Activation of cyclin-dependent kinase 5 (Cdk5) in primary sensory and dorsal horn neurons by peripheral inflammation contributes to heat hyperalgesia

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Abstract

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the CDK family. It is predominantly expressed in postmitotic neurons and has been implicated in neuronal plasticity. The present study showed that Cdk5 and p35 were expressed in primary sensory and dorsal horn neurons, while p25, an N-terminal truncated derivative of p35, could only be detected in the dorsal horn neurons. Importantly, in the case of control rats, the p35 protein level was much higher in small- and medium-diameter DRG neurons than it was in large neurons. Following CFA injection, Cdk5 activity was upregulated in both primary sensory and dorsal horn neurons. Cdk5 activation in DRG neurons required p35, whereas p25 was required in the dorsal horn. Intrathecal pretreatment with Roscovitine, a specific inhibitor of Cdk5 activity, and intrathecal delivery of the DN-Cdk5(N144) gene both alleviated CFA-induced heat hyperalgesia but not mechanical allodynia. In contrast, overexpression of Cdk5, p35 or p25 in primary sensory and dorsal horn neurons significantly enhanced heat hyperalgesia. We conclude that Cdk5/p35 and Cdk5/p25 complexes in primary sensory and dorsal horn neurons may potentially be involved in nociceptive transmission after inflammation and may be employed in synaptic plasticity underlying pain hypersensitization.

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Keywords: Cdk5 activation; Pain hypersensitization; Heat hyperalgesia

1. Introduction

States of nociception to thermal and mechanical stimuli, such as hyperalgesia and allodynia, often occur after tissue injury, inflammation and nerve lesions. In fact, many acute and chronic pain conditions are an expression of neural plasticity (Woolf and Salter, 2000). To date, a large number of studies show that multiple intracellular kinase cascades play a critical role in the modulation of neuronal plasticity by leading to phosphorylation of key membrane receptors and channels, increasing synaptic efficacy and contributing to pain hypersensitivity (Ji and Woolf, 2001).

Cyclin-dependent kinase 5 (Cdk5) is a unique member of the cyclin-dependent kinase (CDK) family. In contrast to other Cdks, Cdk5 activity is at a high level in terminally differentiated neurons that are no longer in the cell cycle. Similar to activation of other Cdks (Nurse, 1990), activation of Cdk5 requires the presence of its activators, p35 and p39 (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995), or their truncated forms p25 and p30 (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995). Presently, the best-characterized activator of Cdk5 is p35.

It is well known that the Cdk5/p35 complex plays a pivotal role in the nervous system. Cdk5 knockout embryos show massive abnormalities in the development and structure of their nervous system (Ohshima...
et al., 1996). Cdk5-mediated phosphorylation of cytoskeletal proteins (Lew et al., 1992; Patrick et al., 1999) has been implicated in neurite outgrowth and controls multiple aspects of axon patterning (Nikolic et al., 1996; Connell-Crowley et al., 2000; Hahn et al., 2005). p35/−/− mice exhibit impaired hippocampal long-term depression, decreased long-term potentiation (LTP), defective spatial learning and memory, and lowered depression, decreased long-term potentiation (LTP), calcitonin gene-related peptide (CGRP) expression, and hyperalgesia but not mechanical allodynia. Taken together, these results identified Cdk5/p35 and Cdk5/p25 as pivotal players in nociceptive signaling. It would be plausible that Cdk5 activation in primary sensory and dorsal horn neurons may likewise contribute to altered pain sensitization after inflammation. In the present study, the potential involvement of Cdk5 in nociceptive transmission was explored using the CFA model. Here, we showed that Cdk5 and p35/p25 were prominently expressed in primary sensory and dorsal horn neurons of adult rats, and that p35/p25-associated Cdk5 kinase activity in primary sensory and dorsal horn neurons increased following CFA treatment. Moreover, our findings revealed that Cdk5 activity was associated with heat hyperalgesia but not mechanical allodynia. Taken together, these results identified Cdk5/p35 and Cdk5/p25 as pivotal players in nociceptive signaling. It would be of interest to search for targets of Cdk5 in primary sensory and dorsal horn neurons under conditions of inflammatory pain.

