Biernat *et al.*, 1992), AT-180 (1 μ g/ml) (Goedert *et al.*, 1994) (Innogenetics, Gent, Belgium), or PHF-1 (1/200) (Otvos *et al.*, 1994) (gift from P. Davies, New York, NY).

Immunoprecipitation and *in Vitro* Kinase Assay

In vitro phosphorylation of histone H1 (Tsai *et al.*, 1993) was performed as follows: mouse brain was homogenized in 3 ml ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet-P40, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin. After centrifugation (100,000g, 35 min, 4°C) the supernatants were stored at -70°C. Before the subsequent immunoprecipitation of cdk5, brain extracts were precleared by protein G-agarose beads (Pierce). Another aliquot of protein G-agarose beads was mixed with 10 μ l of the polyclonal antibody to cdk5 (C-8) (Santa Cruz Biotechnology) before addition of aliquots of the precleared supernatant supplemented with 5% bovine serum albumin. After mixing for 2.5 h at 4°C, and centrifugation (16,000g, 20 min, 4°C), the immunoprecipitates were washed three times with lysis buffer and once with kinase buffer, i.e., 50 mM Hepes (pH 7.0), 10 mM MgCl_2 , 1 mM DTT, and 4 μ M ATP. Pellets were resuspended in 50 μ l kinase buffer containing 6 μ g histone H1 and 5 μ Ci of [γ - 32 P]ATP (NEN-Dupont, Boston, MA). After incubation at 37°C for 20 min, the reaction was stopped by addition of 50 μ l of Laemmli sample buffer (all reagents at double concentration), and the mixture was boiled for 10 min. The samples were analyzed by SDS-PAGE on 4–20% gradient gels (Novex) and radiolabeled histone H1 was measured by autoradiography.

Histochemistry and Immunohistochemistry

Immunohistochemical analysis was performed on sections of paraffin-embedded brain tissue (6 μ m) or on vibratome sections (40 μ m). Mice were anesthetized with nembutal (Abbott, Abbott Park, IL) and transcardially perfused with ice-cold saline for 3 min and with either paraformaldehyde (4% in PBS) or methacarn, i.e., 60% methanol, 30% chloroform, and 10% acetic acid, for 12 min. Mouse heads were postfixed for 20 h by immersion in the same fixatives prior to removal of the

brain from the skull. Coronal sections from paraffin-embedded brain were deparaffinated in xylol, rehydrated, treated with 1.7% H₂O₂, and incubated for 1 h in blocking buffer, containing 10% normal goat serum and 3% bovine serum albumin, 0.15 M sodium chloride in 10 mM Tris (pH 7.5). Primary antibodies were diluted in the same blocking solution and sections were incubated overnight at room temperature. After the sections were washed with TBS, biotinylated secondary antibody diluted in blocking solution was applied for 1 h before reactions were revealed with the streptABComplex/HRP system (DAKO, Glostrup, Denmark) with 3,3'-diaminobenzidine as chromogen. A similar protocol was used for immunohistochemistry on free-floating vibratome sections.

The polyclonal antibodies H-291 (3 µg/ml) and N-20 (5 µg/ml) (Santa Cruz Biotechnology) were used to detect cdk5 and p35, respectively, in mouse brain sections. Monoclonal antibodies Tau-1 (1 µg/ml) (Biernat *et al.*, 1992) (Boehringer Mannheim, Mannheim, Germany), AT-180 (1 µg/ml), and AD-2 (1 µg/ml) (Buée-Scherrer *et al.*, 1996) (gift from B. Pau, Lille, France) were used to determine the phosphorylation state of protein tau on mouse brain sections. Monoclonal antibody Tau-1, which has a specificity that is "complementary" to that of AT-8 in terms of phosphorylation of their epitope (Biernat *et al.*, 1992; Mercken *et al.*, 1992), and AD-2, recognizing the same epitope as PHF-1 (Buée-Scherrer *et al.*, 1996; Otvos *et al.*, 1994), were preferred since they yielded the clearest staining patterns of all antibodies tested on mouse brain sections. Monoclonal antibodies to neurofilament protein NF-200 (Sigma, St. Louis, MO) are phosphorylation-independent, while SMI-31 and SMI-32 (Sternberger, Baltimore, MD) are phosphorylation-dependent.

Garvey silver staining was performed on deparaffinized sections. Briefly, sections were immersed in 20% AgNO₃ at 37°C, washed several times, developed, washed again, incubated in 0.2% AuCl₂ and 5% Na₂S₂O₃, washed, and mounted with dexex.

RESULTS

Generation of Transgenic Mice and Analysis of Expression

Human cdk5 and p35 transgenic founder mice were generated in the FVB genetic background by our standard methods (Moechars *et al.*, 1996, 1999; Spittaels *et al.*, 1999; Tesseur *et al.*, 2000) and offspring of distinct lines were analyzed for expression (see following).

