Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation

Margareta Nikolic,[1] Henryk Dudek,[2] Young T. Kwon,[1] Yolande F.M. Ramos,[1] and Li-Huei Tsai[1,3]

[1]Department of Pathology, [2]Department of Neurology, Division of Neuroscience, Children's Hospital, and Department of Neuroscience, Harvard Medical School, Boston, Massachusetts 02115 USA

Cyclin-dependent kinase 5 (cdk5) is highly homologous to other members of the cdk family that are known to function in proliferating cells. Despite the structural similarity, cdk5-associated histone H1 kinase activity is only detectable in postmitotic neurons of the central nervous system (CNS). p35 is a neuronal-specific cdk5 regulator that activates cdk5 kinase activity upon association. The cdk5/p35 kinase activity increases during the progression of CNS neurogenesis, suggesting a function of cdk5 in neuronal differentiation. Here we show that both cdk5 and p35 proteins are present in the growth cones of developing neurons. The staining pattern of cdk5 in the growth cones is similar to that of actin filaments but not microtubules. To address the functional significance of the cdk5/p35 kinase in neurogenesis, we ectopically expressed wild-type or mutant kinases in cortical cultures. Expression of dominant-negative mutants of cdk5 (cdk5N^{144} and cdk5T^{33}) inhibited neurite outgrowth, which was rescued by coexpression of the wild-type proteins. A similar extent of neurite outgrowth inhibition was obtained by transfection of an antisense p35 construct, which in turn was only rescued by p35 but not cdk5 coexpression. In contrast, longer neurites were elaborated in neurons that coexpressed exogenous cdk5 and p35. These observations suggest that the cdk5/p35 kinase plays a critical role in neurite outgrowth during neuronal differentiation.

[*Key Words*: cdk5; p35; neurite outgrowth; dominant-negative mutants; growth cone]

Received December 20, 1995; revised version accepted February 27, 1996.

Cyclin-dependent kinase 5 (cdk5) was isolated by virtue of its homology with the human cell cycle regulatory kinase cdc2 (Meyerson et al. 1992). It was also independently isolated as a histone H1 kinase activity from bovine brain (Lew et al. 1992a). Despite a wide distribution of basal cdk5 expression (Meyerson et al. 1992; Tsai et al. 1993), cdk5 kinase activity has thus far only been detected in the nervous system (Lew et al. 1992a; Shetty et al. 1993; Tsai et al. 1993) where cdk5 protein is most highly expressed. It has been shown that two members of the intermediate filament family, the medium and heavy neurofilament forms (NF-M and NF-H, respectively) (Lew et al. 1992b; Shetty et al. 1993) can be phosphorylated by cdk5 kinase activity. Neurofilaments are neuron-specific proteins that represent the major cytoskeletal component of axons. Furthermore, the cdk5 kinase complexes were shown to phosphorylate microtubule-associated proteins tau and MAP2 (for review, see Lew and Wang 1995; L.-H. Tsai and K.S. Kosik, unpubl.). These MAPs are important regulators of neuronal polarity and microtubule dynamics (for review, see Kosik and Caceres 1991).

[3]Corresponding author.

p35 was isolated as a brain-specific cdk5-associated protein with the ability to bind and activate the otherwise inert cdk5 kinase (Ishiguro et al. 1994; Lew et al. 1994; Tsai et al. 1994). Interestingly, despite its regulatory role for cdk5, p35 does not exhibit homology with any known cyclins, the regulators of other cdk family members. p35 appears to have unique specificity for cdk5 as it does not activate other members of the cdk family (Tsai et al. 1994). It is expressed exclusively in neurons of the central nervous system (CNS) with highest levels detected in the forebrain. In the developing cerebrum, p35 is excluded from the ventricular zone and thus proliferating cells. In contrast, high levels of cdc2, cdk2, cdk4, and cdk6 accompanied by their regulatory subunits, cyclins A, B, D's and E are evident in the ventricular zone (I. Delalle and V. Caviness, unpubl.). Low expression of p35 is first seen in migrating young postmitotic neurons and rapidly increases as they mature. In mice, p35 expression is maximal between developmental embryonic day 15 (E15) and postnatal day 0 (P0). Therefore, during embryonic development, the temporal pattern of cdk5 and p35 expression and thus, the cdk5/p35 kinase activity, closely parallel the extent of corticogenesis. Subsequent to cortical development, p35 levels gradually decline and remain high only in certain

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

areas of the forebrain characterized by high neuronal plasticity including the hippocampal system, the olfactory cortex, and the pyramidal layers of the neocortex (I. Delalle and L.-H. Tsai, in prep.). Therefore, despite the structural similarity between cdk5 and kinases that play key roles in cell cycle progression, the observed temporal and spatial patterns of the cdk5/p35 kinase suggest that it is involved in neurogenesis rather than cell division.

To determine whether the cdk5/p35 kinase has an essential function in neurogenesis, we set out to examine the effect of elimination or overexpression of cdk5/p35 kinase activity in neurons undergoing terminal differentiation. To date, cultured primary neurons are the only in vitro system in which cdk5-associated kinase activity is present and the cdk5/p35 complexes are evident. In culture, cortical neurons obtained from E17–18 rat embryos undergo morphological maturation within 5–7 days. The cdk5/p35 kinase activity is undetectable in immature cultures. Kinase activity first appears at day 3 postplating and subsequently rises with increased neuronal differentiation, indicated by extensive neurite elaboration and axonal fasciculation (Tsai et al. 1994). Therefore, these cultures serve as an excellent in vitro model system for studying the role of the cdk5/p35 kinase.

To alter endogenous cdk5/p35 kinase activity in differentiating neurons we transfected plasmid DNAs into cultured cortical neurons. We found that cdk5/p35 kinase activity is essential for neurite outgrowth, as overexpression of dominant-negative mutants of cdk5 or antisense p35 (as.p35) constructs caused significantly shorter neurites while coexpression of *ckd5* and p35 induced longer neurites. These results, together with the temporal pattern of cdk5/p35 kinase activity in the CNS and the observation that both cdk5 and p35 proteins are present in the growth cones of young neurons, suggested a role of the cdk5/p35 kinase in neurogenesis. They also provide the first known function of the cdk5/p35 kinase in differentiating neurons.

Results

cdk5 and p35 colocalize in neurons and are present in axonal growth cones

Cultured primary neurons derived from E17–18 rat cortices display a temporal pattern of cdk5 kinase activity that increases with the extent of their maturation. To deduce a function of the cdk5/p35 kinase, the subcellular localization of cdk5 and p35 proteins was examined by indirect immunocytochemistry and confocal microscopy. Three different cdk5 (one polyclonal and two monoclonal) (Tsai et al. 1993) and five different p35 (one polyclonal and four monoclonal) (Tsai et al. 1994) antibodies gave rise to the same staining patterns, indicating that the obtained results were specific for each protein. Antibodies to cdk5 labeled cell soma, axons, and dendrites of neurons in 3-day-old cultures (Fig. 1A,G). In all cases, staining was seen along entire neurites, including the growth cones (Fig. 1D,J,M). The subcellular localization of p35 was similar to that of cdk5 with staining evident in neuronal soma and along the entire length of neurites (Fig. 1B). Both cdk5 and p35 were also evident in the nuclei (Fig. 1A,B,G). Significant p35 levels could be seen in axonal growth cones (Fig. 1E). Interestingly, both cdk5 and p35 were present in the central core and periphery of axonal growth cones (Fig. 1D,E,F). Therefore in neurons, cdk5 and p35 proteins reside in the same subcellular compartments.

