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Tau Phosphorylation by Cyclin-dependent kinase5/p39 during Brain Development Reduces its Affinity for Microtubules

Running title: Tau Phosphorylation by Cdk5 during Brain Development

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SUMMARY

The microtubule-associated protein tau is a developmentally regulated neuronal phosphoprotein. The phosphorylation of tau reduces its ability to bind and stabilize axonal microtubules during axonal growth. Although tau is phosphorylated by cyclin-dependent kinase 5 (Cdk5) *in vitro*, its *in vivo* roles remain unclear. Here, we show that tau is phosphorylated by Cdk5/p39 during brain development, resulting in a reduction of its affinity for microtubules. The activity of Cdk5 is tightly regulated by association with its neuronal activators, p35 or p39. The p35 and p39 expression levels were investigated in the developing mouse brain: the p39 expression level was higher in embryonic hindbrain and spinal cord, and in postnatal cerebral cortex, while that of p35 was most prominent in cerebral cortex at earlier stages of development. The ability of Cdk5 to phosphorylate tau was higher when in association with p39 than in association with p35. Tau phosphorylation at Ser-202 and Thr-205 was decreased in Cdk5-/- mouse brain but not in p35-/- mouse brain, suggesting that Cdk5/p39 is responsible for the *in vivo* phosphorylation of tau at these sites. Our data suggest that tau phosphorylation by Cdk5 may provide the

neuronal microtubules with dynamic properties in a region-specific and developmentally regulated manner.

INTRODUCTION

Neuronal development involves morphogenetic changes of neurons associated with complex regulatory mechanisms, which coordinate at the levels of gene expression and post-translational modifications. Current evidence supports the view that neuronal microtubule-associated proteins (MAPs) determine the microtubule rearrangements underlying neuronal morphogenesis (1). This process can be achieved through the regulation of the expression of particular MAP isoforms at specific subcellular locations and at distinct developmental stages, as well as through the modification of MAPs by phosphorylation and dephosphorylation (2).

Among the neuronal MAPs, tau protein has attracted a particular interest due to its polar distribution in the axon as compared to the somatodendritic compartment, as well as

its developmentally regulated expression and phosphorylation (3, 4). Tau is one of several

MAPs that regulate the assembly and stabilization of the microtubule network (5).

Multiple isoforms of tau are generated from a single gene by alternative splicing, leading to

the developmentally regulated expression of different isoforms (6). Phosphorylation

provides tau with further molecular diversity; tau's function as a microtubule binding

protein is regulated by the phosphorylation of specific residues (7). In general, an increase

in tau phosphorylation correlates inversely with its ability to bind and stabilize

microtubules. Thus, phosphorylation of tau contributes an additional mechanism to

control the balance between microtubule dynamics and stabilization in developing axons (8,

9). Several developmental studies have already shown that phosphorylated tau is present

in neurons at a high level only during the period of intense neuritic outgrowth and that it

becomes barely detectable during the period of neurite stabilization and synaptogenesis (10,

11). Phosphorylated tau is essential during brain development to maintain a certain degree

of microtubule instability, thus affecting the growth of neurites.

Tau phosphorylation at specific residues not only occurs during normal brain

development, but also during pathological conditions such as Alzheimer's disease (AD).

In normal brain, the equilibrium between tau phosphorylation and dephosphorylation

modulates the stability of the axonal cytoskeleton and thereby the axonal morphology (12).

However, the breakdown of this equilibrium under pathological conditions results in tau

dysfunction, which is considered to be one of the critical events leading to neuronal

degeneration (13). It has been shown that hyperphosphorylation of tau reduces its affinity

for microtubules and can contribute to the self association of tau and the formation of

neurofibrillary tangles (NFT), one of the major histopathological hallmarks of AD (14).

The phosphorylation is interpreted as abnormal in the sense that this kind of tau

phosphorylation has never been observed in normal aged human brain (15). Surprisingly,

the phosphorylation of tau in NFT has been found to be very similar to a transient

hyperphosphorylation of tau that occurs during early development of the brain (8, 9, 16-18).

Therefore, the biochemical pathways that play a role during early brain development are

likely to be reactivated in AD brains. Tau is phosphorylated *in vitro* by several protein

kinases,   including   cyclic   AMP-dependent   protein   kinase   (19),

calcium/calmodulin-dependent protein kinase II (20), protein kinase C (21), casein kinase I (22), casein kinase II (21) and proline-directed protein kinases such as MAP kinase (23), glycogen synthase kinase-3β (24) and cyclin-dependent kinase 5 (Cdk5) (3, 25-27). Of these kinases, Cdk5 has been found to phosphorylate tau at sites implicated in AD pathology (13, 28).

Cdk5 is an active enzyme in postmitotic neurons; the activation of Cdk5 requires its association with the neuronal activators, p35 or p39 (29-31). p35 was the first activator identified for Cdk5 (29, 30). Subsequently, an additional Cdk5 activator, p39, was identified based on its sequence homology to p35 (31). p35 and p39 share 57% amino acid identity (31). Cdk5 has been shown to play an important role in the development of the nervous system, including neuronal migration and neurite outgrowth (32-35). Insights into the critical function of Cdk5/p35 in brain development have been gained from gene targeting experiments (32, 35). Cdk5-/- mice display extensive defects in all brain areas (35), while p35-/- mice display defects mostly confined to the forebrain (32). This difference in the phenotypic severity suggests the functional importance of another Cdk5

activator, p39, in brain development.   Although p39-/- mice have no obvious abnormalities

in the brain, p35-/-p39-/- double-knockout mice exhibit phenotypes identical to those of

Cdk5-/- mice (36).   These findings suggest that p35 and p39 are major activators of Cdk5

in the brain, and that their coexistence as Cdk5 activators may contribute to the *in vivo*

activation of Cdk5 in a region-specific or developmentally regulated manner, as proposed

by Wu et al. (37).

Developmental regulation of tau phosphorylation is critical in maintaining the

balance between microtubule plasticity and stability in developing axons.   The

phosphorylation pattern of tau in AD brains closely resembles that of tau in embryonic

brains (8, 9, 16-18).   Thus, the developing brain is a useful experimental system to study

the mechanisms that control tau phosphorylation.   Although Cdk5 has been found to

phosphorylate tau *in vitro*, its *in vivo* roles remain to be examined.   Here, we show that

Cdk5 in association with p39 is involved in the *in vivo* phosphorylation of tau during brain

development and that its phosphorylation reduces the ability of tau to bind to microtubules.

In addition, Cdk5/p35 and Cdk5/p39 were found to exhibit different abilities for tau

phosphorylation, with the higher activity in Cdk5/p39. Considering the temporal and

spatial expression patterns of p35 and p39 reported here, the role of tau phosphorylation by

Cdk5 is discussed with regard to the regulation of microtubule stability during brain

development.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies — Polyclonal antibodies to p35 (C-19) and Cdk5 (C-8) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An affinity purified rabbit polyclonal antibody against p39 was kindly provided by Dr. Tsai (Howard Hughes Medical Institute and the Department of Pathology, Harvard Medical School, Boston, MA). The phosphorylation-dependent tau antibody, AT-8, was obtained from Endogen (Woburn, MA). The phosphorylation-independent mouse monoclonal TAU-5 antibody was purchased from Biosource International, Inc. (Camarillo, CA). An antibody against α-tubulin was purchased from Sigma (Saint Louis, MO). Cell culture reagents were purchased from Life Technologies, Inc. (Rockville, MD).

Animals — The gene-targeting strategy and generation of Cdk5-/- and p35-/- mice have been reported previously (35, 38). Mouse lines of Cdk5 and p35 mutants, as well as wild-type controls, were maintained in a C57BL/6 × 129/SvJ hybrid background.

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Conception was ascertained by the presence of a vaginal plug. The first 24 h following

conception was considered day 0 of embryonic development (E0) and the first 24 h

following birth, day 0 post-partum (P0). Mouse embryonic development takes

approximately 20 days. The genotypes of the mutants were determined by performing

Southern blot and/or PCR analysis on genomic DNA isolated from tail biopsies as

described earlier (35, 38). Animal care and use practices conformed to the *NIH Guide for*

Care and Use of Laboratory Animals.

Preparation of tissue samples at different developmental stages ⎯ Embryonic and postnatal

mouse brains were dissected on ice into various brain regions: cerebral cortex, cerebellum,

brain stem and spinal cord. For Northern blot analysis, total RNA was extracted from the

various brain regions of wild-type mice with TRIzol reagent (GIBCO-BRL, Rockville,

MD), as recommended by the manufacturer. Total RNA concentration and purity were

determined by U.V. absorbance at 260 and 280 nm.   For Western blot analysis, each tissue

from wild-type, p35-/- and Cdk5-/- mice was washed in ice-cold PBS and homogenized in

10 volumes of lysis buffer at 4°C.   The lysis buffer consisted of 50 mM Tris-HCl (pH 7.5)

containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM

phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 20 mM

β-phosphoglycerate, 10 mM sodium fluoride, 1 mM sodium vanadate, and 1 μM okadaic

acid.   Following a 30-min incubation on ice, insoluble material was removed by

centrifugation at 4°C, and the protein concentration of the supernatant was determined as

described (39). For Cdk5 kinase activity assay, the brain extracts were prepared with the

same lysis buffer except with a NaCl concentration of 50 mM, to allow Cdk5 association

with p35 and p39.

