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The Expression of Cdk5, p35, p39, and Cdk5 Kinase Activity in Developing, Adult, and Aged Rat Brains

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The expression of cyclin-dependent kinase 5 (Cdk5) and its regulatory subunits, p35 and p39, was investigated in rat brain from embryonic day 12 (E12) to postnatal 18 months (18M). The Cdk5 protein levels increased from E12 to postnatal day 7 (P7) and remained at this level until 18M. The Cdk5 kinase activity and the levels of both p35 mRNA and protein were low at E12, became prominent at E18-P14 but then decreased in the adult and aged rat brains of 3M to 18M. In comparison, the expression pattern of p39 appeared to have an inverse relationship to that of Cdk5 and p35. In regional distribution studies, p35 protein levels and Cdk5 kinase activity were significantly higher in the cerebral cortex and hippocampus, but lower in the cerebellum and striatum. These results suggested that Cdk5, p35 and p39 might have region-specific and developmental stage-specific functions in rat brain.

KEY WORDS: Cdk5; p35; p39; cerebral cortex; hippocampus; development.

INTRODUCTION

Cyclin-dependent kinase 5 (Cdk5) is unique among cyclin-dependent kinases (Cdks) in that it has no known cell division cycle regulatory function, but appears to play pivotal roles in neuronal function (1,2). In adult animals, the brain is the only organ containing a significant amount of Cdk5-associated kinase activity (3–5). Similar to other Cdks, Cdk5 activity shows an absolute dependence of specific activator proteins. Two Cdk5 activator proteins have been found, both essentially showing a brain and neuron specific expression (6–9). Hence, the two activators are designated neuronal Cdk5 activator (p35) and neuronal Cdk5 activator isoform (p39) (8,9). In vitro, Cdk5 phosphorylates a number of cytoskeletal proteins including the neurofilament proteins and the neuron-specific microtubule associated protein tau (10–13), suggesting that the kinase participate in the regulation of neurocytoskeleton organization and dynamics. Both Cdk5 and the Cdk5 activator p35 have been implicated in neurite outgrowth, neuronal differentiation and neuronal migration (14–46). Loss of the regulation of Cdk5 kinase activity has been implicated in the pathology of Alzheimer's disease (AD) (17–19).

There are a number of reports in the literature documenting the developmental patterns of Cdk5 and

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p35 in embryonic rat brains (20,21), but much less is known about aging rat brain and the developmental pattern of p39. In order to understand more about the physiological and pathological functions of Cdk5 in the central nervous system (CNS), we have investigated the presence and activity of Cdk5 and the levels of its two regulatory protein subunits, p35 and p39, in rat brain during development and aging from embryonic day 12 (E12) to 18 months after birth (18M). The expression of these proteins was also studied in four brain regions, namely the cerebral cortex, hippocampus, striatum and cerebellum.

EXPERIMENTAL PROCEDURE

Animals and Tissue Preparation. Pregnant Sprague-Dawley rats were obtained from the Animal Care Facility, The Hong Kong University of Science and Technology. The pregnant rats were then allowed to gestate their young for varying durations. Following euthanasia by inhalation of 100% carbon dioxide, whole brains were removed from E12, E14, E16, and E18 embryos. Postnatal rats were sacrificed at 1 day (P1), 7 days (P7), 14 days (P14), 1 month (1M), 3 months (3M), 6 months (6M), 12 months (12M) and 18 months (18M). The cerebral cortex, cerebellum, hippocampus and striatum were then surgically removed. Brain samples from 2–4 animals were homogenized in a lysis buffer [25 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml antipain, 0.3 μg/ml benzamidine, pH 7.2] for one experiment. The crude homogenates were centrifuged at 100,000 *g* for 30 min and the resulting supernatants were then subsequently analyzed.