The significance of p35 and cdk5 in neurites and growth cones of cultured cortical neurons was addressed by examining whether the proteins are found in similar subcellular compartments as cytoskeletal elements involved in neurite formation. Actin and microtubule domains are localized to different regions of growth cones. Most of the microtubules (composed of α- and β-tubulin) are concentrated in the central core of the growth cone, whereas actin filaments are seen at the periphery. Both actin and microtubules play crucial roles during the growth and extension of axons and dendrites; therefore, an antibody to tubulin or rhodamine-conjugated phalloidin was used to compare the subcellular localization of actin and tubulin with cdk5 and p35. Figure 1, G–I, show that overall, antibodies to cdk5 and tubulin stained similar regions of cell soma and neurites. However, in the axonal growth cones, while the tubulin antibody stained the central cores, the cdk5 antibodies also stained the periphery (Fig. 1J–L). This demonstrated that cdk5 and actin microfilaments localized to similar growth cone regions (Fig. 1M–O).

These observations indicated that cdk5 and p35 predominantly colocalize with each other in cell soma and processes (Fig. 1C,F). The presence of cdk5 and p35 in the terminals of growing neurites where actin microfilaments reside raised the possibility that this kinase is involved in neurite outgrowth and/or axonal pathfinding.

Transfection of primary cortical neurons

To examine the role of the cdk5/p35 kinase in differentiating neurons, we altered the levels of endogenous kinase by transfection with wild-type or mutant cdk5 and p35 expression vectors. A β-galactosidase (β-gal) construct driven by the cytomegalovirus (CMV) early promoter was used to estimate transfection efficiency. A 1%–2% transfection efficiency was usually achieved in cultured rat E17 cortical neurons (Fig. 4A, below). Immunostaining with an antibody to the glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed by glia but not neurons, showed that on average <1% of the cultured cells were glia. Therefore, the vast majority of transfected cells were neuronal. To examine the subcellular localization of exogenous proteins, a cdk5–green fluorescent protein fusion construct (cdk5–GFP) was made that allowed visualization of the fusion product without further processing. GFP alone was diffusely localized to the nuclei and cell soma and poorly transported down neurites (data not shown). In contrast, the cdk5–GFP fusion product was present in all the processes as was p35, when the cdk5–GFP-expressing cells were stained with p35 antibodies (Fig. 2A,B). Therefore, these experiments showed that the overexpressed proteins in neurons were localized to the appro-

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

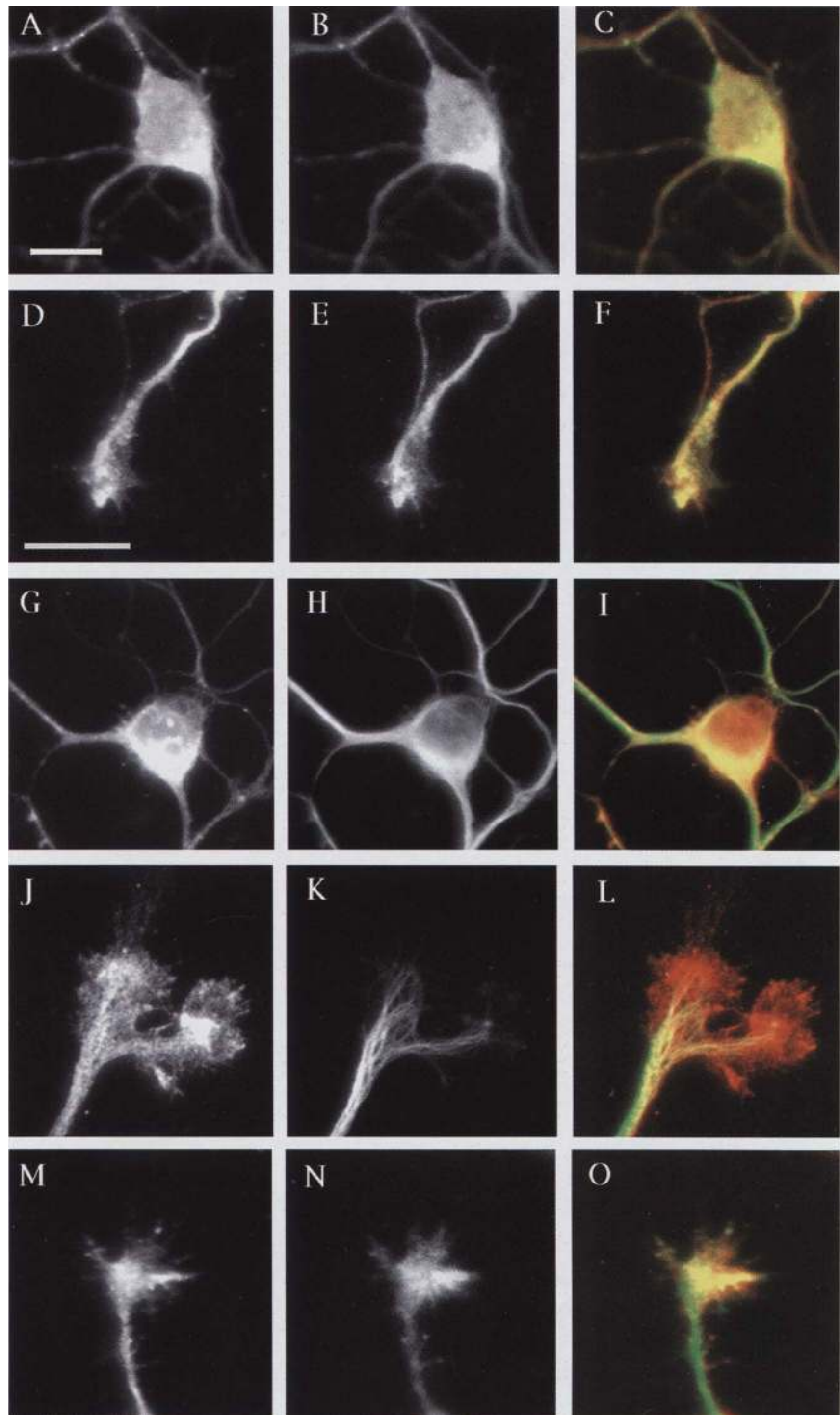

Figure 1. cdk5 and p35 colocalize in cortical neurons. Double immunolabeling of 3-day-old cultures of E17 rat cortical neurons was performed with an anti-cdk5 polyclonal antibody (*A,D,G,J,M*) and an anti-p35 monoclonal antibody (*B,E*), anti-tubulin antibody (*H,K*), or rhodamine-conjugated phalloidin (*N*). cdk5 was detected using a FITC (*A,D,M*) or Texas Red (*G,J*)-conjugated secondary antibody. Tubulin and p35 were visualized using a FITC or Texas Red-conjugated secondary antibody, respectively. Note that cdk5 and p35 or actin are seen in similar subcellular compartments, especially in the growth cones (*C,F,O*). This is in contrast to tubulin, which is only evident in the growth cone central core (*L*). Images were obtained using a Zeiss LSM 410 confocal microscope. The scale bar shown in *A* is 10 μm and applies for *B, C, G, H, I, J, K,* and *L*; the scale bar in *D* is 10 μm and applies for *E, F, M, N,* and *O*. cdk5 and p35 immunostaining in cell soma (*A–C*) and growth cones (*D–F*); cdk5 and tubulin immunostaining in cell soma (*G–I*) and growth cones (*J–L*); cdk5 immunostaining and actin in growth cones (*M–O*).

priate compartments where the endogenous cdk5 and p35 proteins normally reside.