Northern blot analysis ⎯ Each sample (20 μg of total RNA) was electrophoresed in a 1%

agarose-formaldehyde gel and blotted onto a nylon membrane (Schleicher & Schuell, Inc.,

Keene, NH). After UV-cross linking, the membrane was prehybridized for more than 3

hours at 42°C in the presence of 50% formamide, 10× Denhardt's solution, 5× SSPE, 0.1%

SDS, 10% dextran sulfate, and 100 μg/ml boiled fragmented salmon sperm DNA.

^{32}P-labeled probes were added to the prehybridization buffer and the membrane was incubated overnight at 42°C. After hybridization, the membrane was washed 2 times in 2× SSC, 0.1% SDS at 42°C for 10 min, and 2 times in 0.1× SSC, 0.1% SDS at 65°C for 20 min. The washed membrane was exposed to X-ray film with double intensifying screens at –80°C. After stripping the probe, the same membrane was used for hybridization with a new probe. The expression levels of Cdk5, p35 and p39 mRNAs were quantified by measuring the optical densities of specific bands using an image analysis system with NIH Image software, version 1.62.

For detection of p35 mRNA, a 924 bp fragment of mouse p35 cDNA containing the entire coding region was used as a probe as previously described (40). A 275 bp fragment used as a p39 probe (nucleotides 891-1165 of the full-length mouse p39 cDNA) was generated by RT-PCR using the following primers: 5'-CAACGAGATCTCCTACCCGCTC-3' and 5'-TCATAGTCCAGTGCTTGGCTCC-3'. This fragment excludes the region of homology between p35 and p39 cDNAs, thus minimizing the probability of cross-hybridization. A 560 bp fragment used as a Cdk5

probe (nucleotides 331-890 of the full-length mouse Cdk5 cDNA) was produced by RT-PCR using the following primers: 5'-AGCTGCAATGGTGACCTGGACC-3' and 5'-TCCTCTGCTGAGATGCGCTGCA-3'. As an internal control, a 433 bp fragment used as a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was generated as described previously (41).

Western blot analysis — Equal amounts of protein for each experiment were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) before being transferred onto a nitrocellulose membrane. The membranes were blocked in $1\times$ PBS containing 5% skim milk and 0.05% Tween 20 and incubated with primary antibodies overnight at 4°C. Incubation with peroxidase-conjugated anti-mouse or rabbit IgG (1:10,000) was performed at room temperature for 60 min. A signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and relative optical densities of the bands were quantified as described above.

For detection of p35, p39 and Cdk5 protein, membranes were incubated with

anti-p35 antibody (1:1,000), anti-p39 antibody (1:1,000) and anti-Cdk5 antibody (1:1,000).

The phosphorylation state of tau was examined using the AT-8 antibody (diluted to 5 μg/ml) which recognizes tau only when Ser-202 and Thr-205 (numbering based on longest human brain tau isoforms) are phosphorylated (42). To determine total tau levels, the phosphorylation-independent monoclonal antibody TAU-5 (1:5,000) was used (43). The data obtained with the AT-8 antibody were normalized to total tau levels on the stripped and reprobed membranes. For reuse, the membranes were stripped for 30 min at 50°C in 63 mM Tris-HCl (pH 6.8) containing 100 mM 2-mercaptoethanol and 2% SDS. For the detergent extraction assay, the amount of tau was determined with the TAU-5 antibody.

Cdk5 kinase activity assay — Different regions of mouse brain were lysed in ice-cold lysis buffer as described above. The supernatants (brain extracts) were collected after centrifugation at 10,000 x g for 30 min at 4°C and immunoprecipitated with either anti-Cdk5 (C-8), anti-p35 (C-19) or anti-p39 antibodies. The Cdk5 immunoprecipitate was prepared by incubation of 300 μl of the lysate (corresponding to 300 μg of protein)

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with anti-Cdk5 antibody (3 µg) overnight at 4°C followed by further incubation with 25 µl

of Protein A-Agarose beads (50% slurry in lysis buffer; Santa Cruz Biotechnology, Inc.,

Santa Cruz, CA) for 3 hours at 4°C. For the preparation of p35 or p39 immunoprecipitates,

500 µl of the lysate (corresponding to 1 mg of protein) was incubated with either anti-p35

antibody (3 µg) or anti-p39 antibody (3 µg) as described above. The immunoprecipitates

were washed twice with the lysis buffer and twice with a kinase buffer consisting of 50 mM

Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol, and

resuspended in 60 µl of the kinase buffer. Kinase activity assays were performed using

either histone H1 or bacterially-expressed human tau as a substrate as described previously

(44). Tau was purified from the heat-stable supernatant of an Escherichia coli lysate by

phosphocellulose column chromatography (45). Briefly, a total volume of 50 µl of kinase

assay mixture was used, containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA,

1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM microcystin LR, 10 µl of the

immunoprecipitate and either 10 µg of histone H1 or 5 µg of tau protein. The

phosphorylation reaction was initiated by the addition of 0.1 mM [γ-^{32}P] ATP and incubated

at 30°C for 60 min.   The reaction was stopped by the addition of SDS-PAGE sample buffer

and boiled immediately for 5 min.   Samples were separated by SDS-PAGE on a 15%

polyacrylamide gel, and autoradiography was used to detect the phosphorylation of histone

H1 or tau.   To quantify the Cdk5 levels in the p35 and p39 immunoprecipitates, 10 μl of

each immunoprecipitate was immunodetected by Western blotting using an anti-Cdk5

antibody.

Cell culture and detergent extraction — Primary cultures of cerebellar neurons were

prepared as described previously (4).   Briefly, cerebella were dissected from E17 wild-type,

p35-/- and Cdk5-/- mouse brains, dissociated by trituration, counted and plated at 1×10^5

cells/cm^2 onto 6-well culture plates previously coated with polyethylenimine (2 μg/ml in

0.1 M boric acid buffer, pH 7.4).   After 1 hr in 10% horse serum-containing medium, the

cells were maintained with Dulbecco's Modified Eagle Medium/F12 supplemented with the

N2 mixture, 50 units/ml penicillin G and 50 mg/ml streptomycin.   Cultures were

maintained in a 37°C incubator with 5% CO_2.   The cells were used for experiments on the

7th day in culture.

To investigate the effect of tau phosphorylation on its association with microtubules, a Triton X-100 extraction assay was utilized to separate the detergent-insoluble cytoskeletal component from the detergent-soluble cytosolic component. The fractionation of cell lysates was performed by a modification of a previously reported procedure (46). Briefly, cultures were washed with prewarmed (37°C) PBS followed by washing with prewarmed (37°C) microtubule stabilization buffer (0.1 M PIPES, pH 6.8, 1 mM $MgCl_2$, 2 mM EGTA, 30% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 20 mM β-phosphoglycerate, 10 mM sodium fluoride, 1 mM sodium vanadate, and 1 μM okadaic acid) and incubated at 37°C for 10 min in the same buffer containing 0.1% Triton X-100. The lysates were centrifuged and the detergent-soluble supernatants were collected. The supernatants were incubated in a boiling water bath for 5 min following addition of 2× SDS sample buffer. The remaining cellular pellets were solubilized in 2× SDS sample buffer, sonicated and incubated in a boiling water bath as above. The relative protein concentration of these samples was

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determined by densitometry of a Coomassie blue-stained SDS-gel. Equal protein amounts

were subsequently electrophoresed on 10% SDS polyacrylamide gels and immunodetection

was performed with a TAU-5 antibody.

RESULTS

Regional differences in tau phosphorylation during brain development — Tau is

predominantly localized in axons and has a specific function in the formation of the axonal

cytoskeleton. A study that demonstrated the temporal and spatial expression patterns of

tau mRNA in the rat brain suggested that its expression appeared to coincide with the

region-specific onset of axogenesis (47). The onset of tau mRNA expression and the

subsequent decrease in the brain stem occurred earlier than those in the cerebral cortex (47).

While studies have demonstrated the developmental regulation of tau phosphorylation in

the brain, its spatial regulation remains less clear. To address this question, we examined

the developmental changes in the expression and phosphorylation of tau in different brain

regions, the cerebral cortex and brain stem, using the phosphorylation-dependent AT-8 antibody and phosphorylation-independent TAU-5 antibody. It was found that the developmental phosphorylation of tau was also regulated in a spatial manner. Tau phosphorylation in the cerebral cortex was detected at E15, increased to a peak level at P7, decreased by P14 and could not be detected at P28 (Fig. 1A). Interestingly, tau phosphorylation in the brain stem peaked earlier at P0, decreased by P7, and was undetectable at P14 and P28 (Fig. 1B). These results indicate that there are differences in tau phosphorylation in specific brain regions at different developmental stages. It was also noted that the rapid downregulation of phosphorylation coincided with the transition from immature to several adult isoforms of tau, with a regional difference between the cerebral cortex and brain stem (Fig. 1), consistent with the previous report (47).