High-expressing lines were selected for breeding and experiments. All behaved normally, remained healthy, and were breeding normally. Cdk5 and p35 transgenic mice were crossed to generate double transgenic mice, even to double homozygous for both transgenes. Finally, the double transgenic mice were further crossed with the tau transgenic mice (Spittaels *et al.*, 1999) to yield triple cdk5/p35/tau40 transgenic mice, heterozygous for each of the transgenes. In addition to routine PCR, selected mice from each generation were analyzed by elaborate Southern blotting, i.e., hybridization with three probes consecutively on the same blots, to define unequivocally the presence of the concerned transgenes and to demonstrate correct transmission of the integrated transgene with the original restriction pattern of the respective transgenic founder mice (results not shown).

Northern blotting of total brain RNA was used to demonstrate all the relevant transgenic mRNA. The cdk5 transgene yielded a specific transcript of 2.3 kb absent in nontransgenic mice (Fig. 1A) as expected, i.e., 876 bp of the cdk5 cDNA and approximately 1.5 kb of the mouse thy-1 gene expression cassette. The endogenous 1.6-kb murine cdk5 mRNA (Ino *et al.*, 1994) was visible after longer exposure (result not shown). The p35 transgene-specific mRNA was approximately 2.5 kb, containing 924 bp of the p35 cDNA with about 1.5 kb of the mouse thy-1 gene cassette. Endogenous p35 mRNA was visible at around 4.4 kb (Fig. 1B). Blots holding total RNA from wild-type and thy-1 tau40 transgenic mice revealed a 2.8-kb human tau-specific transgenic mRNA (Fig. 1C).

The transgenic human proteins were demonstrated to be expressed in brain tissue of the respective heterozygous transgenic mice by Western blotting of brain homogenates. In the cdk5 transgenic mice, at least 10-fold overexpression of the 31-kDa human cdk5 protein relative to the endogenous protein was evident (Fig. 1D). In brain of p35 transgenic mice, the p35 protein level was estimated to be 50% higher than endogenous mouse p35 (Fig. 1E). As demonstrated previously, brain of tau transgenic mice contained human protein tau (64 kDa) in addition to murine protein tau (50–55 kDa) (Fig. 1F) with an overexpression ratio of about 3 in heterozygous mice (Spittaels *et al.*, 1999).

Immunohistochemical Localization of the Transgenes

The cellular expression of the transgenes was strictly neuronal but widespread throughout the brain

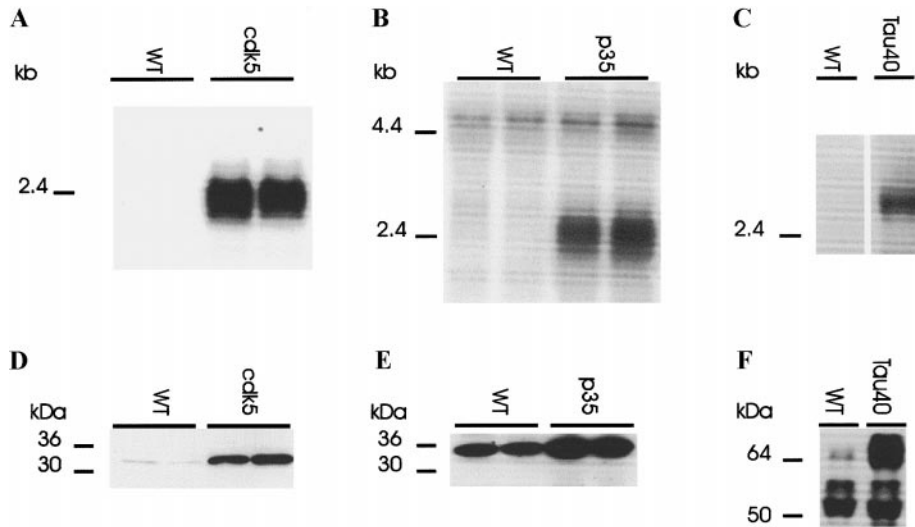


FIG. 1. Analysis of expression of the human transgenes in brain of single transgenic mice. (A–C) Northern blots of total brain RNA. (D–F) Western blots of brain protein extracts. Extracts were derived from wild-type (WT) and transgenic mice as indicated. The apparent mRNA size, based on radioactively labeled markers, is indicated in kilobases. The apparent size of the proteins, based on prestained markers, is indicated in kilodaltons.

of all transgenic mice analyzed. The restriction to neurons was documented by immunohistochemical staining as in previous studies (Spittaels *et al.*, 1999; Tesseur *et al.*, 2000). The H-291 polyclonal antibody to cdk5 specifically stained cell bodies and apical dendrites of neurons in the neocortex, especially in the pyramidal neurons of layer 5 (Fig. 2B), in hippocampal neurons, and in neurons of the thalamus of cdk5 transgenic mice. The N-20 polyclonal antibody to p35 reacted with neurons in the cerebral cortex (Fig. 2D), in the hippocampus, and in the thalamus of p35 transgenic animals. Absent or weak staining was seen in the comparative brain regions of nontransgenic mice (Figs. 2A and 2C). With several distinct monoclonal antibodies directed to protein tau, we confirmed the soma-to-dendritic localization of human protein tau in neurons of the hippocampus and cortex in the tau transgenic mice (Spittaels *et al.*, 1999) (results not shown).