Antibodies. Cdk5 polyclonal antibody (C-8) and p35 polyclonal antibody (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cdk5 monoclonal antibody (Ab-2) was purchased from CALBIOCHEM (Cambridge, MA). The p39 polyclonal antibody was raised against an expressed 30 kDa truncated form of p39 (amino acid residues 115–367) (unpublished data, 1999).

Immunoblotting. 50 μg of protein per lane of the various supernatants was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (22) before being transferred to a polyvinylidene difluoride membrane in a trans-blotting buffer [20 mM Tris, 200 mM glycine, 20% (v/v) methanol]. Membranes were incubated with either a monoclonal anti-Cdk5 antibody or polyclonal anti-p35 or anti-p39 antibodies (2 μg/ml) in a blocking buffer containing 10% BSA for 1 h followed by incubation with peroxidase-conjugated anti-mice IgG or anti-rabbit IgG in a blocking buffer containing 5% skimmed milk for 1 h at room temperature. A signal was detected by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech). Western blots were quantified by a BIO-RAD model GS-670 Imaging Densitometer.

RNA Isolation, Reverse Transcription, and PCR Amplification. RNA extraction and RT/PCR were carried out as described by Gao et al (21). Frozen brain samples (200 mg) were homogenized and extracted in TRIzol Reagent according to the manufacturer's instructions (GIBCO BRL, Bethesda, MD). The mRNAs were reversely transcribed into cDNAs by an Advantage RT-for PCR Kit (CLONTECH, Palo Alto, CA) and the cDNAs were used as templates for the polymerase chain reaction (PCR). A pair of RT/PCR primers was designed from the mouse p39 mRNA sequence (Genebank: AF016393) in order to amplify a 365 bp fragment. Their sequences were

Upstream primer	5′-AGTCGCTTCGTGGGGAC-GAGCT-3′(734–755)
Downstream primer	5′-GTTCATAGTCCAGTGCTTG-GCTC-3′(1076–1098)

Another pair of primers was designed from the rat p35 mRNA sequence (Genebank: U50707) for amplifying a 406 bp fragment. Their sequences were

Upstream primer	5′-CAACAAT GAGAACCTGAA-GAAGTC-3′(243–266)
Downstream primer	5′-CACCTCAGAGGAGATGA-CAT-3′(629–648)

Oligonucleotides were synthesized by GIBCO BRL and PCR was conducted for 25 cycles for both p35 and p39. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was an endogenous control for reverse transcription. A negative control without reverse transcriptase was included to ensure that the PCR products were derived from RNA.

Immunoprecipitation and Kinase Assays. An in vitro Cdk5-associated histone H1 kinase activity (Cdk5 kinase activity) assay was carried out as described by Zheng et al. (22). Immunoprecipitation of active Cdk5 from different rat brain lysates (100,000 x *g* supernatant, 200 μg) was performed using polyclonal anti-Cdk5 (C-8, 5 μl per sample). The antibody was preincubated with protein G-sepharose 4B (GIBCO BRL, Bethesda, MD). The immuno-complexes were used to determine the kinase activity at 30°C for 30 min in a final volume of 50 μl containing a kinase buffer (30 mM MOPS, 10 mM $MgCl_2$, pH 7.4), 2 μg of histone H1 peptide ($P^9KTPKKAKKL^{18}$) and 5 μ Ci of [γ-^{32}P] dATP. The incorporation of phosphate (^{32}P) into labeled peptides was quantified by a liquid scintillation counter.

