

# Synaptic localization of p39, a neuronal activator of cdk5

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The expression and kinase activity of cyclin dependent kinase 5 (cdk5) parallels the extent of neuronal differentiation. Cdk5 activity has been shown to be required for neurite outgrowth, cortical lamination and the overall development of the nervous system. p35 was identified as the first regulatory activator of cdk5 whose presence is required for cdk5 activation. p39 is a homolog of p35, and the only one identified in mammals thus

far. We show here that p39 expression is mainly postnatal. In addition, we provide evidence for the presence of p39 at synaptic junctions through co-fractionation experiment, electron microscopy and immunostaining. The temporal and spatial expression of p39 indicate a possible role of the p39/cdk5 kinase at the synapse. *NeuroReport* 11:2213–2216 © 2000 Lippincott Williams & Wilkins.

**Key words:** cdk5; expression pattern; p35; p39; synapse

## INTRODUCTION

Cyclin-dependent kinase 5 (cdk5) is highly related to the family of cdk known to function throughout the cell division cycle. Cdk5 and associated kinase activity are detected at high level in the nervous system [1]. Cdk5 requires association with a regulatory subunit for activation. p35 was isolated as the first cdk5 regulator through its physical interaction with cdk5 in brain lysates, and by its ability to activate cdk5 upon direct binding [2–4]. Expression of p35 is restricted to post-mitotic neurons of the CNS [5]. Gene targeting experiments have been performed for both p35 and cdk5. Mice lacking p35 are viable but have increased sensitivity to seizures [6]. Perhaps the most intriguing phenotype these mice display is the inverted layering of cortical neurons due to an inability of later born neurons to migrate past their predecessors [6,7]. Cdk5 null mice show late embryonic or perinatal lethality [8]. Extensive defects were found in these animals, including disordered lamination of the cerebral cortex, abnormal hippocampal formation and disrupted cerebellar development. The more severe phenotype of cdk5 knockout reveals the existence of regulatory subunits for cdk5 other than p35.

The search for an additional cdk5 regulator led to the identification of p39 by virtue of its sequence homology to p35 [9]. p39 and p35 share 57% amino acid identity. p39 is similar to p35 in terms of displaying cdk5 histone H1 kinase activity *in vitro* [9]. Expression of p39 during rat embryonic development was investigated by *in situ* hybridization, which indicated that like p35, p39 is specific to post-mitotic neurons of the nervous system [10,11]. Using a

p39-specific antibody, we show here that p39 protein expression peaks postnatally and demonstrate the presence of p39 protein at the synapse.

## MATERIALS AND METHODS

**Antibodies, Western blot:** The rabbit polyclonal p39 [12] was raised by immunization of rabbits with an N-terminal truncation of human p39 (amino acids 111–367) fused to a 6-histidine tag and was affinity purified on a GST-p39 column. The actin rabbit polyclonal antibody (Sigma), the p35 antibody C19 (Santa Cruz), the cdk5 mouse monoclonal antibody DC17 (Kinetek Biology Corporation) and the monoclonal PSD-95 antibody (Upstate Biotechnology) were used for Western blot. Cerebellum or cortices from mice at different stages were lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA pH 8, 0.1% Nonidet P-40, 5 mM DTT, 10 mM NaF, 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin.

**Synaptic preparation:** Subcellular fractions of adult whole rat brain were prepared as described [13]. Briefly, rat brain Dounce homogenates were centrifuged at 1000 × g to remove nuclei and other large debris (pelleted in P1). The supernatant (S1) was centrifuged at 10 000 × g to obtain a crude synaptosomal fraction (P2), which was subsequently lysed hypo-osmotically and centrifuged at 25 000 × g to pellet a synaptosomal membrane fraction (LP1). The supernatant (LS1) was then centrifuged at 165 000 × g to obtain a crude synaptic vesicle-enriched fraction (LP2). Concurrently, the supernatant (S2) above the crude synaptosomal

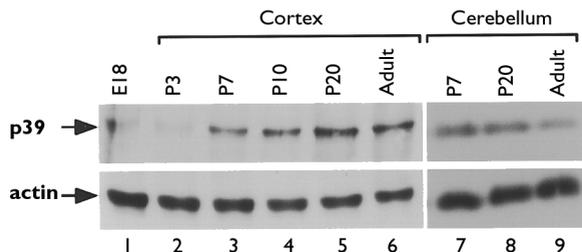
fraction pellet (P2) was centrifuged at  $165\,000 \times g$  to obtain a cytosolic fraction (S3) and a light membrane fraction (P3).

