VGLUT2/ Cdk5/p25 Signaling Pathway Contributed to Inflammatory Pain By CFA

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Abstract

Vesicular glutamate transporter type 2 (VGLUT2) is known to play an important role in mediating the heat hyperalgesia induced by inflammation. However, the underlying mechanism for this activity is poorly understood. Cyclin-dependent kinase 5 (Cdk5), serving as a key regulator in mediating release of glutamate, contributed to the inflammatory heat . It remains unknown whether there is a bridge between Cdk5 and VGLUT2 for mediating inflammatory pain. Therefore, we designed the experiment to determine whether VGLUT2 signaling pathway is involved in Inflammatory pain mediated by Cdk5 and the heat hyperalgesia induced by complete Freund's adjuvant (CFA) can be reversed by roscovitine, a selective inhibitor for Cdk5 through inhibition of VGLUT2 expression. Immunohistochemistry results suggest that when compared with rats in a control group, rats in an experimental group showed significant coexpression of Cdk5/VGLUT2 in small and medium-sized neuronal cells of the dorsal root ganglion (DRG) and spinal cord between days 1 and 3 following subcutaneous injection of CFA. Moreover, our study revealed that the expression of VGLUT2 protein in DRG and spinal cord was remarkably increased between days 1 and 3 following CFA injection. Additionally, p25 but not p35, a activator of Cdk5,protein was significantly increased and reduced by roscovitine.The increased expressions of VGLUT2 protein was significantly reduced by roscovitine as well. Our study showed that VGLUT2/Cdk5 signaling pathway contributed to the inflammatory pain medicated by Cdk5/p25.

Introduction

Pain caused by inflammation in the peripheral or central nervous system remains a significant clinical problem, and is often resistant to treatment with conventional analgesics. Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and plays a key role in processing of nociceptive pain [1]. Prior to its release from excitatory synapses, glutamate is transported into synaptic vessels by vesicular glutamate transporters (VGLUTs) composed of VGULT proteins 1-3. Previous studies have established that VGLUT1, 2, and 3 proteins are expressed by largely non-overlapping and functionally distinct populations of glutamatergic neurons, and play multiple roles in the CNS [2-4].

However, only VGLUT 2 plays a key role in mediating pain hypersensitivity induced by inflammation and peripheral nerve injury [5-12]. Co-expression of VGLUT2 and two main nociceptors of calcitonin gene related peptide (CGRP) and transient receptor potential channel (TRPV1) was observed in small and medium-sized neurons of the dorsal root ganglion (DRG) in mice [6, 7]. Additionally, VGLUT2 knock-out mice challenged with CFA demonstrate a complete loss of heat hyperalgesia, whereas CFA-induced mechanical hypersensitivity remains intact. Nevertheless, a detailed mechanism for VGLUT2 mediation of inflammation-induced heat hyperalgesia remains elusive.

Emerging evidence suggests that the serine/threonine kinase Cdk5 plays an important role in mediating inflammation-induced heat hyperalgesia [13-16]. A previous study showed that Cdk5 can modulate CFA-induced heat hyperalgesia by controlling membrane trafficking of TRPV1 and phosphorylating vanilloid receptor 1 (VR1) in the dorsal root ganglion (DRG) of rats [14, 15]. The activities of Cdk5 and p35 in the spinal cord were significantly increased following peripheral injection of CFA. Furthermore, both Cdk5 kinase activity and heat hyperalgesia were inhibited by Cdk5/p35 knockdown or intrathecal administration of roscovitine. Previous studies have suggested that presynaptic Cdk5 is the primary regulator of neurotransmitter release in the CNS [17]. Synapsin II is a synaptic vesicle protein associated with the release of neurotransmitters, and a recent study revealed that expression of both VGLUT1 and VGLUT2 was strongly reduced in the spinal cord of synapsin II knockout mice [18]. Moreover, our previous studies revealed that increased levels of synaptophysin protein, an important presynaptic vesicle membrane protein that functions in release of neurotransmitters, are involved in mediating CFA-induced heat hyperalgesia mediated by Cdk5 in rats. Furthermore, the recent study showed that VGLUT2-pH fluorescence co-localizes with synaptophysin at synaptic boutons and was involved in the trafficking of synaptic vesicles [19]. This suggests that Cdk5 may mediate inflammation-induced heat hyperalgesia by controlling the release of neurotransmitters [20]. Here, we report that the VGLUT2/Cdk5 signaling pathway contributed to the inflammatory pain mediated by Cdk5.