RESULTS

Expression Profiles of Cdk5, p35, and p39 in Rat Brain. Western blot analysis using Cdk5 monoclonal antibody revealed a single band of 33 kDa in rat brains of different ages (Fig. 1A). The protein level of Cdk5 increased progressively during the embryonic brain development from E12 to E18 and reached its highest level at P7. This level was maintained in adult and aged rat brains up to 18M. The immunoblot using the p35 antibody indicated a 35 kDa band, which corresponds to p35 (Fig. 1B). The expression level of p35 was barely detectable at E12 and E14, but its level became detectable in E16 rat brains and gradually increased to its peak level at P7–P14. Thereafter, it declined, remaining at 30–40% of the P7 level in aged rat brains from 6M to 18M. The p39 protein was detected in E12 and at high levels between E14 to P7, thereafter declining at

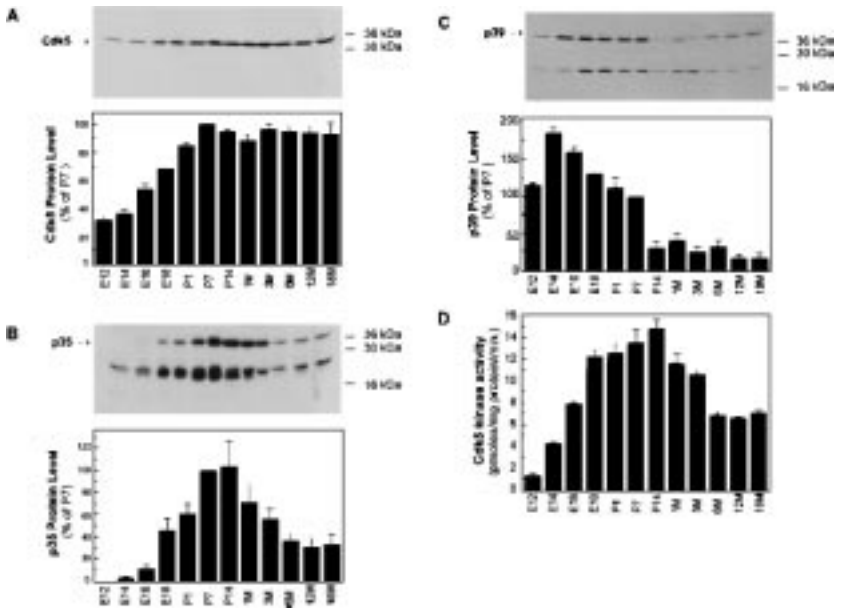

Fig. 1. Expression of Cdk5, p35, 39 and Cdk5 kinase activity in rat brains. Whole brain extracts (50 μg of protein per lane) from rats at embryonic day 12 (E12), 14 (E14), 16 (E16), 18 (E18), postnatal ages of 1 day (P1), 7 days (P7), 14 days (P14), 1 month (1M), 3 months (3M), 6 months (6M), 12 months (12M) and 18 months (18M) were analyzed by immunoblotting with (**A**) anti-Cdk5 antibody, (**B**) anti-p35 antibody and (**C**) anti-p39 antibody. Western blots at the top of each panel are from a typical experiment. (**D**) Cdk5 kinase activity. Quantified results are the mean ± SEM from three independent experiments.

P14 but remaining detectable throughout the experimental period (Fig. 1C). The Cdk5 kinase activity was low at E12. It gradually increased to its highest level between P7 to P14 and then declined from 1M to 3M. In aged rat brains from 6M to 18M, the kinase activity remained at approximately 40% of the P14 level (Fig. 1D).

Expression of p35 and p39 mRNA. The expression of p35 and p39 mRNA was measured in rat brains from E12 to 18M by RT/PCR. The developmental changes associated with the p35 mRNA transcripts (Fig. 2A) were similar to the protein expression pattern (Fig. 1B). The mRNA for p35 was detected at E12 and gradually reached a high level at E18 and P1. This high level was maintained throughout the measuring period and only slightly decreased in aged brains (Fig. 1B). A band for p39 mRNA was not detected at E12. It was slightly expressed at E14 and E16. The expression was prominent at P7 to 1M but gradually declined to a low level in the 18M rat brain (Fig. 2B). Levels of GAPDH were determined in parallel as an external control (Fig. 2C). No PCR product was detected in the negative controls.