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (200–250 g) were obtained from the animal center of Peking University Health Science Center. Rats were housed in climate-controlled rooms on a 12-hour (h) light–dark cycle with free access to food and water. Animals were acclimated for 5 days (d) before any experimental procedures began. All experimental procedures conformed to the guidelines of the Animal Care and Use Committee of Peking University. Complete Freund’s adjuvant (CFA, 100 μl, Sigma, St. Louis, MO, USA. F5881) was injected into the plantar surface of the left hindpaw. 2 h, 6 h, 1 day, 3 days, 5 days and 7 days after injection, rats were perfused or tissues were dissected out. For intrathecal drug delivery, implantation of i.t. cannulas was performed following the method of Storkson et al. (1996). Briefly, rats weighing 200–220 g were anesthetized with 10% chloral hydrate (0.3 g/kg, i.p.). The back skin of the rats was incised and the spinal column was exposed. The intraspinal space between lumbar vertebrae 4 and 5 (L4 and L5) was chosen as the site for insertion of the needle. Slight movements of the tail indicated proper insertion of the needle into the subarachnoid space. PE-10 polyethylene catheters were implanted 4.0 cm using a catheter-through-needle technique to reach the lumbar enlargement of the spinal cord. The outer end of the catheter was plugged and fixed onto the skin with a single suture. The rats were housed individually after surgery and allowed 4–5 days for recovery before being tested. Animals with neurological damage after catheter implantation were excluded from the study. 3 μl of a Cdk5 inhibitor, Roscovitine (1, 10 and 100 μg, Sigma, St. Louis, MO, USA. R7772), followed by 5 μl sterilized saline was administered 30 min before CFA treatment and pain responses were measured at times from 6 h to 1 week after CFA injection. DMSO was used as the vehicle control for the intrathecal injection. For the experiments involving gene delivery, 100% CFA was diluted to 25% in incomplete Freund’s adjuvant (IFA) in order to avoid excessive spontaneous pain behavior. Noxious responses after intrathecal injection or delivery of genes were measured in a blinded fashion.

2.2. Assessment of inflammation

Thermal hyperalgesia was assessed in unrestrained rats using a procedure adapted from published reports (Hargreaves et al., 1988). Animals were allowed to become accustomed to the environment for 20 min prior to being tested. Paw withdrawal latency to radiant heat was recorded. A cut-off time of 30 s was used to prevent tissue damage. The paw withdrawal latency of each rat was measured four times at intervals of 5 min and the median score was recorded. Mechanical allodynia was tested by Von Frey filaments (Stoeltting, USA) (Chaplan et al., 1994). The paw withdrawal threshold was calculated using the up-down method.

2.3. Plasmids and constructs

For mammalian expression, the open reading frames of Cdk5, p35 and a truncated form of p35 (p25) corresponding to amino acid residues Ala99 to Arg307 (Lee et al., 2000) were amplified by PCR and subcloned into pEGFP-N1 and pDs-Red1-C1 vectors (Clontech), respectively. The dominant-negative (kinase inactive) Cdk5 (N144) (Nikolic et al., 1996) was prepared by mutating an aspartic acid in the 144 position to an asparagine in the pEGFP-N1 Cdk5 plasmid. Mutagenesis was performed with the Quick Change Site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All constructs were verified by sequencing performed by Shanghai Sangon Biological Engineering and Technology and Service CO. Ltd (Shanghai, China).

2.4. Delivery of genes into the DRG and spinal dorsal horn

After 4–5 days of recovery from surgery for placement of the intrathecal catheter, rats were subjected to intrathecal injection of the Cdk5-pEGFP-N1, DN-Cdk5(N144)-pEGFP-N1, p35-pDsRed1-C1 and p25-pDsRed1-C1 genes. The rats were randomized into the following experimental groups: N-S/Lipofectamine 2000, pEGFP-N1/Lipofectamine 2000, pDsRed1-C1/Lipofectamine 2000, Cdk5-pEGFP-N1/Lipo-
fectamine 2000, DN-Cdk5(N144)-pEGFP-N1/Lipofectamine 2000, p25-pDsRed1-C1/Lipofectamine 2000 and p25-pDsRed1-C1/Lipofectamine 2000. Complexes with 10 μg DNA were injected slowly over 5 min. After the injection, the needle remained in situ for 2 min before being withdrawn. The basal PWL or PWT was measured 4 days after gene delivery and then 25% CFA was injected into the plantar surface of the left hindpaw. Nociceptive responses were again measured 6 h after CFA administration. To prepare DNA complexes, 10 μg plasmid DNA, pEGFP-N1, Cdk5-pEGFP-N1, DN-Cdk5 (N144)-pEGFP-N1, pDsRed1-C1, p25-pDsRed1-C1 and p25-pDsRed1-C1 was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in sterile saline by adding Lipofectamine 2000 solution into the DNA solutions. The ratio of DNA (in μg) to Lipofectamine 2000 (in μl) was 1:2. The total volume of injection was 40 μl.