To unambiguously identify transfected cells and observe their morphology in the experiments described below, CMV–β-gal expression vectors were cotransfected with excess amounts of other plasmids (1:5 ratio to total DNA), followed by indirect immunostaining using an anti-β-gal antibody. Use of this DNA ratio ensures that most of the β-gal-positive cells are also transfected with the other expression vectors. It has been shown that β-gal uniformly distributes throughout neurons and is transported down the axons and dendrites (Fig. 4A, below; Cepko et al. 1990; Halliday and Cepko 1992), allowing clear visualization of overall cell morphology. Alkaline phosphatase (AP) expression from the Moloney leukemia virus (MLV) long terminal repeat (LTR) was

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

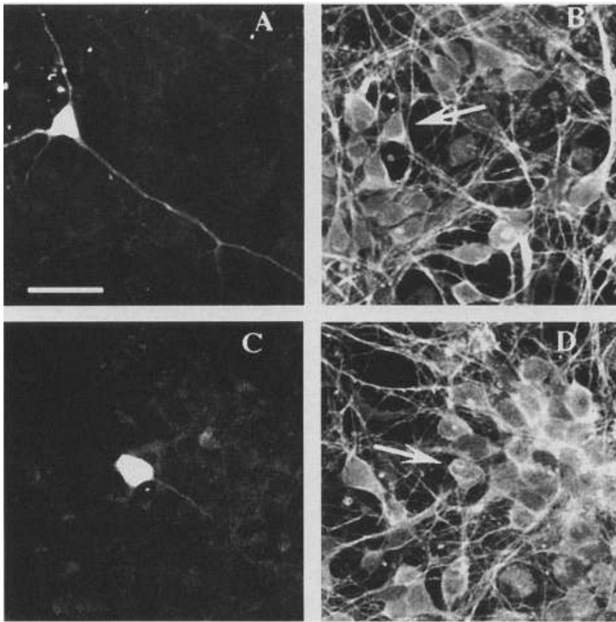

Figure 2. GFP fusion proteins are evident in the appropriate subcellular compartment. Cultured cortical neurons obtained from E17 rat embryo cortices were transfected 3 days post-plating with cdk5–GFP (*A*) or cdk5N^{144}–GFP (*C*). The cultures were fixed 2 days post-transfection and costained with an anti-p35 monoclonal antibody (*B,D*). Arrows in *B* and *D* point to cdk5–GFP or cdk5N^{144}–GFP-expressing cells, respectively). Images were obtained using a Zeiss LSM 410 confocal mircrscope. Scale bar, 25 μm.

used as an alternative marker of transfected cells and yielded comparable results as β-gal (data not shown; Halliday and Cepko 1992; Reid et al. 1995). The transfected cells were also examined by Hoechst 33258 and MTT staining (see Materials and methods). Hoechst 33258 is a DNA-specific dye that revealed no chromosomal abnormalities or cell death associated with any of the transfections. The MTT dye also distinguished living from dead cells. Using this reagent we observed no obvious nonspecific cell toxicity in neuronal populations transfected with any of the expressed vectors.

Expression of cdk5 dominant-negative mutants in differentiating neurons causes distinct morphological consequences

To address the requirement for cdk5/p35 kinase activity during neuronal differentiation we analyzed the consequences of altered kinase levels in differentiating neurons by overexpression of cdk5 dominant-negative mutants. An Asp (D) to Asn (N) change at codon 144 of *ckd5* was constructed previously (van den Heuvel and Harlow 1994) that was shown to abolish activation of endogenous cdk5 by p35 when overexpressed (Tsai et al. 1994). Structural analyses of protein kinases suggested that the equivalent position to cdk5D^{144} on protein kinases is involved in the phosphotransfer reaction (De Bondt et al. 1993; Taylor et al. 1993; Jeffrey et al. 1995). To establish whether cdk5N^{144} behaves as a dominant-negative mutant, we coexpressed cdk5N^{144} and p35 in C33A cells (derived from a cervical carcinoma) and scored for levels of histone H1 kinase activity and strength of association between p35 and mutant cdk5. As reported previously, expression of p35 activated the endogenous cdk5 kinase (Fig. 3A). Coexpression of p35 with wild-type cdk5 gave rise to a higher level of kinase activity. However, coexpression of cdk5N^{144} with p35 completely abolished kinase activity associated with p35. A second mutant of cdk5 with a Lys (K) to Thr (T) change at codon 33 was also tested in this experiment. The equivalent position to K33 on other kinases was shown previously to be involved in ATP binding (De Bondt et al. 1993; Taylor et al. 1993; Jeffrey et al. 1995). Coexpression of cdk5T^{33} with

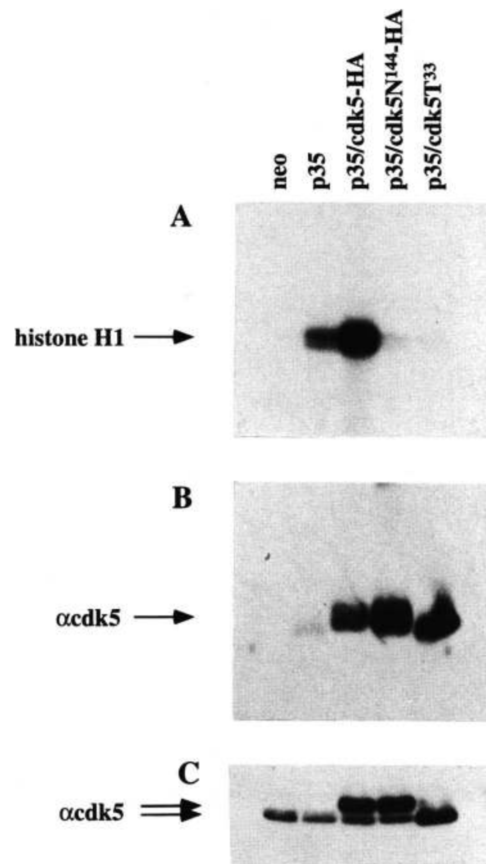

Figure 3. cdk5 mutants are dominant over wild-type cdk5. C33A cervical carcinomas were transfected with CMV backbone vector (neo); CMV–p35; CMV–p35 and CMV–cdk5–HA; CMV–p35 and CMV–cdk5N^{144}–HA; or CMV–p35 and CMV–cdk5T^{33} as described in Materials and methods. (*A*) One hundred micrograms of cleared protein extracts was subjected to p35 (anti-p35 antibody 4E3) immunoprecipitations and histone H1 kinase reactions. Total protein extracts (3.5 mg) were subjected to anti-p35 immunoprecipitations and Western blot analysis (*B*), or (20 μg) directly to Westen blot analysis with an anti-cdk5 antibody (DC17) (*C*). The samples were separated on denaturing 12% acrylamide gels.