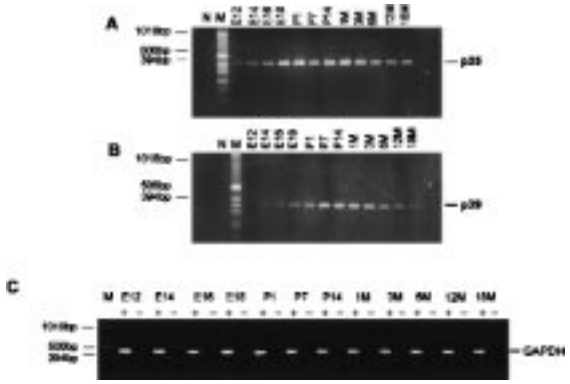

Fig. 2. Expression of p35 and p39 mRNA in rat brains. (**A**) RT/PCR of p35 from whole rat brains from embryonic day 12 (E12) to postnatal ages of 18 months (18M). (**B**) RT/PCR of p39 from whole rat brains from E12 to 18M. Lane "N": Negative control using P1 rat brain RNA without reverse transcriptase; lane "M": 1kb marker. (**C**) RT/PCR of GAPDH as external control. (+) With reverse transcriptase, (–) without reverse transcriptase.

Cdk5, p35, and p39 Expression and Cdk5 Kinase Activity in the Cerebral Cortex and Hippocampus. Since it is difficult to obtain enough samples of the hippocampus and striatum from embryonic rat brains, the measurement was performed only on postnatal rat brains. The expression of Cdk5, p35, and p39 proteins as well as Cdk5 kinase activity in the two regions was similar to that in the whole brain. The expression level of Cdk5 was high in the P1 cerebral cortex (Fig. 3A)

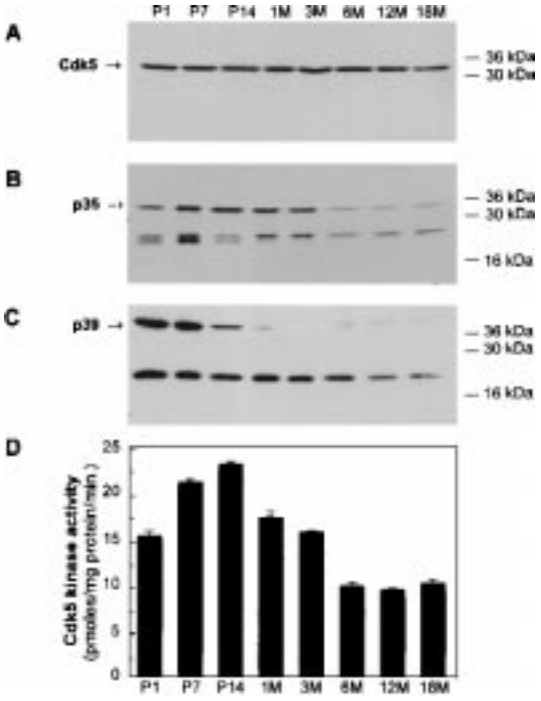

Fig. 3. Expression of Cdk5, p35, p39 and Cdk5 kinase activity in the cerebral cortex of P1-18M rat brain. Whole cerebral cortex extracts (50 μg of protein per lane) from rats at postnatal ages of 1 day (P1), 7 days (P7), 14 days (P14), 1 month (1M), 3 months (3M), 6 months (6M), 12 months (12M) and 18 months (18M) were analyzed by immunoblotting with (**A**) anti-Cdk5 antibody, (**B**) anti-p35 antibody and (**C**) anti-p39 antibody. (**D**) Cdk5 kinase activity was expressed as the mean ±SEM of 3–4 experiments.