### 2.5. Western blot

Rats were deeply anesthetized with 10% chloral hydrate (0.3 g/kg, i.p.) and then the L4/5 DRG and dorsal horn of the L4/5 spinal cord were removed and immediately homogenized in ice-chilled lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 1% Triton X-100, 0.5 mM Na3VO4, 1 mM PMSF, 1 mM dithiothreitol, 0.1 mM EGTA and 0.1 mM dithiothreitol). The final pellet was resuspended in reactive buffer (20 mM Tris–HCl (pH 7.5), 20 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 0.08 μg/ml histone H1 protein (Sigma, St. Louis, MO, USA, H5505), 0.2 μCi/μl γ-32P-ATP) to yield a total volume of 40 μl. The mixture was incubated at 30 °C for 30 min, and the reaction was terminated by the addition of SDS–PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS–PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed to X-ray film for autoradiography.

### 2.7. Tissue preparation and immunofluorescence

After anaesthesia, rats were perfused transcardially with 200 ml saline at 37 °C, followed by 200 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4/5 DRG and spinal cord were removed, postfixed in the same fixative agent for 2–4 h, and then transferred to a 30% sucrose solution and incubated until the sample sank to the bottom of the container. Then, frozen tissue sections were cut coronally in a cryostat at a thickness of 10 μm. For staining, sections were blocked for 1 h at room temperature with 0.3% Triton X-100/10% normal serum of the species in which the secondary antibody was raised. Subsequently, sections were incubated overnight at 4 °C with rabbit polyclonal anti-p35 antibody (1:100, Santa Cruz Biotechnology, CA, USA. sc-820), monoclonal anti-Cdk5 antibody (1:200, Upstate Biotechnology, Lake Placid, NY, USA, 05-364), or monoclonal anti-β-actin (1:2000, Sigma, St. Louis, MO, USA. A5316). The blots were washed in TBST for 10 min for three times. Then, they were incubated with HRP-conjugated secondary antibody (1:2000 dilution, goat anti-rabbit or mouse; Bio-Rad Laboratories) for 1 h at room temperature. Finally, the blots were developed with the lightening chemiluminescence kit (Santa Cruz Biotechnology, CA, USA. sc-2048).

### 2.6. In vitro kinase assay

Cdk5 kinase activity was measured as described using an immune complex kinase assay and histone H1 as substrate (Nikolic et al., 1998). Briefly, tissue lysates were immunoprecipitated with anti-Cdk5 antibody (1:50, Santa Cruz Biotechnology, CA, USA. sc-173) or rabbit control IgG (Santa Cruz Biotechnology, CA, USA) at 4 °C for 3 h. Protein A-Sepharose CL-4B resin (Amersham Biosciences, Sweden) was added to the samples and incubation was continued for a further 12 h, after which samples were washed four times with TBS/0.1% Triton X-100 and two times with assay buffer (20 mM Tris–HCl (pH 7.5), 20 mM MgCl2, 1 mM EDTA, 1 mM EGTA and 0.1 mM dithiothreitol). The final pellet was resuspended in reactive buffer (20 mM Tris–HCl (pH 7.5), 20 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 0.08 μg/ml histone H1 protein (Sigma, St. Louis, MO, USA, H5505), 0.2 μCi/μl γ-32P-ATP) to yield a total volume of 40 μl. The mixture was incubated at 30 °C for 30 min, and the reaction was terminated by the addition of SDS–PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS–PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed to X-ray film for autoradiography.

