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Reactive oxygen species (ROS) modulate AMPA receptor phosphorylation and cell-surface localization in concert with pain-related behavior

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Abstract

Sensitization of dorsal horn neurons (DHNs) in the spinal cord is dependent on pain-related synaptic plasticity and causes persistent pain. The DHN sensitization is mediated by a signal transduction pathway initiated by the activation of NMDA receptors (NMDA-Rs). Recent studies have shown that elevated levels of reactive oxygen species (ROS) and phosphorylation-dependent trafficking of GluA2 subunit of AMPA receptors (AMPA-Rs) are a part of the signaling pathway for DHN sensitization. However, the relationship between ROS and AMPA-R phosphorylation and trafficking is not known. Thus, this study investigated the effects of ROS scavengers on the phosphorylation and cell-surface localization of GluA1 and GluA2. Intrathecal NMDA- and intradermal capsaicin-induced hyperalgesic mice were used for this study since both pain models share the NMDA-R activation-dependent DHN sensitization in the spinal cord. Our behavioral, biochemical, and immunohistochemical analyses demonstrated that: 1) NMDA-R activation *in vivo* increased the phosphorylation of AMPA-Rs at GluA1 (S818, S831, and S845) and GluA2 (S880) subunits, 2) NMDA-R activation *in vivo* increased cell-surface localization of GluA1 but decreased that of GluA2, and 3) reduction of ROS levels by ROS scavengers PBN or TEMPOL reversed these changes in AMPA-Rs, as well as pain-related behavior. Given that AMPA-R trafficking to the cell surface and synapse is regulated by NMDA-R activation-dependent phosphorylation of GluA1 and GluA2, our study suggests that the ROS-dependent changes in the phosphorylation and cell-surface localization of AMPA-Rs are necessary for DHN sensitization and thus pain-related behavior. We further suggest that ROS reduction will ameliorate these molecular changes and pain.

Keywords

glutamate receptor; synaptic plasticity; reactive oxygen species; persistent pain; chronic pain; central sensitization

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1. INTRODUCTION

Sensitization of spinal dorsal horn neurons (DHNs) is considered one of the bases of the persistent pain that follows prolonged nociceptive inputs. The nociceptive input to the spinal cord activates DHN NMDA receptors (NMDA-Rs) causing Ca^{2+} influx into the cytoplasm. This transient spike of cytosolic Ca^{2+} initiates DHN sensitization by activating cellular signal transduction pathways for pain-related synaptic plasticity [15,55]. As a part of the signaling pathways for the induction of persistent pain, reactive oxygen species (ROS) are also critically involved in DHN sensitization [23,51,54]. Thus increased production of ROS is observed in DHNs in various pain models including neuropathic pain (spinal nerve ligation) [23], inflammatory pain [54], and capsaicin-induced hyperalgesia [44]. Artificial elevation of spinal ROS induces pain-related behavior in normal mice without nerve injury or tissue inflammation [23,24,44]. Spinal long-term potentiation (LTP), induced by high frequency stimulation of the dorsal root, is greatly reduced by ROS scavengers [28].

Modulation of the trafficking and activity of AMPA receptors (AMPA-Rs) is known as a key mechanism for synaptic plasticity, which in turn is the basis of various functions of the central nervous system (CNS), such as learning and memory [45]. Recent studies demonstrated the critical roles of AMPA-R subunits, GluA1 and GluA2, in pain-related synaptic plasticity [53]. For example, facilitated nociceptive plasticity and enhanced long-lasting inflammatory hyperalgesia were observed in GluA2 knock-out (KO) mice [12]. Spinal LTP was significantly enhanced in GluA2 KO mice [56]. In GluA1 KO mice, on the other hand, acute inflammatory hyperalgesia was reduced [12]. Furthermore, a recent study demonstrated that the internalization of GluA2-containing AMPA-Rs in the spinal dorsal horn is dependent on phosphorylation at Serine 880 (S880) on GluA2, and this internalization is essential for the development of inflammatory pain [40]. Similarly, phosphorylation at GluA2-S880 by PKC is essential for GluA2 internalization during long-term depression (LTD) in the cerebellum [6,50]. These studies suggest that synaptic plasticity in the brain and the spinal cord may share the same signaling pathways that are mediated by AMPA-R phosphorylation. During the induction of hippocampal LTP and LTD, phosphorylation of GluA1 plays a critical role for the trafficking of AMPA-Rs [13,26,27,35,45]. Given that GluA1 plays a critical role in pain [12,53] and GluA1 is a substrate of kinases (e.g. on S831 by CaMKII, on S845 by PKA, and on S818 by PKC) involved in DHN sensitization in persistent pain [9,11,17,31,47], it is likely that GluA1 phosphorylation by these kinases may play important roles for the induction of persistent pain.

In this study, we investigated if AMPA-R phosphorylation and cell-surface localization can be regulated by ROS using two animal models of pain. Our study provided several lines of evidence that removal of ROS leads to reduced AMPA-R phosphorylation in concert with decreased GluA1 cell-surface localization and pain-related behavior. This suggests that ROS-dependent changes in the phosphorylation and cell-surface localization of AMPA-Rs are essential parts of the signaling pathway for synaptic plasticity in the spinal cord and thus for persistent pain.