Materials and methods

Animals

All adult male Sprague-Dawley rats (200-250g) used in this study were obtained from the animal center of Nanjing Medical University (Nanjing China). All experimental procedures were verified and approved by the Committee of Animal Use for Research and Education of Nanjing Medical University. Moreover, these procedures were performed in accordance with

1 guidelines developed by the International Association for the Research on Pain [21].
 2 According to the guidelines mentioned before, rats were placed with room temperature of 22
 3 $\pm 2^{\circ}\text{C}$ and a standard 12/12 hours light/dark cycle, and food and water were available ad
 4 libitum. To minimize the animals we used in this research and avoid any unnecessary stress
 5 and pain, all animals were permitted to adapt to the housing facilities for 1 week before the
 6 experiments. Complete Freund's Adjuvant CFA (100 μL ; Sigma, St. Louis, MO, USA) and
 7 saline (100 μL) were injected into the plantar surface of the ipsilateral and contralateral hind
 8 paws of rats respectively. The saline group was settled as a control ($n = 6/\text{group}$). The same
 9 volume of saline as used for control group was injected into ipsilateral and contralateral paws
 10 of rats in the control group ($n = 6/\text{group}$).

11 *Surgery and drug administration*

12 Drugs used in this study were intrathecal injection as described before, with a slight
 13 modification [22]. Briefly, a catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d.; Clay Adams,
 14 Parsippany, NJ, USA) was inserted at the lumbar level of the spinal cord between lumbar
 15 vertebrae 4 and 5 (L4 and L5) of the rats which were anesthetized before with the 4%
 16 pentobarbital (40 mg/kg). 5 μL of 2% lidocaine was confirmed to be injected through the
 17 catheter for the recovery after the anesthesia and surgery, followed by flushing with 15 μL of
 18 saline. Following this procedure, successful insertion of the catheter was further confirmed if
 19 the animal presented with impaired motor function of their hind legs at 10 s post-lidocaine
 20 administrations. Following surgery, 5 μL of roscovitine (100 μg) (Sigma, St. Louis, MO,
 21 USA R7772) dissolved in 10% DMSO was delivered for 5 consecutive days to the rats in the
 22 experimental group, followed by rinsing of 15 μL of sterile saline 0.5 h before a single
 23 treatment with CFA ($n = 6/\text{group}$). The same concentration of roscovitine and DMSO was
 24 injected into the rats in the vehicle control group ($n = 6$) and the experimental group.

25 *Behavioral test*

26 Heat hyperalgesia was quantified by measuring paw withdrawal latencies (PWL) in
 27 response to radiant heat stimulation as previously described [23]. Briefly, radiant heat was
 28 directed to the plantar surface of each hind paw through a 1 mm thick glass plate. In order to
 29 prevent tissue damage, a 20 second cut-off limit was radicate for heat exposure. The time
 30 between the onset of the heat stimulus and a manifestation of paw withdrawal response was

1 recorded as the thermal nociceptive latency period. Rats were allowed to adapt to the room
2 for 30 min before the behavioral test. The thermal stimulus was then delivered to the
3 ipsilateral and contralateral hind-paws of rats in both groups after adaption. The time of PWL
4 measurements was at 0 d, 6h, 1 d, 3 d, and 5 d post-CFA or saline injection, which was
5 recorded as the average result of 3 trials for each hind paw. The interval time between PWL
6 measurements taken with the ipsilateral and contralateral paws was 5 minutes. So, again PWL
7 measurements of the rats in the DMSO and roscovitine-pretreatment groups were taken by the
8 same method. The behavioral results showed that the heat hyperalgesia induced by CFA
9 significantly decreased from 6h to 5 d and reversed by roscovitine ,which is in accordance
10 with our previous studies [21].