In Vitro Kinase Activity

Since the human cdk5 and p35 proteins were clearly present in neurons in the brain of the respective transgenic mice, we undertook a semiquantitative determination of the enzymatic activity of cdk5, following immunoprecipitation from extracts of brain from wild-type and cdk5 and p35 transgenic mice. The *in*

vitro phosphorylation of histone H1 by immunoprecipitated cdk5 was increased in both cdk5 and p35 transgenic mice in comparison with brain of nontransgenic mice (Fig. 3). This assay proved to be subject to variation in our hands and could not be used to define more exactly the increase in cdk5 enzymatic activity.

Analysis of tau Phosphorylation in Brain of Transgenic Mice

We analyzed the brain from double homozygous cdk5/p35 transgenic mice aged up to 14 months and from triple heterozygous cdk5/p35/htau40 transgenic mice aged up to 6 months. Western blotting with three different anti-tau antibodies, i.e., AT-8, AT-180, and PHF-1, all recognizing phosphorylated epitopes, failed to reveal any extra reaction in transgenic mouse brain compared to wild-type and thy-1 tau40 mice (Fig. 4). Monoclonal antibody TAU-5 is phosphorylation-independent and was used to normalize for the amount of protein tau loaded on the gels, but failed to reveal any change in electrophoretic migration of protein tau. Taken together, these observations indicated that the cdk5/p35 kinase failed to phosphorylate murine endogenous as well as human transgenic protein tau in brain of double and triple transgenic animals, respectively.

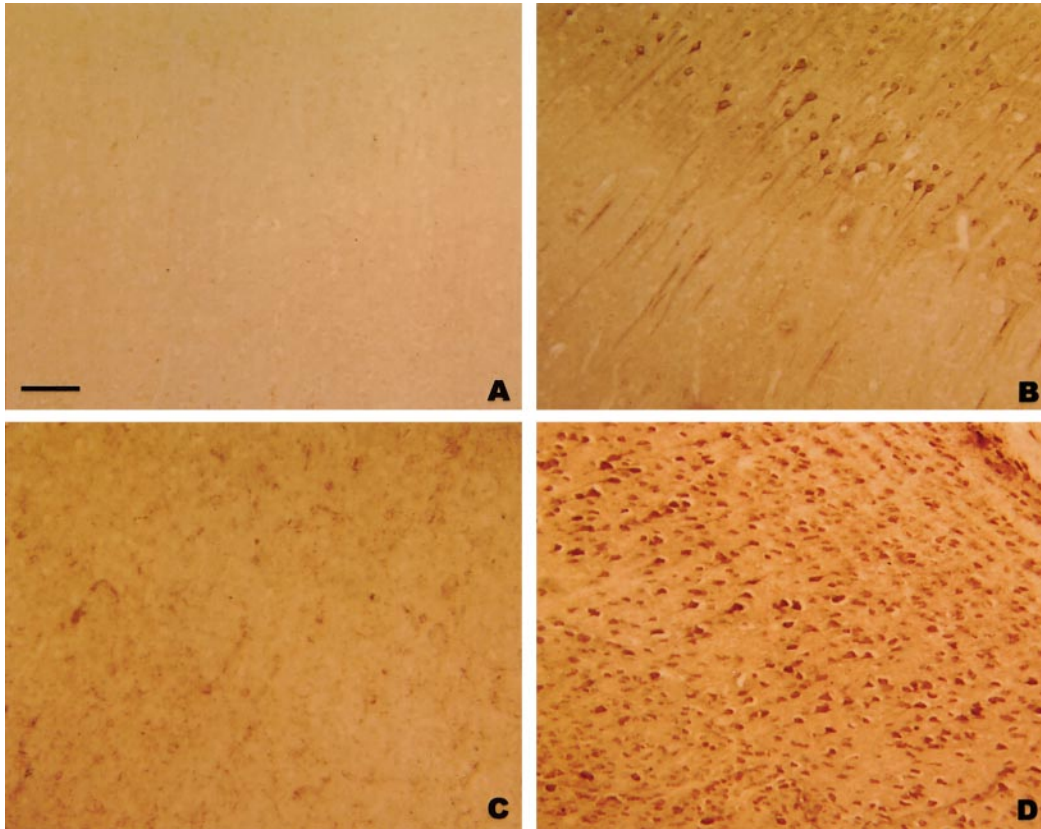


FIG. 2. Immunohistochemical localization of the human transgenic proteins in brain of transgenic mice. Demonstration of cdk5 in neurons in the neocortex of WT (A) and cdk5 transgenic mice (B). Demonstration of p35 in neurons in the neocortex of WT (C) and p35 transgenic mice (D). Scale bar, 50 μ m.