2. Methods

2.1 Animals, pharmacological treatments, and behavior tests

Young male C57BL/6J mice (8–10 weeks old, Jackson Laboratory) were used for this study. Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and performed accordingly. Guidelines published by the International Association for the Study of Pain for the ethical

care and use of laboratory animals were followed [58]. Care was taken to minimize both the number of animals used and their discomfort.

N-methyl-D-aspartic acid (NMDA) was injected (5 μ L of 50 μ M) intrathecally at the L5 and L6 intervertebral space in mice by a direct transcutaneous intrathecal (i.t.) injection method [25]. N-tert-butyl- α -phenylnitron (PBN) was injected (150 μ L of 110 mM, 150 mg/kg) intraperitoneally (i.p.) 20 minutes before the NMDA injection. Capsaicin was injected (5 μ L of 16.37 mM or 0.5%) intradermally (i.d.) into both hindpaws [43]. An i.p. injection (150 μ L of 150 mM, 200 mg/kg) of 4-hydroxy-2, 2, 6, 6-tetramethylpiperidin-1-oxyl (TEMPOL) was done 1 hour after the capsaicin injection [43].

Our previous study showed that i.d. capsaicin induces both primary and secondary hyperalgesia that last approximately 5–8 h [44]. To see the TEMPOL effects on the secondary hyperalgesia, we used a post-treatment paradigm. On the other hand, the i.t. NMDA induces hyperalgesia that peaks at 1 hr and then quickly subsides [25]. Since, the analgesic effect of i.p. PBN also takes 1 hr to show the maximum effect, it was necessary to inject PBN before NMDA to see the maximum effect of PBN on the i.t. NMDA induce hyperalgesia.

Mechanical hyperalgesia of the hindpaw was assessed by measuring the paw withdrawal frequency of each mouse with 10 repetitive stimuli using Von Frey (VF) filaments (VF #3.61, 0.4g, and VF #3.22, 0.16g, North Coast Medical) at 40 and 60 min after the NMDA injection.

Open-field exploratory activity tests of mice were performed using a photobeam activity system (PAS, San Diego Instruments) as described in previous studies [36,37]. Briefly, each mouse was placed in a locomotor activity cage 15 min after the NMDA injection and the activity of the mouse was monitored for 30 minutes by collecting data in 5-minute time blocks. The frequency and duration of horizontal movements were analyzed by measuring the frequency of beams broken, distance moved, active time, and rest time. The frequency and duration of rearing (standing with only the hind legs on the ground) were measured as vertical movements. The development of mechanical hyperalgesia in the hindpaw tends to decrease rearing behavior (since that will put high pressure to their hind paws) as well as the horizontal movements. Therefore, an increase in rest time or decreases in all the other measured parameters would indicate an increase of pain-related behavior.

Both of the VF and activity tests were done in a blind manner thus the tester was not aware of the manipulations done on the testing animals. Two separate groups of animals were used for each behavioral testing. Thus, only one behavior test was conducted for each animal. Immediately after the behavioral testing, spinal cords were dissected for either Western blot or immunohistochemistry analyses.

2.2 Immunohistochemistry

Animals were deeply anesthetized with isoflurane and perfused via the heart with cold artificial cerebrospinal fluid (ACSF, 0.15 M NaCl, 10 mM HEPES, 3 mM KCl, 0.2 mM CaCl_2 dihydrate, 10 mM glucose), followed by a fixative containing 4% paraformaldehyde in phosphate buffer solution (PBS: 0.1 M NaCl, 2.5 mM KCl, 10mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.4). The L4/5 regions of the spinal cord were removed 70 min after the NMDA injection or 120 min after the capsaicin injection. The spinal cord segments were post-fixed for at least 4 hours in the same fixative at 4°C and then cryoprotected overnight in PBS including 30% sucrose at 4°C. Transverse sections (30 μ m thick) of the L4/5 spinal cord were cut on a cryostat (Leica CM1900) and processed for immunostaining on gelatin-coated coverslips. The sections were first blocked with PBS including 5% normal goat

serum (NGS) and 0.2% Triton X-100. The sections were then incubated overnight at 4°C with gentle shaking in PBS including 5% NGS, 0.2% Triton X-100, and primary antibodies. After washing in PBS, the sections were then incubated for 2 hours at room temperature (RT) with gentle shaking in PBS including 5% NGS, 0.2% Triton X-100, and secondary antibodies. After washing in PBS, the coverslips were mounted onto slides with Permafluor mounting medium (Thermo-Pierce, TA030FM). The immunostained dorsal horn sections were examined and imaged using a confocal microscope (Nikon A1, with 20x and 60x oil immersion objectives). The images were analyzed by using ImageJ software (NIH, Washington, DC, USA) and Element Software (Nikon).