11 *Immunofluorescence*

12 4% paraformaldehyde was injected into the animals which were deeply anesthetized with
13 sodium pentobarbital (> 100 mg/kg. i.p). Tissues collected from ipsilateral segments L4-L6 of
14 the DRG and spinal cord were immediately cryoprotected in 30% sucrose, and sectioned
15 transversely at 16 μ m thickness within a cryostat afterwards. The tissue sections were
16 embedded with Tissue-Tek (Sakura Finetek, Torrance, CA, USA) and frozen in dry ice
17 powder. The cross sections were sliced into 16 μ m thick sections at -28°C using a cryostat.
18 Using double immunofluorescence for the examination, sections were incubated overnight at
19 4°C with appropriate antibodies (anti-Cdk5 1:200, ab115812, and anti-VGLUT2, 1:200,
20 ab79157; Abcam, Cambridge, MA, USA), followed by a second overnight incubation at 4°C
21 with the corresponding secondary antibodies (1:200; Invitrogen, Carlsbad, CA, USA). An
22 Axiovert/LSM 510 confocal scanning microscope (Carl Zeiss Microimaging, Inc., Germany)
23 was used to exam the double-stained sections. After that, these sections were washed and the
24 fluorescence-labeled secondary antibodies (Goat anti-Mouse; goat anti- rabbit) were used for
25 the 1 h incubation at room temperature, respectively and 9 slices from 3 different mice in each
26 group. For each slice, Cdk5-positive and VGLUT2-positive [Cdk5(+)/VGLUT2(+)] cells
27 from 4 randomly selected sections (250 μ m $\times$ 250 μ m) within DRG and spinal cord were
28 manually counted [24].

29 *Protein extraction and western blot analysis*

1 DRG and spinal cord tissues from the L4-L6ipsilateral sides of each treatment groups were
2 collected respectively and immediately stored in ice-chilled lysis buffer (50 mM Tris, pH 7.4,
3 150 mM NaCl, 1.5 mM MgCL₂, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 0.5 µg/ml
4 leupetin, 1 mM PMSF, 1 mM Na₃VO₄, 10 mM NAF, and a proteinase inhibitor cocktail).
5 Homogenates were centrifuged at 12,000rpm for 15 min at 4°C, and BCA assay kit (Pierce,
6 Rockland, IL, USA) was used to determine the protein concentrations. Each of the samples
7 containing 30 µg of protein were identified by sodium dodecyl sulfate polyacrylamide gel
8 electrophoresis (SDS-PAGE) to determine the estimated molecular mass of the protein and
9 the gel were then transferred onto PVDF membranes. The membranes were then blocked with
10 1% bovine serum albumin (BSA) in TBST (50 mM Tris-HCL, pH 7.5; 150 mM NaCl, 0.05%
11 Tween 20) for 1 h at room temperature, and incubated overnight at 4°C with the appropriate
12 primary antibody (anti-VGLUT2, 1:500; Abcam, Cambridge, MA, USA, ab79157;
13 anti-p35/p25, anti-p35, 1:300, Cell Signaling, MA, USA, 2680). Membranes were washed in
14 TBST between each of the incubations. Blots were then incubated with a 1:1000 dilution of
15 horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit or -mouse,
16 respectively) for 1.5 h at room temperature. The individual bands were visualized by
17 incubation with enhanced chemiluminescence reagent (Boehringer Mannheim, Indianapolis,
18 IN, USA). All bands were exposure to x-ray film which was later digitally analyzed using
19 NIH Image software, version 1.60.

20 *Statistical analysis*

21 All data are presented as the mean ± SEM. SAS (version 8.01 software for Windows SAS
22 Institute Inc., Cary, North Carolina, USA) was used to calculate statistical results. The time
23 courses of effects were analyzed by two-way analysis of variance, followed by the Dunnett's
24 test. VGLUT2 expression was analyzed using one-way analysis of variance, followed by the
25 Student Newman–Keuls' test and P-value < 0.05 was used to indicate statistical significance.

