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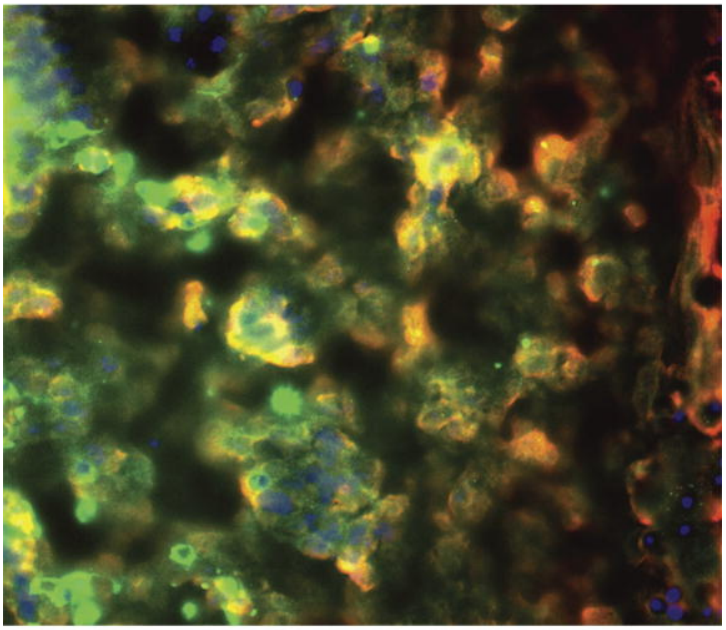

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Research Report

Role of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunit GluR1 in spinal dorsal horn in inflammatory nociception and neuropathic nociception in rat

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ABSTRACT

The present study aims to investigate changes of spinal cord AMPA receptor GluR1 and its phosphorylation in inflammatory and neuropathic pain. Complete Freund's adjuvant (CFA) injection into the hind paw produced inflammatory thermal hyperalgesia that was assessed by decreased response latency to radiant heat; spinal nerve ligation (SNL) was used to induce mechanical allodynia that was evaluated with von Frey hairs. By method of Western blot, expression of GluR1 (the main subunit of the AMPA receptor) and its phosphorylated forms at serine 845 (pGluR1-Ser845) and at serine 831 (pGluR1-Ser831) in the spinal dorsal horn was observed. It was found that the expression of pGluR1-Ser845 and pGluR1-Ser831 increased significantly at 1 h after CFA injection, reached peak at 4 h and returned to the normal control level at 24 h, while no significant change was detected in GluR1 itself. In contrast, neither GluR1 nor pGluR1 showed any significant change in rats following SNL. These results suggest that phosphorylated GluR1 (pGluR1-Ser845 and pGluR1-Ser831) might play a role in the induction of inflammatory but not neuropathic pain.

1. Introduction

Ionotropic glutamate receptors are involved in excitatory synaptic neurotransmission in the central nervous system (Mayer and Westbrook, 1987; Hollmann and Heinemann, 1994). There are three types: *N*-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, and kainate receptor. The AMPA receptor is composed of GluR1 — 4 subunits including homo- and hetero-multimers. Immunohistochemical and *in situ* hybridization studies indicate that the four subunits of the AMPA receptor are all expressed in spinal dorsal horn. GluR1 mRNA and protein expression are mainly in laminae I and II of the spinal dorsal horn (Garry and Fleetwood-Walker, 2004; Jakowec et al., 1995). It is well known that the superficial dorsal horn is the first synaptic relay of afferent fibers from skin and is regarded as the initial processing site for signals directly

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate; CFA, complete Freund's adjuvant; SNL, spinal nerve ligation; PWL, paw withdrawal latency; PWT, paw withdrawal threshold; CCI, chronic constriction injury

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related to the transmission and modulation of pain (Garry et al., 2004). So GluR1 is plausibly considered to be an important factor in pain processing.

Competitive antagonists of AMPA/kainate receptors exhibit antinociceptive effects in both inflammatory and neuropathic pain models (Bennett et al., 2000; Kondo et al., 2002; Yoon et al., 2005; Yoshimura and Yonehara, 2006). Intrathecal injection of the highly selective AMPA receptor antagonists NS-257 and SYM 2206 attenuated CCI-induced thermal hyperalgesia (Garry et al., 2003). Systemic or intrathecal administration of selective AMPA receptor antagonist YM872 reduced hyperalgesic responses in both inflammatory and neuropathic nociception (Nishiyama et al., 1999; King and Barr, 2007).