Expression patterns of Cdk5, p35 and p39 in the developing mouse brain — Although Cdk5 has been shown to phosphorylate tau *in vitro*, its contribution to a temporal and spatial regulation of tau phosphorylation remains to be determined. Moreover, the fact that the

phenotypic abnormalities in p35-/- mice involved fewer brain regions and were of lesser

magnitude as compared to those in Cdk5-/- mice, suggested the functional importance of

another Cdk5 regulatory subunit, p39, in the developing brain and led us to examine the

possible differences in the temporal and spatial expression of Cdk5 and its regulatory

subunits, p35 and p39, during brain development.   We have determined their mRNA levels

in various brain regions at E15, E17, P0, P7, P14 and P28.   Northern blot analysis revealed

bands of approximately 4.4 kb, 2.4 kb and 1.6 kb for p35, p39 and Cdk5, respectively (Fig.

2).   Interestingly, analysis of various brain regions at different developmental stages

revealed distinct p35 and p39 expression patterns.   The p35 mRNA level was higher during

embryonic development in all regions of the brain, and then decreased postnatally.   A

significant difference was observed in the developmental expression pattern of p39 in the

cerebral cortex as compared to other brain regions.   In contrast to p35, the p39 mRNA

level in the cerebral cortex was markedly lower before birth and then gradually increased to

its peak level at P7.   Thereafter, this high level was maintained throughout the period

analyzed up to P28.   In the cerebellum, brain stem and spinal cord, the p39 mRNA was

maintained at high levels during embryonic development and then gradually decreased postnatally. Thus, except in the cerebral cortex, the developmental expression pattern of p39 in these brain regions was similar to that of p35. The Cdk5 mRNA expression was maintained at high levels in all regions of the brain throughout the period analyzed.

Furthermore, we have determined the regional distribution patterns of p35 and p39 at different developmental stages and also observed a prominent difference in the regional distribution pattern of p39 in the embryonic and postnatal brain (Fig. 3). The expression of p35 mRNA was most prominent in the cerebral cortex as compared to other regions of the brain throughout brain development. In contrast, the regional distribution pattern of p39 mRNA changed after birth. At E15, the p39 mRNA level was higher in the cerebellum, brain stem and spinal cord, but very low in the cerebral cortex. This pattern of expression continued at P0. The regional distribution pattern of p39 mRNA shifted by P14, with the highest expression level now in the cerebral cortex, similar to that of p35. The protein levels of p35, p39 and Cdk5 correlated with their mRNA levels in a temporally and spatially regulated manner as shown by Western blot analysis of wild-type mouse brain

regions (Fig. 4). We have found that the p39 protein level was upregulated in the p35-/-

mouse brain, while the Cdk5 level was unaffected, suggesting a possible compensatory

mechanism in the absence of p35 (Fig. 4). This finding was consistent with a previous

report by Ko et al. (36).

Different substrate preference of Cdk5/p35 and Cdk5/p39 — It was of interest to see if the

different distribution patterns of p35 and p39 correlated with different effects on Cdk5

activity in the developing brain. To address this question, the substrate preference of

Cdk5/p35 and Cdk5/p39 was examined using the different substrates, histone H1 and tau

protein. The kinase activities for these substrates were determined with either p35 or p39

immunoprecipitates from brain extracts of P14 mouse cerebral cortex. The Cdk5 kinase

assay revealed that Cdk5/p35 phosphorylated histone H1 more than did Cdk5/p39, while

the kinase activity using tau as a substrate was higher with Cdk5/p39 than with Cdk5/p35

(Fig. 5). The p35 and p39 immunoprecipitates used in this assay did not contain the same

amount of Cdk5. The p35 immunoprecipitate contained about three times as much Cdk5

than did the p39 immunoprecipitate (Fig. 5A). Thus, we cannot conclude that Cdk5/p35

preferentially phosphorylates histone H1 as compared to Cdk5/p39 because a larger amount

of Cdk5 may phosphorylate any substrate better than the smaller amount.   However, these

results clearly indicate that the smaller amount of Cdk5 phosphorylates tau better in the

presence of p39 than it does in the presence of p35, suggesting that p39 preferentially

activates Cdk5 phosphorylation of tau as compared to p35.   To confirm this observation,

Cdk5 immunoprecipitates from different brain regions of wild-type and p35-/- mice at E17

and P14 were subjected to Cdk5 kinase assay using either histone H1 or tau as a substrate

(Fig. 6).   Cdk5 immunoprecipitates from wild-type mouse brain consisted of Cdk5/p35 and

Cdk5/p39, while those from p35-/- mouse brain contained only Cdk5/p39.   The kinase

activity using histone H1 as a substrate in p35-/- mouse brain was less than 20% in all brain

regions tested as compared to wild-type mouse brain (Fig. 6A and B), however, p35-/-

mouse brain exhibited 40-80% of the kinase activity of wild-type mouse brain when using

tau as a substrate (Fig. 6C and D).   These results further indicated that Cdk5/p39

preferentially phosphorylates tau.   Regional distribution of the kinase activity using tau as

a substrate varied between embryonic (Fig. 6C) and postnatal brain (Fig. 6D). Higher

activity was observed in embryonic cerebellum, brain stem and spinal cord. However, this

activity pattern changed postnatally with the highest activity now found in the cerebral

cortex, consistent with the temporal and spatial expression pattern of p39 protein (Fig. 4).

Involvement of Cdk5 in the in vivo phosphorylation of tau — Although Cdk5 has been

reported to phosphorylate tau *in vitro* (25-27), its ability to phosphorylate tau *in vivo*

remains to be determined. To address this question, we have examined the

phosphorylation state of tau in p35-/- and Cdk5-/- mouse brains using a

phosphorylation-dependent tau antibody, AT-8 (Fig. 7). This antibody recognizes the sites

on tau that Cdk5 has been shown to phosphorylate *in vitro*. A decrease in AT-8

immunoreactivity was seen in Cdk5-/- mouse brain but not in p35-/- mouse brain. It is of

interest to note that there was no difference in AT-8 immunoreactivity in the cerebral cortex

where the p39 protein level was very low, which is consistent with a previous observation

that AT-8 immunoreactivity was observed in the brain stem of E18 rats but not in the

cerebral cortex (10). These results indicate that Cdk5, especially in association with p39, is involved in the *in vivo* phosphorylation of tau during brain development.

Effect of tau phosphorylation by Cdk5 on its binding affinity for microtubules — As shown above, Cdk5 was found to be involved in the *in vivo* phosphorylation of tau, and therefore, may regulate tau function in axonal development. Tau function is regulated through its association with microtubules. To investigate the physiological role of tau phosphorylation by Cdk5, the ability of tau to bind to microtubules was examined by a detergent extraction assay using cultured cerebellar neurons from E17 wild-type, p35-/- and Cdk5-/- mouse embryos (Fig. 8). The detergent-insoluble cytoskeletal component was separated from the soluble cytosolic component to determine the amount of tau associated with microtubules. Although tau was present both in the soluble component and in the insoluble component, tau in the insoluble component migrated faster than tau in the soluble component, suggesting that tau in the insoluble component was phosphorylated to a lesser extent than tau in the soluble component. In addition, the amount of tau in the insoluble

component (microtubule-associated tau) was greater in cultured neurons from Cdk5-/- mice than those from wild-type and p35-/- mice. Concomitantly, the soluble component from the Cdk5-/- mouse neurons contained a lesser amount of tau. There was no apparent difference in the amount of tau associated with microtubules between wild-type and p35-/- mouse neurons. Thus, these results suggest that tau phosphorylation by Cdk5/p39 reduces the ability of tau to bind to microtubules.

DISCUSSION

The functions of tau as a microtubule-associated protein are modulated by its phosphorylation state. The phosphorylation of tau may be essential during brain development to maintain a certain degree of microtubule instability, thus affecting neurite outgrowth. This study reports that Cdk5/p39 is involved in the *in vivo* phosphorylation of tau during brain development, as evidenced by the observation that tau phosphorylation at Ser-202 and Thr-205 was decreased in Cdk5-/- mouse brain but not in p35-/- mouse brain.

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Cdk5/p35 and Cdk5/p39 exhibited different preferences for tau as a substrate. The ability

of Cdk5 to phosphorylate tau was higher when in association with p39 rather than in

association with p35. Furthermore, during brain development, the p39 expression and the

Cdk5/p39 activity as a tau kinase were varied in a spatial manner. Higher expression and

kinase activity were observed in embryonic cerebellum, brain stem and spinal cord, but

their distribution changed postnatally with the highest expression and activity in the

cerebral cortex. Additionally, the phosphorylation of tau by Cdk5/p39 reduced the

affinity of tau for microtubules. These results described here suggest that the *in vivo*

phosphorylation of tau by Cdk5 may regulate microtubule dynamics in a region-specific

and developmentally regulated manner.

