Intrathecal SB366791 Prevents Spinal EphrinB1/EphB1 Signaling Activation-induced Acute Thermal Hyperalgesia

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**Abstract:** Recent studies reported that activation of spinal ephrinB1/EphB1 signaling induced pain-like behaviors, although the underlying mechanisms remain largely unknown. The present study investigated the functional role of transient receptor potential vanilloid receptor 1 (TRPV1) in intrathecal ephrinB1-Fc-induced acute thermal hyperalgesia. Intrathecal ephrinB1-Fc provoked significant decrease in thermal paw withdrawal latency (PWL) behavioral response, while intrathecal pretreatment with SB366791, a selective TRPV1 antagonist, markedly reversed this decrease in a dose-dependent manner. Immunohistochemical staining of spinal c-Fos activation, a widely-used neuronal marker in the central nervous system, further confirmed the behavioral finding. Taken together, these findings, for the first time, indicate that TRPV1 may be a potential downstream effector in spinal ephrinB1/EphB1 system activation-induced thermal pain behavior in the mature nervous system.

**Key words:** TRPV1; thermal hyperalgesia; ephrinB1; EphB1; spinal cord; c-Fos protein.

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**Received:** October 31 2015; **Accepted:** November 17 2015; **Published:** November 21 2015


**Introduction**

Thermal hyperalgesia, or thermal pain is a pathological statement where thresholds of pain sensation are decreased to noxious thermal stimuli (1). EphB receptors and their ligands ephrins contribute to neuronal development, including synapse formation and neural circuitry assembly (2, 3), although their functional roles in the mature nervous system remain largely unknown. Recent studies suggest that alternation of spinal EphrinB1/EphB1 signaling might have a pivotal role in pain modulation in animal models (3-8). For example, intrathecal injection of ephrinB1-Fc induces acute thermal hyperalgesia and mechanical allodynia in mice (6), and spinal ephrinB1/EphB1 signaling is significantly up-regulated in bone cancer pain and neuropathic pain models in rat (4, 7, 8). However, the underlying downstream mechanisms are still open to interpretation and largely unknown.

TRPV1 is also called capsaicin receptor, or vanilloid receptor (VR1). TRPV1 is a member of transient receptor potential (TRP) channels and a noxious heat-activated cation channel that acts as an endogenous transducer of noxious heat, which contributes to pain modulation (9). TRPV1 has a robust expression in the spinal cord dorsal horn. Compelling and increasing evidence indicates that the TRPV1 receptor, especially on the spinal level, plays a key role in acute and persistent pain modulation (10-12). For example, mice lacking this ion channel exhibited significant impairment in their ability to detect and respond to noxious heat stimuli (9). Intrathecal administration of TRPV1 antagonists reversed the thermal hyperalgesia in inflammatory and neuropathic pain (13-16). Given the evidence above, the present study will investigate the role of spinal TRPV1 in intrathecal injection of ephrinB1-Fc-induced acute thermal pain by administrating the TRPV1 antagonist SB366791 and observing its effects on spinal c-Fos protein expression.

**Materials and Methods**

**Animals and experimental design**

Male, adult Kunming mice, 20–25g, employed in the present study were provided by the Animal Center of Nanjing Medical University. All mice were at their age of ~7 weeks at time of experimental protocols. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Nanjing Medical University and enacted according to the guidelines of the International Association for the Study of Pain (17).

**Drugs and treatments**

EphrinB1-Fc was purchased from R&D Systems Inc., and dissolved in artificial cerebral spinal fluid(aCSF). SB366791, a potent and selective vanilloid receptor antagonist, was purchased from Sigma company, and dissolved in DMSO (DMSO terminal concentration <1% in aCSF). Mice received intrathecal injection of SB 366791 (0.04, 0.20 or 1.00µg) or DMSO followed by ephrinB1-Fc (0.5µg)or aCSF at a time interval of 1 hour. All drugs or vehicles were injected in a volume of 5µl. Mice were...
Intrathecal injection

The intrathecal injection was performed using a micro-syringe following the method previously reported (18, 19). A stainless needle is attached to a 25 µl micro-syringe and inserted between the L5 and L6 vertebrae of conscious mice. A sudden slight flick of the tail indicated the needle entered into the subarachnoid space. Five microliters of drug solution or vehicle was injected over a period of 30 seconds. After injection, there was a wait period of 15 seconds before removing the needle to ensure retention.