Genetic knockout of the GluR1 led to the decrease of hyperalgesia in mice. For example, a reduction in number of Ca^{2+}-permeable AMPA receptors and density of AMPA channel currents in spinal neurons in GluR1-deficient mice was accompanied by a loss of nociceptive plasticity *in vitro* and a reduction in inflammatory hyperalgesia *in vivo* (Hartmann et al., 2004). Up-regulation of phosphorylated GluR1 at serine 831 and 845 sites was found in the superficial laminae of the spinal dorsal horn shortly after capsaicin treatment (Fang et al., 2003; Nagy et al., 2004), suggesting that the GluR1 subunit and its phosphorylation may play a role in inflammation.

To our knowledge, all reports about the change of AMPA receptors have only been following acute pain and assessed within no longer than 1–2 h after the insult. However, clinical inflammatory pain can be chronic and it is important to study the role of AMPA receptors at timepoints greater than 1–2 h. For example, in our previous study, subcutaneous injection of complete Freund's adjuvant (CFA) induced thermal hyperalgesia lasting as long as 28 d post injection (Luo et al., 2004). Currently we aim to investigate the possible role of the AMPA receptor in chronic inflammatory pain during this chronic state of hyperalgesia.

Studies have suggested that the AMPA receptor may be involved in neuropathic pain. For example, AMPA receptors were reported to be involved in loose sciatic nerve ligation-induced neuropathic allodynia (Harris et al., 1996; Garry et al., 2003). Peripheral application of an NMDA, but not an AMPA receptor antagonist was shown to reduce neuropathic pain (Jang et al., 2004). In the present study, spinal nerve ligation (SNL) model, one of the most frequently used neuropathic pain models, was used to examine the possible role of the AMPA receptor in neuropathic pain.

Using Western blot and behavioral sensitivity, the present study aims to observe the changes of spinal cord AMPA receptor subunit GluR1 and its phosphorylated form both in CFA-produced inflammatory nociception and L5 SNL-produced neuropathic nociception in rats.

2. Results

2.1. *Thermal hyperalgesia and mechanical allodynia in rats*

Thermal hyperalgesia of the left hind paw was tested before and 1 h, 4 h, 12 h, 1 d, 3 d, 7 d, 14 d, and 28 d after CFA injection. Results are shown in Fig. 1A. The contralateral hind paw was used as a control. At 1 h post CFA injection, paw withdrawal latency (PWL) of the ipsilateral average (5.4±0.7 s) was significantly shorter than that of the contralateral side (15.0±4.0 s) ($n=8$, $p<0.001$). The average PWL of the injection side shortened to 2.6±0.5 s at 12 h post CFA injection, but that of the contralateral side (12.7±3.5 s) exhibited no significant decrease ($n=8$). The average PWL was kept around 3.6±1.5 s to 8.6±1.3 s until 14 d after injection ($n=8$). At 28 d after injection, the average PWL returned to 15.6±2.4 s which compared with the PWL of the contralateral side (17.7±3.0 s) ($n=8$, $p>0.05$). In a word, relative to the contralateral side, the paw withdrawal latency began to shorten at 1 h ($p<0.001$), reached maximum difference around 12 h ($p<0.001$), remained decreased relative to control until 14 d ($p<0.001$), and was no longer significantly different at 28 d after CFA injection.

Mechanical allodynia of the left hind paw was evaluated by von Frey hairs before and 8 h, 12 h, 1 d, 3 d, 14 d and 28 d after lumber 5 (L5) SNL. Results are shown in Fig. 1B. Within 8 h after SNL, PWT did not show obvious decrease compared with that