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

p35 also abolished p35-associated kinase activity (Fig. 3A). Western blot analysis was performed to examine the association between p35 and the cdk5 mutants. Figure 3B shows that as reported, p35 and cdk5 formed complexes upon coexpression. A notably higher level (about two- to threefold) of association was observed between p35 and cdk5N^{144}, despite equivalent levels of protein expression (Fig. 3C). A high level of p35 association was also observed with cdk5T^{33} (Fig. 3B). These experiments demonstrated clearly that cdk5N^{144} and cdk5T^{33} are kinase dead mutants, both of which bind p35 more efficiently than wild-type cdk5. Similar results were obtained using several cell lines of neuronal origin such as PC12 (rat pheochromocytoma), SKNSH (neuroblastoma), and HiB5 (SV40 T antigen-immortalized rat hippocampal neurons). We therefore conclude that both cdk5N^{144} and cdk5T^{33} are dominant-negative mutants when expressed in cells.

Cortical neurons obtained from E17–18 rat embryos mature morphologically during the course of culturing, which is accompanied by a rapid increase in cdk5-associated kinase activity from undetectable levels in immature cultures to very high activity in 7-day-old cultures (Tsai et al. 1994). We utilized the transfection approach to examine the requirement for cdk5/p35 kinase activity during the maturation of these cells. Neurons transfected with β-gal or AP were morphologically indistinguishable from each other and from untransfected neurons (Fig. 4A; data not shown). Coexpression of cdk5 or p35 with β-gal did not significantly alter the morphology of cells in comparison to β-gal expression alone (Figs. 4A,B, and 5A). Remarkably, expression of cdk5N^{144} caused extensive axonal and dendritic shortening (Figs. 4C and 5A) and the same effects were evident upon cdk5T^{33} overexpression (Fig. 4D).

A proportion of dominant-negative cdk5-expressing cells hardly displayed any visible neurites (e.g., see Fig. 2C). On the other hand, occasional neurons with long neurites also existed (Fig. 5; see Discussion). The morphological alterations were equally evident when the amount of cdk5N^{144} was reduced to half of the total DNA transfected and the rest supplemented with carrier DNA, further demonstrating the dominant effects of this mutant (Fig. 5A; Table 1).

To quantitate the differences between neurons with wild-type or reduced levels of cdk5 kinase activity, the

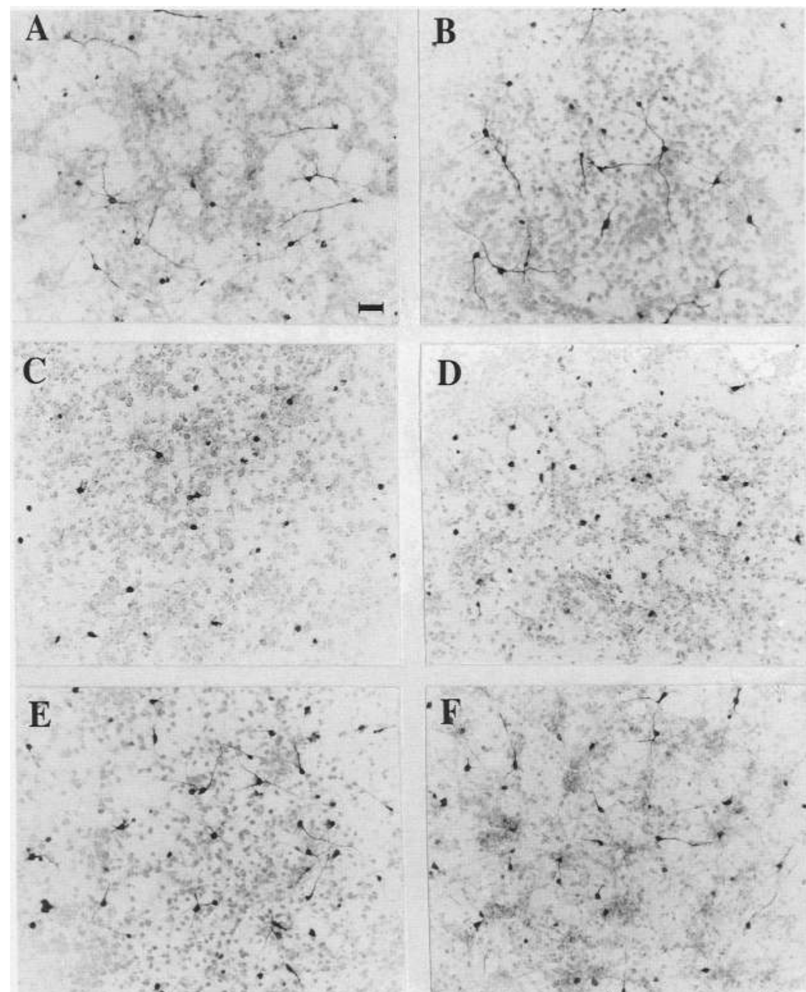

Figure 4. Expression of cdk5N^{144} in developing neurons specifically caused inhibition of neurite outgrowth. Cultured cortical neurons were transfected with the following expression vectors: CMV–β-gal alone (*A*); CMV–p35 (*B*); CMV–cdk5N^{144} (*C*); CMV–cdk5T^{33} (*D*); equal amounts of CMV–cdk5N^{144} and CMV–cdk5 (*E*); or equal amounts of CMV–cdk5N^{144} and CMV–p35 (*F*). In *B, C, D, E,* and *F*, CMV–β-gal was included at a 1:5 ratio to total DNA tranfected. Targeted cells were immunocytochemically visualized using specific anti-β-gal antibodies (see Materials and methods). Only expression of CMV–cdk5N^{144} or CMV–cdk5T^{33} caused a visible morphological alteration, causing neurons to elaborate shortened neurites (*C,D*). The phenotype was reversed to normal by cotransfection of CMV–cdk5N^{144} with CMV–cdk5 or CMV–p35, revealing that the alterations were due specifically to the loss of wild-type cdk5 in complex with p35. Scale bar, 40 μm.

