The effects of spinal NMDA receptor activation on KCC2, EphB2 and n-cadherin and its implications for hyperalgesic priming

JiYoung Kim, Elaine K. Murad, Galo Mejia, Theodore J. Price

Department of Medical Pharmacology, College of Medicine, The University of Arizona, Tucson, AZ 85724-504

Abstract

N-Methyl-D-aspartate glutamate receptor (NMDAR) function is crucial to synaptic plasticity in the CNS, including nociceptive sensitization in the pain pathway. One way that NMDAR function affects synaptic function is by interacting with membrane proteins such as potassium-chloride transporter 2 (KCC2) and the ephrine B 2 receptor (EphB2R) (Song *et al*). Recent studies show that NMDAR activation or peripheral nerve injury leads to a μ-calpain-mediated decrease in KCC2 expression (Zhou *et al*). Additionally, blockade of spinal NMDAR was shown to prevent EphB2-induced thermal sensitivity in naïve rats. While these data clearly indicate that this activity is important to pain processing in the spinal cord, the coincidence of KCC2 and EphB2R expression in this processing has not been previously investigated. In our study, we hypothesized that NMDAR activation can lead to concurrent changes of KCC2 and EphB2R expression, both of which are expressed in excitatory synapses. Using spinal synaptoneurosomes (SNS) from mice, we demonstrated that NMDAR activation with 300μM NMDA led to a decrease in the monomeric form of KCC2 and EphB2R within 15 min. These effects were completely blocked by co-incubation of NMDA with physiological levels of Mg^{2+} (2mM). Subsequently, we tested the effects of *in vivo* activation of NMDA by injecting brain-derived neurotrophic factor (BDNF) into the spinal cord. We showed that BDNF-induced allodynia is prevented by the NMDAR blocker D-AP5, demonstrating that BDNF requires NMDAR activation for the establishment of mechanical allodynia. Moreover, at 24 hours after BDNF injection, we observed reduced expression of EphB2R but not KCC2. Interestingly, even 7 days after BDNF injection, EphB2R expression remains altered. Furthermore, our investigation has expanded beyond the behavior of NMDAR-modulated KCC2 and EphB2R expression into exploration of additional interactions with n-cadherin, an important synaptic adhesion molecule. During NMDA SNS experiments, we observed that full-length n-cadherin decreased simultaneously with monomeric KCC2 and full-length EphB2R. We also saw that n-cadherin expression decreased 24 hours after BDNF injection but returned to baseline by Day 7 after injection. Our discovery suggests that NMDA-mediated pro-nociceptive changes in spinal synapses may have profound implications in the initiation and persistence of pain.

Conclusions and Future Directions

1. Acute NMDA exposure causes downregulation of KCC2, EphB2R and n-cadherin in the spinal cord synaptoneurosomes.
2. BDNF-mediated NMDAR activation acutely reduces EphB2R and n-cadherin expression in the mouse spinal dorsal horn. EphB2R expression was found changed even 7 days after BDNF injection.

Our lab has previously demonstrated that spinal cord plasticity is an important component of hyperalgesic priming, which is a model of acute to chronic transition of pain (Asiedu *et al*). Our current finding suggests that the expression of certain pro-nociceptive proteins such as EphB2R changes with intrathecal injection of BDNF, which establishes hyperagesic priming. Such changes seem to be mediated by NMDAR activation as the blockade of this receptor prevented the effects of BDNF. We plan to further investigate whether upregulation of EphB2R can serve as a marker for hyperagesic priming by looking at EphB2R expression using other priming agents (i.e., IL-6 and carrageenan).

Materials and Methods

Synaptoneurosome Preparation and Treatment

Spinal cord synaptoneurosomes (SNS) were isolated from 3-weeks-old (11-13g) male ICR mice. The collected tissues were homogenized in an artifical cerebrospinal fluid (aCSF) containing NaCl, KCl, $MgSO_4$, $CaCl_2$, KH_2PO_4 and glucose. Samples were filtered through 100μm and 11μm nylon mesh filters (Millipore) and centrifuged at 1,000 × g for 20 min. The pellet was resuspended in the homogenization buffer without $MgSO_4$ containing 300μM NMDA (Tocris) for 15 min at 37ºC. The cellular fraction was collected by centrifugation at 20,000 x g for 2 min. The pellet was resuspended in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4), ultrasonicated and centrifuged at 200,00 x g for 10 min. The supernatant was collected and assayed using Western blot analysis.

Behavioral Testing

Male ICR mice were placed in acrylic boxes with wire mesh floors, and baseline mechanical withdrawal thresholds of the left hind paw were measured after habituation for 1 h using the up-down method (Chaplan et al., 1994). The experimenter making measurements was always blinded to the experimental conditions. 0.1ng BNDF was injected into the spinal cord in a volume of 5μl. For D-AP5 experiment, the drug was injected 15 minutes prior to the BDNF intrathecal injection.