2.3 Western blot analyses

Either 70 min after the NMDA injection or 120 min after the capsaicin injection, mice were perfused via the heart with cold ACSF. The L4/5 regions of the spinal cord were dissected, snap-frozen with liquid nitrogen, and stored at -80°C. The spinal cord segments were solubilized with PBS including 1 µM EDTA, 1 µM EGTA, 1% Triton X-100, 0.2% SDS, and protease inhibitor cocktail (Sigma, S-8830). Lysates were centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatants were collected and protein concentration was determined using a bicinchoninic acid protein assay kit (BIO-RAD) to equilibrate the total protein amount in each group. The samples were analyzed through SDS-PAGE and Western blotting as described before [20]. Briefly, PBS including 1% bovine serum albumin (BSA) and 0.1% Tween-20 was used for blocking, incubating with antibodies, and washing processes. Western blots were imaged with a gel imaging system (ChemiDoc XRS, Bio-Rad). Multiple blots were produced from the same set of samples, and each blot was probed with a specific antibody. The density of each phospho-specific protein signal (e.g. pS818) was normalized to the density of the total protein signal (e.g. GluA1). To verify equal loading of proteins in each well, each blots was probed with tubulin antibodies. For quantification, densitometry was done using Image Lab (Bio-Rad) and Image J (NIH) software.

2.4 Labeling of surface proteins using a membrane-impermeable cross-linking reagent BS³

To label surface proteins, a cross-linking reagent BS³ (bis-(sulfosuccinimidyl) suberate, Pierce) was used as described in previous studies [4,57] with some modifications. After perfusion of mice via the heart with cold ACSF, the L4/5 regions of the spinal cord were removed 70 min after NMDA injection, and placed into cold ACSF oxygenated with mixed gas (95% O₂ and 5% CO₂). The tissue was cut into 5 slices at approximately 1 mm in thickness with scissors and allowed to float in the oxygenated ACSF. The slices were incubated in BS³ (Thermo-Pierce) solution (1.25 mM BS³ dissolved in ACSF) for 40 minutes at 10°C with gentle shaking. After quenching with ACSF including 100 mM glycine three times for 5 min each, the slices were processed for Western blotting as described above.

2.5 Antibodies

For immunohistochemistry, commercial antibodies were used: GluA1 (1:500, Millipore, MAB2263) GluA1-pS831 (1:500, Millipore, 04-823), NeuN (1:500, Millipore, MAB377), MAP2 (1:1000, Invitrogen, 13-1500), and Alexa Fluor 488, 546 or 647 goat anti-mouse, rabbit, and/or chicken antibodies (1:500, Invitrogen). For Western blot analyses, commercial antibodies were used: GluA1 (1:3,000, Millipore, MAB2263), GluA2 (1:3,000, Millipore, AB10529), α-tubulin (1:300,000, Millipore, MAB1637), GluA1-pS831 (1:3,000, Millipore, 04-823), GluA1-pS845 (1:3,000, Millipore, AB5849), GluA2-pS880 (1:3,000, Millipore, 07-294), and ECLTM Horseradish Peroxidase-linked donkey anti-mouse or rabbit antibodies (1:3,000, GE Healthcare). GluA1-pS818 antibody (1:1,000) was made and its specificity was tested in a similar way as previously described [3].

2.6 Statistical analyses

One-way analysis of variance (ANOVA) was performed to compare data from the behavior tests, Western blottings, and immunohistochemistry. Von Frey data were analyzed non-parametrically through Kruskal-Wallis one-way ANOVA. When significant F-values were encountered, the different treatments were compared using the Tukey multiple comparison test. AMPA-R phosphorylation (at sites of pS818, pS831, pS845, and pS880) in the L4/5 spinal cord was measured after NMDA injection and Von Frey tests. Thus, AMPA-R phosphorylation values were correlated with the paw withdrawal frequency of each mouse by using the Pearson product-moment correlation. Linear regression was used to obtain a best-fit line for each plot. The p-values were derived from regression. Data are expressed as Mean $\pm$ Standard Error of the Mean (SEM). The n-number refers to the number of animals used for experiments. Only one n number was shown in case where the n number is the same for all groups in a set of experiments. For all statistical analyses, probability (p) of 0.05 or less was considered significant. All statistical analyses were performed using SigmaPlot (Ver 12, SYSTAT Software).

3. RESULTS

3.1 Intrathecal NMDA induced pain-related behavior and AMPA-R phosphorylation in the spinal dorsal horn in a ROS-dependent manner

Pain-related synaptic plasticity in the spinal cord is mediated by the activation of post-synaptic NMDA-Rs in physiological conditions [55]. To evaluate pain development, paw withdrawal responses to Von Frey (VF) stimuli and open-field exploratory activity tests were performed after intrathecal (i.t.) NMDA injection (Fig. 1 and 2).