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

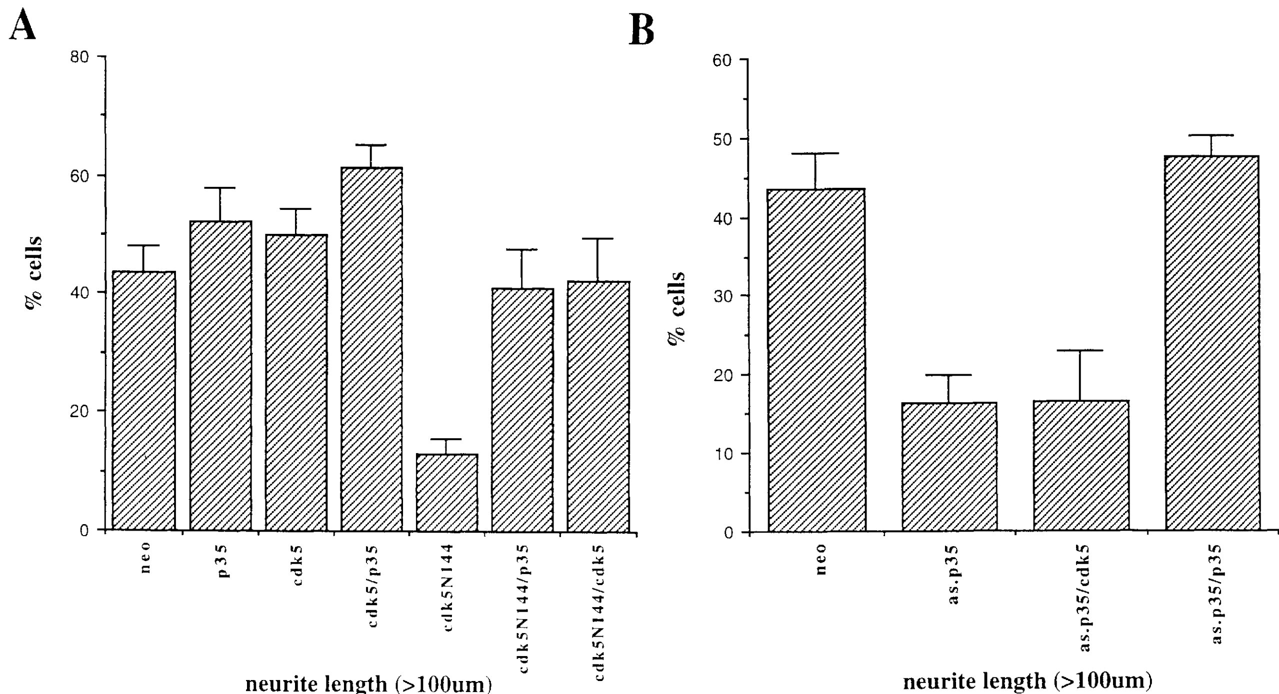

Figure 5. Loss of cdk5/p35 kinase activity causes neurite truncation in developing neurons. Cultured cortical neurons were transfected with the following expression vectors in *A*: CMV–neo, CMV–p35, CMV–cdk5, CMV–p35 and CMV–cdk5, CMV–cdk5N^{144}, CMV–cdk5N^{144} and CMV–p35, or CMV–cdk5N^{144} and CMV–cdk5. Vectors in *B*: CMV–neo, CMV–as.p35, CMV–as.p35 and CMV–p35, or CMV–as.p35 and CMV–cdk5. The precise length of the longest neurite of each transfected neuron was measured as described in Materials and methods. More than 120 neurons were examined for every transfection, and the lengths of neurites were compared to mock-transfected cells. The data are presented as the percentage of examined neurons elaborating neurites longer than 100 μm.

neurite lengths of transfected cells were examined using a cooled charged-coupled device (CCD) camera and Metamorph image analysis software (Universal Imaging). For each transfection 120–240 positive cells were examined, as described in Materials and methods. Analysis of the obtained data revealed that only ~13% of cdk5N^{144}- expressing neurons elaborated neurites longer than 100 μm in contrast to 44% of mock-transfected cells (Fig. 5A; Table 1).

Dominant-negative cdk5 expression specifically affects neurite outgrowth

To rule out the possibility that loss of cdk5/p35 kinase caused a general protein transport defect, we visualized actin filaments in the transfected cells using rhodamine-conjugated phalloidin, which revealed a clear shortening of neurites upon cdk5N^{144} or cdk5T^{33} expression (data not shown). Furthermore, use of AP as a transfection marker yielded the same results as β-gal. No difference was observed in the levels of marker protein expression (β-gal or AP) between different transfections, indicating that expression of cdk5N^{144} or cdk5T^{33} did not cause poorly visible neurites in this manner. Transfection of cdk5N^{144}–GFP into cortical neurons also caused extensive process truncation (Fig. 2A,C), further indicating that the observed effect was not specific to β-gal or AP.

We also considered that loss of cdk5/p35 kinase activity might result in neurite regression. This is also unlikely, as analysis of dominant-negative cdk5-expressing neurons 3 days post-transfection did not reveal shorter neurites than neurons from the same experiment 1 or 2 days post-transfection. In contrast, neurites of all the other targeted neurons had extended during this period on average 20%. We thus favor the explanation that the cdk5/p35 kinase regulates neurite outgrowth; therefore, loss of the kinase activity in neurons prevents or reduces neurite elaboration.

Inhibited neurite outgrowth is specific to the loss of cdk5/p35 kinase activity

Next, we sought to determine whether the morphological consequences of dominant-negative cdk5 expression in neurons was a specific effect attributable to loss of cdk5 kinase. Cotransfection of cdk5N^{144} or cdk5T^{33} with wild-type cdk5 successfully reversed the neurite outgrowth inhibition; thus, ~42% of transfected neurons elaborated neurites longer than 100 μm (Figs. 4E and 5A). This suggested that the morphological effects were attributable to loss of cdk5 kinase.

We further examined whether neurite outgrowth inhibition was the result of the loss of active cdk5/p35 kinase by cotransfection of cdk5N^{144} or cdk5T^{33} with p35. Remarkably, expression of equal amounts of p35 and cdk5N^{144} vectors prevented the inhibition of neurite

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

Table 1. *The effect of mutant or wild-type cdk5/p35 kinase on neurite outgrowth in differentiating neurons*

Transfection	(no. cells)	Percent cells > 150 μm (S.E.)	Percent cells > 100 μm (S.E.)	Percent cells > 50 μm (S.E.)
CMV–neo	(208)	12.5 (2.1)	43.6 (4.5)	86.5 (3.9)
p35	(201)	19 (3.3)	52.3 (5.6)	88.4 (2.8)
cdk5	(242)	18.6 (3.1)	50 (4.4)	90 (2.4)
cdk5/p35	(194)	24 (3.3)	61.6 (3.8)	95 (2.2)
$cdk5N^{144}$	(124)	5.1 (2.2)	13.2 (2.5)	39 (4.2)
$cdk5N^{144}$/p35	(180)	13.1 (3.5)	41 (6.7)	77 (7.7)
$cdk5N^{144}$/p35	(127)	13.4 (3.6)	42.5 (7.2)	83.6 (4.7)
as.p35	(137)	4.1 (0.9)	16.2 (3.6)	52 (8.0)
as.p35/p35	(120)	16.7 (2.3)	47.5 (2.8)	88 (1.7)
as.p35/cdk5	(120)	4.1 (2.5)	16.6 (6.3)	54 (8.4)

outgrowth observed with $cdk5N^{144}$ alone (Fig. 4F). Thus, the percentage of neurons elaborating neurites longer than 100 μm was fully restored to 41% (Figs. 4F and 5A; Table 1). Similarly, the effects caused by $cdk5T^{33}$ were fully rescued by p35. As shown in Figure 3, the dominant-negative cdk5 mutants are more efficient at binding p35 than wild-type cdk5 are. Thus we postulate that when coexpressed, p35 sequesters the mutant forms of cdk5 efficiently, preventing them from affecting the differentiating neurons. The ability to prevent the $cdk5N^{144}$- or $cdk5T^{33}$-induced morphological defects with the regulatory subunit of the cdk5/p35 kinase argues strongly for a role of the active kinase in differentiating neurons.

We gained further support for the role of the cdk5/p35 kinase function in neurite outgrowth by transfecting cells with as.p35 expression vectors. p35 expression levels correlate with the temporal pattern of cdk5-associated kinase activity during cortical differentiation. Expression is absent from proliferating neuronal precursor cells and increases with the extent of terminal differentiation (Tsai et al. 1994). At the time of transfection, the cdk5/p35 kinase levels in cortical cultures are low, therefore providing a good time point for reducing or eliminating endogenous p35 protein by overexpression of as.p35 vectors. Primary neurons expressing as.p35 elaborated truncated neurites similar to that seen with $cdk5N^{144}$ or $cdk5T^{33}$ (Fig. 5, cf. A and B). Quantification revealed that only 16% of as.p35-transfected cells elaborated neurites longer than 100 μm, in contrast to 44% of mock transfectants (Fig. 5B; Table 1). Coexpression of as.p35 with p35 fully restored the percentage of neurons with neurites longer than 100 μm (47.5%), confirming the specificity of the as.p35 effects (Fig. 5B; Table 1). On the other hand, we were unable to prevent the consequences of as.p35 expression with wild-type cdk5 (Fig. 5B). Therefore, neurite outgrowth inhibition caused by as.p35 was attributable to specific loss of the p35 protein.