26

27 **Results**

28 *Heat hyperalgesia induced by intraplantar CFA injection was significantly reduced by*
29 *roscovitine*

1 The effects of roscovitine on heat hyperalgesia induced by injection of CFA into the
2 ipsilateral hind paws of rats was determined by measuring heat hyperalgesia on days 1-5
3 following CFA injection. Compared to the contralateral hind paw of animals in the same
4 group during the same time span, the average thermal PWL threshold (seconds) of the
5 ipsilateral hind paw was significantly reduced 6 hours and on days 1-5 following CFA
6 injection (**P < 0.01, Figure 1 A, n = 6/group). However, no pronounced difference was
7 observed between the ipsilateral and contralateral paws of rat in the control group (P > 0.05,
8 Figure 1B, n = 6/group). Furthermore, on days 1 and 3 after CFA injection, the average PWL
9 thresholds of ipsilateral paws pretreated with roscovitine for 30 min prior to intraplantar CFA
10 injection were remarkably increased, when compared to the ipsilateral paws of animals
11 pretreated with DMSO (**P < 0.01, Figure 1 B, n = 6/group).

12 ***Upregulated expression of Cdk5/VGLUT2 in DRG neurons***

13 To investigate the possible morphologic relationships between Cdk5 and VGLUT2, light
14 microscopy examinations of the DRG from the L4-L6 segments of spinal cord was to
15 illustrate a dense plexus of immunoreactive elements in the small and medium diameter
16 neuron cells. Compared with the control group injected with saline, coexpression of Cdk5 and
17 VGLUT2 was clearly elevated on both day 1 post-CFA injection (Figure 2 **P < 0.01 n =
18 3/group). These results demonstrated that Cdk5 mediated inflammatory pain by closely
19 interacting with VGLUT2.

20 ***Upregulated expression of Cdk5/VGLUT2 in spinal cord neurons***

21 Subsequently, we observed the co-expression of Cdk5 and VGLUT2 from the segments of
22 L4-L6 spinal cord using double-labeled immunofluorescence. The co-expression of
23 Cdk5/VGLUT2 was preferentially distributed in the gray matter area of spinal cord, which
24 mainly transmitted the pain message from peripheral to central. Compared with the control
25 group challenged with saline, the co-expression of Cdk5 and VGLUT2 was significantly
26 increased on both day 1 post-CFA injection (Figure 3 **P < 0.01 n = 3/group).

27 ***Increased VGLUT2 protein expression in DRG was significantly reduced by roscovitine***

28 We next used western blot analysis to investigate VGLUT2 protein expression in DRG
29 neurons from the L4-L6 ipsilateral sides of spinal cord on days 0, 1, 3, and 5 post-CFA
30 challenges. The results showed that levels of VGLUT2 protein in DRG neurons were

1 significantly increased between days 1 and 3 post-CFA injection (Figure 3 and Figure 5, *P <
2 0.05, n = 4/group). Furthermore, the elevated levels of VGLUT2 protein found between days
3 1 and 3 were obviously reduced by roscovitine. (Figure 4, **P < 0.01 n = 4/group).

4 ***Increased VGLUT2 protein expression in spinal cord neurons was significantly reduced by***
5 ***roscovitine***

6 In accordance with the results of VGLUT2 protein expression in DRG, the VGLUT2 protein
7 from the L4-L6 ipsilateral spinal cord neurons challenged with CFA was significantly
8 increased on days 1 and 3 as compared with the control group. Moreover, the increased
9 VGLUT2 protein was obviously reduced by roscovitine. (Figure 5 **P < 0.01, n = 4/group).