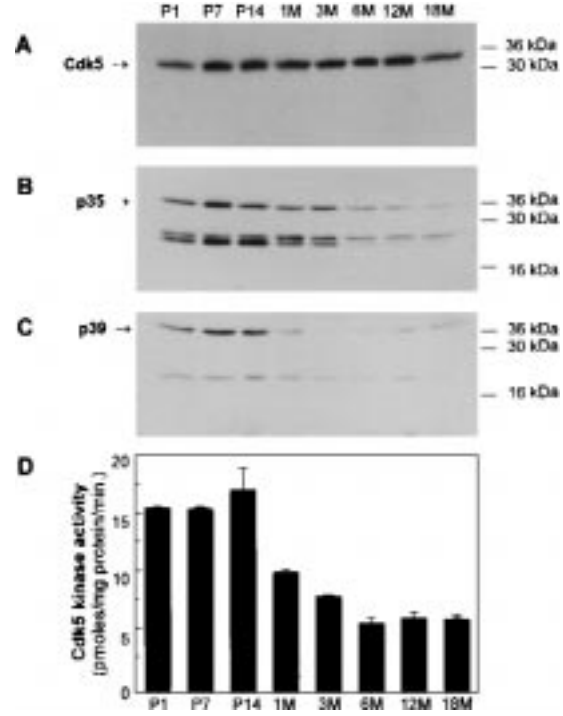

Fig. 4. Expression of Cdk5, p35, p39 and Cdk5 kinase activity in the hippocampus of rat brains. Whole rat hippocampus extracts (50 μg of protein per lane) from rats at postnatal ages of 1 day (P1), 7 days (P7), 14 days (P14), 1 month (1M), 3 months (3M), 6 months (6M), 12 months (12M) and 18 months (18M) were analyzed by immunoblotting with (**A**) anti-Cdk5 antibody, (**B**) anti-p35 antibody and (**C**) anti-p39 antibody. (**D**) Cdk5 kinase activity was expressed as the mean ± SEM of 3–4 experiments.

and the hippocampus (Fig. 4A) and remained at this level in adult and aged brains. Immunoblot analysis of p35 in the cerebral cortex and hippocampus revealed that the expression level of p35 reached the maximal level at P7 and P14 and then declined slightly between 1M and 3M, further decreasing to approximately 10% of the maximal level upon entering the aged stages from 6M to 18M (determined by scanning densitometry of the immunoblots; data not depicted here) (Figs. 3B and 4B). The expression of p39 was high in the cerebral cortex and hippocampus between P1 and P7, but was very low in later adult and aged stages (Figs. 3C and 4C). Cdk5 kinase activity was prominent from P1 to P14 and gradually decreased in the adult stages (1M to 3M) while 30%–40% of the maximal level was detected in the aged brain (Figs. 3D and 4D).

Changes of Cdk5 Expression and Kinase Activity in the Cerebellum and Striatum of Rat Brain. Cdk5 expression patterns in the cerebellum (Fig. 5A) and striatum (Fig. 5B) were similar to those in the cerebral cortex and hippocampus (Figs. 3A and 4A). In contrast, Cdk5 kinase activity measured in the cerebellum and striatum was the highest at P1 and gradually decreased throughout the adult and aged periods (Fig. 5C). Western blot analysis revealed that the p35 expression profile in the cerebellum and striatum parallels the changes in Cdk5 kinase activity.

Comparison of the Expression of Cdk5 and p35 with Cdk5 Kinase Activity in Rat Brain at P14. The expression of Cdk5 and p35 proteins, and Cdk5 kinase activity were compared in the cerebral cortex, hippocampus, cerebellum and striatum of P14 rat brain. Western blot analysis revealed that k5 expression was similar in all four regions studied (Fig. 6A). The expression level of p35 was found to be the highest in the cerebral cortex, moderate in the hippocampus, and lowest in the cerebellum and striatum (Fig. 6B). Similar to the expression of p35, the Cdk5 kinase activity in the cerebral cortex and hippocampus was higher than in the cerebellum or striatum (Fig. 6C). Rat brains from P14 to 18M were also investigated and the results