Immunohistochemical localization of protein tau in brain of triple cdk5/p35/htau40 transgenic mice aged 14 months was compared to that in age-matched single tau transgenic mice. No differences in staining pattern were obvious with monoclonal antibodies Tau-1, AT-180, AD-2 (Fig. 5), and AT-8 (not shown).

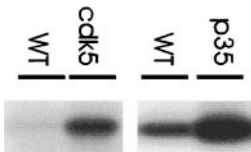


FIG. 3. Kinase activity of immunoprecipitated cdk5 toward histone H1. Phosphorylation of histone H1 by cdk5, immunoprecipitated from brain of cdk5 and p35 transgenic mice as indicated and from nontransgenic wild-type mice (WT). The autoradiographs shown are different, representative experiments with different exposures and do not allow a direct comparison of the intensity of the radiolabeled histone as a measure of the cdk5 kinase activity. See text for more details and interpretation.

Neurons in the neocortex and in the hippocampus reacted in single as well as in triple transgenic mice. Antibody Tau-1, which recognizes a nonphosphorylated epitope, clearly stained neurons in the hippocampus and pyramidal neurons in the cortex, in both single and triple transgenic mice (Figs. 5A and 5B). Antibody AT-180, recognizing a PHF-specific, phosphorylated epitope, stained these neurons in hippocampus and cortex less clearly (Figs. 5C and 5D). Antibody AD-2 also recognizes a phosphorylated epitope and stained only some neurons in the cortex in the single and triple transgenic mice (Figs. 5E and 5F). All antibodies also reacted with neurons in the thalamus (results not shown).

No major qualitative or quantitative differences became apparent with any of these antibodies on sections of brain of different transgenic mice of 14 months, by judging cell type or regional number of cells that reacted positively. Even when we took into account the subcellular (re-)localization of the human

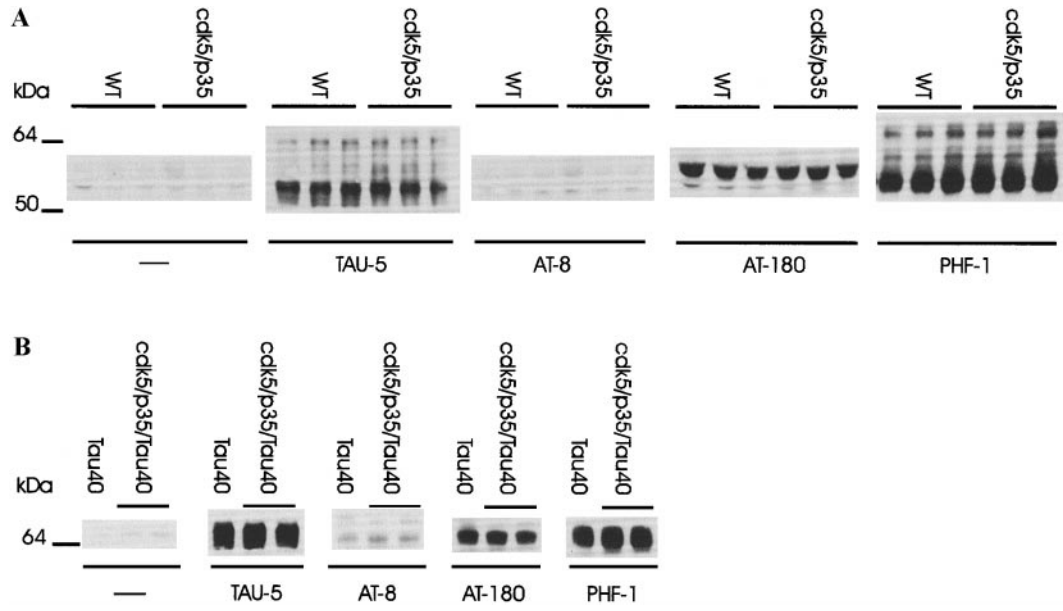


FIG. 4. Analysis of tau phosphorylation in brain of transgenic mice by Western blotting. Western blotting of mouse brain extracts with monoclonal antibodies TAU-5, AT-8, AT-180, and PHF-1 as indicated or without first antibody (–) as control of nonspecific reaction of the secondary antibody. (A) cdk5/p35 double transgenic mice as indicated compared to nontransgenic mice (WT); in each instance three distinct mice were analyzed. (B) cdk5/p35/tau40 triple transgenic mice were compared to single tau transgenic mice that were extensively characterized previously (Spittaels *et al.*, 1999). Only human protein tau is shown. The signal obtained with the phosphorylation-independent antibody TAU-5 was used to normalize the quantitation of the reaction with the phosphorylation-dependent antibodies AT-8, AT-180, and PHF-1. The apparent size of prestained molecular weight markers is indicated at the left in kilodaltons.