In the VF testing paradigm, the i.t. NMDA caused a significant increase in the number of paw withdrawal responses, indicating the development of mechanical hyperalgesia in the hindpaw (Fig. 1A). This result was similar to that observed in other pain models, such as neuropathic pain and intradermal (i.d.) capsaicin-induced hyperalgesia [23,43]. Intraperitoneal (i.p.) injection of PBN, a non-specific ROS scavenger, 20 min before the NMDA injection, blocked the NMDA-induced hyperalgesia (Fig. 1A). In a different group of animals, the levels of open-field exploratory activity were measured by using a photobeam activity system. The i.t. NMDA also significantly decreased the levels of open-field exploratory activity compared to that with i.t. saline (Fig. 1B). Additional analyses of several other activity parameters provided supportive data indicating that the NMDA-injected mice were less active compared to saline-injected mice (Fig. 2). The changes in spontaneous exploratory behavior (increase in rest time or decreases in frequency of beams broken, distance moved, active time, the frequency and duration of rearing) would indicate an increase of pain-related behavior. Again, i.p. PBN blocked the reduction in open-field exploratory activity by i.t. NMDA (Fig. 1B and 2). Particularly, the reverses of NMDA effects by PBN were statistically significant in active time, rearing frequency, and rest time (Fig. 1B and 2). The reverses in rearing duration, beams broken, and distance moved were also observed although they were not statistically significant (Fig. 2). Together, these results suggest the essential role of ROS for NMDA-induced pain-related behavior.

Thus, both behavioral tests demonstrated that the i.t. NMDA induces pain-related behavior in mice, and that ROS is essential for the behavior.

Next, Western blotting (Fig. 3) and immunohistochemistry (Fig. 4) were performed to analyze the changes in AMPA-R phosphorylation in the L4/5 spinal cord. Spinal cords were sampled at about 70 min after i.t. NMDA because mice showed the peak pain response around this time [25]. For this experiment, the spinal cord samples were obtained from those animals that were used for mechanical hyperalgesia testing. Mice were perfusion fixed

immediately after mechanical hyperalgesia testing at 60 min after i.t. NMDA and L4/5 spinal cord were sampled.

To test if GluA1 phosphorylation is involved in pain-related synaptic plasticity, three phosphorylation sites, serine (S) 818, S831, and S845 of GluA1, were analyzed since they are known to be essential for LTP induction in the hippocampus [13,26,27,35,45]. The change in the phosphorylation of GluA2-S880 was also analyzed since the increase of GluA2-S880 phosphorylation is essential for synaptic plasticity in the inflammatory pain model [40].

One hour after the NMDA injection, the phosphorylation on S818, S831, and S845 of GluA1 and S880 of GluA2 was significantly increased (Fig. 3). On the other hand, when ROS levels were reduced by PBN, the NMDA-induced increase of phosphorylation of GluA1 and GluA2 was reversed significantly (Fig. 3). The data thus suggest that elevated levels of ROS contributed, at least partially, to the increase of AMPA-R phosphorylation after NMDA injection.

Since the samples for AMPA-R phosphorylation analyses were obtained from the behaviorally tested mice, the changes in phosphorylation were correlated to the pain-related behaviors. As shown in the most right hand side panels in Fig. 3, a significant positive correlation was found between the phosphorylation and paw withdrawal responses to VF stimuli (Fig. 3C), suggesting that the phosphorylation is a part of the signaling pathway for induction of pain-related behavior.

After i.t. NMDA injection, our immunohistochemical analyses of the phosphorylated GluA1-S831 showed a significant increase in the superficial dorsal horn of the L4/5 spinal cord (Fig. 4), where NMDA-R activation plays a critical role in induction of pain-related synaptic plasticity [14,55]. Again, PBN significantly reduced the elevated GluA1-pS831 by i.t. NMDA thus suggesting that ROS was necessary for the phosphorylation change in the superficial dorsal horn (Fig. 4A). Furthermore, our triple labeling with neuronal marker (NeuN) and dendritic marker (MAP2), as well as GluA1-pS831, demonstrated that the phosphorylation increase was predominantly in neurons (Fig. 4B).

3.2 Intradermal capsaicin increased AMPA-R phosphorylation in the spinal dorsal horn in a ROS-dependent manner

We clearly understood that i.t. NMDA injection is an artificial condition. Thus, to check if peripheral nociceptive inputs (that are closer to physiological condition) also induce similar ROS-dependent increases in GluA1 and GluA2 phosphorylation in the spinal cord, our study was expanded to the capsaicin pain model. Consistent with NMDA-treated animals, i.d. capsaicin injection into the hindpaw significantly increased the phosphorylation of GluA1 and GluA2 in the L4/5 spinal cord (Fig. 5A and 5B). In this experiment, TEMPOL, a superoxide scavenger, was used as a ROS scavenger since superoxide is essential for capsaicin-induced pain [44]. TEMPOL was clearly effective in reversing the increased AMPA-R phosphorylation induced by i.d. capsaicin (Fig. 5 and 5B). Again, after i.d. capsaicin injection, our immunohistochemical analyses of the phosphorylated GluA1-S831 showed a significant increase in the superficial dorsal horn of the L4/5 spinal cord in a ROS-dependent manner (Fig. 5C). Taken together, these data indicate that the pattern and ROS dependency of AMPA-R phosphorylation in the spinal dorsal horn are almost identical in both i.d. capsaicin- and i.t. NMDA-induced pain models.