**Electron microscopy:** P11 mice were perfused with a 4% paraformaldehyde solution. Cerebella were dissected, processed with tannic uranyl acetate as described [14], embedded in Unicryl (BBI international/EMS, Fort Washington, PA) and polymerized using u.v. light at 4°C. For immunogold labeling, 60–80 nm sections were picked up on nickel grids and incubated in 0.5% fish skin gelatin/0.1% Triton X-100 (BB) for 30 min to reduce non-specific staining. Purified p39 polyclonal antibody diluted 1:50 in BB was applied for 16 h at 4°C. After three 5 min washes in BB, bound antibody was visualized by incubation with 10 nM protein A–gold for 1 h at room temperature. Sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL 1200EX transmission electron microscope.

**Immunocytochemistry:** Primary hippocampal neurons were prepared from E16 mouse as described for E18 rat [15]. After 3 weeks cells were fixed in 4% paraformaldehyde/PBS, blocked with 10% BSA/1× PBS, and permeabilized with 0.2% Triton X-100/1× PBS. Cells were stained with monoclonal anti-synapsin II (a gift from Andrew Czernick) and polyclonal anti-p39 antibodies followed by alexa-488 goat anti-rabbit (Molecular Probes) and Texas-red donkey anti-mouse (Jackson ImmunoResearch) antibodies. Coverslips were mounted with DABCO in polyvinyl alcohol and imaged using a Deltavision™ deconvolution imaging microscope.

## RESULTS

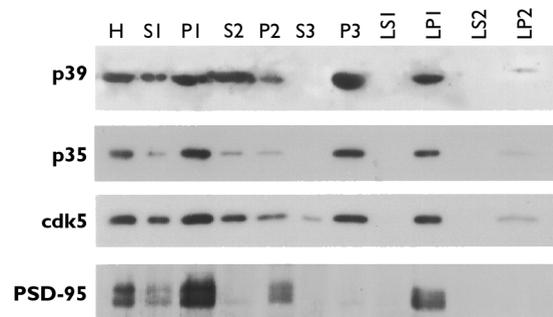
**p39 protein expression peaks postnatally:** To investigate the distribution of p39 protein in brain, equal amount of extracts from mouse cortex and cerebellum isolated at various developmental ages were analyzed by Western blot (Fig. 1). p39 protein is barely detectable in E18 total brain preparations (Fig. 1, lane 1). In cortical extracts, p39 levels gradually increase from P3 and peak at P20 (Fig. 1, lanes 2–5). In adult cortex, the protein level decreases to a level comparable to that at P10 (Fig. 1, lane 6). In cerebellum, p39 protein is most prominent between P7 and P20 (Fig. 1, lanes 7 and 8). Northern blot analysis of RNA isolated from mouse cortex and cerebellum at different stages of