protein tau, which depends on the level of phosphorylation, differences were not found, indicating that the phosphorylation state of the protein was not altered in the triple transgenic mice, compared to that in single protein tau transgenic mice.

Histochemistry, Immunohistochemistry, and Western Blotting

Garvey silver staining was performed on paraffin-embedded sections of brain from 20-month-old single and double transgenic mice. A consistent increase in silver staining of the apical dendrites of pyramidal neurons in the cortex of cdk5/p35 double transgenic mice was evident (Figs. 6A and 6B), which was, to a lesser extent, also noted in single cdk5 and p35 transgenic mice (results not shown). Since neurofilament proteins are argyrophilic and known substrates for cdk5, additional immunohistological analysis was performed with antibodies specific for neurofilaments. More prominent dendritic staining of neurofilaments became evident with antibodies NF-200 (Figs. 6C and 6D) and SMI-31 and SMI-32 (results not shown) in the

neocortex of cdk5, p35, and double cdk5/p35 transgenic mice. In particular, the apical dendrites of the pyramidal neurons of cortical layer V were more immunoreactive in the transgenic mice than in nontransgenic littermates. This immunoreactivity paralleled the increased argyrophilic reactivity of these apical dendrites, indicating that cdk5 exerted activity in these neurons of the transgenic mice *in vivo*.

In contrast with p25 overexpressing mice recently reported (Ahlijanian *et al.*, 2000), no argyrophilic cell bodies or axons were observed in the cdk5/p35 transgenic mice. To determine the cause of this difference, we analyzed by Western blotting with antibody C-19 the presence of p25 next to p35 in the brain of the transgenic mice. Human brain samples were analyzed in parallel, allowing the unequivocal demonstration of the presence of the 25-kDa proteolytic derivative of the p35 protein (Fig. 7). Some p25 was evident in the brain extracts of p35 transgenic mice, but was virtually absent in brain of nontransgenic mice (Fig. 7). A marked difference in levels and in the ratio of p25/p35 was estimated to be at least 2 orders of magnitude lower in mouse brain relative to human brain samples. Only very long exposure of the Western blots revealed