Cdk5 displays ubiquitous tissue distribution (48), however, its kinase activity has

been found mainly in brain where its regulatory subunits, p35 and p39, are predominantly

expressed (29, 49, 50). Insight into the critical functions of Cdk5 in brain development has

been gained from gene targeting experiments for Cdk5, p35 and p39 genes. It is of interest

to note that there are prominent differences in the phenotypic severity of these three mice

(32, 35, 36, 51). Cdk5-/- mice display late embryonic and/or perinatal lethality and extensive defects in the central nervous system (35). Whereas, p35-/- mice are viable and display defects mostly confined to the forebrain, with cortical lamination defects similar to those observed in Cdk5-/- mice (32). In the cerebellum, the typical foliation and tripartite layering are observed in p35-/- mice but not in Cdk5-/- mice (32, 35). In the brain stem, p35-/- mice show no apparent abnormalities, whereas Cdk5-/- mice display migration defects in facial branchiomotor and inferior olive neurons (51). In the spinal cord, abnormal motor neurons with ballooned perikarya, characteristic of chromatolytic changes, are observed in Cdk5-/- mice but not in p35-/- mice (32, 35). In comparison, p39-/- mice do not show any noticeable defects in the brain while p35-/-p39-/- double-knockout mice display a phenotype similar to that of Cdk5-/- mice (36). Our results presented here clearly demonstrate that p39 expression is higher during embryonic development in the cerebellum, brain stem and spinal cord, where the pathological defects are mostly absent in p35-/- mice. However, the level of p39 expression is very low in the embryonic cerebral cortex, where p35-/- mice have lamination defects similar to those observed in Cdk5-/-

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mice. These results suggest that Cdk5/p39 may compensate for a lack of Cdk5/p35 activity in p35-/- mice, indicating the overlapping roles of p35 and p39. Furthermore, the fact that p39-/- mice do not show any noticeable abnormalities is also compatible with our observation that the p35 expression level is high in all regions of the brain during embryonic development, suggesting the compensatory role of p35 in the absence of p39.

It has not been clear, however, whether p35 and p39 can each confer a distinct function to Cdk5. The specific subcellular localization of p35 and p39 has been reported: p39 is both cytosolic and membrane associated while p35 is mainly membrane associated, suggesting a distinct role of each regulatory subunit to determine the subcellular localization of the Cdk5 complex (52). Our observation that the developmental expression pattern of p39 in the cerebral cortex appears to be the inverse to that of p35 may be related to a hypothesis that each regulatory subunit targets Cdk5 to different substrates during brain development. We show here that Cdk5/p35 and Cdk5/p39 exhibit different preference for tau as a substrate: p39 preferentially activates Cdk5 phosphorylation of tau than does p35. The preference of Cdk5/p39 for tau was also confirmed by the observation that p35-/-

mouse brain showed higher Cdk5 activity using tau as a substrate as compared to histone

H1. Furthermore, p35-/- mouse brain, which contains the upregulated amounts of p39

protein, does not show any differences in tau phosphorylation as compared to wild-type

mouse brain. In contrast, a significant reduction of tau phosphorylation is observed in

Cdk5-/- mouse brain, except in the cerebral cortex, where the p39 expression level is very

low. These results indicate that Cdk5/p39 is involved in the *in vivo* phosphorylation of tau

during brain development, while the remaining AT-8 immunoreactivity in Cdk5-/- mouse

brain suggests the contribution of other protein kinases. Thus, the regulatory subunits, p35

and p39, appear to play important roles in determining the substrate specificity of Cdk5 as

well as its subcellular localization.

To gain insight into the functional consequences of tau phosphorylation by

Cdk5/p39, the binding ability of tau to mictotubules was examined using cultured

cerebellar neurons from wild-type, p35-/- and Cdk5-/- mice. Cultured cerebellar neurons

were used because the p39 expression level is higher in the embryonic cerebellum. A

detergent extraction assay revealed that the amount of tau associated with microtubules was

greater in cultured neurons from Cdk5-/- mice than those from wild-type and p35-/- mice.

Moreover, cerebellar neurons from p35-/- mice showed no difference in the amount of tau

associated with microtubules as compared to those from wild-type mice. These results

suggest that tau phosphorylation by Cdk5/p39 reduces the ability of tau to bind to

microtubules. The observations that the regional distribution in the p39 expression level

and the Cdk5/p39 activity as a tau kinase is drastically changed during the perinatal period

further suggest that Cdk5/p39 may contribute to a developmental regulation of tau

phosphorylation and its microtubule-binding ability. Phosphorylated tau has been shown

to be less efficient than dephosphorylated tau in promoting microtubule assembly, while the

dephosphorylated tau promotes significantly more rapid and more extensive polymerization

of microtubules (53). Thus, tau phosphorylation by Cdk5/p39 in the developing brain may

provide the microtubules with more dynamic properties to allow microtubule

rearrangements during axonal growth in a region-specific and developmentally regulated

manner. It has been previously described that the extent of Cdk5 kinase activity is

coincident with neurite outgrowth in cultured cerebellar neurons (54). Moreover, distinct

roles of p35 and p39 have been observed in neurite outgrowth (55). Although both p35

and p39 induced neurite outgrowth in cultured hippocampal neurons, only antisense p39

prevented basic fibroblast growth factor - induced neurite outgrowth. This observation

suggests that p39 is required for neurite outgrowth, consistent with our present

observations.

A transient hyperphosphorylation of tau during the early development of the brain

has been found to be very similar to the abnormal phosphorylation of tau in AD brains (9,

17, 18). The hyperphosphorylation of tau in mature neurons may contribute to its loss of

microtubule binding ability and aggregation into NFT. Cdk5 has been proposed to be

implicated in the hyperphosphorylation of tau observed in AD brains in association with

p25 but not with p35 (27). p25 is generated from p35 upon neurotoxic insults activating

the Ca^{2+}- dependent cysteine protease, calpain (45, 56, 57). This proteolytic conversion

gives p25 altered biological properties as compared to p35. p25 is more stable and has a

different subcellular localization, leading to mislocalization and prolonged activation of

Cdk5 (45, 56, 57). Treatment of cultured neurons with amyloid-β peptide, a primary

constituent of the amyloid plaques in AD brains, causes the conversion of p35 to p25, concomitant with the activation of calpain (56). Furthermore, recent *in vitro* and *in vivo* studies demonstrated that the conversion of p35 to p25 promotes phosphorylation of tau at Ser-202 and Thr-205 (57, 58, 59). These results suggest that Cdk5/p25 may be involved in the abnormal phosphorylation of tau in AD brains. Thus, it seems that Cdk5 not only acts as a physiological tau kinase in association with p39 but also as a pathogenic tau kinase in association with p25. Recently, p39 has also been found to be a substrate for calpain (60). Similar to the conversion of p35 to p25, calpain-mediated cleavage of p39 generates the C-terminal fragment p29, with biological properties that cause the deregulation of Cdk5 (60). It will be important to examine whether Cdk5/p29 may act as a pathogenic tau kinase in certain pathological conditions. Cdk5 may display diverse functions through tau phosphorylation in developing brain as well as in pathogenic brain depending on its association with different partners, p35/p25 or p39/p29.

ACKNOWLEDGMENTS

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Abbreviations

The abbreviations used are: AD, Alzheimer's disease; NFT, neurofibrillary tangles; Cdk5, cyclin-dependent kinase 5; WT, wild-type; KO, knockout; E, days of embryonic development; P, days post-partum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE LEGENDS

Fig. 1.

Regional differences in tau phosphorylation during brain development.

Brain lysates were prepared from cerebral cortex (*A*) and brain stem (*B*) of wild-type mice on the 15th day of embryonic development (E15), 17th (E17), day 0 post-partum (P0), 7th (P7), 14th (P14) and 28th (P28); the first 24 h following conception was considered E0 and the first 24 h following birth, P0. Developmental changes in tau phosphorylation were examined by SDS-PAGE and Western blotting with phosphorylation-dependent (AT-8) and –independent (TAU-5) tau antibodies. The amount of protein loaded on the gels was determined by reprobing the same membrane with an anti α-tubulin antibody. The rapid downregulation of phosphorylation of tau coincided with the transition in the composition of the isoforms. Note that the downregulation in the brain stem precedes that seen in the cerebral cortex.

Fig. 2.

Developmental expression patterns of p35 and p39 in various brain regions.

A, Total RNA was prepared from various brain regions of wild-type mice on the 15th day of embryonic development (E15), 17th (E17), day 0 post-partum (P0), 7th (P7), 14th (P14) and 28th (P28), and subjected to Northern blot analysis. The p35 mRNA level was higher during embryonic development in all regions of the brain. The developmental expression pattern of p39 was similar to that of p35 in the cerebellum, brain stem and spinal cord. However, in the cerebral cortex, p39 expression pattern was inverse to that of p35. *B*, Quantitative results indicate the optical densities of p35, p39 and Cdk5 mRNA relative to that of GAPDH mRNA, expressed as a percentage of the value for the P0 group and are shown as the mean ± s.d. from three independent experiments.

Fig. 3

Regional distribution patterns of p35 and p39 at different developmental stages.