3.3 The changes in cell-surface localization of GluA1 and GluA2 accompany pain-related behavior and depend on ROS

In hippocampal and cerebellar neurons, the cell-surface localization of AMPA-Rs is regulated by phosphorylation of GluA1 and GluA2, respectively [6,7,30,38,50]. The cell-surface localization of GluA2 in the spinal cord is also negatively regulated by S880 phosphorylation [40]. AMPA-R trafficking to the cell surface is known to increase AMPA-R trafficking to the synapse. This phenomenon is called as priming effect of AMPA receptor surface localization on its way to synapses [30,34,38]. Given that the phosphorylation sites on GluA1 and GluA2 were significantly increased by the NMDA injection (Fig. 3 and 4), it is important to see whether the cell-surface localization of GluA1 and GluA2 is changed after i.t. NMDA injection.

The changes in the AMPA-R localization on DHN surface (trafficking) was analyzed through a biochemical assay with BS³ that can be used to cross-link subunits of ion channels or receptors consisted of multiple subunits. Since BS³ cannot penetrate the plasma membrane, receptors that are only at the cell surface will be cross-linked. In a SDS-PAGE, the cross-linked surface proteins will show up as a protein band with higher molecular weight compared to that of the intracellular pool of the proteins that are separated into single subunits by protein gel loading buffer. The cross-linked GluA1 subunits on the cell surface were detected in Western blot analyses as tetramers (> 250 kDa) compared to intracellular monomers (~105 kDa) (Fig. 6A). However, tubulin that is not present on the cell surface, was not detected as a protein band with higher molecular weight after the cross-linking with BS³ (Fig 6B and 6C), demonstrating that the BS³ in our assay selectively cross-linked proteins only at the cell surface.

The amount of surface GluA1 was significantly increased after i.t. NMDA injection, while that of surface GluA2 was significantly decreased (Fig. 7). Reduction of ROS levels with PBN significantly reversed the NMDA-induced increase of surface GluA1 and decrease of surface GluA2 (Fig. 7). The data suggest that ROS increase is essential for the NMDA-induced trafficking of GluA1 and GluA2 in and out of the DHN surface, respectively. A significant correlation was also found between the cell-surface localization of AMPA-R subunits and the paw withdrawal responses to Von Frey (VF) stimuli (data not shown).

Taken together, these results strongly suggest that the ROS-dependent changes in AMPA-R phosphorylation and localization at the cell surface are parts of a signaling pathway for the induction of pain-related synaptic plasticity in DHNs (Fig. 8).

4. DISCUSSION

Persistent pain is a devastating state that often destroys the quality of life. To understand the molecular and cellular mechanisms underlying persistent pain, we studied the signal transduction pathway for pain-related synaptic plasticity in the spinal dorsal horn. Using two animal models of persistent pain, i.t. NMDA and i.d. capsaicin, our study showed that the phosphorylation and cell-surface localization of GluA1 are significantly increased after NMDA-R activation and that these increases were blocked by the removal of ROS. ROS-dependent increase of GluA2-S880 phosphorylation and thus decreased cell-surface localization of GluA2 were also parts of the signaling pathway. Thus, our study suggests a signal transduction pathway that consists of NMDA-R activation, ROS production, AMPA-R phosphorylation, and AMPA-R trafficking to and from the cell surface, plays a critical role in pain-related synaptic plasticity in DHNs.

In this study, we used i.t. NMDA injection to induce pain-related behavior of mice for the following reasons: 1) the i.t. NMDA injection produces pain-related behavior in animals

[1,25,32,46], 2) i.t. NMDA induces pain-related behavior in animals without the involvement of other factors such as inflammation, 3) i.t. NMDA model allows us to study central mechanism with minimal peripheral involvement, and 4) the i.t. NMDA injection could activate a large population of DHNs through immediate, direct, strong, and homogenous stimulation. Thus the changes in the signaling pathway could be large enough to be detected by biochemical and optical imaging analyses, as shown in our study.

Since NMDA is not an endogenous neurotransmitter, the AMPA-R phosphorylation was also examined in i.d. capsaicin-injected mice, and then compared to that of the i.t. NMDA mice. Capsaicin will activate peripheral nerve endings of nociceptors and will cause glutamate release from nociceptor terminals in the spinal cord and then activate NMDA-Rs, as well as, other glutamate receptors on DHNs [48]. Furthermore, blocking NMDAR activation by an NMDAR inhibitor, AP7, prevents the development of the sensitization of spinothalamic tract neurons in the spinal cord after i.d. capsaicin [8]. Thus the activation of NMDA-Rs in the spinal cord by i.d. capsaicin is closer to a physiological condition compared to that produced by i.t. NMDA. Despite these differences, the results are similar: ROS-dependent increases in phosphorylation of GluA1 and GluA2 are in concert with pain-related behavior. Therefore, this parallel study suggests that the ROS-dependent AMPA-R phosphorylation is a common component for the pain-related signaling pathway at least in these two pain models.