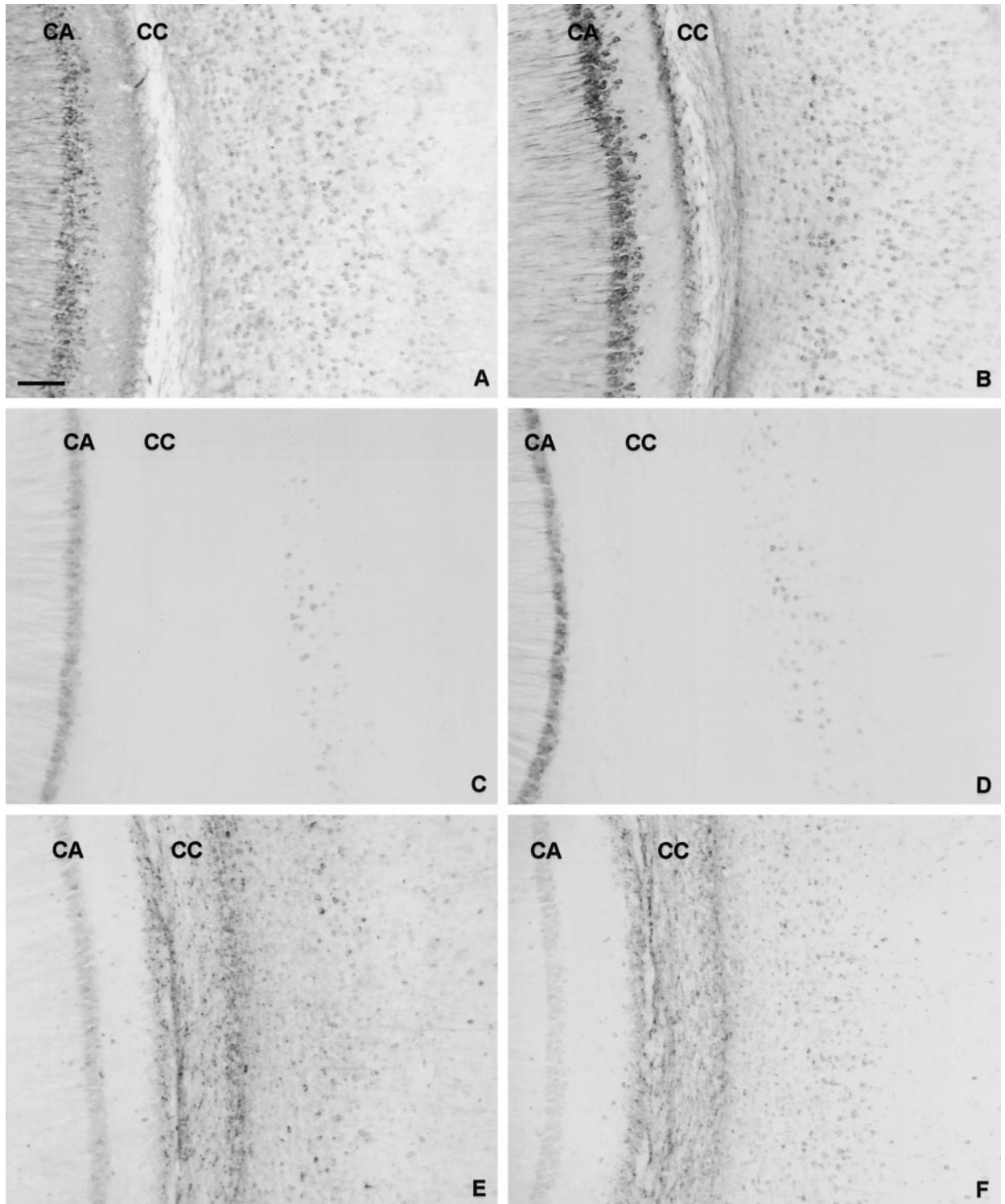


FIG. 5. Immunohistochemistry of brain of tau and of cdk5/p35/tau triple transgenic mice. (A, C, E) Single tau transgenic mice. (B, D, F) Triple cdk5/p35/tau40 transgenic mice. Antibodies: Tau-1 (A, B), AT-180 (C, D), AD-2 (E, F). All are vibratome sections of paraformaldehyde-fixed brain. CA, Ammon's horn, CC, corpus callosum. Scale bar, 100 μ m.