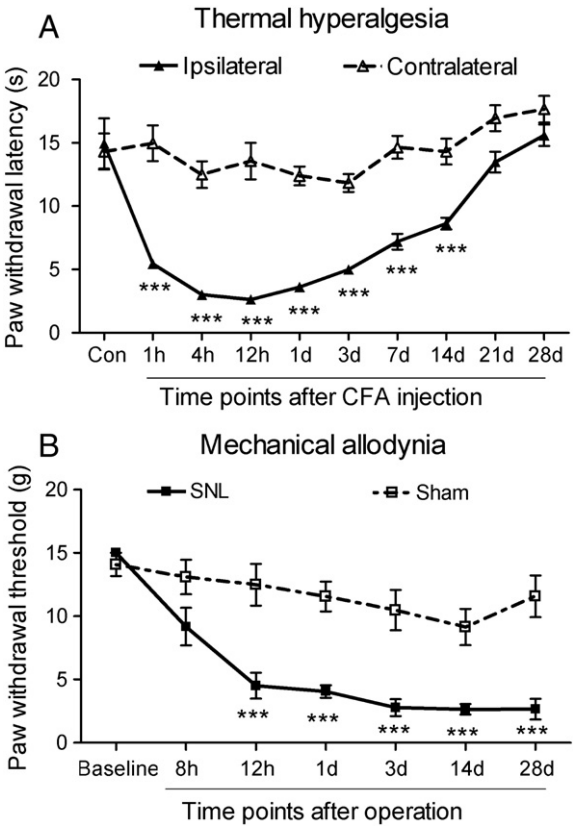

Fig. 1 – Development of thermal hyperalgesia and mechanical allodynia in rats. (A) Development of thermal hyperalgesia in CFA rats. Thermal hyperalgesia was tested with radiant heat and paw withdrawal latency (PWL) was recorded. PWLs decreased began at 1 h after CFA injection, reached a maximum at 12 h after injection, began to decrease after 14 d and nearly returned to normal level at 28 d. $^{***}p<0.001$ compared with the contralateral side, $n=8$. (B) Development of mechanical allodynia in SNL rats. Mechanical allodynia was tested with von Frey hair during the 28 d after SNL. 50% paw withdrawal threshold (PWT) relative to the sham-operation group significantly decreased 12 h after SNL and continuously existed until 28 d . No obvious allodynia was observed in the sham-operation rats. $^{***}p<0.001$ compared with the sham-operation group, $n=9$.