We do not believe that the DHN sensitization after i.t. NMDA is the same as central sensitization induced by a nerve or tissue injury or inflammation. The reasons include that NMDA is not an endogenous neurotransmitter although it is specific for NMDA-Rs, and it activates non-synaptic NMDA-Rs as well as synaptic NMDA-Rs. However, our present data and previous studies strongly suggest that a similar mechanism is involved at least for the initial phase of DHN sensitization regardless of animal models of pain [23,25,28,43] *in vivo* or *in vitro* [28].

To analyze pain behavior, we performed open-field exploratory activity tests in addition to Von Frey tests. Exploratory activities require brain functions and do not entirely rely on spinal reflexes. Furthermore, the spontaneous activity test can also be used for the detection of spontaneous pain, which is difficult to measure in most animal models of pain. Consistent results from two types of pain-related behavior tests provided us a solid foundation for our analyses of pain-related signaling pathway.

A main finding from our study is the link from ROS to AMPA-R phosphorylation and cell-surface localization in the L4/5 spinal cord, as a part of the signaling pathway for the induction of pain-related synaptic plasticity. ROS is known as a critical component for the signaling pathway [23,51,54]. However, molecules downstream of ROS are not known. It has been well known that increases of PKC, CaMKII, and PKA activities are essential for the induction of pain-related behavior [9,11,31,47]. Since all these kinases have target sites on AMPA-Rs [2,3,7,19,42] and AMPA-Rs play a role in pain [12,40], we analyzed the changes of AMPA-R phosphorylation (S818, S831, and S845 of GluA1 and S880 of GluA2) in two animal models of pain. Our data demonstrated that phosphorylation on the all four examined sites was significantly increased by NMDA-R activation in concert with pain-related behavior. Importantly, the changes in AMPA-R phosphorylation after NMDA-R activation were dependent on ROS levels. Furthermore, the changes of AMPA-R localization on the cell surface in the L4/5 spinal cord were also dependent on ROS. It has been shown that the cell-surface localization of AMPA-Rs is regulated by phosphorylation of AMPA-R in the brain [6,7,30,38,50] and spinal cord [40]. AMPA-R trafficking to the cell surface has the priming effect on AMPA-R trafficking to the synapse [30,34,38]. Taken together, it is likely that the ROS-dependent changes of AMPA-R phosphorylation and

subsequent AMPA-R trafficking in and out of the cell surface are essential parts of signaling pathways for pain-related synaptic plasticity.

This is the first study to demonstrate the potential role of GluA1-pS818 in the pain-related synaptic plasticity in the spinal cord. Our data demonstrated co-variance of GluA1-pS818 with pain behavior. This agrees with the role of GluA1-pS818 in synaptic plasticity in the brain which was established recently [3,16,30]. The ROS-dependent increase in GluA1 (S831 and S845) and GluA2 (S880) phosphorylation during the induction of pain is a novel finding in our study, although the increases of the phosphorylation have been reported in some pain models [5,10,22,40].

Both GluA1-S818 and GluA2-S880 are substrates of PKC and they are essential for hippocampal LTP [3] and inflammatory pain [40], respectively. Our study with animal models of pain clearly demonstrated that the GluA1/GluA2 phosphorylation at the PKC sites is significantly increased during the induction of pain, and then almost completely reversed when ROS were removed. The data thus suggest that ROS are critical for activation of PKC during the induction of pain. Similarly, the phosphorylation of S845 and S831, the sites of PKA and CaMKII, respectively, was also significantly increased during the induction of pain and regulated by ROS levels. However, the ROS scavenger effect on reversing phosphorylation at S831 is smaller compared to that at S818, S880, and S845. Thus the data suggests that at least some part of CaMKII activity increase during the induction of pain is not dependent on ROS. The small differences between control and NMDA+PBN might have been caused by either an insufficient ROS scavenging by PBN and TEMPOL or an imbalance between scavenging and production of ROS.

Our multidisciplinary data consistently demonstrated a significant reversal of NMDA or capsaicin effects on pain behavior and AMPA-R cell-surface localization, as well as on AMPA-R phosphorylation, by ROS scavengers. These data strongly suggested that ROS is a necessary intermediate element for induction of pain behavior, which is also consistent with previous studies from our group [23–25,28,44]. Our data thus suggest that ROS is working as an intermediate signaling molecule between NMDA-R activation and AMPA-R phosphorylation by regulating kinases (and/or phosphatases) activities during development of pain related synaptic plasticity. Previous studies support our idea. ROS-dependent activation of PKA increases excitability of amygdala neurons and pain behavior [29]. ROS are also known as signaling molecules for various kinases [33].