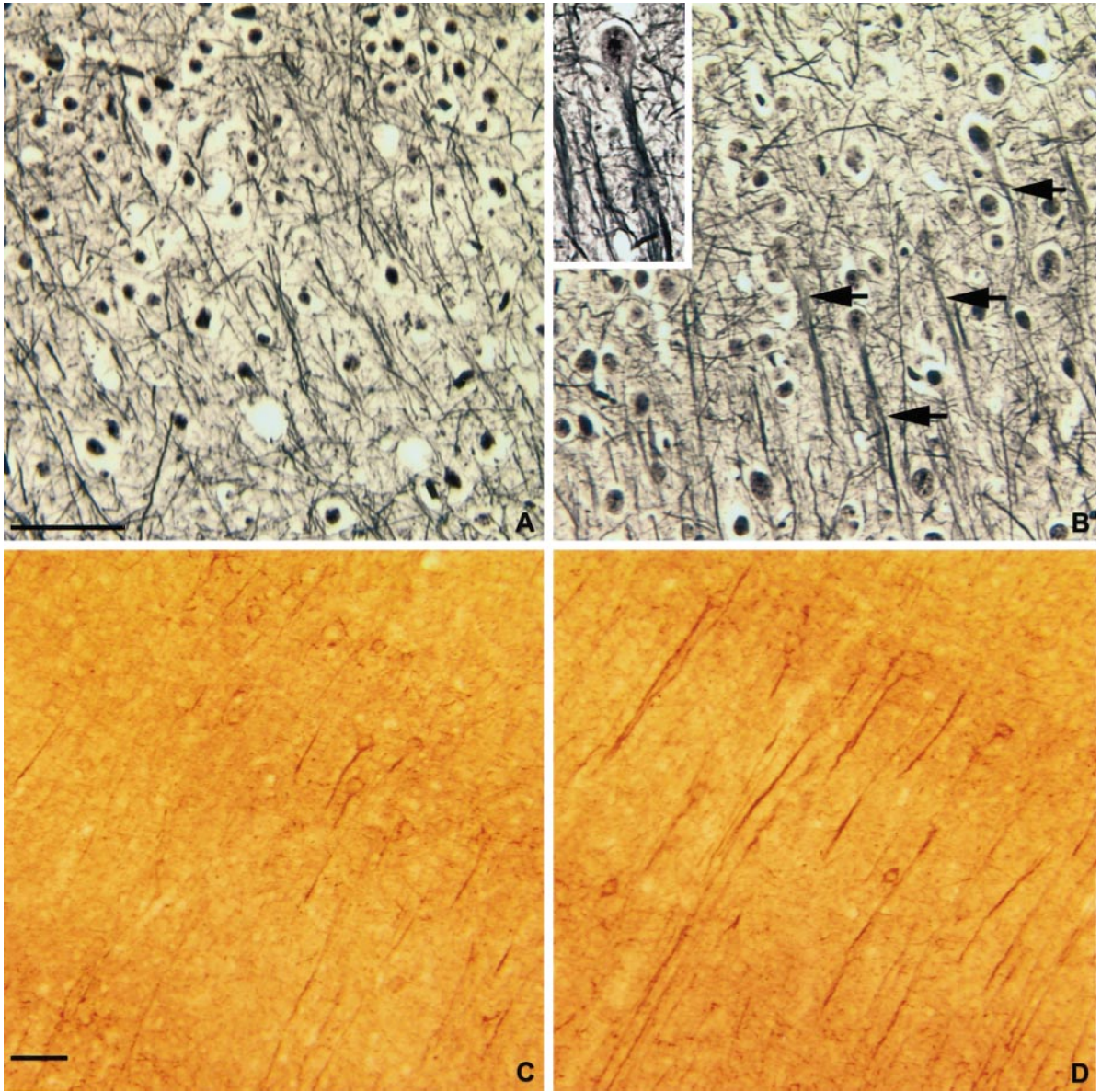


FIG. 6. Histochemistry and immunohistochemistry for neurofilaments in brain of *cdk5/p35* double transgenic mice. (A, B) Silver staining of the cortex of a nontransgenic mouse (A) and of a double-homozygous *cdk5/p35* transgenic mouse (B). Arrows point to the argyrophilic apical dendrites of pyramidal neurons of the cortex. The inset in B is a 2.5-fold higher magnification of one of these apical dendrites. (C, D) Immunohistochemical staining for the heavy neurofilament protein NF-H with antibody NF-200 in the cortex of a nontransgenic mouse (C) and a double homozygous *cdk5/p35* transgenic mouse (D). Scale bar, 50 μm .

the 25-kDa protein in brain extracts of nontransgenic mice (results not shown).

DISCUSSION

The relationship of the *cdk5* kinase activity to protein tau and its state of phosphorylation in AD is still a matter of great concern and importance.

Recent observations on the increased kinase activity of *cdk5* in brain lysates of AD patients were interpreted to confirm and extend earlier claims of *cdk5* as a putative tau kinase *in vivo* (Lee *et al.*, 1999; Patrick *et al.*, 1999). To investigate directly whether *cdk5* indeed acts *in vivo* as an effective protein tau kinase, we have generated multiple transgenic mice that overexpress human *cdk5* and its activator p35, in addition to its

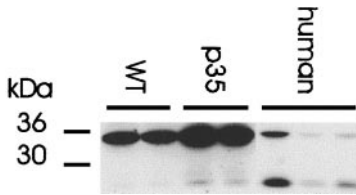


FIG. 7. Western blotting for p35 and p25 in human brain and in p35 transgenic mouse brain. Protein extracts from brain of nontransgenic, wild-type mice (WT), and p35 transgenic mice (two distinct mice each) and of three distinct human brain extracts were blotted with antibody C-19, detecting both mouse and human p35 and its processing product p25 (see text for details). The apparent protein size, based on prestained protein markers, is indicated in kilodaltons on the left.

substrate under investigation, human protein tau. We used the mouse *thy-1* gene promoter for all constructs to ensure high neuronal coexpression of all transgenes (Moechars *et al.*, 1996, 1999; Spittaels *et al.*, 1999; Tesseur *et al.*, 2000).

Biochemically, *cdk5* activity was demonstrated to be increased in *cdk5* and p35 transgenic mice as measured by *in vitro* phosphorylation of histone H1. This confirmed the activity of the transgenic proteins and additionally indicated that endogenous p35 and *cdk5* responded also to their transgenic partners. This was as expected, based on the high primary sequence identity, i.e., more than 98%, of human and mouse *cdk5* (Meyerson *et al.*, 1992; Ino *et al.*, 1994) and p35 (Tsai *et al.*, 1994; Oshima *et al.*, 1996). The altered distribution of neurofilaments as evidenced by immunohistochemical analysis with different antibodies demonstrated increased *in vivo* activity in the *cdk5* and p35 transgenic mice. This finding is in agreement with the capacity of *cdk5* to phosphorylate NF-H *in vitro* (Shetty *et al.*, 1993; Lew *et al.*, 1992; Hisangana *et al.*, 1993; Veeranna *et al.*, 1995) and in cells (Bajaj and Miller, 1997; Guidato *et al.*, 1996; Sun *et al.*, 1996). Nevertheless, in Western blotting, the neurofilament-specific antibodies SMI-31, SMI-36, and RT-97 failed to reveal differences in phosphorylation of neurofilament proteins in transgenic and wild-type mice (results not shown). The already high phosphorylation grade of neurofilaments *in vivo* (Julien and Mushynski, 1982; Elhanany *et al.*, 1994) is likely to mask additional phosphorylation, if any, in neurons of *cdk5*/p35 transgenic mice.