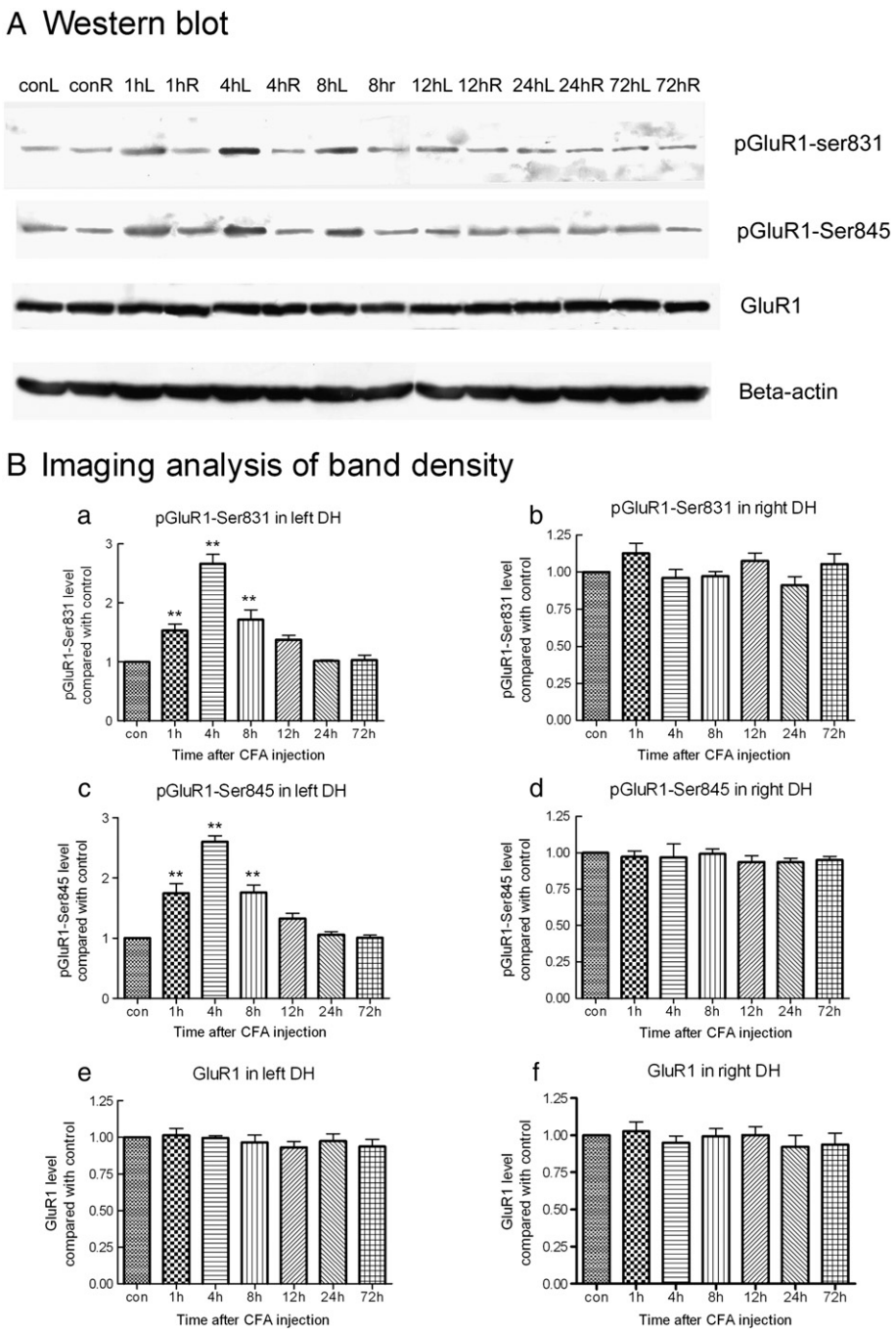

Fig. 2 – Expression of GluR1 and phosphorylated GluR1 (pGluR1-Ser831 and pGluR1-Ser845) in spinal dorsal horn in CFA rats. (A) Western blot detection of expression of GluR1 and pGluR1. The four rows are pGluR1-Ser831, pGluR1-Ser845, GluR1 and β-actin respectively. (B) Band density analysis results of Western blot. Compared with the normal control, pGluR1-Ser831 and pGluR1-Ser845 in the ipsilateral side began to increase at 1 h after CFA injection, reached its peak at 4 h and then returned to the normal control level at 24 h (the first and the second row in A and a, c in B), while no significant change was found in the contralateral side (b and d in B). The expression of GluR1 did not show significant change in both sides of the spinal dorsal horn after CFA injection (the third row in A; e and f in B). β-actin was used as internal reference (the fourth row in A). CFA: complete Freund's adjuvant, Con: control group, L: left side, R: right side. $p<0.01$, $n=5$.**

in the sham-operation group, indicating that no allodynia occurred. The rats in the SNL group exhibited allodynia since 12 h following surgery. At 12 h post operation, the 50% PWT (4.5±3.1 g) in the SNL group was significantly shorter than that in the sham-operation group (12.1±4.9 g) ($n=9$, $p<0.001$). The 50% PWT decreased to 2.8±2.0 g at 3 d post surgery in the SNL group but that in the sham-operation group was still 10.5±4.5 g ($n=9$). Until 28 d post surgery, the 50% PWT of SNL group remained 2.7±2.5 g ($n=9$) ($p<0.001$). Therefore, 50% PWT was significantly decreased relative to that in the sham-operation

group 12 h following surgery and remained significantly different through 28 d post surgery.

2.2. *Change of GluR1 and pGluR1 in the spinal dorsal horn in CFA rats*

Western blot analysis was performed to test whether there is any change in the expression of AMPA receptor subunit GluR1 or its phosphorylated forms following intraplantar injection of CFA. Expression of GluR1 and pGluR1 in the spinal dorsal horn was measured at 1 h, 4 h, 8 h, 12 h, 1 d and 3 d after CFA injection. The GluR1 expression in bilateral spinal dorsal horn did not show obvious change after CFA injection as compared with that in normal rats. The phosphorylated GluR1 (at sites of Ser831 and Ser845) increased significantly in the ipsilateral side of the spinal dorsal horn, although no significant change was observed in the contralateral side of the spinal dorsal horn. Results of Western blot and band density analysis are shown in Fig. 2. Compared