10 ***p25 but p35 protein expression in spinal cord neurons was significantly increased by CFA***

11 To verify the role of p25 or p35 as a activator of Cdk5 in mediating the heat hyperalgesia by
12 CFA, the p25 and p35 protein from the L4-L6 ipsilateral spinal cord neurons were
13 examined, respectively. p25 protein from the L4-L6 ipsilateral spinal cord neurons challenged
14 with CFA was significantly increased on days 1 and 3 as compared with the control group.
15 However, p35 remains markedly unchanged as compared with the control group. (Figure 6
16 **P < 0.01, n = 4/group)

17 ***Heat hyperalgesia was significantly inhibited by roscovitine via p25 rather than p35***

18 To explore the pathway inhibiting heat hyperalgesia by roscovitine, we respectively detected
19 the protein changing of p25 and p35 after spinal intrathecal administration of roscovitine in
20 the inflammation model induced by CFA. Basically, in agreement with the results of FIG 6, the
21 expression of p25 was significantly reduced by roscovitine but not p35, suggesting that
22 inhibition of heat hyperalgesia by Cdk5 inhibitor roscovitine was via inhibiting the activity of
23 p25 not p35. (Figure 7 **P < 0.01, n = 4/group)

24 **Discussion**

25 The present study preliminarily illustrated that the VGLUT2 signaling pathway was involved
26 in CFA-induced heat hyperalgesia mediated by Cdk5 in the DRG and spinal cord neurons. It
27 is well established that glutamate is the major excitatory neurotransmitter in the CNS, and
28 plays a key role in nociceptive processing in inflammatory and neuropathological pain [1].
29 Storage of glutamate within vesicles is controlled by VGLUTs, and small DRG neuron cells
30 accumulate 6-fold more glutamine, a precursor in the synthesis of L-glutamate, than larger

1 DRG neurons [25]. As currently determined, the family of VGLUTs consisting of VGLUTs
2 1-3, is not overlapped, and only VGLUT 2 plays a key role in mediating the heat hyperalgesia
3 induced by inflammation [6, 7]. Previous studies conducted using a CFA-induced
4 inflammatory pain model has shown that the thresholds of heat hyperalgesia in mice with
5 knocked out VGLUT2 were markedly increased compared with wild mice. However, the
6 mechanisms by which VGLUT2 mediates inflammation-induced heat hyperalgesia remains to
7 be explored in every detail.

8 Considerable evidence demonstrated that Cdk5 acts a key role in mediating heat
9 hyperalgesia induced by inflammation [13-16]. Moreover, previous results obtained by other
10 investigators suggest that Cdk5 is a key regulator in mediation of neurotransmitters, including
11 glutamate in the CNS [17]. Our previous studies demonstrated that synaptophysin, an
12 important presynaptic vesicle membrane protein which mediates release of neurotransmitters,
13 was involved in heat hyperalgesia mediated by Cdk5, suggesting that Cdk5 may mediate heat
14 hyperalgesia induced by inflammation by controlling the release of neurotransmitters. A
15 recent study further indicated that VGLUT2 was involved in the trafficking of synaptic
16 vesicles by interacting with synaptophysin at synaptic boutons [19], which further show that
17 VGLUT2 mediates the heat hyperalgesia via Cdk5 pathway. In addition, another study made
18 it clear that the expression of VGLUT1 and VGLUT2 gene was strongly reduced in synapsin
19 II (another key vesicle protein involved in release of neurotransmitters) knockout mice [18].
20 Thus, we examined the possible linkage between Cdk5 and VGLUT2 in mediating heat
21 hyperalgesia induced by CFA using our current model.

22 In our research, the immunohistochemistry results showed that the co-expression of Cdk5
23 and VGLUT2 was obviously in small and medium-sized neuronal cells of the DRG and spinal
24 cord. It had been established that small- and medium-sized neuronal cells of the DRG and
25 spinal cord are important mediators of inflammation and neuropathologic pain. Moreover, the
26 small and medium-sized neuronal cells of the DRG and spinal cord have been shown to
27 express a variety of nociceptive ion channels and receptors. The results from our current study
28 align with findings from previous studies, in that Cdk5 and VGLUT2 are prominently
29 distributed in the small and medium-sized neuronal cells of the DRG and spinal cord . More
30 importantly, our results demonstrate that Cdk5 and VGLUT2 are co-expressed in small and
31 medium-sized neuronal cells of the DRG and spinal cord. Additionally, the co-expression of
32 Cdk5 and VGLUT2 was found to be significantly increased between days 1 and 3 following