Most importantly, however, no major effects were observed regarding the phosphorylation of murine protein tau in double *cdk5*/p35 mice. Although co-overexpression of human protein tau considerably fa-

cilitated the biochemical and immunohistochemical analyses with antibodies that are directed to human protein tau, no differences were observed in *cdk5*/p35/*htau40* triple transgenic mice, with any of the antibodies in immunohistochemistry or Western blots. The increased immunoreactivity observed with antibodies AT-8, SMI-31, and AT-180 *in vitro* (Baumann *et al.*, 1993; Paudel *et al.*, 1993) was not recapitulated *in vivo* in the brain of these transgenic mice. The effective demonstration of additional phosphorylation of protein tau with antibodies AT-8, AT-180, PHF-1, and AD-2 in brain homogenates of double tau/*GSK-3 β* transgenic mice (results not shown) served as the methodological validation of the procedures applied.

Although *cdk5*/p35 has been proven to phosphorylate protein tau *in vitro*, *in vivo* data are all but lacking. In COS-7 cells transfected with p23, the bovine homologue of human p25, in combination with *htau40*, increased protein tau phosphorylation was observed at Ser-202 and Ser-404 and to a lesser extent at Ser-205, Thr-231, and Ser-235 (Michel *et al.*, 1998). Moreover p25/*cdk5* but not p35/*cdk5* was shown to phosphorylate protein tau in COS-7 cells on the AT-8 and PHF-1 epitope (Patrick *et al.*, 1999). This was not due to lack of enzymatic activity, because both p35 and p25 are equally potent to activate *cdk5* kinase activity toward histone H1. Differences in subcellular localization and the longer half-life of p25 compared to p35 appeared to be the cause (Patrick *et al.*, 1999). Indeed, in contrast to our p35 and *cdk5* mice, transgenic mice overexpressing human p25 showed increased protein tau and NF-H phosphorylation (Ahlijanian *et al.*, 2000). That was, however, not the case in the cerebellum where expression of p25 was substantially lower than in amygdala, thalamus/hypothalamus, and cortex. This could mean that the threshold level of p25, needed for effective activation of *cdk5* to act as a "tau kinase," was not reached in our p35 mice. The p25/p35 ratio was much higher in the human brain samples that we analyzed and in the p25 transgenic mice (Ahlijanian *et al.*, 2000) than in our p35 mice. Therefore, we cannot exclude the possibility that the ratio of p25/p35 and their different subcellular localizations may be determinant factors.

Silver staining and immunohistological staining revealed minor cytoskeletal defects in the apical dendrites of *cdk5*/p35 transgenic mice. This was not comparable to the brain pathology observed in p25 overexpressing mice, in which cell bodies and axons were affected (Ahlijanian *et al.*, 2000). Our data are further concordant with results obtained in transfected primary cortical neurons, in which silver-positive reac-

tion was frequently seen in cdk5/p25 but never in cdk5/p35 transfected neurons (Patrick *et al.*, 1999). This then indicates that the cdk5/p25 kinase activity is more potent to induce morphological degeneration and cytoskeletal disruption in neurons.

In contrast with reports from others (Ahlijanian *et al.*, 2000), we detected some p25 protein in mouse brain extracts using antibody C-19 in Western blotting, but only by careful blocking and preclearing of the samples, allowing much longer exposures. Our data demonstrate that conversion of p35 to p25 is evident in mouse brain, although the p25 product is much less abundant than in human brain. The calpain-mediated conversion of p35 to p25 thereby appears to be the most important determinant for cdk5 kinase activity to act as a tau kinase and to induce pathological alterations in neurons *in vivo* (Lee *et al.*, 2000). Our current *in vivo* data derived from the brain of transgenic mice are in complete agreement with cell biological data obtained in COS7 cells, demonstrating a lack of phosphorylation of protein tau by p35/cdk5 (Patrick *et al.*, 1999). Despite an increase *in vivo* of the cdk5 activity, no signs or markers of emerging pathology were evident, either in cell bodies or in axons. That the cdk5/p35 kinase activity is not detrimental or is less detrimental than the cdk5/p25 kinase activity in cerebral neurons is an important conclusion and opens new possibilities for fundamental and applied studies as well as therapeutical strategies to regulate *in vivo* calpain activity and p25 production.

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