Examination of thermal paw withdrawal latency

Paw withdrawal latency was measured with the IITC Plantar Analgesia Meter (IITC Life Science Inc., CA) in a temperature-controlled room (23–2°C). Mice were placed on the glass floor of the plantar analgesia meter in separated transparent acrylic enclosures with lids, and PWL was tested after a 2 hour-habitation. PWL was considered as the time from the start of the radiant heat stimulus to paw withdrawal or licking. The base line was set to 10–12 s by adjusting the heat source intensity, and an automatic 25 s cutoff was used to prevent tissue damage (Plantar analgesia meter parameter: 5025* for cutoff time, 3030* for light intensity). PWL was tested the day before the experiment to determine baseline responses. On the experimental day, PWL was tested at the following time points: 0 hour after SB injection, 0, 2, 4, 8, 12, 24 hour after ephrinB1-Fc injection. The interval between two injections was 1 hour.

Immunohistochemistry

For immunohistochemical experiments, mice were fixed with 4% (vol/vol) paraformaldehyde followed by PBS. Spinal cord were removed and kept in 10% formalin at 4°C for 24 h, then transferred to 30% sucrose for 48 h and sliced into coronal sections using a vibrating blade microtome (Leica Microsystems, model VT1000S). The tissue was blocked in PBS-T (0.3% Triton X-100) including 2% BSA (Bovine Serum Albumins Sigma) and then exposed overnight to antibodies against c-Fos (Cell Signaling Technology, 1:2,000). The sections were then incubated in biotinylated goat anti-rabbit (1:200) followed by avidin–biotin–peroxidase complex (1:100). Finally, the sections were treated with 0.05% diaminobenzidine (DAB), then mounted on gelatin-coated slides, air-dried, dehydrated with 70–100% alcohol, cleared with xylene, and covered for examination. For each animal, one slice was collected from every 5 continuous slices for immunostaining, and all c-Fos-positive neurons in the spinal dorsal horn from 5 slices were used for analysis.

Statistics

All data are expressed as mean ± s.e.m and analyzed with Graph PAD 5.0 software. One-way or Two-way ANOVAs followed by Bonferroni post-test to determine significance. Effects were considered to be significant at P < 0.05.

Results

Intrathecal ephrinB1-Fc induces thermal hyperalgesia and spinal c-Fos activation

To examine the effects of intrathecal ephrinB1-Fc on thermal pain behavior, we injected 0.5 µg of ephrinB1-Fc (in 5 µl) (8), and observed the paw withdrawal latencies to thermal stimulation. We found that injection of spinal ephrinB1-Fc induced a significant decrease of paw withdrawal latencies, which could last for at least 8 hours (Figure 1). Spinal c-Fos protein is a widely-used molecular marker for neuronal activation under acute and chronic pain states, so we examined the c-Fos protein expression with immunohistochemistry to further confirm the pro-hyperalgesia effect of intrathecal ephrinB1-Fc, and found an increased spinal c-Fos protein level in ephrinB1-Fc group when compared with DMSO control group (Figure 2).