A, Total RNA was prepared from various brain regions of wild-type mice on the 15th day of embryonic development (E15), day 0 post-partum (P0) and 14th (P14), and subjected to Northern blot analysis. The expression of p35 was most prominent in the cerebral cortex throughout brain development. In contrast, the regional distribution pattern of p39 changed after birth: the p39 mRNA level was first higher in embryonic (E15) and perinatal (P0) cerebellum, brain stem and spinal cord, and then higher in postnatal cerebral cortex (P14). *B*, Quantitative results indicate the optical densities of p35, p39 and Cdk5 mRNA relative to that of GAPDH mRNA and are shown as the mean ± s.d. (n=3).

Fig. 4

Compensatory increase in p39 protein in the absence of p35.

Brain lysates from the different regions of wild-type (*WT*) and p35-/- (*KO*) mice at the 15th day of embryonic development (*A*) and the 14th day post-partum (*B*) were examined by SDS-PAGE and Western blotting with anti-p35, anti-p39 and anti-Cdk5 antibodies. Note that the p39 protein level was upregulated in the p35-/- mouse brain.

Fig. 5

Differences in substrate preference of Cdk5/p35 and Cdk5/p39 complexes.

A, One milligram of brain lysate from 14th day post-partum mouse cerebral cortex was immunoprecipitated with anti-p35 or anti-p39 antibody and then subjected to Cdk5 kinase assay using either histone H1 or tau as a substrate. Autoradiography was used to detect the phosphorylation of histone H1 and tau. These immunoprecipitates were also examined to determine the amount of the associated Cdk5 by Western blotting (WB) with an anti-Cdk5 antibody. *B and C*, Quantitative results are shown by normalizing the kinase activity to the amount of Cdk5 in each immunoprecipitate and represent the mean ± s.e.m. from three independent experiments. These data were analyzed using the Student's *t*-test and considered to be significantly different when $p < 0.05$ (*).

Fig. 6

Developmental change in the regional distribution of Cdk5/p39 kinase activity.

46

Lysates from different brain regions of wild-type (*WT*) and p35-/- (*KO*) mice at the 17th day of embryonic development (E17) and the 14th day post-partum (P14) were immunoprecipitated with an anti-Cdk5 antibody (C-8) and subjected to the kinase assay using either histone H1 or tau as a substrate. Autoradiography was used to detect the phosphorylation of histone H1 and tau. Quantitative results indicate the relative Cdk5 kinase activity expressed as a percentage of the value for the cerebral cortex of a wild-type mouse, and are shown as the mean ± s.e.m. from three independent experiments. *A*, Cdk5 kinase activity using histone H1 in the E17 mouse brain. *B*, Cdk5 kinase activity using histone H1 in the P14 mouse brain. *C*, Cdk5 kinase activity using tau in the E17 mouse brain. *D*, Cdk5 kinase activity using tau in the P14 mouse brain. Note that Cdk5 in the p35-/- mouse brain shows a higher kinase activity using tau as a substrate, as compared to that using histone H1, with a distinct regional distribution during brain development.

Fig. 7

***In vivo* phosphorylation of tau by Cdk5/p39 during brain development.**

Brain lysates were prepared from different brain regions of wild-type, p35-/- and Cdk5-/-

mouse embryos on the 15th day of embryonic development. The samples were examined

by SDS-PAGE and Western blotting using the phosphorylation-dependent tau antibody,

AT-8, and the phosphorylation-independent tau antibody, TAU-5. *A*, Decrease in AT-8

immunoreactivity was observed in Cdk5-/- mouse brain but not in p35-/- mouse brain.

Note that there was no difference in the immunoreactivity in the cerebral cortex, where the

p39 protein level was very low. *B*, To quantitatively determine the phosphorylation state

of tau, the data obtained with the AT-8 antibody for wild-type, p35-/- and Cdk5-/- mice

were normalized to total tau levels in the samples. The data are presented as a

densitometric ratio of AT-8 immunoreactivity to TAU-5 immunoreactivity from three

independent experiments. These data were analyzed using the Student's *t*-test and

considered to be significantly different when $p<0.05$ (*).

Fig. 8

Reduced ability of tau phosphorylated by Cdk5/p39 to bind to microtubules.

Cultured cerebellar neurons from wild-type, p35-/- and Cdk5-/- mouse embryos were subjected to the Triton X-100 extraction assay on the 7th day after plating, and the detergent-insoluble cytoskeletal component was separated from the soluble cytosolic component to determine the amount of tau associated with microtubules.   An equal amount of protein from each component was subjected to SDS-PAGE and Western blotting using the phosphorylation-independent tau antibody, TAU-5.   *A*, The amount of tau in the detergent-insoluble component (associated with microtubules) was greater in cultured neurons from Cdk5-/- mice as compared to those from wild-type and p35-/- mice. Concomitantly, the soluble component from Cdk5-/- mouse neurons contained a lesser amount of tau.   *B*, The amounts of tau in the soluble component (upper panel) and the insoluble component (lower panel) were quantitatively determined by densitometry and presented as the relative level of TAU-5 immunoreactivity to that from the wild-type mouse

neurons. All values are shown as the mean ± s.e.m. from three independent experiments.

Figure 1 (Takahashi, et al.)

Figure 2 (Takahashi, et al.)
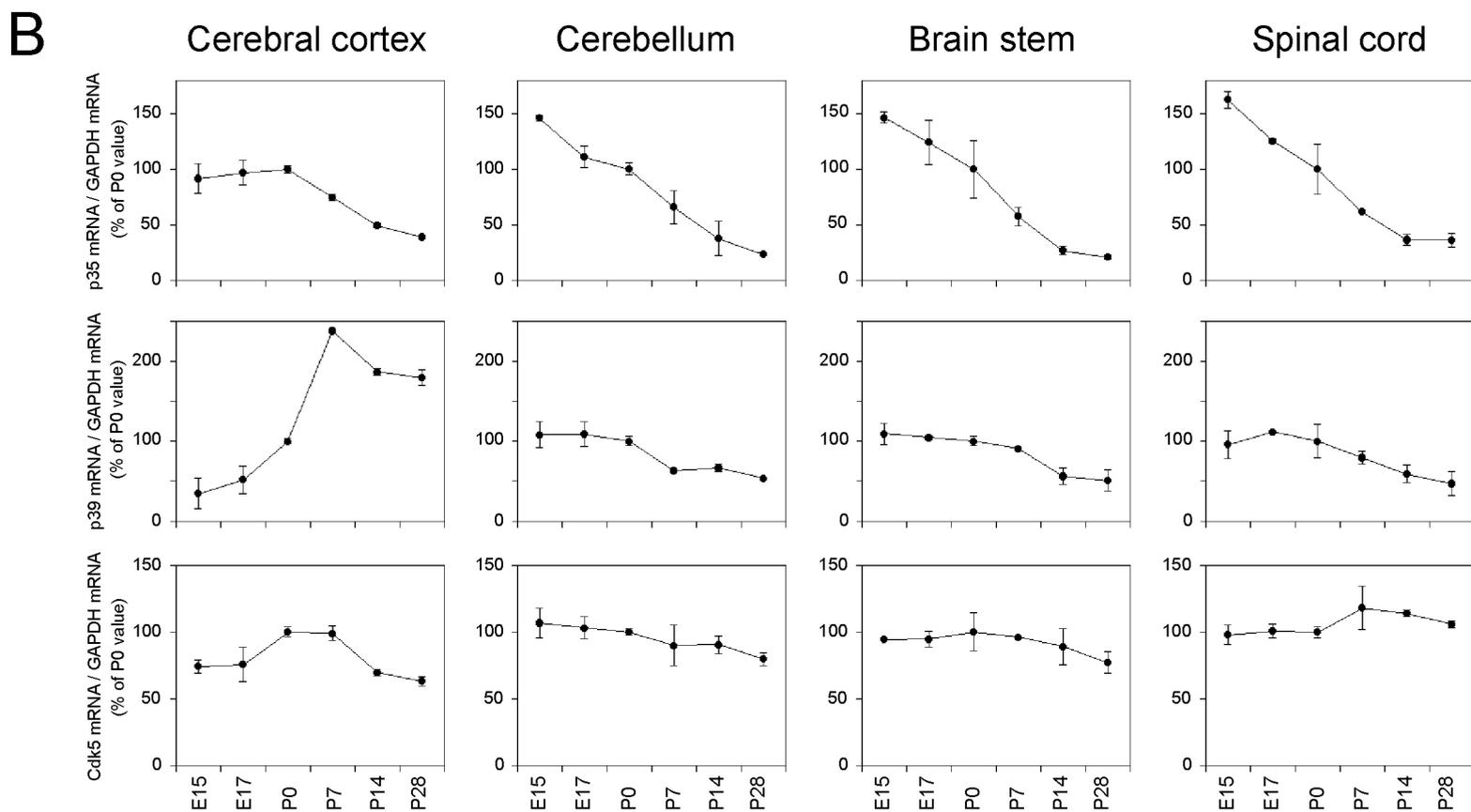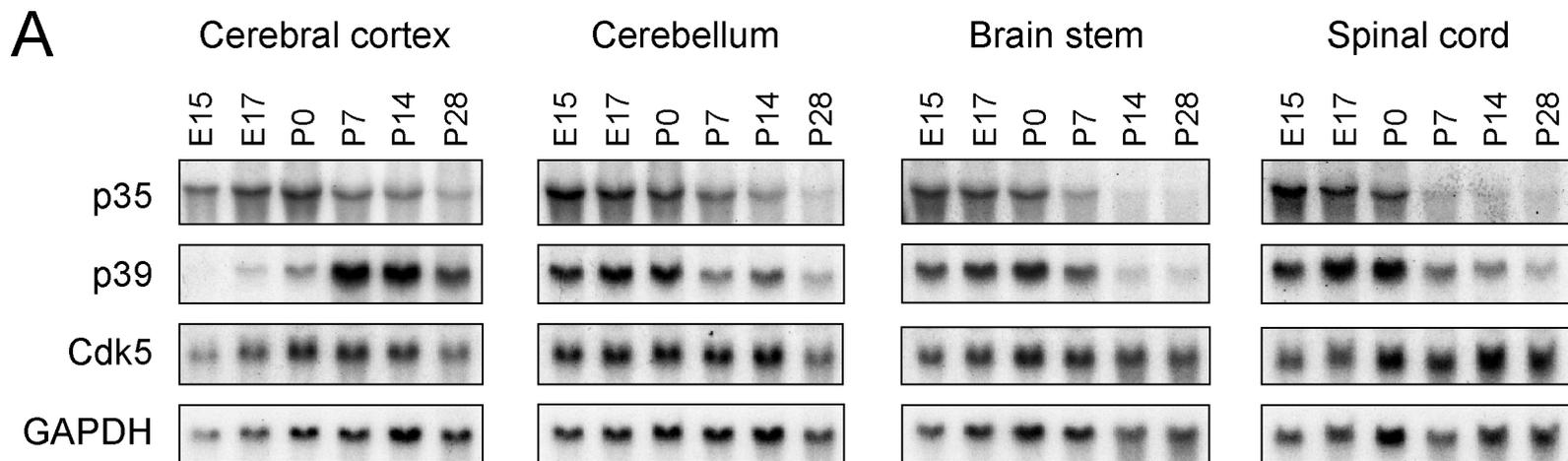
Figure 3 (Takahashi, et al.)