### 2.8. Image analysis

For DRG analysis, five randomly selected sections from the L4 and L5 DRG of normal rats were used. All immunoreactive positive profiles in a section were outlined, creating an artificial overlay. The average intensity and ferret area of each object identified by the overlay were then measured automatically by MetaMorph software. Proportions of neurons labelled for p35 per total immunoreactive positive neurons and the average intensity of p35 immunoreactivity were calculated according to the size of the cell body.

### 2.9. Statistical analysis

For Western blotting and the in vitro kinase assay, the films were scanned, and the densities of specific bands were measured and normalized with an internal loading control band. All data were represented as means ± SEM. Differences between groups were compared using either the Student’s t test or ANOVA test. The criterion for statistical significance was $P < 0.05$. 

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111
3. Results

3.1. Expression and localization of p35/p25 and Cdk5 proteins in the DRG and spinal cord

It has been reported that expression of the Cdk5 protein is ubiquitous in the nervous system (Tsai et al., 1993). As we know, tissue distribution and subcellular localization help determine the function of a protein kinase. Thus, we explored the expression of Cdk5 and its activators in pain transmission pathways in normal rats. Immunoblot analysis of Cdk5 expression in DRG and spinal dorsal horn showed a single band of 33 kDa (Fig. 1a), and the expression level of Cdk5 was higher in dorsal horn than in DRG (Fig. 1a). A single band of 35 kDa in DRG representing p35 and two bands of 35 and 25 kDa, respectively, representing p35 and p25 in dorsal horn were visualized by a p35 antibody (c-19) which could recognize both p35 and p25 (Fig. 1a). The results demonstrated that Cdk5 and p35 were expressed both in DRG and the dorsal horn, while p25 could only be detected in the dorsal horn. Moreover, the expression level of Cdk5 and p35 was higher in the dorsal horn than in DRG (Fig. 1a). P35 is the most important Cdk5 activator and is required for Cdk5 kinase activity (Tsai et al., 1994). In this study, we detected the distribution of p35 in DRG and p35/p25 in dorsal horn, which are the sites where the processes leading to pain hypersensitization take place. In order to define the cellular distribution of p35 in DRG and p35/p25 in dorsal horn, sections from adult rat DRG and spinal cord were stained with anti-p35 (c-19) (Figs. 1b and e). It was shown that DRG neurons of all sizes were immunoreactive. In addition, the immunoreactivity of p35 was more intense in small-(200–600 μm²) and medium-diameter (600–1200 μm²) neurons than in large-diameter (>1200 μm²) neurons, although the intensity of immunoreactivity in individual neurons varied substantially (Figs. 1b, b’ and d). Consistent with the immunofluorescent data, analysis of size

Fig. 1. Western blot and immunolocalization analysis of normal adult rat DRG and spinal dorsal horn (SDH) lysate and sections probed with anti-p35 (C-19) and anti-Cdk5 antibodies. (a) Total protein of different tissues was extracted and subjected to 12% SDS–PAGE. β-Actin was used as a loading control. (b) Immunolocalization of p35 in DRG neurons of normal adult rat. Scale bar, 20 μm. (b’) Enlargement of the boxed area in (b). Small arrows and large arrows represent characterized small- and large-diameter neurons. Scale bar, 20 μm. (c) Size-frequency distribution of p35-immunoreactive neurons. (d) Average intensity of p35 immunoreactivity in DRG neurons of different sizes. (e) Localization of p35/p25 in spinal dorsal horn of normal adult rat. p35/p25 were stained by p35 antibody (c-19). Scale bar, 20 μm. (e’) Enlargement of boxed area in (c). Scale bar, 20 μm. DRG, dorsal root ganglion; SDH, spinal dorsal horn. Data represent three independent experiments.
frequency revealed that p35 predominantly expressed within small- and medium-diameter neurons (Fig. 1c).

In dorsal horn, immunofluorescent analysis indicated that the p35/p25 protein was prominently expressed in neurons in almost all laminae of the dorsal horn (Fig. 1e). Furthermore, at a higher magnification, we found that the staining of p35 in primary sensory neurons and of p35/p25 in dorsal horn neurons was strongly concentrated in the cytoplasm, although there was slight staining in the nucleus (Figs. 1b and e). Taken together, the results implied that p35 or p35/p25 protein was prominently distributed in small- and medium-diameter primary sensory and dorsal horn neurons.