By labeling surface proteins with a membrane-impermeable cross-linking reagent BS³, we clearly demonstrated the increase of GluA1 on the cell surface after NMDA-R activation. Furthermore, the finding of ROS-dependency of GluA1 cell-surface localization was also a novel finding in this study. The insertion of the GluA1 into the plasma membrane has been reported in some pain models [5,11,21,39,40,52,53]. Most previous studies, however, have shown the increase of GluA1 in a membrane fraction that could include not only the plasma membrane but also the internal membrane, and thus they have a contamination problem. GluA1 trafficking to the cell surface has the priming effect on AMPA-R trafficking to the synapse during hippocampal LTP [30,34,38]. Therefore, the increase of cell-surface localization of GluA1 in the spinal cord after i.t. NMDA injection would likely contribute to the increase of synaptic AMPA-Rs, thus resulting in the pain-related behavior observed in our study. The ROS-dependent decrease of GluA2 cell-surface localization is also novel and consistent with a previous study which showed GluA2 internalization during inflammatory pain [40].

The opposite movement of GluA1 and GluA2 in and out of the DHN surface would change surface GluA1/GluA2 ratio. Considering that only GluA2 subunit can block Ca²⁺ influx

through AMPA-Rs, the NMDA induced trafficking of GluA1 and GluA2 would increase Ca^{2+} -permeable AMPA-Rs on the cell surface, and thus at the synapse of DHNs. Previous studies have shown that Ca^{2+} -permeable AMPA-Rs contribute to DHN sensitization and pain-related behavior [5,12,18,41,49]. The increase of Ca^{2+} influx through AMPA-Rs may activate some signaling pathways in the micro domain around the Ca^{2+} -permeable AMPA-Rs. However, molecular mechanisms downstream of the Ca^{2+} influx through the Ca^{2+} -permeable AMPA-Rs and their contribution in the pain-related signaling pathway are yet to be revealed. Furthermore, the relationship and relative contribution between Ca^{2+} influx through AMPA-Rs and Ca^{2+} influx through NMDA-Rs is not yet clear. Further studies are warranted for resolving these issues.

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SUMMARY

Removal of reactive oxygen species (ROS) leads to reduced AMPA receptor phosphorylation in concert with decreased GluA1 cell-surface localization and pain-related behavior.

Fig. 1. Pain-related behavior induced by intrathecal NMDA is dependent on ROS

(A) Intrathecal (i.t.) NMDA injection (50 μ M, 5 μ L) induced mechanical hyperalgesia, as demonstrated by increased paw withdrawal responses to Von Frey (VF) stimuli applied to the mouse hindpaw (measured at 40 & 60 min after i.t. NMDA). The control group with i.t. saline (Cont) showed ~10% response rate. In the third group (NMDA+PBN), pre-injection of a ROS scavenger, PBN (110 mM, 150 μ L, i.p., 20 min before NMDA), significantly reduced the NMDA-induced mechanical hyperalgesia. $n = 6$ per group. (B) Open-field exploratory activity tests were conducted using activity cages. Time spent while active (Active time) was measured in 5-minute time blocks for a total of 30 min. The active time was significantly decreased after i.t. NMDA compared to the control group, indicating the development of pain. The PBN treated (NMDA+PBN) group was as active as the control group during the entire testing period. $n = 8$ per group. (A–B) Quantified data are shown as mean $\pm$ SEM. Statistical analyses were done through ANOVA followed by Tukey multiple comparisons. *: $p < 0.05$ (respect to control), †: $p < 0.05$ (respect to NMDA).

Fig. 2. Decrease of open-field exploratory activity after intrathecal NMDA is dependent on ROS
To analyze pain-related behavior of mice, various parameters in the open-field exploratory activity tests were measured and plotted in accumulated bar graphs. All parameters consistently indicated that mouse activity was decreased after i.t. NMDA and PBN reduced the NMDA effect on activity. n=8, Statistical analyses and labeling were as described as in Fig. 1.

Fig. 3. The phosphorylation of AMPA-R subunits in the spinal cord was increased after i.t. NMDA, and it was dependent on ROS

After i.t. NMDA (NMDA), mice were subjected to the Von Frey test (VF #3.61, 70 min after i.t. NMDA). After the behavioral test of mice, their L4/5 segments of the spinal cord were dissected out and used for Western blot analyses of AMPA-R phosphorylation. (A) The i.t. NMDA significantly increased the phosphorylation of serine (pS) 818, 831, and 845 of GluA1 and pS880 of GluA2. Pre-injection with PBN significantly reversed the NMDA-induced increase in the phosphorylation of GluA1 and GluA2. The α -tubulin signal was used as a control verifying the same amount of total proteins in each blot (not shown). The treatments are indicated at the top of lanes. The antibodies used for Western blotting are indicated at the bottom of each panel. (B) For quantification, the intensity of each phospho-specific protein signal (e.g. pS818) was normalized to the total protein signal (e.g. GluA1). The normalized intensity is presented as a relative intensity to control (Cont) for each phosphorylation site. Data are shown as mean $\pm$ SEM. $n = 8$ per group. Statistical analyses were done through ANOVA followed by Tukey multiple comparisons. *: $p < 0.05$ (respect to control), **: $p < 0.01$ (respect to control), †: $p < 0.05$ (respect to NMDA), ††: $p < 0.01$ (respect to NMDA). (C) The phosphorylation changes were significantly correlated with behavioral alterations as shown in scattered plots.