SB366791 neutralizes intrathecal ephrinB1-Fc-induced thermal hyperalgesia and spinal c-Fos expression

To test the role of TRPV1 in intrathecal EphrinB1-Fc-induced thermal hyperalgesia, we pretreated mice with three different doses of SB366791(0.04, 0.20 or 1.00μg/5μl), a TRPV1 antagonist, or vehicle (DMSO) 30 minutes before EphrinB1-Fc injection. Behavioral tests shown that SB366791, but not DMSO significant-
ly neutralized intrathecal ephrinB1-Fc-induced thermal hyperalgesia in a dose-dependent manner (Figure 1, for SB1.00 + ephrinB1-Fc group, P<0.05 at 0 hour, P<0.01 at 2 hour, P<0.05 at 4 hour time point after ephrinB1-Fc injection, when compared with DMSO + ephrinB1-Fc group, for SB0.20 + ephrinB1-Fc group, P<0.05 at 2 hour time point when compared with DMSO + ephrinB1-Fc group). However, no significant effects were observed in aCSF + SB1.00 group when compared with DMSO + aCSF group.

To further confirm the behavioral results, we examined the c-Fos protein expression from different groups 2 hours after EphrinB1-Fc injection. Immunohistochemical staining showed that 1.0 µg of SB366791 pretreatment prevented the c-Fos protein activation-induced by intrathecal ephrinB1-Fc (Fig.2A and B, **P<0.01, compared with DMSO + ephrinB1-Fc group). No significance was observed between DMSO + aCSF group and SB1.00 + ephrinB1-Fc group.

Discussion

EphB receptors and their ligands ephrinBs contribute to neuronal development, although their functional roles in the mature nervous system remains largely unknown (2, 3). Recent studies suggest that spinal ephrinB1/EphB1 signaling contributes to pain modulation in animal models (3-8). However, its underlying mechanisms remain largely unknown. TRPV1 is a well-investigated and widely-accepted endogenous transducer of noxious stimulation in different pain models, and has a robust expression in the spinal cord dorsal horn, especially in the superficial laminae (20). In this study, we pretreated intrathecal ephrinB1-Fc mice with three different doses of SB366791, a potent and selective TRPV1 antagonist, to investigate the potential functional role of spinal TRPV1 in ephrinB1/EphB signaling activation-induced acute thermal pain behavior. Behavioral data found that intrathecal ephrinB1-Fc induced significant decreases in thermal paw withdrawal latency, which could last for at least 8 hours and was consistent with recent animal studies. Notably, SB366791 pretreatment markedly prevented this decrease in a dose-dependent manner.

c-Fos protein, the product of the c-fos immediate early gene, has been used as a maker for neuronal activation in the central nervous system for more than 3 decades. c-Fos was found to express robustly in different acute or persistent pain models while relief of pain behaviors would decrease its expression (21). To further confirm the effects of pretreatment with intrathecal SB366791 on intrathecal ephrinB1-Fc-induced thermal pain behavior, we selected 1.00µg SB366791 to observe its effect on spinal c-Fos protein expression and found that pretreatment with 30 minutes before ephrinB1-Fc administration markedly prevented the activation of spinal c-Fos expression at 2 hour time point after ephrinB1-Fc injection. This finding strongly supports the behavioral data. Taken together, this data suggests that spinal TRPV1 might contribute to spinal ephrinB/ephB system activation-induced thermal pain behavior.

TRPV1 is a ligand-gated non-selectively cation channel. When activated, TRPV1 channels are permeable only to cations like Na+, K+, and Ca2+ to produce a net inward current, which depolarizes the membrane and increases the probability of action potential generation. However, we still do not know the contribution of each cations in this process. Increasing translocation of TRPV1 from the cytoplasm to the cell membrane was found in other studies (20). A limitation in this study is that we did not investigate the alternation of TRPV1-mediated current, or the expression and translocation of TRPV1. Future investigation will hope to these issues and the functional role of TRPV1 in other ephrinB/ephB-related pain models.

The present results found that intrathecal injection of SB366791 to block TRPV1 showed potent analgesic effects in spinal ephrinB/EphB signaling activation-induced acute pain. This result suggests that TRPV1 may be a potential downstream effector in spinal ephrinB/ephB system activation-induced thermal pain behavior in the mature nervous system.

References