A

E15

P0

P14

B

E15

P0

P14
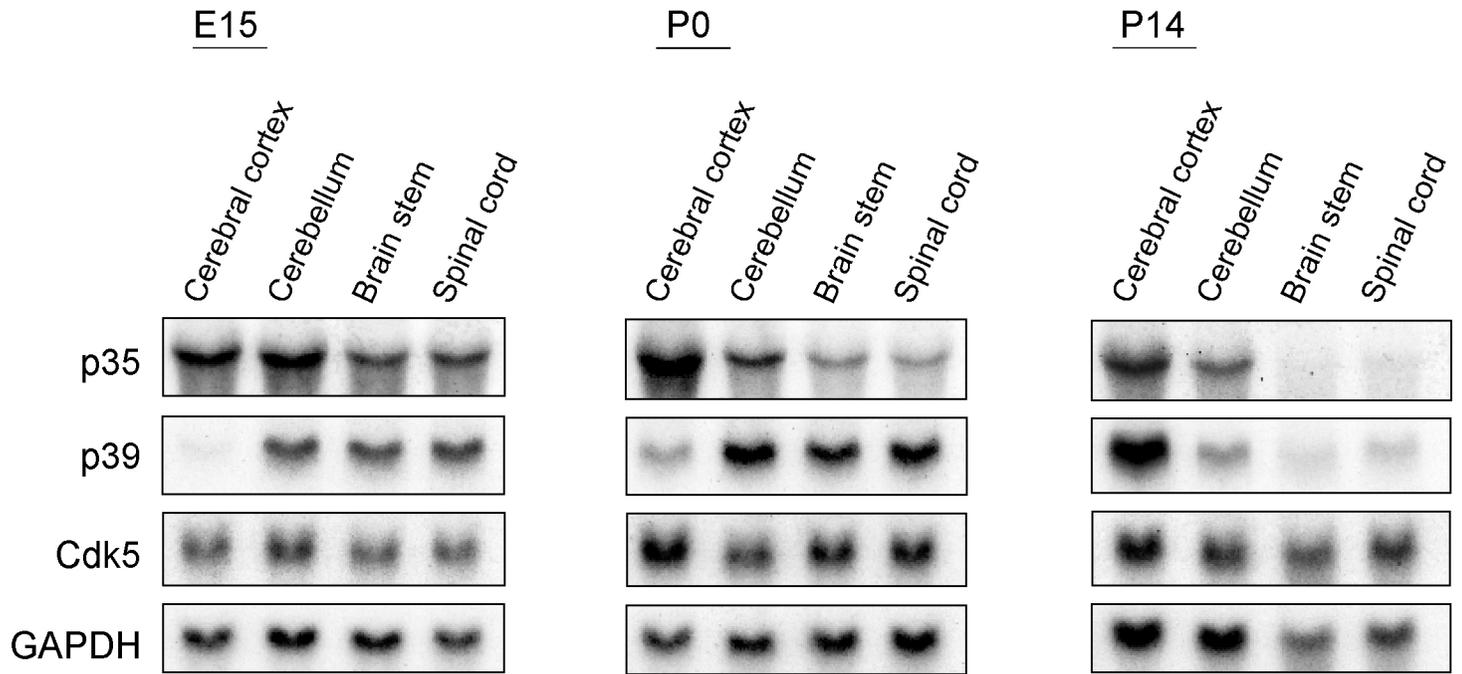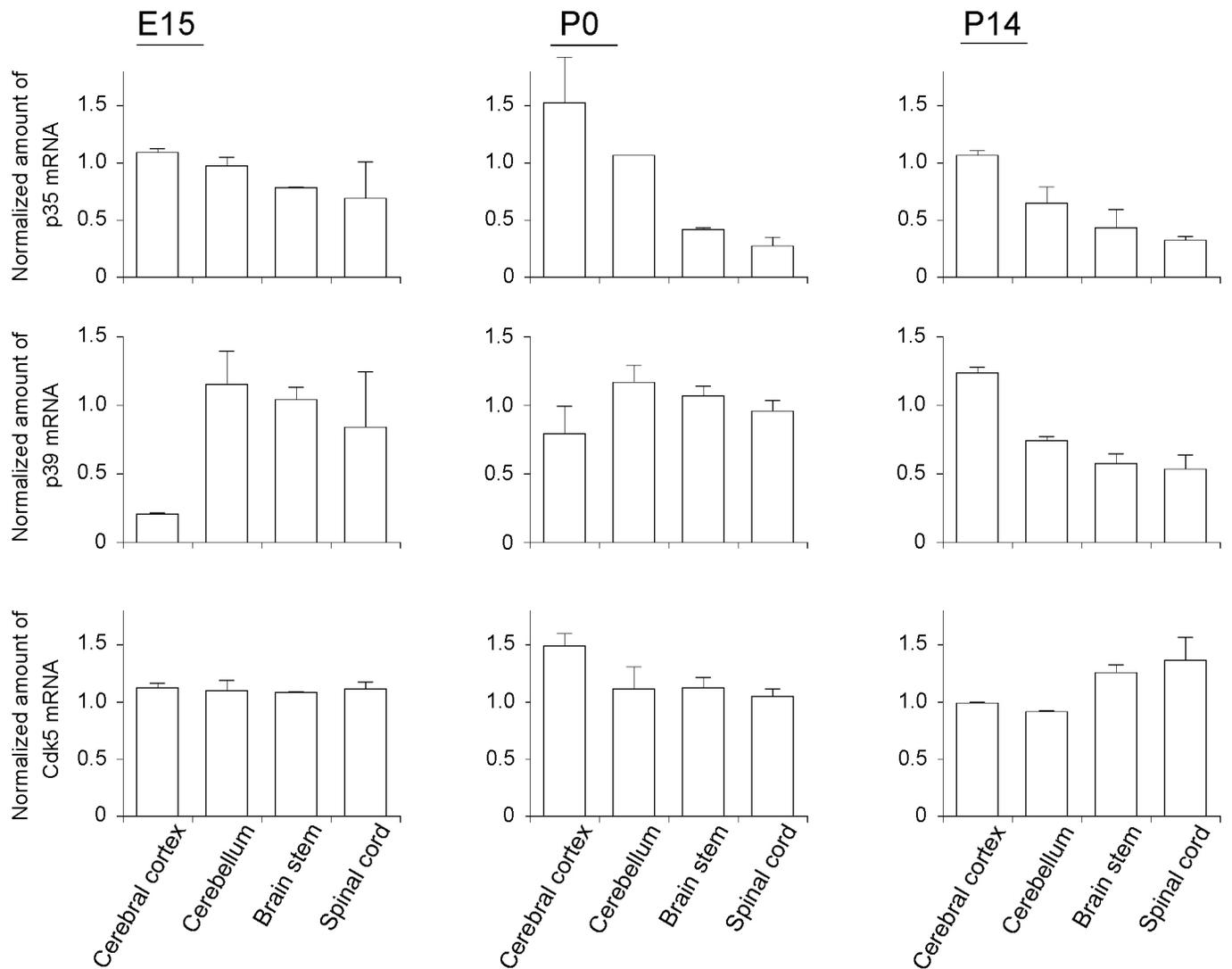
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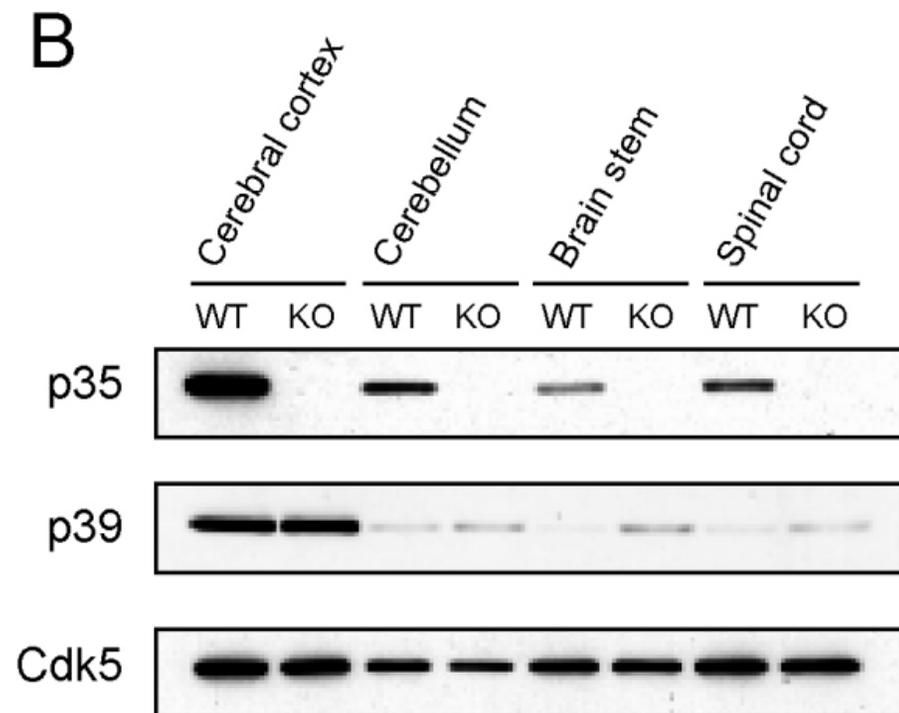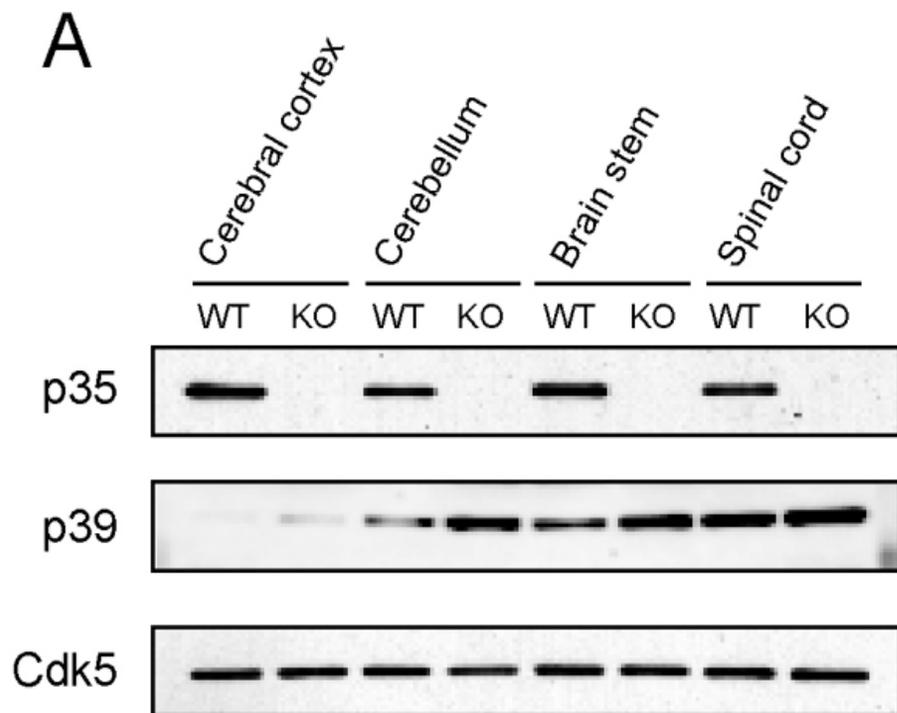