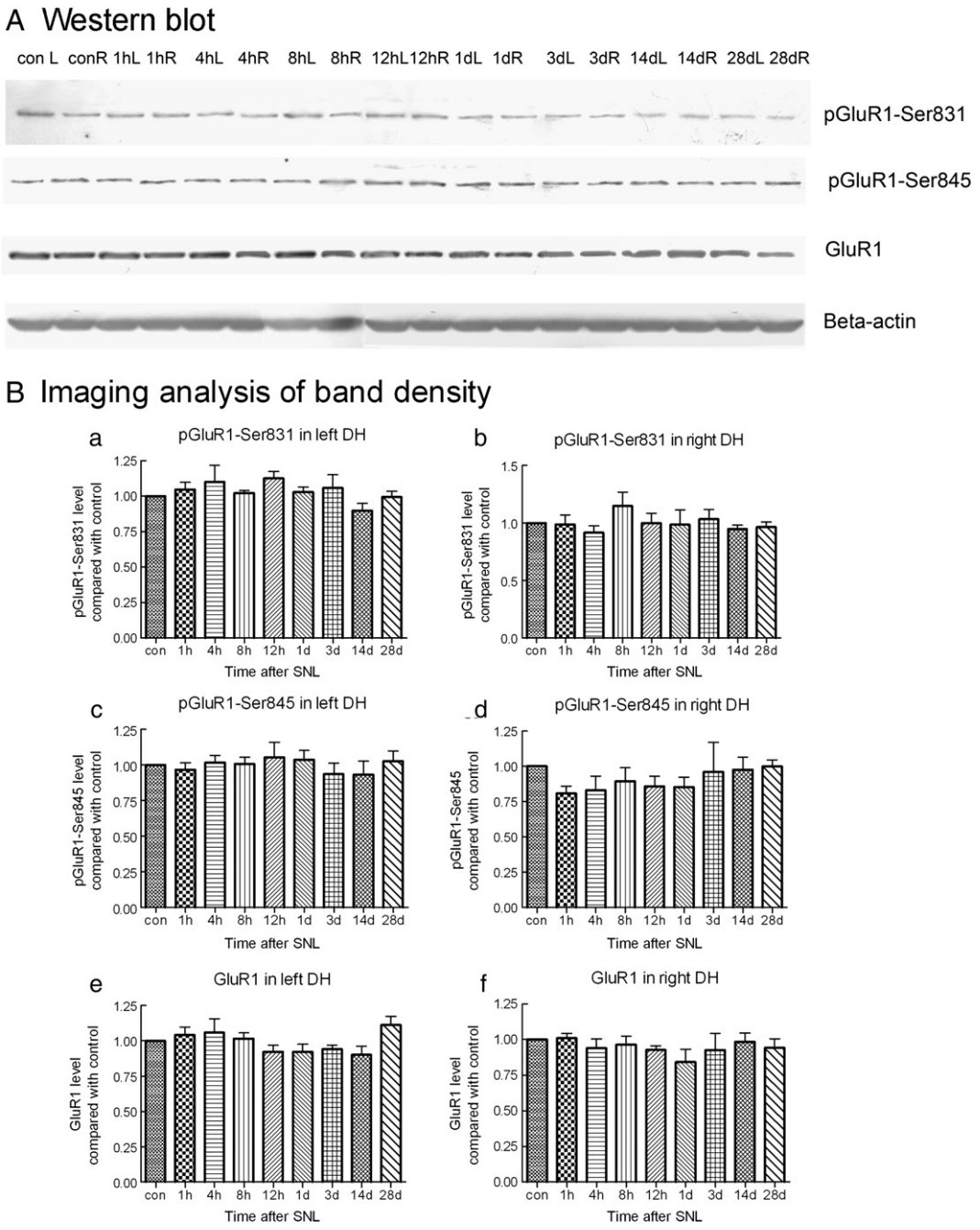

Fig. 3 – Expression of GluR1 and phosphorylated GluR1 (pGluR1-Ser831 and pGluR1-Ser845) in spinal dorsal horn in SNL rats. (A) Western blot detection of expression of GluR1 and pGluR1. The four rows are pGluR1-Ser831, pGluR1-Ser845, GluR1 and β-actin respectively. (B) Band density analysis results of Western blot. a, c and e are statistical analysis results of the ipsilateral side of pGluR1-Ser831, pGluR1-Ser845 and GluR1 respectively; b, d and f are results of the contralateral side. Compared with normal control, the expression of GluR1 itself, pGluR1-Ser831 and pGluR1-Ser845 did not show significant change in both sides. SNL: spinal nerve ligation, Con: control group, L: left side (the nerve ligature side), R: right side. $n=3$.

with the normal level, the expression of pGluR1-Ser831 and pGluR1-Ser845 began to increase at 1 h after CFA treatment, reached its peak at 4 h, began to decrease from 8 h and came back to the normal control level at 24 h. Statistical analysis of these data showed that the increase of phosphorylated GluR1 was around 1.5- (1.5±0.25, $p<0.05$), 2.6- (2.6±0.37, $p<0.01$) and 1.7-folds (1.7±0.37, $p<0.01$) at 1 h, 4 h and 8 h after CFA injection, when compared with the normal level ($n=5$).