Acknowledgements

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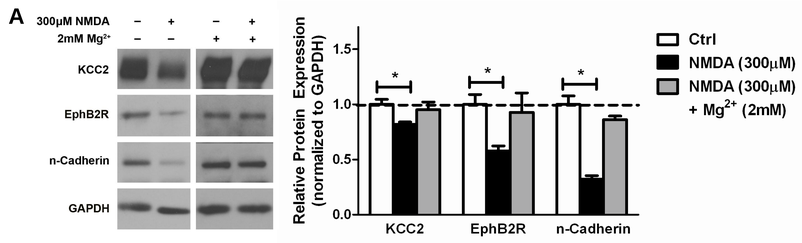
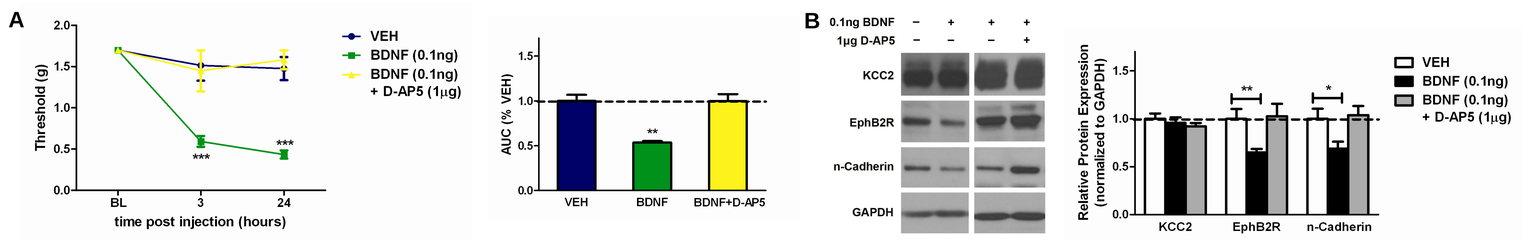
Results

1. Acute exposure to NMDA causes selective loss of KCC2, EphB2 receptor and n-cadherin expression in the mouse spinal cord synaptoneurosomes.



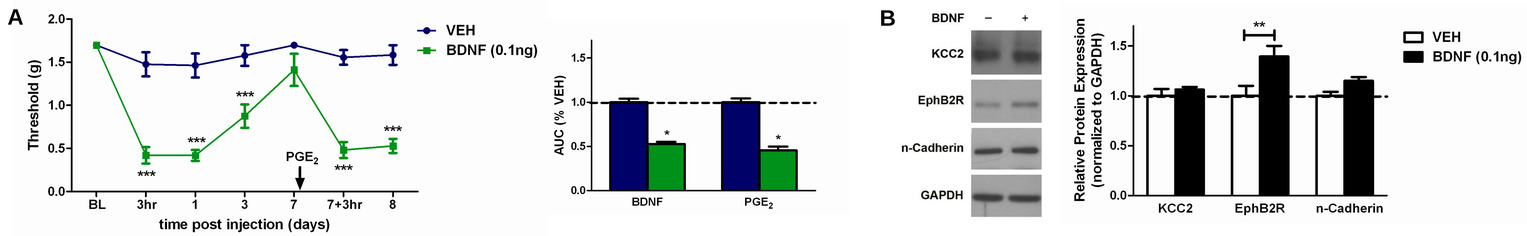
(a) Western blot of SNS fraction treated with 300μM NMDA for 15 minutes in Mg^{2+} free aCSF. Stimulation with NMDA significantly decreased monomeric form of KCC2, n-cadherin and EphB2R ($p< 0.05$). When the same experiment was done in 2mM Mg^{2+} containing aCSF, all the NMDA-induced changes were blocked.
(b) μ-calpain inhibitor, MDL28170, prevented NMDA-induced downregulation of KCC2 but not EphB2R and n-cadherin.
(c) 300μM NMDA treatment does not affect the expression of other membrane proteins such as GluA1, nIgn1 and trkB.

2. Intrathecal injection of BDNF causes changes in the expression of EphB2R and n-cadherin but not KCC2 at 24hours.



(a) D-AP5, a competitive NMDAR antagonist, prevented mechanical allodynia induced by intrathecal injection of BDNF. D-AP5 was injected intrathecally 15 minutes prior to BDNF injection (n=6). Animals that received BDNF and D-AP5 were significantly did not develop mechanical allodynia ($p< 0.01$).
(b)EphB2R and n-cadherin expressions in the spinal dorsal horn were significantly reduced in animals that received BDNF 24 hours before (*$p< 0.05$; **$p< 0.01$). However, these changes were not seen in animals that received both BDNF and D-AP5. On the other hand, KCC2 expression was found unchanged in both BDNF and BDNF+D-AP5 treated groups.

3. Intrathecal injection of BDNF causes persistent changes in the expression of EphB2R.



(a) Intrathecal injection of BDNF established hyperalgesic priming. Animals that received BDNF showed mechanical allodynia that lasted 3 days. On day 7, we injected 100ng PGE_2 to their left hindpaws (n=8). Animals that previously received BNDF showed mechanical allodynia that lasted at least 24 hours when the vehicle received animals did not respond to interplantar injection of PGE_2 ($p< 0.05$).
(b) Western blot images and quantification of protein expression of KCC2, EphB2R and n-cadherin in the spinal dorsal horn on day 7 post-BDNF administration. We observed that EphB2R expression was significantly elevated whereas KCC2 and n-cadherin expressions were similar to the vehicle-treated group ($p< 0.01$).

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