Coexpression of wild-type cdk5 and p35 promotes neurite outgrowth

Neurons overexpressing cdk5 or p35 alone appeared to elaborate slightly longer neurites than mock-transfected cells, which was evident following the exact measurement of neurite lengths. Approximately 19% of cdk5 or p35-transfected neurons elaborated neoneurites longer than 150 μm in comparison to 12.5% of mock transfectants (Table 1). Interestingly, this phenomenon was greatly enhanced following coexpression of both p35 and cdk5. In this case, 60% of transfected cells elaborated neurites longer than 100 μm in contrast to only 44% of mock-transfected cells (Fig. 5). The effects on outgrowth were most apparent in the longest range of measured neurite lengths. Thus, the proportion of neurons transfected with p35 and cdk5 elaborating neurites longer than 150 μm was double that seen in mock transfectants (cf. 24% of p35 and cdk5 transfectants with 12.5% of neo transfectants in Table 1). Together, these results strongly suggested that the cdk5/p35 kinase plays an important role during neurite outgrowth as both loss- and gain-of-function experiments yielded specific and complementary phenotypes.

Discussion

On the basis of its unique expression pattern in the developing CNS, the cdk5/p35 kinase has been speculated to play a role in neurogenesis. In this study we addressed the role of the cdk5/p35 kinase in maturation of cultured neurons derived from cortices of E17–18 rat embryos. We altered the levels of the cdk5/p35 kinase in cultured neurons by transfection with wild-type or mutant forms of cdk5 or p35 expression vectors. Two independent cdk5 dominant-negative mutants, $cdk5N^{144}$ and $cdk5T^{33}$, together with an antisense p35 construct, were used to assess the consequence of loss of cdk5/p35 kinase activity in neurons undergoing differentiation. Results from these experiments strongly suggest that the cdk5/p35 kinase plays an essential role in neurite outgrowth.

Primary cultures derived from cortices of rat E17–18 embryos have been demonstrated to be a good in vitro source of cdk5/p35 kinase activity. These cultures undergo morphological maturation within ~5–7 days, which is closely paralleled by an increase in the level of cdk5/p35 kinase activity (Tsai et al. 1994). We altered the levels of cdk5/p35 kinase activity in these cultures by transfection with mutant or wild-type cdk5 and p35

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

constructs. The morphological consequences were observed post-transfection. β-Gal uniformly distributes throughout neurons and is transported down the axons and dendrites; in addition, AP is a membrane-associated protein (Cepko et al. 1990; Halliday and Cepko 1992; Reid et al. 1995). These two markers enabled clear visualization of overall cell morphology. Therefore, cotransfection of marker proteins such as β-gal or AP was used to identify transfection-positive cells. Only small amounts of β-gal or AP plasmids were needed for detection of positive cells via indirect immunocytochemistry or direct staining. A cdk5–GFP fusion construct was used to demonstrate a similar subcellular localization of exogenously expressed and endogenous proteins.

To eliminate cdk5 kinase activity, we first took advantage of the existing $cdk5N^{144}$ mutant. This mutant was shown previously to block endogenous cdk5 activation in C33A cells (Tsai et al. 1994). In this report we show that while $cdk5N^{144}$ is no longer an active kinase, it binds to p35 at least two to three times more efficiently than wild-type cdk5. Another mutant that we created, $cdk5T^{33}$, harboring a K → T alteration at condon 33, behaved similarly to $cdk5N^{144}$. Because these mutants were kinase inactive yet displayed stronger affinity for p35 than wild-type cdk5, we considered them to be dominant negative. The molecular mechanisms that underlie the higher levels of association between mutant cdk5 and its regulatory subunit remain unclear, especially as no other cdk mutants have been documented to display similar phenotypes. Nonetheless, when expressed in cells, $cdk5N^{144}$ and $cdk5T^{33}$ are thought to efficiently block activation of endogenous cdk5 by titrating out available p35 in cells derived from both neuronal and non-neuronal origins.

Expression of $cdk5N^{144}$, $cdk5T^{33}$, or as.p35 in differentiating neurons caused drastic morphological changes, especially in neurite maturity. We have eliminated the possibility that the observed effects were attributable to alterations in β-gal expression or transport down existing neurites by examining actin distribution in the transfected neurons. In addition, the $cdk5N^{144}$–GFP-fused construct when expressed in neurons also caused a high degree of outgrowth inhibition. In contrast, expression of cdk5–GFP, cdk5, or p35 resulted in normal looking neurites. Use of AP as a transfection marker also yielded the same results as β-gal. We also do not believe that loss of cdk5/p35 kinase activity might cause neurite regression, as demonstrated by observing no increase in the degree of neurite shortening in neurons expressing $cdk5N^{144}$ or $cdk5T^{33}$ with time post-transfection. The fact that two independent dominant-negative mutants resulted in the same phenotype and that this effect could be fully rescued by cotransfection with the wild-type kinase strongly suggest that the phenotype observed is attributable to specific loss of active cdk5 kinase in neurons. Expression of as.p35 in neurons also caused a profound effect on neurite outgrowth that was reverted by p35 but not cdk5 coexpression. We further tested the possibility that it is the cdk5/p35 kinase that is required for neurite outgrowth by rescuing the effect of cdk5 dominant-negative mutants with p35. Coexpression of excess amounts of p35 fully reversed the neurite truncation effect of the dominant-negative mutants. These results provided the most significant evidence for the specificity of the observed morphological consequences of $cdk5N^{144}$ or $cdk5T^{33}$ expression in differentiating neurons and therefore the importance of the cdk5/p35 kinase in these cells. Finally, coexpression of cdk5 and p35 resulted in longer neurites, which further supported the notion that the cdk5/p35 kinase regulates neurite outgrowth.

We consistently observed a small percentage of $cdk5N^{144}$ or as.p35-transfected neurons exhibiting long neurites. It is possible that these neurons already possessed long neurites by the time of transfection and were refractory to the loss of cdk5 kinase activity. Alternatively, although highly unlikely, a few transfected cells might have only received the marker plasmids but not $cdk5N^{144}$ or as.p35 and therefore displayed unaltered phenotypes.

We have shown by indirect immunostaining and confocal microscopy that both cdk5 and p35 co-localize in neurite terminals where they are in some cases clearly enriched. Immunodetection of tubulin or visualization of actin revealed that cdk5 and p35 reside not only in the growth cone central core but also in the periphery. The mechanisms by which the cdk5/p35 kinase may regulate neurite outgrowth are not yet clear. However, the similar subcellular localization as actin suggests that the kinase may regulate the dynamics of actin microfilaments by phosphorylation of actin-associated proteins. We have been able to detect direct association between p35 and α-actinin using the yeast two-hybrid system (L.-H. Tsai, unpubl.). α-Actinin is one of the major players connecting the cell membrane-spanning integrins and intracellular actin microfilaments, in this way enabling cells to move by contraction of actin microfilaments against extracellular matrix proteins (Wang et al. 1993). Our results may suggest one of the ways that the cdk5/p35 kinase regulates actin microfilament dynamics and therefore neurite outgrowth. The subcellular localization of cdk5 and p35 indicates that the kinase may play a role in axonal pathfinding; however, our experimental design did not allow us to directly address this question. Our data provide the first known in vivo role of the cdk5 kinase, unique among other known cdk family members by its function in nonproliferating cells.