Several negative controls were included to confirm the specificity of the positive antibody responses. Normal rabbit IgG was used in place of primary antibody and produced non-specific staining in DRG and spinal cord sections. The blocking peptide preincubation reduced the staining intensity to background levels and no primary antibody control produced background staining (data not shown) in DRG and spinal cord sections.

3.2. Induction of Cdk5 activation in primary sensory neurons following CFA-induced peripheral inflammation

To test whether peripheral noxious stimuli induce Cdk5 activation in DRG, 100 µl of CFA was injected into the plantar surface of the rat left hindpaw. The injection caused localized inflammation, associated with swelling and erythema. Hyperalgesia and allodynia were evoked at 2 h, reached a peak 1 day after inflammation, and were maintained for more than 1 week in the ipsilateral paw. Pain hypersensitization did not occur in the contralateral paw (data not shown). Using an in vitro phosphorylation assay, we found that Cdk5 activity was relatively low in normal rats, began to increase at 2 h, peaked at 1 day, and then remained at a high level for up to 1 week after CFA injection. The elevation of Cdk5 activity was apparent at 2 h, peaked at 1 day, and remained stable for up to 1 week following CFA injection (Fig. 3a). Quantification indicated that Cdk5 activity was elevated 4-fold at 6 h and 5-fold at 1 day after CFA injection, compared with that of naïve rats (Fig. 3b). Similarly, Cdk5 activity in spinal dorsal horn showed a linear correlation with nociceptive responses of the rats after inflammation ($R^2 = 0.4657$, $P < 0.001$) (Fig. 3g).

To determine what was responsible for Cdk5 activation, we assessed protein levels of Cdk5 and its activators by Western blot as a function of time after CFA injection. P35 antibody (c-19), which can recognize both p35 and p25 (a N-terminal truncated product of p35), was used in the present study (Patrick et al., 1999). Fig. 3c shows that p35 and Cdk5 protein levels did not vary significantly over time following CFA injection (Figs. 3d and f). However, the p25 protein level showed a significant time-dependent change, being elevated at 2 h (4-fold), reaching a maximal level at 1 day (6-fold) and retaining a high level 1 week after CFA injection (Figs. 3c and e). The same results were obtained in the contralateral spinal dorsal horn (data not shown).

Since p25 lacks the conserved myristoylation sequence, conversion of p35 to p25 can change its cellular location and alter substrate specificity. Moreover, p25 has a longer turnover rate and is not readily degraded, thus p25 can deregulate Cdk5 activity (Patrick et al., 1999). We find that the p25 protein level was linear related to the Cdk5 activity in spinal dorsal horn following peripheral inflammation ($R^2 = 0.7026$, $P < 0.001$) (Fig. 3h), and conclude that Cdk5 activation may mediate pain central sensitization.

3.4. Possible involvement of Cdk5 activity in pain hypersensitivity

We confirmed that Cdk5 activity was markedly heightened both in primary sensory and dorsal horn neurons following peripheral inflammation. To investigate whether Cdk5 activity was involved in nociceptive transmission, a Cdk5-specific inhibitor, Roscovitine
intrathecally injected 30 min before CFA administration, and nociceptive responses were examined from 6 h to 1 week after CFA injection. Intrathecal injection of 100 µg Roscovitine did not affect rat movement before CFA injection as detected by the inclined plane test (Rivlin and Tator, 1977) (Fig. 4b), but it significantly depressed the elevation of Cdk5 activity both in primary sensory and dorsal horn neurons 6 h after CFA administration (Fig. 4a). Likewise, heat hyperalgesia was dose-dependently inhibited 6 h after inflammation. Furthermore, intrathecal injection of 10 µg Roscovitine still prevented the heat hyperalgesia 1 day after inflammation. The effect of 100 µg Roscovitine lasted for 5 days and was absent at 7 days after CFA injection. However, pretreatment with DMSO or 1 µg Roscovitine had no effect (Fig. 4d). In contrast, pretreatment with Roscovitine failed to reduce mechanical allodynia (Fig. 4c) or inflammatory swelling (Fig. 4c).