2.3. *Change of GluR1 and pGluR1 in the spinal dorsal horn in SNL rats*

To investigate the change of GluR1 and its phosphorylated forms in neuropathic pain , we observed the expression of GluR1 and phosphorylated GluR1 in SNL rats. The change of expression of GluR1 and pGluR1 in the spinal dorsal horn in SNL neuropathic pain rats is shown in Fig. 3A, and the band density analysis results are shown in Fig. 3B. At all time points (1 h, 4 h, 8 h, 12 h, 1 d, 3 d, 7 d, 14 d and 28 d after treatment), the expression of GluR1 and pGluR1 did not show any significant change in both ipsilateral and contralateral spinal dorsal horn ($p>0.05$).

3. Discussion

The present study investigated the change of GluR1 subunit and phosphorylated GluR1 in rat spinal dorsal horn both in inflammatory nociception and neuropathic nociception models of rats by method of Western blot. To determine whether these changes were related to hyperalgesia, we also investigated nociceptive behavior after CFA injection or L5 SNL. Following CFA-induced inflammatory hyperalgesia in rats, the expression of GluR1 did not show significant change, while pGluR1 (pGluR1-Ser831 and pGluR1-Ser845) increased significantly. Neither GluR1 nor pGluR1 showed significant change in neuropathic rats after L5 SNL.

3.1. *Role of GluR1 subunit and its phosphorylated forms in CFA inflammatory nociception*

In our study, rats exhibited thermal hyperalgesia at 1 h after CFA injection, reached its peak at around 12 h, began to decrease at 1 d, and did not fully return to normal levels until 14 d after injection (Fig. 1A). We observed that GluR1 had no significant change, while phosphorylated GluR1 (pGluR1-Ser831 and pGluR1-Ser845), as the activated form, increased significantly in the first 24 h after CFA injection. The change of pGluR1 began at 1 h, reached its peak level at 4 h and almost came back to normal level at 1 d after injection (Fig. 2). From these data, we can see interestingly that the expression of pGluR1 changed significantly during the acute phase of thermal hyperalgesia. Phosphorylated GluR1 began to increase at the same time when thermal hyperalgesia was apparent (1 h after the treatment); when the expression of phosphorylated GluR1 reached its peak (4 h after the treatment), thermal hyperalgesia also became most obvious (around 4–12 h after the treatment). When the expression of pGluR1 returned to the normal level (24 h after the treatment), the thermal hyperalgesia began to decrease. Taken together these, we propose that the AMPA receptor may be involved in the induction of inflammatory nociception through GluR1 phosphorylation at sites of serine 831 and 845. Rats exhibited thermal hyperalgesia until 14 d after CFA injection, while the expression of pGluR1 returned to normal level since 1 d after CFA injection. These results suggest that phosphorylated GluR1 may not contribute to the maintenance of inflammatory nociception. We propose that phosphorylation of GluR1 is important for the induction, but not maintenance, of CFA-produced inflammatory pain.

GluR1 can be phosphorylated on multiple sites that are all located on the C-terminus of the protein. Most of the phosphorylated sites are on serine residues and a few on threonine and tyrosine residues (Blackstone et al., 1994; Roche et al., 1996; Rong et al., 2001; Wu et al., 2004; Boehm et al., 2006; Wang et al., 2005). Phosphorylation on threonine and tyrosine residues might serve to regulate receptor integrity and location, while phosphorylation on serine residues is believed to be involved in modulating channel properties and receptor trafficking (Derkach et al., 1999; Banke et al., 2000; Ehlers, 2000; Derkach, 2003).

It is suggested that the AMPA receptor is activated through phosphorylation on serine residues of GluR1, such as serine 831 and serine 845, and phosphorylation of GluR1 at serine 831 and 845 residues is very important in AMPA activity modulation. For example, in hippocampus, the switch between LTP and LTD was associated with phosphorylation and dephosphorylation of AMPA receptors respectively (Barria et al., 1997; Lee et al., 2000, 2003).