Materials and methods

Cell cultures

Time pregnant rats (E17) of the Long Evans strain were purchased from Harland Sprague-Dawley. Embryos were surgically removed from the mothers, and the cortices dissected from their brains, removing the meningies in dissociation media (82 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM HEPES at pH 7.3, 20 mM glucose, 0.001% phenol red, 0.2 mM NaOH). Cells were dissociated with papain and plated on to laminin and poly-D-lysine-coated glass coverslips. For higher density cultures (2×10^5 to 4×10^5), cells were maintained in basal medium of Eagle (BME), supplemented with penicillin,

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

streptomycin, 1 mM L-glutamine, 2.5 mg/ml of transferrin, 0.8 mg/ml of putrescine, 5% fetal bovine serum, 0.12 μM progesterone, 2.5 μg/ml of insulin, 2.5 μg/ml of human transferrin, 2.5 ng/ml of sodium selenate, 35 mM glucose, and 4.5 μl/ml of stable vitamin mix (3 mg/ml of L-proline, 3 mg/ml of L-cystine, 1 mg/ml of *p*-aminobenzoic acid, 12.5 μg/ml of vitamin B12, 2 mg/ml of *i*-inositol, 2 mg/ml of choline chloride, 5 mg/ml of furmaric acid, 80 μg/ml of coenzyme A, 2 μg/ml of *d*-biotin, 0.5 mg/ml of DL-6,8-thiocitic acid). For culturing at low density (0.5×10^5 to 1×10^5), cells were maintained in neurobasal media supplemented with factor B27.

Generation and analysis of cdk5 mutants

The cdk5T[33] mutant was generated by in vitro mutagenesis using the Promega Altered Sites II kit. Transient transfections into C33A cells were performed as described previously (Graham and Van der Eb 1973). The total amount of transfected DNA was always 20 μg, of which only 5 μg of CMV–p35 was used while the rest was supplemented where appropriate with 15 μg of carrier DNA, 15 μg of CMV–cdk5 or 15 μg of the dominant-negative cdk5 mutant expression vectors. Cells were lysed 2 days post-transfection in 250 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.1% NP-40, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 1 μg/ml of pepstatin, and 100 μg/ml of PMSF. Precleared lysates (100 μg) were subjected to immunoprecipitation with an anti-p35 antibody (mAb 4E3) or an anti-SV40 large T antigen antibody (used as a control), and the precipitates examined for their ability to phosphorylate histone H1 as described previously (Tsai et al. 1993). Total lysates (3.5 mg) were subjected to immunoprecipitation with antibody 4E3 or anti-cyclin A antibody (used as a control) both cross-linked to protein A–Sepharose beads and were examined further by Western blot analysis. In all cases, samples were separated on denaturing 12% polyacrylamide gels. Western blot analyses were performed with an anti-cdk5 antibody (mAb DC17, 1:5) as described previously (Tsai et al. 1994).

Generation of as.p35 constructs

*Bam*HI DNA fragments containing the p35 cDNA were subcloned into CMV expression vectors in the 3'>5' orientation. The constructs were verified by transient transfection into C33A cells. Cotransfection of sense and as.p35 expression vectors prevented generation of the p35/cdk5 kinase activity normally seen by expression of p35 protein in these cells.

Transfection of primary cortical cultures

Transfections were carried out on 3-day-old cortical cultures as follows. Cells were incubated for 1 hr in serum-free Dulbecco's modified Eagle medium (DMEM) containing 1 mM kynurenic acid, 0.02 mM HEPES (pH 7.3), 10 mM $MgCl_2$ at 37°C, and 5% CO_2. Precipitates were made by mixing 60 μg of DNA in 500 μl of 0.25 mM $CaCl_2$ with 500 μl of 2× HEPES-buffered saline (HBS) (pH 7.05–7.07) (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-glucose, 42 mM HEPES), and 40–60 μl used per coverslip. Subsequent to 30 min to 1 hr of transfection, cells were washed extensively in DMEM/kynurenic acid and cultured in conditioned media at 37°C and 5% CO_2. A 1:2 ratio of marker (β-gal or AP expression vectors) to remaining DNA on average results in 70% of the transfected cells expressing all of the constructs. We therefore used a 1:5 ratio of marker to remaining DNA in all of the experiments to ensure that most of the transfected cells contained all of the expression vectors. The total amount of DNA was always 60 μg. In CMV–cdk5, CMV–p35, CMV–as.p35, or CMV–neo transfections alone, fifty micrograms of the expression plasmid was used. In the case of dominant-negative cdk5 expression, only 25 μg of the vector was used and the rest supplemented with carrier DNA (25 μg). Transfections with more than one construct (CMV–p35 and CMV–cdk5, CMV–cdk5N[144] and CMV–cdk5, CMV–cdk5N[144] and CMV–cdk5, CMV–as.p35 and CMV–p35, or CMV–as.p35 and CMV–cdk5) were always carried out so that 25 μg of each plasmid was used.

Potential transfection toxicity was assessed by exposing transfected neuronal cultures to MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue] and color detection as described in the Sigma catalog. The neuronal cultures were also stained during immunocytochemical analysis with the DNA dye Hoechst 33258 [2'-(4-hydroxyphenyl]-5-(4-methyl-1-piperazinyl]-2-5'-bi-1H-benzimidazole) trihydrochloride] at a 2 μg/ml concentration, to confirm the presence of healthy (nonapoptotic) nuclei. A more detailed description of the protocol for neuronal transfection will be presented elsewhere (Z.X. Xia, H. Dudek, C.K. Miranty, and M.E. Greenberg, *in prep.*).

Immunocytochemistry

Primary neuronal cultures were fixed for 10 min in 4% paraformaldehyde and 4% sucrose in 1× PBS prewarmed to 37°C for 10 min. Fixed cells were washed three times in PBS containing 0.1% Triton X-100 and 10 mM glycine, once in PBS containing 0.1% Triton X-100 and incubated with 10% goat serum, 3% BSA, and 2% Tween 20 in PBS (1 hr at room temperature) to block nonspecific binding of antibodies. The indicated primary antibodies diluted in blocking buffer (listed below) were added in 3% BSA/PBST (PBS containing 2% Tween-20; 3 hr at room temperature or 16 hr at 4°C). Subsequently, the cells were washed in PBST and exposed to FITC, Texas Red, or biotin-conjugated secondary antibodies in 3% BSA/PBST for 1 hr at room temperature. When biotin-conjugated antibodies were used, after extensive washing PBST, the cells were exposed to the A/B solution (Vector Laboratories) for 1 hr, at room temperature and Ni–DAB (0.07% $NiCl_2$ and 0.025% diaminobenzidine-tetrahydrochloride and 0.02% hydrogen peroxide in PBS) for 5–10 min. After washing with PBST, the coverslips were mounted in gel/mount (Biomeda).