Roscovitine substantially inhibits Cdk5 activity in the nervous system. It is reported that Roscovitine also depresses Cdk1 and Cdk2 activity; moreover, ERK activity is inhibited with a 100-fold IC50 compared with Cdk5 (Meijer et al., 1997). As we know, Cdk1 and Cdk2 are expressed at a very low level in postmitotic neurons of adult rats. However, ERK1/2 play an essential role in pain hypersensitization, and p-ERK1/2 increases in the early phase of inflammation (Ji et al., 1999). In order to exclude the effect of pretreatment with Roscovitine on p-ERK, we examined p-ERK at 15 min and 6 h after CFA injection. By Western blot analysis, the level of p-ERK did not vary in primary sensory or dorsal horn neurons pretreated with 100 µg Roscovitine or DMSO at either 15 min and 6 h after inflammation (Fig. 4a).
All results indicated that pretreatment with Roscovitine, a specific inhibitor of Cdk5 activity, reduced CFA-induced heat hyperalgesia but not mechanical allodynia. Additionally, a single pretreatment with Roscovitine before CFA injection decreased the nociceptive responses for a long time. We conclude that Cdk5 activity in primary sensory and dorsal horn neurons may contribute to peripheral sensitization and central sensitization, respectively.

3.5. The effects of overexpression of Cdk5, of a dominant-negative mutant of Cdk5, and of its activators on pain hypersensitivity

Inhibition of endogenous Cdk5 activity by pretreatment with Roscovitine decreased heat hyperalgesia. To elucidate further the roles of Cdk5 activity in pain transmission, we employed the intrathecal delivery of the Cdk5-pEGFP-N1, DN-Cdk5(N144)-pEGFP-N1, p35-pDsRed1-C1, and p25-pDsRed1-C1 genes to enhance or depress Cdk5 activity, respectively. Lipofectamine serves as a non-viral plasmid vector that has been widely used to transfer foreign genes into a variety of mammalian cell types, including both dividing and non-dividing cell types (Yao et al., 2002; Wang et al., 2005b). Firstly, we intrathecally delivered the Cdk5-pEGFP-N1 and DN-Cdk5(N144)-pEGFP-N1 genes, Lipofectamine and pEGFP-N1 gene were used as controls. In order to avoid excessive inflammation and spontaneous nociceptive behavior, 25% CFA (diluted with incomplete Freund’s adjuvant) was injected into the plantar surface of the rat left hindpaw 4 days after gene delivery. It was found that delivery of genes did not affect rat movement as detected by the inclined plane test (Fig. 5 c) and failed to affect the basal nociceptive responses (Figs. 5 d and e). Stronger green fluorescence was observed in primary sensory neurons (Fig. 5a) and some diffuse spots in spinal
dorsal horn after delivery of the pEGFP-N1, Cdk5-pEGFP-N1 and DN-Cdk5(N144)-pEGFP-N1, compared to after treatment with Lipofectamine alone (data not shown). As determined by an in vitro phosphorylation assay, Cdk5 activity was upregulated or inhibited by intrathecal delivery of the Cdk5-pEGFP-N1 or DN-Cdk5(N144)-pEGFP-N1 genes, respectively, both in DRG and dorsal horn 6 h after inflammation, compared with the delivery of the pEGFP-N1 gene and Lipofectamine (Fig. 5b). At this time, the PWL was obviously reduced or increased by the intrathecal delivery of Cdk5-pEGFP-N1 or DN-Cdk5(N144)-pEGFP-N1 genes, respectively, in the ipsilateral paw, and was not affected by the delivery of pEGFP-N1 gene and Lipofectamine. The contralateral nociceptive responses were not altered upon inflammation (Fig. 5f). Unlike its effects on thermal hyperalgesia, transfection of the exogenous genes did not change mechanical allodynia (Fig. 5h) or the local inflammatory reaction, compared with control groups (data not shown). We delivered the p35-pDsRed1-C1 and p25-pDsRed1-C1 genes in the same manner as the Cdk5-pEGFP-N1 gene. Surprisingly, the delivery of the p35-pDsRed1-C1 gene heightened Cdk5 activity both in DRG and dorsal horn, whereas delivery of the p25-pDsRed1-C1 gene increased Cdk5 activity only in dorsal horn, which was similar to the endogenous expression pattern. Cdk5 activity achieved by delivery of the p35-pDsRed1-C1 gene was much higher than that by delivery of the p25-pDsRed1-C1 gene (Fig. 5g). Different transfection efficacies might account for the distinct upregulation of Cdk5 activity. We demonstrated that transfection of p35-pDsRed1-C1 and p25-pDsRed1-C1 genes did not affect rat movement or the local inflammatory reaction (data not shown). Heat hyperalgesia was potentiated by transfection of both p35 and p25 after inflammation, compared with transfection of pDsRed1-C1 and Lipofectamine alone (Fig. 5i). The mechanical allodynia did not vary among the experimental groups (Fig. 5j).
In summary, intrathecal transfection with Cdk5 and its activators could induce an elevation of Cdk5 activity and cause heat hyperalgesia, whereas there was no effect on mechanical allodynia and local inflammation. Conversely, transfection of DN-Cdk5(N144) suppressed Cdk5 activity and desensitized heat hyperalgesia.