1 plantar injection of CFA, as compared with the control group of rats that was challenged with
2 saline. The increased co-expression of Cdk5 and VGLUT2 lasted for more than 3 days, until
3 day 5, which suggests a key role of these molecular factors in the induction stage of chronic
4 inflammatory pain. Subsequently, our study explored the effects of Cdk5 on VGLUT2 by
5 administering roscovitine via intrathecal injection on day 1 and day 5 prior to CFA injection,
6 which allowed us to observe the changes in protein of VGLUT2 under the heat hyperalgesia
7 condition.

8 Previous studies conducted using a CFA-induced inflammatory pain model demonstrated
9 that thresholds of heat hyperalgesia in mice with VGLUT2 knockout in the DRG were
10 significantly increased compared to thresholds in wild-type mice, suggesting that VGLUT2
11 plays a key role in mediating the heat hyperalgesia induced by peripheral injection of CFA. In
12 our study, both VGLUT2 and Cdk5 expression in the DRG and spinal cord were significantly
13 increased between days 1 and 3 following CFA injection, and returned to baseline levels on
14 day 5. In the current study, an intrathecal injection of roscovitine was used to determine
15 whether endogenous VGLUT2 was inhibited by roscovitine in DRG and spinal cord neurons.
16 The results showed that increased VGLUT2 protein expressions were significantly reduced by
17 roscovitine. Although the activities of Cdc2, Cdk2, and Cdk5 kinase are all inhibited by
18 roscovitine [26], our previous findings suggested that in adult rats, roscovitine mainly affects
19 the activity of Cdk5, and does not affect the activity of other Cdks [21]. In addition, p25 but
20 not p35 as the activator of Cdk5 contributed to the CFA-induced heat hyperalgesia. It was
21 well established that Cdk5' activity mainly depends on its activator. Thus, our data suggests
22 that inhibition of Cdk5 kinase activity by p25 led to the decreases in VGLUT2 protein
23 expression observed in our current model. Taken together, our findings suggest that in our
24 current model, Cdk5 functions as the key regulator of VGLUT2.

25 It is important to note that the results from our model system failed to pinpoint exactly how
26 Cdk5 mediates the neurotransmitters release, e.g. glutamate (excitatory) interaction with
27 VGLUT2, or how Cdk5 mediates the synaptic vesicles transportation, e.g. by closely
28 interacting with VGLUT2. In addition, we only used VGLUT2 antibody for
29 immunohistochemistry and protein expression changing in our model to show the role of pain
30 transmission mediated by VGLUT2, lacking the more specific method in our model. Future

experiments will be designed to determine the mutual relationship between Cdk5 and VGLUT2 in mediating the release of excitatory neurotransmitters of glutamate in this model of knockout of VGLUT2 as control.

Our studies demonstrate that Cdk5 mediates CFA-induced heat hyperalgesia by regulating VGLUT2 protein expression in DRG and spinal cord neurons. Increased co-expression of Cdk5 and VGLUT2 was observed in small and medium-sized neuronal cells of the DRG and spinal cord induced by CFA. Spinal administration of roscovitine markedly alleviated heat hyperalgesia induced by CFA. Furthermore, increased VGLUT2 protein expression in DRG and spinal cord neurons was reduced by roscovitine, suggesting that Cdk5/p25, as the upstream regulator of VGLUT2, plays an important role in inducing and developing the early stage of chronic inflammatory pain. Consequently, we propose that severing the linkage between Cdk5/p25 and the VGLUT2 signaling pathway may present a promising therapeutic strategy for diminishing inflammatory pain.

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Competing Interests statement:

All authors declare no competing interests.