Fig. 4. The phosphorylation of GluA1-S831 in the spinal dorsal horn was increased after i.t. NMDA, and it was dependent on ROS

Transverse sections (30 μm) of L4/5 regions of spinal cord were immunostained with antibodies including one specific for GluA1 with phosphorylation of serine 831 (GluA1-pS831), and imaged with a 20 $\times$ (A) or 60 $\times$ (B) objective by confocal microscopy. (A) The GluA1-pS831 immunostaining was particularly prominent in the superficial areas of the dorsal horn. The i.t. NMDA significantly increased pS831-stained area. PBN treatment significantly reversed the NMDA-induced increase in the phosphorylation. However, the GluA1 signal was not changed by NMDA or PBN. Scale bar = 20 μm . For quantification, the area with GluA1 and pS831-staining above a threshold was measured in the superficial dorsal horn and the pS831 signal was normalized to the GluA1 signal. The normalized value was presented as relative value to control. (B) Representative images of spinal cord slices immunostained for GluA1-pS831, NeuN (Neuronal marker), and MAP2 (neuronal dendrite marker) and imaged with a 60 $\times$ objective at the superficial dorsal horn. This triple labeling demonstrated that the ROS-dependent increase of GluA1-pS831 was predominantly in neurons. Scale bar = 10 μm . For quantification, area with pS831- and MAP2-staining above a threshold was measured independently, the pS831 area was normalized to the MAP2 area, and presented as relative value to the control. (A & B) Quantified data are shown as mean $\pm$ SEM. $n = 4$ per group, Statistical analyses were done through ANOVA followed by Tukey multiple comparisons. *: $p < 0.05$ (respect to control), **: $p < 0.01$ (respect to control), ††: $p < 0.01$ (respect to NMDA).

Fig. 5. The increased phosphorylation of AMPA-R subunits after i.d. capsaicin was also dependent on ROS

(A) Western blot analyses of AMPA-R phosphorylation in the L4/5 spinal segments of the mice treated with intradermal capsaicin (0.5%, 5 μ L, i.d.). Capsaicin treatment significantly increased the phosphorylation of serine (pS) 818, S831, and S845 of GluA1 subunit and pS880 of GluA2 subunit. Treatment with a superoxide scavenger, TEMPOL (150 mM, 150 μ L, i.p.), significantly reversed the capsaicin-induced increase in the phosphorylation. (B) Quantification of the phosphorylation changes in Western blotting. The quantification of Western blotting, statistics (n=6), and labeling of figures were done as described in Fig. 3. (C) L4/5 spinal cord slices immunostained for GluA1-pS831 demonstrated that the capsaicin injection significantly increased the phosphorylation of serine 831 (pS831) of GluA1 subunit in the superficial dorsal horn compared to the control group injected with saline. Injection of TEMPOL (Temp), 1hr after capsaicin, reversed the capsaicin-induced increases in the phosphorylation. The quantification, statistics (n = 8), and labeling of immunostaining figures were done as described as in Fig. 4. The scale bar in the set of representative images is 10 μ m.

Fig. 6. Cross-linked AMPA-Rs on the cell surface were detected as tetramers in a Western blot analysis

The spinal cord slices were incubated with membrane impermeable BS³ to cross-link surface proteins. (A) In addition to the low molecular weight bands (Monomer) of non-cross-linked GluA1, the cross-linked GluA1 subunits on the surface of neurons were detected as high molecular weight bands (Tetramer) only when BS³ was added. A band above the tetramer is a non-specific band due to aggregated proteins in the well of protein gel. (B & C) Tubulin was used as negative control for this BS³ mediated cross-linking of surface proteins since tubulin is not on the cell surface. Although low molecular weight bands of α-tubulin were detected as monomers with low exposure during imaging of a Western blot with cross-linked protein samples (B), no high molecular weight bands of α-tubulin were detected even with high exposure (C). Molecular weights of standards are indicated in kDa on the left (A–C).

Fig. 7. ROS-dependent changes in the cell-surface localization of AMPA-R subunits

Cell surface proteins of the L4/5 spinal cord slices were cross-linked with a membrane impermeable cross-linker, BS³. The cross-linked GluA1 on the surface (GluA1 tetramer) was significantly increased after i.t. NMDA injection. On the other hand, the cross-linked GluA2 on the surface (GluA2 tetramer) was significantly decreased after i.t. NMDA injection. This data strongly suggest that GluA1 trafficking into the surface was increased while GluA2 was internalized after injection of NMDA. PBN partially but significantly reversed the changes in the cell-surface localization of both subunits of AMPA-Rs. α -tubulin signal was used as a loading control. The quantification, statistics ($n = 4$), and labeling of Western blots figures were done same as described as in Fig. 3.

Fig. 8. The signal transduction pathway involved in the pain-related synaptic plasticity in dorsal horn neurons

The activation of NMDA receptors (NMDA-R) results in Ca^{2+} influx and production of ROS. This leads to the phosphorylation of AMPA receptors (AMPA-R) and trafficking of AMPA-Rs into and out of the cell surface, respectively. Either or both kinase and phosphatase should be involved in the phosphorylation of GluA1/2 subunit by ROS production.