4. Discussion

It is well known that Cdk5 is expressed ubiquitously in the central nervous system (Meyerson et al., 1992) and that p35 is a brain-specific Cdk5 activator. Additionally, a few groups have reported that Cdk5 and p35 were expressed in the primary sensory and spinal
cord neurons of both developing and adult mice or rats (Ito et al., 1994; Zhang et al., 1997; Terada et al., 1998; Pareek et al., 2006). In the present study, we showed that Cdk5 and p35 were prominently expressed in primary sensory and dorsal horn neurons, two important sites for sensory processing. Most importantly, p35 was much more highly expressed in small- and medium-diameter DRG neurons than in large ones.

As mentioned above, Cdk5 activators are essential for Cdk5 function. In general, upregulation of p35, not Cdk5, was responsible for elevated Cdk5 activity in various conditions (Paglini et al., 1998; Li et al., 2000). It has also been well demonstrated that Cdk5 activity is directly associated with the p25 protein level in brain tissues from Alzheimer patients and in amyotrophic lateral sclerosis (ALS) tissues (Patrick et al., 1999; Harada et al., 2001b; Nguyen et al., 2001). We found that the Cdk5 protein level was not altered in DRG and dorsal horn, whereas the p35 protein level in DRG and the p25 protein level in dorsal horn showed similar increases to that of Cdk5 activity, with a highly positive linear correlation with nociceptive responses induced by inflammation in rats. Elsewhere, it was reported that Cdk5 and p25 levels in both primary sensory and dorsal horn neurons increased after peripheral inflammation induced by the injection of carrageenan, causing an upregulation of Cdk5 activity (Pareek et al., 2006). The distinct animal models may be responsible for the discrepancy.

To assess the function of Cdk5 in pain hypersensitivity, we performed intrathecal injection of a Cdk5 inhibitor, Roscovitine, and intrathecal liposome-mediated delivery of Cdk5, DN-Cdk5(N144), and its activator (p35 and p25) genes. The non-viral plasmid vector has advantages of reproducibility and safety (nearly no immune response or toxic effects). It has been widely used to transfer foreign genes into a variety of mammalian cell types, including both dividing and non-dividing cell types in vitro and in vivo (Luo et al., 2005). It was found that Roscovitine could dose-dependently alleviate heat hyperalgesia induced by inflammation with a decrease of Cdk5 activity, which was consistent with the results of others (Wang et al., 2005a). Furthermore, our results revealed that potentiation or depression of Cdk5 activity by overexpression of Cdk5, p35 and p25 or DN-Cdk5(N144) in DRG and dorsal horn affected pain responses by noxious thermal stimuli but not by noxious mechanical stimuli after inflammation, whereas the basal pain responses did not vary among the different groups. In contrast, Pareek et al. reported that Cdk5 activity is important for the basal thermal response in mice by using p35 gene knockout (p35−/−mice) and transgenic mice overexpressing p35 (Tg(p35 mice) (Pareek et al., 2006). The discrepancy may be due to the different approaches. Accordingly, inhibition of Cdk5 activity may provide a promising clinical treatment of pain. Furthermore, our study demonstrated that a single pre-CFA intrathecal injection of Roscovitine persistently attenuated heat hyperalgesia for at least a few days in a chronic pain model, implying that Cdk5 activity plays an important role in the initiation or early phase of inflammatory pain (Amin et al., 2002), although not excluding an effect on maintenance of pain sensitization. Thus, in the clinic it would be advantageous for the control of chronic pain development to prevent or treat pain as soon as possible.