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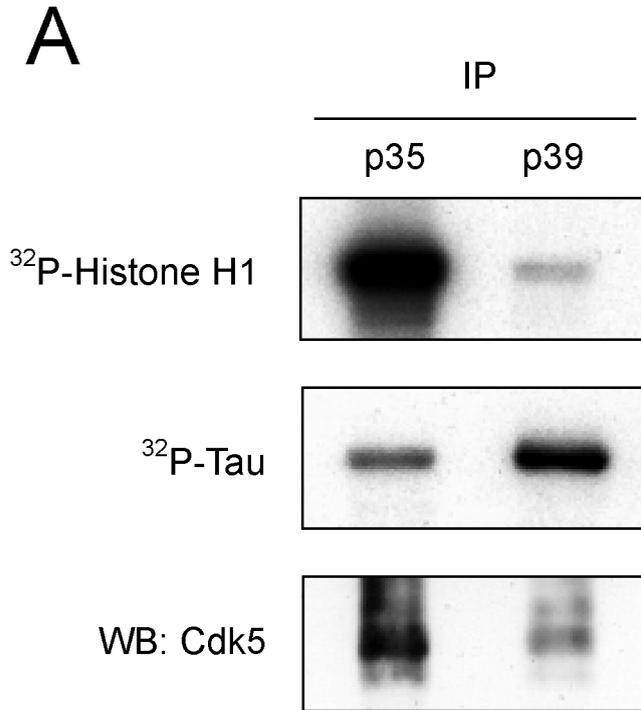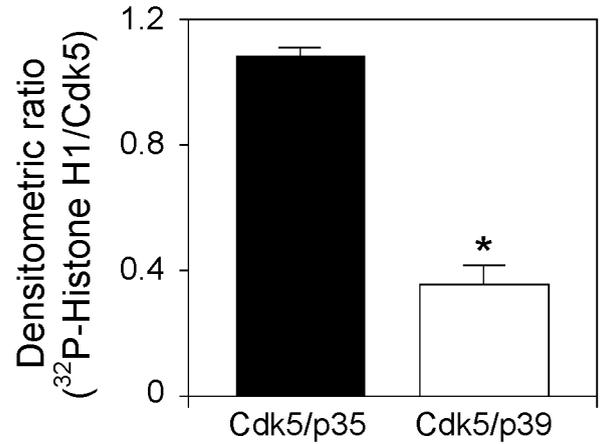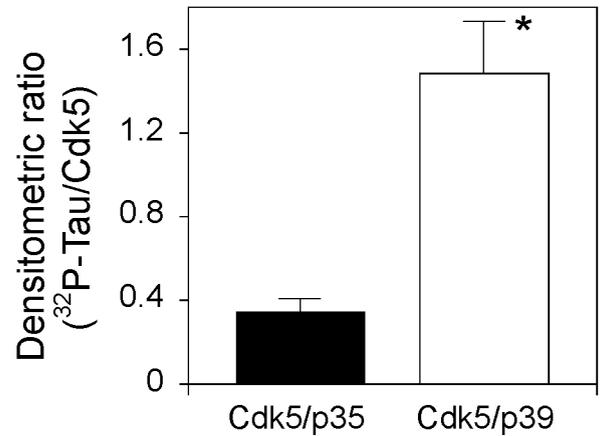
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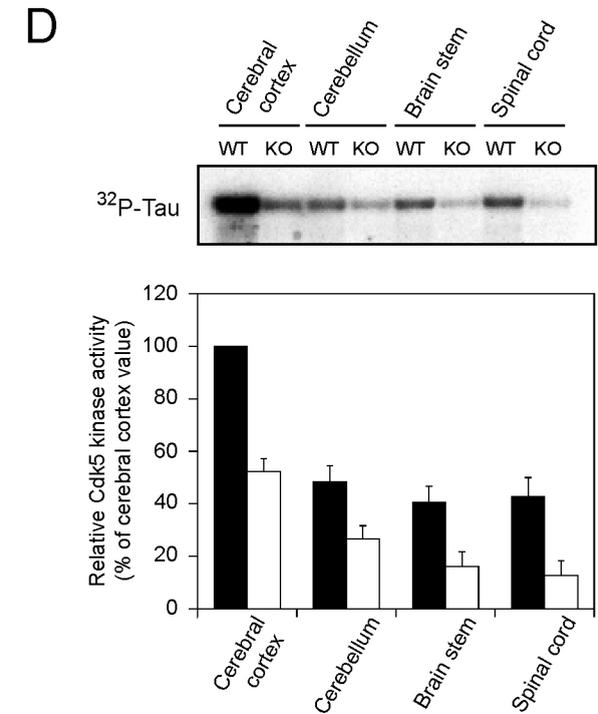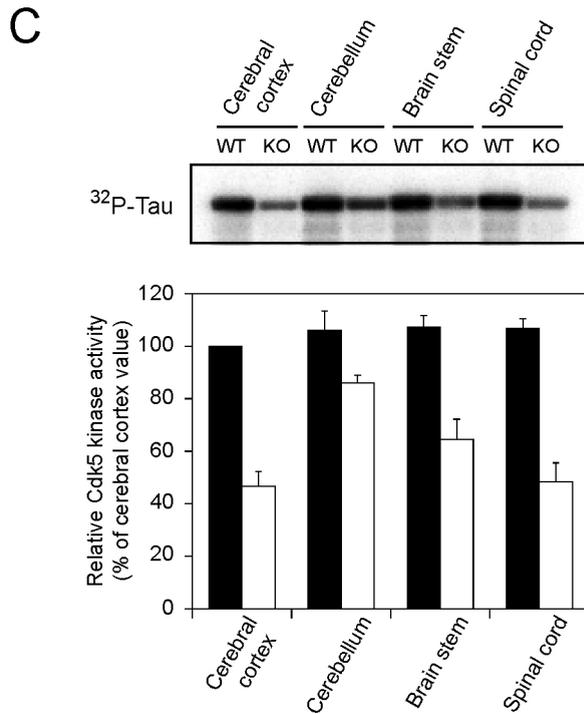