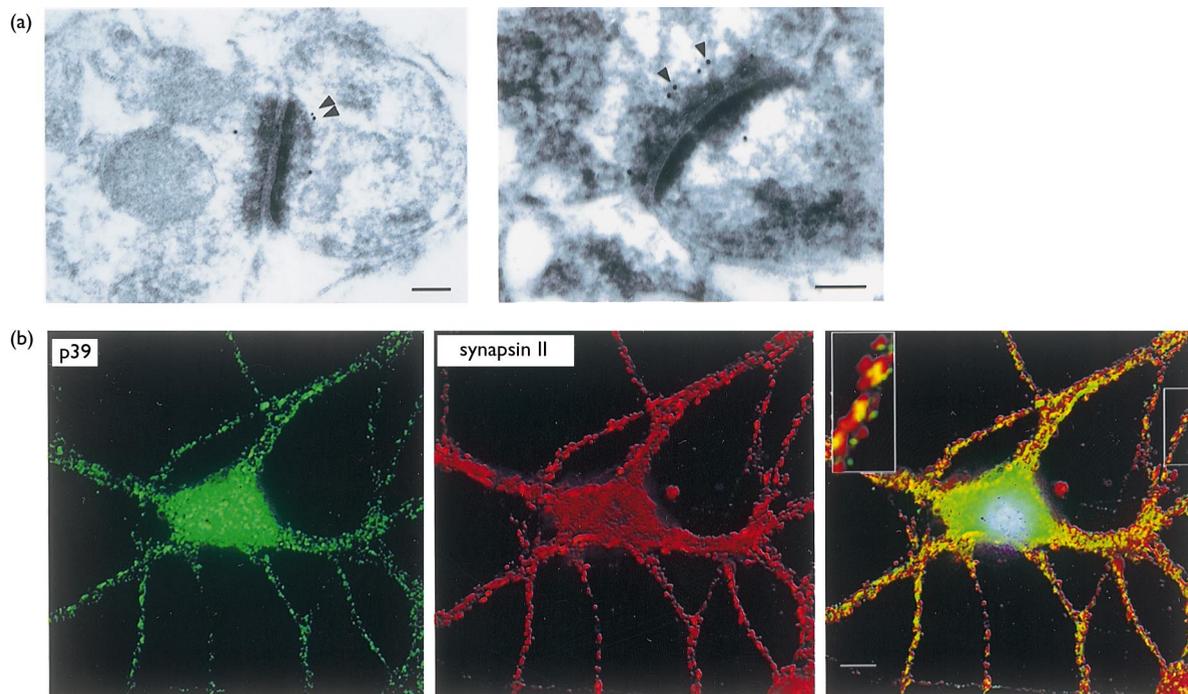
**Fig. 1.** p39 protein expression peaks postnatally. Western blot analysis of whole tissue extracts obtained from the cortex of embryonic day 18 (lane 1), postnatal days 3 (lane 2), 7 (lane 3), 10 (lane 4), 20 (lane 5), adult (lane 6) mice or from the cerebellum of postnatal days 7 (lane 7), 20 (lane 8) or adult (lane 9) mice. Each lane was loaded with 30 µg of total protein. The blot was probed with anti-p39 antibody (upper panel) or anti-actin antibody (lower panel) as a control.

development show that p39 mRNA levels correlated with those of the protein (data not shown). These results demonstrate that p39 is mostly present postnatally in the CNS. Both mRNA and protein levels are maintained at relatively high levels into the adult, especially in the cortex. The age of p39 peak expression is similar in both cortex and cerebellum, although levels of p39 are lower in cerebellum than in cortex as demonstrated by both Western blot and Northern analysis (data not shown).

**Synaptic localization of p39:** The temporal expression pattern of p39 suggests a role for p39 in synaptogenesis. Using different and established approaches for studying synaptic protein localization, we observed p39 expression in synaptic compartments of the cell. Through subcellular fractionation of adult rat brain, we characterized the distribution of p39, p35, cdk5 and the post-synaptic density protein PSD-95, as a control (Fig. 2). Interestingly, p39 is found in the membrane fractions LP1 (synaptic membranes) and LP2 (crude synaptic vesicles). p35 and cdk5 significantly co-fractionate with p39 during this separation process and are also present in LP1 and LP2. p39, p35 and cdk5 are also enriched in P3 which consists of golgi, endoplasmic reticulum and light membranes. A major difference in the subcellular fractionation of these three proteins is the presence of p39 and cdk5 in the soluble fractions S1 and S2 whereas p35 is almost depleted from these fractions. As expected, PSD95 is enriched in LP1. To further confirm p39 localization to the synapses, we immunogold labeled p39 on P10 mouse cerebellar sections and examined the distribution of the gold particles using transmission electron microscopy. Eighteen of 22 synapses analysed show consistent labelling at or near synaptic junction. Density of gold particles is  $42.93/\mu\text{m}^2$  at the synapse and  $1.03/\mu\text{m}^2$  in non-synaptic regions ( $t(29) = 10.8$ ;  $p < 0.0001$ ) revealing the specific presence of p39 at the synapse. Finally, by immunocytochemistry, we double immunostained cultured hippocampal neurons for p39 and synapsin II, a protein present on synaptic vesicles. As shown in Fig. 3b, co-localization is observed between p39 and synapsin II, in particular along the neurites. Indeed, as previously described [12], p39 is present in both the soma



**Fig. 2.** p39 is present in synaptic fractions of whole adult rat brain. H, whole extract; S1, P1 supernatant (S1) and cells and nuclei enriched pellet (P1); S2, P2, supernatant (S2) and crude synaptosomal fraction (P2); S3, cytosolic fraction; P3, Golgi, ER and light membranes enriched fraction; LS1, supernatant of synaptic membrane fraction; LP1, synaptic membrane fraction; LP2, vesicles and small membrane fraction. 50 µg of each fraction was loaded on a 12% polyacrylamide gel before p39, p35, cdk5 and PSD-95 Western blot analysis.



