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.

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 3α and β (GSK-3α 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-3β 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., p39cdc5a (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...
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 neural 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., 1998) and increased cdk5 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; Tesseret et al., 2000). Routine genotyping of offspring of founders carrying human cdk5, p35, and tau40 was performed on tail-biopt DNA by PCR. The probes used were obtained by PCR: for cdk5 the forward primer was 5’-CCCCACACAGAATCCA-3’ and the reverse primer was 5’-TAAACCGGACGGGAATC-3’; for p35 the forward primer was 5’-CCCCACACAGAATCCA-3’ and the reverse primer was 5’-CAAGGTCCCGTTTCTCC-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’-GCAGGCCGCTTACTAG-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

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, Tesseret et al., 2000). The linearized PvuI–NotI restriction fragment of the construct was isolated and microinjected into 0.5-day-old mouse prenuclear embryos from the FVB/N strain as described (Hogan et al., 1994; Moechars et al., 1996, 1999; Spittaels et al., 1999; Tesseret et al., 2000). Southern blotting of Stul-digested mouse tail biopt DNA identified transgenic founders. The probes used were obtained by PCR: for cdk5 the forward primer was 5’-CCCCACACAGAATCCA-3’ and the reverse primer was 5’-TAAACCGGACGGGAATC-3’; for p35 the forward primer was 5’-CCCCACACAGAATCCA-3’ and the reverse primer was 5’-CAAGGTCCCGTTTCTCC-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.

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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 μM Na₃VO₄, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.7 μg/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 μg 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) as indicated.

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 μg/ml aprotinin, and 1 μg/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 μl 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 MgCl₂, 1 mM DTT, and 4 μM ATP. Pellets were resuspended in 50 μl kinase buffer containing 6 μg histone H1 and 5 μCi of [γ-32P]ATP (NEN-Dupont, Boston, MA). After incubation at 37°C for 20 min, the reaction was stopped by addition of 50 μl 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 μm) or on vibratome sections (40 μm). 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 deparaffinized 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 streptABCComplex/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) (Buee-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 (Buee-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 (Stemberger, 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% AuCl₃, and 5% Na₂S₂O₃, washed, and mounted with depex.

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; Teseur 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 mouse (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
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.
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). 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).

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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...
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. 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 pre-stained molecular weight markers is indicated at the left in kilodaltons.
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
substantive 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 facilitated 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-3β 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-

![Graph showing protein bands for p35 and p25 in human brain and p35 transgenic mouse brain.](image-url)
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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