The following antibodies were used for immunocytochemical detection at the indicated dilutions: Primary antibodies were β-gal (mAb 1:300, Promega; pAb 1:200, 5 prime-3 prime) cdk5 (mAb DC39, 1:5; mAb DC27, 1:5; pAb 50, 1:200, Kinetek Biology Corporation), p35 (mAb 5H8, 4E3, 2D12, and 3E7 all raised against whole protein, 1:5 of hybridoma supernatant; pAb neucyc, raised against whole protein, purified, and concentrated against protein A, 1:200), tubulin (mAb 1:200, Sigma), and rhodamine-conjugated phalloidin (Molecular Probes). Secondary antibodies were fluorescein (FITC)-conjugated, affinity-purified goat anti-mouse IgG (whole molecule), 1:150 (Cappel); fluorescein (FITC)-conjugated, affinity-purified goat anti-rabbit IgG (whole molecule), 1:150 (Sigma); Texas Red-conjugated, affinity-purified goat anti-rabbit IgG (whole molecule), 1:150 (Cappel); Texas Red, affinity-purified F(ab')$_2$ sheep anti-mouse IgG (whole molecule), 1:150 (Cappel); biotinylated, affinity-purified horse anti-mouse IgG (H+L), 1:100 (Vector Laboratories); and biotinylated, affinity-purified goat anti-rabbit IgG (H+L), 1:100 (Vector Laboratories). Cells were analyzed using a Zeiss LSM 410 confocal scanning microscope.

GFP–fusion proteins

A novel GFP mutant (a kind gift from F. McKeon, Harvard Medical School, Boston, MA) lacking an ATG was subcloned into the

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

*Bam*HI–*Xho*I sites of the pcDNA3 vector (Stratagene) 3′ to cdk5 or cdk5N[144] fragments inserted into the *Bam*HI site and lacking a stop condon. This resulted in the formation of carboxy-terminally fused cdk5 and cdk5N[144]. Prior to visualization the transfected neurons were treated successively for 3 hr with small quantities of 0.1 N NaOH to enhance GFP fluorescence. Cultures were fixed as described above.

Neurite length measurements

Immunostained neurons were analyzed using a Zeiss Axioplan microscope, a CCD camera, and Metamorph image analysis software (Universal Imaging). Transfected neurons and their neurites were distinguished by intense fluorescence because of immunodetection of expressed marker proteins such as β-gal. Measurements were usually carried out within 1 week of immunostaining. The axon and dendrites were measured for each neuron; however, only one longest neurite per cell was used for comparative purposes with other neurons. Lengths were determined as the distance between the edge of the cell body and the tip of the growth cone. Only clearly visible cells were subjected to analysis to prevent inaccurate scoring. The analyses were carried out "blind" to avoid any subjective influences during the measurements. The data obtained were examined statistically using the *t*-test.

Acknowledgments

We are in debt to Michael E. Greenberg for his valuable advice and guidance in preparing this manuscript. We thank Marc Kirschner, Le Ma, and Robert Davis for unlimited use of and help with the CCD camera imaging and Metamorph image analysis software; the confocal facility at the Dana Farber Institute Department of Pathology, especially Lan Bo Chen and Stine K. Kraeft for extensive help and use of the Zeiss LSM 410 confocal microscope; Frank McKeon for providing us with a novel GFP protein, Paige Williams for help with the statistical analysis; and Teresa Chae, Elena Porro, Michael Rivkin, and Peter Tsai for the reading and discussion of the manuscript. This work was supported by a grant (to L.-H.T.) from the National Science Foundation (MCB-9507109).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Cepko, C.L., C.P. Austin, C. Walsh, E.F. Ryder, A. Halliday, and S. Fields-Berry. 1990. Studies of cortical development using retrovirus vectors. *Cold Spring Harb. Symp. Quant. Biol.* **55:** 265–278.

DeBondt, H.L., J. Rosenblatt, J. Jancarik, H.D. Jones, D.O. Morgan, and S.-H. Kim. 1993. Crystal structure of cyclin-dependent kinase 2. *Nature* **363:** 595–602.

Graham, F.L. and A.J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52:** 456–467.

Halliday, A.L. and L.C. Cepko. 1992. Generation and migration of cells in the developing striatum. *Neuron* **9:** 15–26.

Ishiguro, K., S. Kobayashi, A. Omore, M. Takamatsu, S. Yonekura, K. Anzai, K. Imahori, and T. Uchida. 1994. Identification of the 23kDa subunit of tau protein kinase II as a putative activator of cdk5 in bovine brain. *FEBS Lett.* **342:** 203–208.

Jeffrey, P.D., A.A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague, and N.P. Pavletich. 1995. Mechanism of cdk activation revealed by the structure of a cyclin A-cdk2 complex. *Science* **376:** 313–320.

Kosik, K.S. and A. Caceres. 1991. Tau protein and the establishment of an axonal morphology. *J. Cell Sci.* (Suppl.) **15:** 69–74.

Lew, J. and J.H. Wang. 1995. Neuronal cdc2-like kinase *Trends Biochm. Sci.* **20:** 33–37.

Lew, J., K. Beaudette, C.M.E. Litwin, and J.H. Wang. 1992a. Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J. Biol. Chem.* **267:** 13383–13390.

Lew, J., R.J. Winkfein, H.K. Paudel, and J.H. Wang. 1992b. Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. *J. Biol. Chem.* **267:** 25922–25926.

Lew, J., Q.-Q. Huang, R.J. Winkfein, Z. Qi, R. Aebersold, T. Hunt, and J.H. Wang. 1994. Neuronal cdc2-like kinase is a complex of cyclin-dependent kinase 5 and a novel brain-specific regulatory subunit. *Nature* **371:** 423–425.

Meyerson, M., G.H. Enders, C.-L. Wu, L.-K. Su, C. Gorka, C. Nelson, E. Harlow, and L.-H. Tsai. 1992. A family of human cdc2-related protein kinases. *EMBO J.* **11:** 2909–2917.

Reid, C.B., I. Liang, and C. Walsh. 1995. Systematic widespread clonal organization in cerebral cortex. *Neuron* **15:** 299–310.

Shetty, K.T., W.T. Link, and H.C. Pant. 1993. Cdc2-like kinase from rat spinal cord specifically phosphorylates KSPXX motifs in neurofilament proteins: Isolation and characterization. *Proc. Natl. Acad. Sci.* **90:** 6844–6848.

Taylor, S.S., D.R. Knighton, J. Zheng, J.M. Sowadski, C.S. Gibbs, and M.J. Zoller. 1993. A template for the protein kinase family. *Trends Biochem. Sci.* **18:** 84–89.

Tsai, L.-H., T. Takahashi, V.S. Caviness Jr., and E. Harlow. 1993. Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119:** 1029–1040.

Tsai, L.-H., I. Delalle, V.S. Caviness Jr., T. Chae, and E. Harlow. 1994. p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371:** 419–423.

van den Heuvel, S. and E. Harlow. 1994. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262:** 2050–2054.

Wang, N., J.P. Butler, and D.E. Ingber. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260:** 1124–1127.

Downloaded from genesdev.cshlp.org on August 22, 2022 - Published by Cold Spring Harbor Laboratory Press

The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation.

M Nikolic, H Dudek, Y T Kwon, et al.

Genes Dev. 1996, **10:**
Access the most recent version at doi:10.1101/gad.10.7.816

References This article cites 19 articles, 6 of which can be accessed free at:
http://genesdev.cshlp.org/content/10/7/816.full.html#ref-list-1

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or **click here.**

Dharmacon Reagents
Infinite Reliability
More
horizon
a PerkinElmer company

Copyright © Cold Spring Harbor Laboratory Press