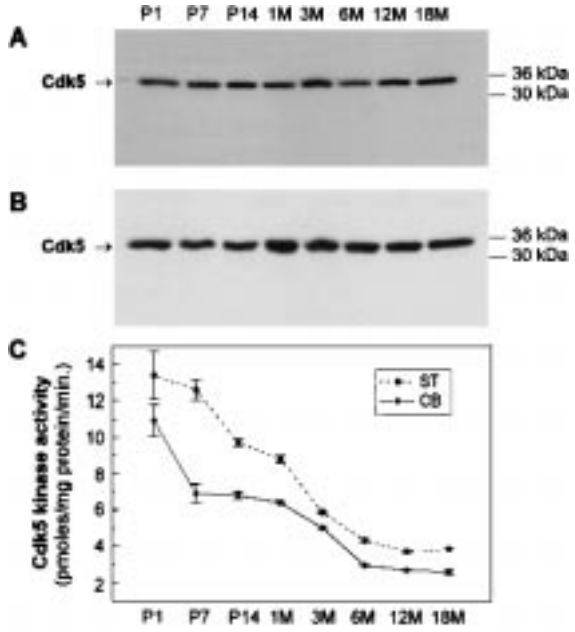

Fig. 5. Expression of Cdk5 and Cdk5 kinase activity in the cerebellum (CB) and striatum (ST) of different ages of rat brain. (**A**) Whole rat cerebellum and (**B**) striatum extracts (50 μg of protein per lane) from rats at postnatal ages of 1 day (P1), 7 days (P7), 14 days (P14), 1 month (1M), 3 months (3M), 6 months (6M), 12 months (12M) and 18 months (18M) were analyzed by immunoblotting with anti-Cdk5 antibody. (**C**) Cdk5 kinase activity was expressed as the mean ±SEM of 3–4 experiments.

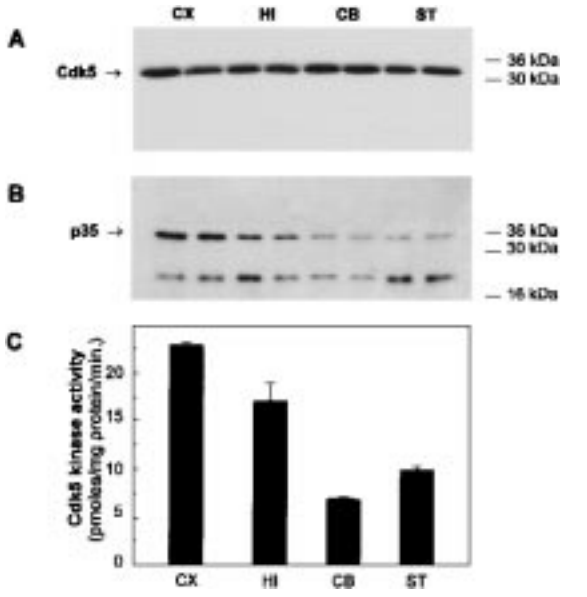

Fig. 6. Expression of Cdk5, p35 and Cdk5 kinase activity in four brain regions of P14 rats. Western blot analysis of (**A**) Cdk5 and (**B**) p35 in the cerebral cortex (CX), hippocampus (HI), cerebellum (CB) and striatum (ST) of P14 rat brains. (**C**) Cdk5 kinase activity was expressed as the mean ± SEM of 3–4 experiments

were similar to those in P14 rat brain. However, in P1 and P7 rat brain, the difference between the p35 expression and Cdk5 kinase activity among these four regions is less obvious (data not shown).