**Fig. 3.** p39 is localized at synaptic junctions. (a) Thin mouse cerebellar sections were incubated with affinity purified p39 polyclonal antibody and viewed by transmission electron microscopy. Arrowheads point to gold particles revealing the presence of p39 near the synaptic junction. (b) 3 weeks old hippocampal cultures were stained with anti-p39 and anti-synapsin II antibodies. Partial co-localization of p39 and synapsin is observed. Bar = 100 nm (a), 10  $\mu$ m (b).

and in punctate areas along the neurites. Some of these puncta also stain for synapsin II, suggesting that p39 is partially pre-synaptic.

## DISCUSSION

By Western blot analysis, we examine p39 expression during development. p39 expression in the CNS is very low before birth and gradually increases postnatally, peaking 3 weeks after birth. p39 remains present at the adult stages with a particularly high level in the cortex. With these results, we show that the peak of p39 expression occurs much later during development than that of p35 in the brain. Our observations are consistent with previous reports showing lower p39 mRNA levels in the developing cerebral wall [10,11].

Expression studies reveal a complementary distribution of p35 and p39 in the developing nervous system. p39 is expressed at high levels in the peripheral sensory organs such as dorsal root ganglia and hindbrain structures such as the pons, inferior colliculus, and cerebellar peduncle during embryonic development [10,11,16]. p35 expression in these compartments is low or undetectable. Instead, p35 expression is most conspicuous in the developing cerebral wall and mesencephalon [5], where p39 was hardly detectable. In the developing cerebellum, robust p39 mRNA expression is seen in the Purkinje cell layer and in the internal granule layer [10]. Since much milder cerebellar abnormality is seen in p35 null mice [6] than in mice lacking cdk5 [8], it is conceivable that p39 might be compensating for p35 during cerebellar development in p35 knockout mice. Similarly, p39 might also play a role in

the differentiation of brain stem neurons, as brain stem defects are observed in *cdk5*<sup>-/-</sup> but not in *p35*<sup>-/-</sup> mice.

The temporal expression pattern of p39 in the cerebral cortex (peaking 2–3 weeks postnatally) strikingly coincides with the wave of synaptogenesis during development. Taken together with the presence of p39 in growth cones [12], it suggests a possible role for p39 in synaptogenesis. Indeed, differential fractionation of brain lysates revealed an enrichment of p39 in the synaptosomal membranes as well as synaptic vesicle membranes fractions. The segregation of p39 in these fractions closely followed that of cdk5 and p35, although p39 and cdk5 were also present in the soluble fractions S1 and S2, whereas p35 was solely membrane associated. In addition, immunogold labeling and transmission electron microscopy allowed visualization of p39 at synaptic junctions, where the protein was present in both pre- and post-synaptic compartments. Finally, p39 and synapsin II, a synaptic marker, partially co-localize in hippocampal neurons. The broader subcellular distribution of p39 and cdk5 compared to that of p35, as revealed by the fractionation experiments, again indicates distinct functions for p39 and p35.

## CONCLUSION

Activation of cdk5 in the brain requires the presence of one of two related proteins, p35 and p39. Interestingly, the temporal and spatial distribution patterns of p39 differ from that of p35 suggesting that the p39/cdk5 kinase has a distinct and complementary role to the p35/cdk5 kinase. In particular, p39 is expressed later in the development compared to p35. Our study reveals the presence of p39 protein

at the synapse and shows that while p35 is mainly membrane associated, p39 and cdk5 are cytosolic and membrane associated. The cdk5 knockout animals display a much more severe and extensive phenotype than the p35 nullizygous animals, indicating additional cdk5 functions not regulated by p35. The analysis of a mouse strain lacking p39 will reveal the function of the p39/cdk5 kinase in the development of the nervous system.

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