In the present study, with Western blotting method, we cannot tell directly that pGluR1 changed pre-synaptically or post-synaptically, though pGluR1 containing AMPA receptors are believed to locate mostly post-synaptically and functional AMPA receptors are predominantly located there (Sprengel, 2006). Most functional postsynaptic AMPA receptors play roles in the activation of synapses and in induction of LTP. GluR1, as the important composer of the AMPA receptor, is abundantly distributed in laminae I and II of the spinal dorsal horn (Garry and Fleetwood-Walker, 2004; Jakowec et al., 1995) which is the first synaptic relay of afferent fibers and is regarded as the initial processing site for signals directly related to the transmission and modulation of pain (Garry et al., 2004).

In our experiment, pGluR1 increased acutely (within 24 h) after CFA injection. It is reported that pGluR1 increased during 1 h after capsaicin injection (Fang et al., 2003; Nagy et al., 2004). This difference possibly is attributive to different pain models. It is not known whether the pGluR1 changes in other inflammatory nociception models such as lipopolysaccharide or formalin induced inflammatory nociception, although GluR1 expression was reported to decrease in the spinal dorsal horn in these two models (Pellegrini-Giampietro et al., 1994; Florenzano and De, 1999). Whether the involvement of pGluR1 in inflammatory nociception is CFA specific or not needs further investigation.

We did not detect the protein expression of GluR1 change in CFA model rats, but Zhou et al. (2001) reported that GluR1/2 mRNA in spinal cord reached peak levels at around 2–5 h after CFA injection. This may indicate the difference in protein and mRNA. The change in mRNA may not predict the change at protein level.

AMPA receptor subunit GluR2 is also reported to be important in pain induction. Some researchers reported that GluR2 was distributed in the superficial spinal dorsal horn (mostly in lamina II) and the expression of GluR2 had been shown to increase following loose ligation of the sciatic nerve (Garry et al., 2003; Lim et al., 2006). However, other people found that an increase in spinal Ca^{2+}-permeable AMPA receptors in GluR2-deficient mice facilitated nociceptive plasticity and enhanced long lasting inflammatory hyperalgesia (Hartmann et al., 2004).

Although our results cannot show directly which factor is important in phosphorylation of GluR1, GluR1 can be phosphorylated by several kinds of protein kinases and this phosphorylation can be regulated by phospholipase A2 and IL-1β (Menard et al., 2005; Lai et al., 2006). Which regulatory factors take effects in GluR1 phosphorylation is worthy of further investigation.

3.2. *Role of GluR1 subunit and its phosphorylated forms pGluR1 in SNL neuropathic nociception*

In this study, we tried to compare the change of AMPA receptor subunit GluR1 in inflammatory and neuropathic nociception. We observed that although phosphorylated GluR1 changed significantly in CFA rats, it showed almost no change in SNL model.

AMPA receptors were reported to be involved in neuropathic pain and its selective antagonists reduced hyperalgesic responses in neuropathic nociception (Garry et al., 2003; Nishiyama et al., 1999). The expression of GluR1 and GluR2 increased in spinal cord after the sciatic nerve was loosely ligated (Lim et al., 2006). These reports suggest that the AMPA receptor, specifically the GluR1 subunit might be involved in neuropathic nociception, which is discrepant to our observation. We noticed that all these findings are from CCI model, not from SNL model as in our study. Some reports also showed that the antagonists of AMPA/kainate receptor had no significant effect on alleviating hyperalgesia produced by SNL or spinal cord ischemia (Hao and Xu, 1996; Jang et al., 2004). Although these findings seem to show that the AMPA receptor has a differential role in different neuropathic pain models, we cannot explain this difference only by different models.

In summary, our present study demonstrated that increased phosphorylated GluR1 subunit of AMPA receptor at sites of Ser831 and Ser845, not GluR1 itself, in the spinal dorsal horn was found in the induction of CFA inflammatory nociception in rats. However, neither GluR1 itself nor phosphorylated GluR1 at Ser831 and Ser845 showed significant change in SNL-produced neuropathic nocicepition. These results suggest that phosphorylated GluR1 of the AMPA receptor in spinal dorsal horn may have a role in inflammatory, but not in neuropathic nociception in rats.

4. Experimental procedures

4.1. *Experimental animals*

Male Sprague–Dawley rats weighing 200–250 g were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. They were housed four to five per cage under diurnal light–dark cycles with food pellets and water *ad libitum*. Measures were taken to minimize pain and/or discomfort in rats. All protocols were approved by our university and followed *the University Guidelines for Animal Care and Use* adapted from NIH, USA.