Since Cdk5 is a member of serine/threonine kinase family, the specific effect of Cdk5 on heat hyperalgesia is presumably determined by phosphorylation of its substrates. Cdk5 phosphorylates serines/threonines in S/TPXK/R-type motifs as do other Cdks (Beaudette et al., 1993). To date, approximate two dozen proteins with diverse functions have been identified as substrates of Cdk5. These include multiple proteins involved in neuronal plasticity such as synaptic vesicle and presynaptic membrane proteins, P/Q type voltage-dependent calcium channels (VGCC), NMDA2A subunits, and PSD-95 (Dhavan and Tsai, 2001). The vanilloid receptor-1(VR1), essential for inflammatory thermal hyperalgesia (Davis et al., 2000), has a consensus sequence for Cdk5 phosphorylation. Cdk5 may play a role in pain hypersensitivity by regulating VR1 function following inflammation. As a protein kinase, some of the well-characterized roles of Cdk5 during nociceptive transmission, such as interactions with distinct cytoplasmic and synaptic target molecules in primary sensory and dorsal horn neurons, may be employed in synaptic plasticity underlying peripheral sensitization and central sensitization (Woolf and Salter, 2000). The entire hypothesis identified above deserves further study to search for specific substrates of Cdk5 in primary sensory and dorsal horn neurons following inflammation.

As for p25, it has been reported that alternative splicing cannot account for the generation of p25 since the open reading frame of p35 does not contain introns, and internal initiation of translation of p35 messenger RNA is also unlikely to produce p25 since no internal methionines are near the beginning of the p25 sequence (Lee et al., 2000). Rather, p25 is produced by cleavage of p35. It has been proven that the conversion of p35 to p25 lies downstream of ubiquitin–protease and calpain activation (Patrick et al., 1998; Lee et al., 2000). Consequently, the upregulation of p25 protein after inflammation may be attributed to the elevation of p35 mRNA transcription and translation, simultaneous with accelerated cleavage of p35. Concerning the elevated level of p35, it has been reported that the ERK pathway mediated the induction of p35 in PC12 cells through the transcription factor Egr1 in the presence of NGF (Harada et al., 2001a). While it has been well documented that NGF and ERK are both involved in pain hypersensitivity (Woolf et al., 1994; Ji et al., 1999, 2002), ERK is
activated in both primary sensory and dorsal horn neurons in the early phase of inflammation (Ji et al., 1999; Zhuang et al., 2004). Hence ERK and NGF may contribute to the induction of p35 in primary sensory and dorsal horn neurons to enhance Cdk5 activity. On the other hand, it has been shown that ubiquitin–protease and calpain activation in primary sensory and dorsal horn neurons were responsible for heat hyperalgesia and mechanical allodynia following inflammatory or neuropathic pain (Moss et al., 2002; Kunz et al., 2004), which might facilitate the cleavage of p35 to p25 following CFA injection. Thus, transcription and posttranslational modification might be implicated in the induction of p35 and p25, but this hypothesis needs to be further investigated. Moreover, it was reported that conversion of p35 to p25 could change its cellular location and alter its substrate specificity (Patrick et al., 1999). P25 was present in dorsal horn neurons but absent in primary sensory neurons in both normal and inflamed rats, this might result in different target proteins phosphorylated by Cdk5 to produce heat hyperalgesia after inflammation.

In summary, Cdk5 and p35, not only in the central nervous system, were extensively expressed in primary sensory and dorsal horn neurons, whereas p25 was only present in dorsal horn neurons. Cdk5 activity, associated with p35 or p25 protein levels, was upregulated in primary sensory or dorsal horn neurons following inflammation. Inhibition or potentiation of Cdk5 activity in primary sensory and dorsal horn neurons efficiently altered heat hyperalgesia, with no effect on mechanical allodynia. Therefore, Cdk5 and its activators played a crucial role in heat hyperalgesia. Regulation of Cdk5 activity may be a potential new pharmaceutical target for the management of inflammatory pain.

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References