DISCUSSION

The main findings of this study are summarized as follows: (1) Cdk5 and p35 protein expression and Cdk5 kinase activity significantly increased during embryonic development from E12 to P1. (2) The p39 protein expression was higher from E12 to P7, which is different from the Cdk5 and the p35 expression patterns. (3) Cdk5 kinase activity correlated well with p35 but not p39 expression. (4) In aged brains, the Cdk5 level remained the same as in an adult brain, but the expression of p35, p39 and Cdk5 kinase activity were at lower levels. (5) Cdk5 kinase activity and p35 expression were much higher in the cortex and hippocampus than that in the cerebellum and striatum from P14 through 18M, but there was no obvious difference in the Cdk5 protein expression across these regions.

Our results demonstrate that Cdk5 protein is expressed in brain at least as early as E12. This observation is in agreement with the detection of Cdk5 mRNA transcripts in E12 mouse and rat brain reported by others (3,21). This study also shows that p35 mRNA and protein could be detected at E12 and E16, respectively. The significant increases in Cdk5 and p35 expression levels from E12 to P1, together with Cdk5 kinase activity, are in general parallel to the neurodevelopment timetable when neurons undergo massive remodeling, network formation and dramatic morphological changes (23). The morphological changes are accompanied by changes in the neuronal cytoskeleton molecules. In vitro, Cdk5 purified from nerve tissue is capable of phosphorylating neuronal cytoskeletal components (10–13,24). The significant increase of Cdk5 and p35 expression and Cdk5 kinase activity during this period suggests that the activated Cdk5 kinase complex is available early in brain development and that it is essential for neurogenesis.

In the present study, the highest level of p39 protein expression was detected from E14 to P7, which is different from Cdk5 and p35 expression. The changes of Cdk5 kinase activity correlate well with the expression pattern of p35 protein but not p39 protein. As an isoform of p35, p39 was reported to have

a similar affinity with and ability to activate Cdk5 as p35 has (9). The significance of the co-existence of these two structurally and functionally homologous proteins in the CNS is still unclear. Our results suggest that p35 and p39 may have different functional roles in the developmental stages of the brain. The regulation of Cdk5 kinase activity by p35 or p39 may be temporally different. Whether Cdk5 and p39 form a complex and what their physiological substrates are still need to be clarified.

In order to examine the functions of Cdk5 in adult and aged CNS, which are often susceptible to debilitating neurodegenerative disorders, in this study we also investigated the expression of Cdk5, p35 and p39, as well as Cdk5 kinase activity in rat brain from 1M to 18M. It was found that the Cdk5 expression level remains the same in adult and aged brain, although p35 and p39 expression and Cdk5 kinase activity decreased and remained at very low levels in aged brains. It is possible that Cdk5 activated by other activators present at this stage does not possess kinase activity against histone H1. Our data suggest that the Cdk5 that does not form a complex with p35 might carry out other physiological and pathological functions.

The expression level of Cdk5 exhibited no obvious difference in the cerebral cortex, hippocampus, cerebellum and striatum of rat brain. However, p35 expression and Cdk5 kinase activity were seen to be higher in the cerebral cortex and hippocampus than in the cerebellum or striatum in rats from P14 to 18M, though no obvious difference existed among the four regions from P1 to P7. Our results indicate that the Cdk5/p35 complex may play physiological roles related to the region-specific function of the cerebral cortex and hippocampus. Another possibility is that the maturation of the cerebellum and striatum takes place earlier than that of the cerebral cortex and hippocampus, and that the maturation process in these regions may rely on Cdk5 kinase activity. Further studies are needed to investigate whether or not these suggestions are probable. We also investigated the p39 expression in the cerebellum and striatum from P1 to 18M. The band was very weak (data not shown). It is possible that the p39 expression levels in these two regions are lower and perhaps the antibody sensitivity is also low.

In this study, immunoblot using anti-p35 and anti-p39 antibodies also revealed a 25 kDa band. In the case of p35, the band is highly likely to be p25, an N-terminal truncated fragment of p35. Further work using different antibodies is needed to confirm this. However, in the case of p39, whether the 25 kDa band represents either a cleavage product of p39 or is derived from a related protein is still not clear.

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