4.2. *CFA inflammatory nociception model*

One hundred microliters of CFA (each ml contains 1 mg mycrobacterium tuberculosis, heat killed and dried, 0.85 ml mineral oil and 0.15 ml mannide monooleate. Sigma-Aldrich, St. Louis, USA) was injected into the plantar surface of the left hind paw of rat to produce inflammatory pain following our previous report (Luo et al., 2004).

4.3. *SNL neuropathic nociception model*

SNL neuropathic nociception model in rats was firstly described by Kim and Chung (1992). Left L5 spinal nerve ligation was ligated as in our previous report (Sun et al., 2005). Briefly, rat was anesthetized with 10% chlorohydrate (0.3 ml/100 g body weight) and placed in a prone position. An incision was made into the left spine at L4–S2 level. The L5 spinal nerve was carefully isolated and tightly ligated with 6-0 silk suture 5–10 mm distal to the dorsal root ganglion (DRG). In the sham-operation rat, the L5 spinal nerve was left intact.

4.4. *von Frey hair test for mechanical allodynia*

Mechanical allodynia was assessed by von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medial Inc., San Jose, CA) applying to the left hind paw (Sun et al., 2005). Briefly, mechanical sensitivity of the left hind paw was tested at 4 h, 12 h, 1 d, 3 d, 14 d and 28 d after L5 nerve ligation. Rats were placed on a metal mesh floor covered with an inverted clear plastic cage (18×8×8 cm) and allowed a 15-min period for habituation. Each trial started with a von Frey force of 2.00 g, following the up and down method and increased or decreased force (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g) was applied when a negative or positive response (paw lifting or licking) was obtained.

4.5. *Radiant heat test for thermal hyperalgesia*

Thermal hyperalgesia was determined using a commercially available thermal paw stimulator described by Hargreaves et al. (1988). The thermal hyperalgesia of the left hind paw was tested before and 1 h, 4 h, 12 h, 1 d, 3 d, 7 d, 14 d and 28 d after CFA injection. The contralateral side hind paw was used as control. Animals were habituated in the test room for 2–3 h, then placed into an individual plastic compartments (18×8×8 cm) mounted on a glass surface maintained at 25±1 °C and allowed a period of 15 min for habituation. A thermal stimulus, in the form of radiant heat emitted from a focused projection bulb, was then applied to the plantar surface of each hind paw. The intensity was adjusted to maintain the paw withdrawal latency of normal rats at 15±2 s and a cut off of 30 s was imposed.

4.6. *Western blot*

Rats were over anaesthetized and killed at different time points after CFA injection or left L5 spinal nerve ligation. Normal rats

are used as control (5 animals in CFA model and 3 animals in SNL model). The L5 lumber segment of the spinal cord was carefully removed and the ipsilateral side was separated from the contralateral, the dorsal horn was separated from the ventral horn. The lumber segment was placed into liquid nitrogen and then homogenized. The concentration of protein in homogenate was determined using the bicinchoninic acid (BCA) kit (BCA™ protein assay Kit; Pierce, USA). Equal amounts of protein (60 μg) were size fractionated by 10% (W/V) gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The blots were placed in blocking buffer for 1 h at room temperature and then incubated with primary polyclonal antibodies to GluR1 (1:2000, Upstate), phosphor-GluR1 at Ser845 (1:1000, Sigma) or phospho-GluR1 at Ser831 (1:1000, Zymed) overnight at 4 °C. The blots were washed three times for 10 min each with washing buffer and then incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) in 5% (W/V) nonfat milk in washing buffer. The membranes were washed with buffer three times for 10 min again and enhanced with a chemiluminescence reagent (Santa Cruz). Then the blots were exposed to autoradiographic film (Kodak, Rochester, NY, USA) and the intensity of specific immuno-reactive bands was quantified using Scanwizard 5.0 scanning analyses and density detection software (TotalLab 1.0). As an internal control, the expression of β-actin (mouse anti-rat antibody from Sigma, USA) was also examined in every group.

4.7. *Statistical analysis*

Data are expressed as mean±standard deviation (SD). Repeated measures analysis of variance (two-way ANOVA) was used for behavior data analysis and *Bonferroni* post hoc test was carried out. In the analysis of Western blot data, repeated measures analysis of variance (one-way ANOVA) followed by Dunnett's test was used. p values less than 0.05 are considered to be statistically significant.

Acknowledgments

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