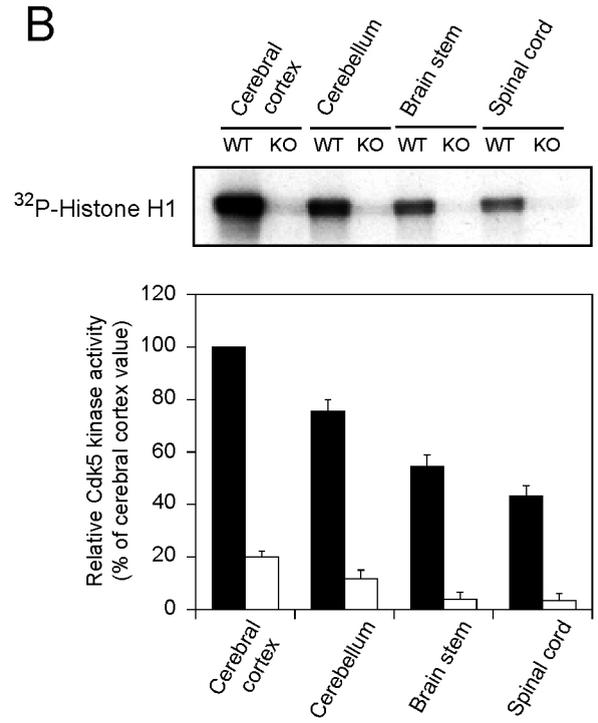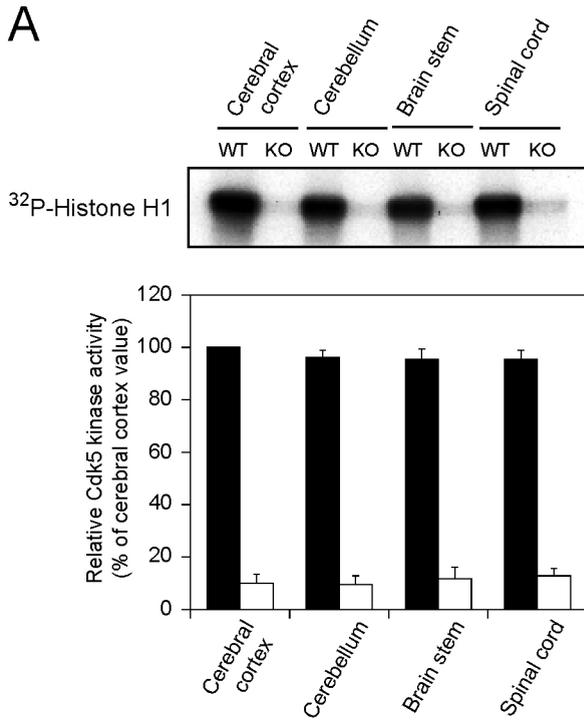
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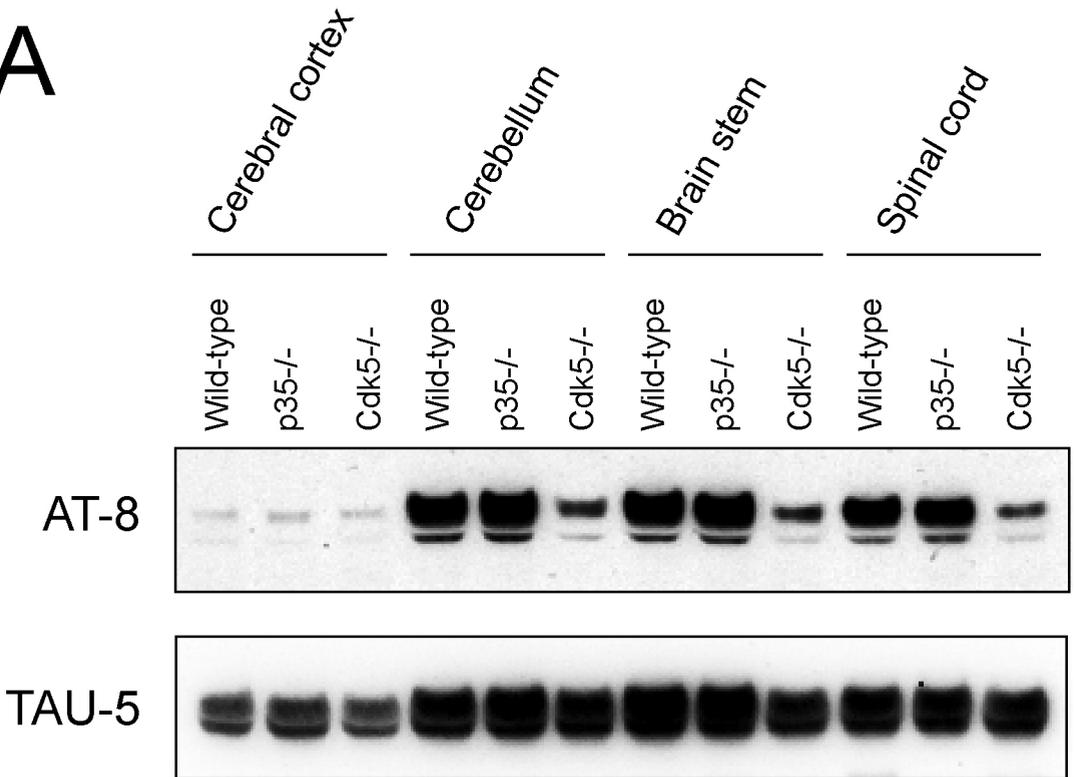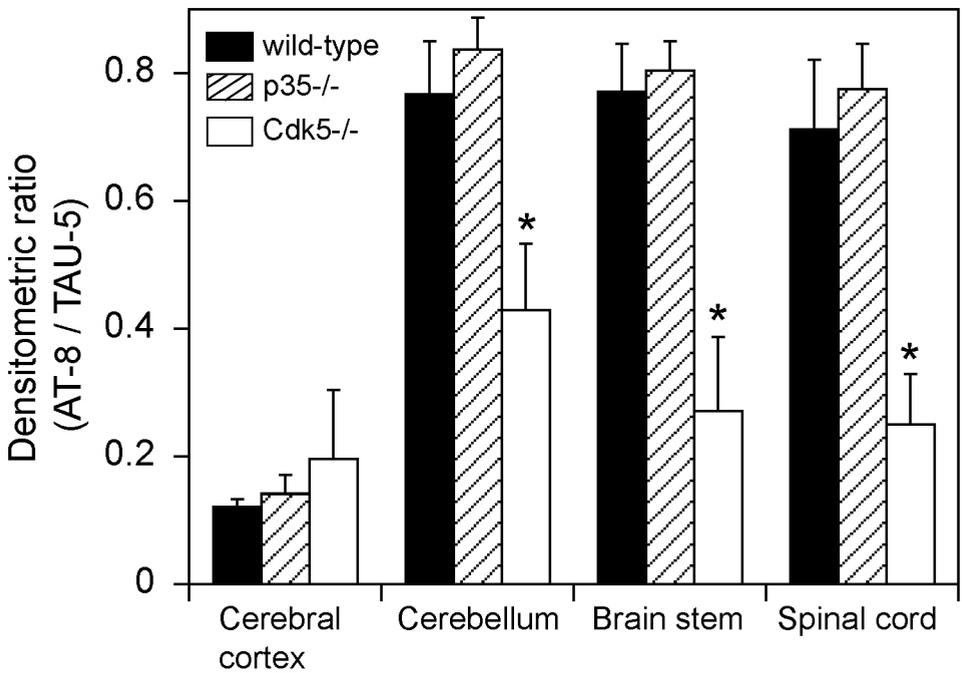
Figure 8 (Takahashi, et al.)

A

B