Author Contributions statement:

Conceived and designed the experiments: HongHai Zhang, ZhiYing Feng
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 Analyzed the data: Peng Xu, Qing Hua Li
 Contributed reagents/materials/analysis tools: GongLu Liu, XianZhe-Luo, Yuan Chen, Yue Shen, HaiXiang Ma

1

2 **Figure legends:**

3 Figure 1. Heat hyperalgesia induced by peripheral injection of CFA was significantly
4 inhibited by intrathecal administration of roscovitine. PWL in response to thermal stimuli
5 significantly decreased following intraplantar injection of CFA. Cont (contralateral) hind paw
6 was injected with saline. Ipsi (ipsilateral) hind paw was injected with CFA. **P < 0.01, n =
7 6/group (A). Compared to PWLs in the control group pretreated with DMSO, PWL response
8 to thermal stimuli significantly was increased following intrathecal injection of roscovitine
9 0.5 h prior to CFA injection. Data shown represent the Ave $\pm$ SEM, **P < 0.01, n = 6/group
10 (B).

11 Figure 2. Co-expression of Cdk5 and VGLUT2 was increased in DRG neuronal cells.
12 Double-immunofluorescence staining for Cdk5 (red) ,VGLUT2(green). Compared with
13 control group (A-F) and day 1(G-L) following intraplantar injection of CFA, co-expression of
14 Cdk5 and VGLUT2 was significantly increased in DRG neurons on day 1 following CFA
15 injection, Data shown represent the Ave $\pm$ SEM,*P < 0.05, **P < 0.01, n = 6/group.

16 Figure 3. Co-expression of Cdk5 and VGLUT2 was increased in spinal cord neuronal cells.
17 Double-immunofluorescence staining for Cdk5 (red) ,VGLUT2(green). Compared with
18 control group (A-F) and day 1 (G-L) following intraplantar injection of CFA, co-expression of
19 Cdk5 and VGLUT2 was significantly increased in DRG neurons on day 1 following CFA
20 injection, Data shown represent the Ave $\pm$ SEM,*P < 0.05, **P < 0.01, n = 6/group.

21 Figure 4. VGLUT2 protein expression was markedly increased in the DRG, and reduced by
22 roscovitine. Compared to the control group, VGLUT2 expression was significantly increased
23 from day 1 to day 3 following intraplantar injection of CFA. *P < 0.05, ** P < 0.01 n =
24 3/group (A). Compared to DMSO-treated controls, the increased expression of VGLUT2
25 protein was significantly reduced by intrathecal injection of roscovitine between day 1 and
26 day 3. Data shown represent the Ave $\pm$ SEM, *P < 0.05 n = 3/group (B).

27 Figure 5. VGLUT2 protein expression was markedly increased in the spinal cord, and reduced
28 by roscovitine. Compared to the control group, VGLUT2 expression was significantly
29 increased from day 1 to day 3 following intraplantar injection of CFA. *P < 0.05, ** P < 0.01
30 n = 3/group (A). Compared to DMSO-treated controls, the increased expression of VGLUT2

1 protein was significantly reduced by intrathecal injection of roscovitine between day 1 and
2 day 3. Data shown represent the Ave $\pm$ SEM, $*P < 0.05$ $n = 3$ /group (B).

3 Figure 6. p25 but not p35 was markedly increased in the spinal cord

4 Compared with the control group, p25 expression was significantly increased from day 1
5 following intraplantar injection of CFA. However, p35 expression was not significantly
6 increased day 1 as compared with the control group. $*P < 0.05$ $n = 3$ /group .

7 Figure 7. Heat hyperalgesia was significantly inhibited by roscovitine via p25 rather than p35

8 Compared to DMSO-treated controls, the increased expression of p25 protein was
9 significantly reduced by intrathecal injection of roscovitine between day 1. However, the
10 effects of administration of on p35 protein expression. Data shown represent the Ave $\pm$
11 SEM, $*P < 0.05$ $n = 3$ /group (B).

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Fig1

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Figure1

Fig2

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Figure2

Fig 3

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Figure3

Fig 4

Figure4

Fig5

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Figure5

Fig 6

Figure6

Fig 7

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Figure7