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EphrinB2 induces pelvic-urethra reflex potentiation via Src kinase-dependent tyrosine phosphorylation of NR2B

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Wu HC, Chang CH, Peng HY, Chen GD, Lai CY, Hsieh MC, Lin TB. EphrinB2 induces pelvic-urethra reflex potentiation via Src kinase-dependent tyrosine phosphorylation of NR2B. *Am J Physiol Renal Physiol* 300: F403–F411, 2011. First published December 8, 2010; doi:10.1152/ajprenal.00520.2010.—Recently, the role of EphB receptor (EphBR) tyrosine kinase and their ephrinB ligands in pain-related neural plasticity at the spinal cord level have been identified. To test whether Src-family tyrosine kinase-dependent glutamatergic *N*-methyl-D-aspartate receptor NR2B subunit phosphorylation underlies lumbosacral spinal EphBR activation to mediate pelvic-urethra reflex potentiation, we recorded external urethra sphincter electromyogram reflex activity and analyzed protein expression in the lumbosacral (L_6-S_2) dorsal horn in response to intrathecal ephrinB2 injections. When compared with vehicle solution, exogenous ephrinB2 (5 μg/rat it)-induced reflex potentiation, in associated with phosphorylation of EphB1/2, Src-family kinase, NR2B Y1336 and Y1472 tyrosine residues. Both intrathecal EphB1 and EphB2 immunoglobulin fusion protein (both 10 μg/rat it) prevented ephrinB2-dependent reflex potentiation, as well as protein phosphorylation. Pretreatment with PP2 (50 μM, 10 μl it), an Src-family kinase antagonist, reversed the reflex potentiation, as well as Src kinase and NR2B phosphorylation. Together, these results suggest the ephrinB2-dependent EphBR activation, which subsequently provokes Src kinase-mediated *N*-methyl-D-aspartate receptor NR2B phosphorylation in the lumbosacral dorsal horn, is crucial for the induction of spinal reflex potentiation contributing to the development of visceral pain and/or hyperalgesia in the pelvic area.

pelvic pain; urethra; Src-family kinase; *N*-methyl-D-aspartate

IN THE LUMBOSACRAL DORSAL horn, neurotransmission mediated by glutamatergic *N*-methyl-D-aspartate receptors (NMDARs) has been implicated in processing nociceptive afferent inputs coming from the lower urinary tract (4, 10). Spinal administration of NMDAR agonists has dose-dependently facilitated the visceromotor reflex, together with pressor responses to pelvic noxious stimulation (14). Conversely, blockage of NMDAR using pharmacological antagonists has inhibited pain behavior caused by irritation of the pelvic viscera (24, 43). Among subunits of NMDAR, the role of the NR2B subunit in pain development has been intensively investigated, as electrophysiological studies have demonstrated that phosphorylation of specific NR2B tyrosine residues is an important determinant for NMDAR-mediated currents (22), which defines the role of NMDARs in pain-related neural plasticity (3, 17, 18, 20). The signaling of Src kinases, a family of protooncogenic tyrosine kinases, has been shown to modulate NMDAR-mediated synaptic transmission and plasticity (1). In inflammatory animal models, the intrathecal administration of Src-family kinase inhibitors prevented spinal NR2B phosphorylation and behavior hyperalgesia, implying that Src-dependent phosphorylation of NMDAR NR2B subunits plays a crucial role in the development of postinflammatory pain and/or hyperalgesia (35).

EphB receptors (EphBRs), transmembrane molecules, were initially identified as guidance cues during neural development (40). In the last decade, studies have demonstrated that the interactions between EphBRs and their ephrinB ligands modulate neural plasticity induction in the mammalian central nervous system, mainly, but not exclusively, via exhibiting effects on NMDAR (8, 12). A recent study also demonstrated that, in association with thermal hyperalgesia, EphBR activation caused by immunoglobulin fusion protein of ephrinB2 (ephrinB2-Fc)-induced, Src kinase-dependent NR2B phosphorylation in the spinal cord (2), suggesting that ephrinB2-EphBR tyrosine kinase interactions could probably modulate pain signaling via spinal Src-dependent NMDAR phosphorylation (2).

Although further proof is still needed for the physiological and/or pathophysiological relevance, the induction of pelvic-urethra reflex potentiation (15, 16), a form of NR2B phosphorylation-dependent neural plasticity (5, 25, 31, 41), has been linked to the development of visceral pain from pelvic organs (26, 27, 30, 32, 33). Studies have shown that the activation of nociceptive afferent fibers expressing transient receptor potential vanilloid/transient receptor potential ankyrin by the instillation of irritants into the uterus (32, 33) and the descending colon (29) facilitated pelvic-urethra reflex activity in a cross-organ manner. Recently, we reported that acute colonic nociceptive stimulation sensitized pelvic-urethra reflex activity via the upregulation of endogenous ephrinB2 expression, which activates EphB1 and EphB2 receptors and leads to Src-family kinase-dependent NR2B phosphorylation in the lumbosacral spinal cord (26). Accordingly, we hypothesized that the interactions between ephrinB2 and EphBRs, as well as downstream Src-dependent NR2B phosphorylation, are involved in the induction of pelvic-urethra reflex potentiation. We tested this hypothesis with direct spinal application of ephrinB2 and simultaneous recordings, which evoked urethra reflex activity in intact animal preparations, and we analyzed the protein expression/phosphorylation of the lumbosacral spinal cord. Our results dem-

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onstrated that the activation of EphBR can initiate downstream phosphorylation of Src-dependent NMDAR NR2B Y1336 and Y1472 tyrosine residues in the lumbosacral dorsal horn to mediate the induction of pelvic-urethra reflex potentiation, which is presumed to underlie the development of pelvic visceral pain/hyperalgesia.

MATERIALS AND METHODS

Animal Preparations

One hundred and ninety-eight female Sprague-Dawley rats (205–290 g) were used in the present experiment, which was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University in Taiwan. Rats were anesthetized with urethane (1.2 g/kg ip). A PE-10 catheter was inserted through a slit made at the atlanto-occipital membrane and passed caudally to the L_6-S_2 spinal cord, which is the spinal level regulating urogenital system, for the dispensing of test agents. The left pelvic nerve was dissected and mounted on a pair of wire electrodes for stimulation. Oligo-/single unit action potentials in the external urethra sphincter electromyogram (EUSE) activity were recorded by a pair of wire electrodes and were continuously recorded on a recording system (MP30, Biopac, Santa Barbara, CA). Single shocks at a fixed suprathreshold strength were repeated at 1 stimulation/30 s [test stimulation (TS)] and given through the stimulation electrodes.

Application of Drugs

Drugs administered included the following: *N*-methyl-D-aspartic acid (NMDA; 10 μM, 10 μl it, Sigma) (27), a selective glutamatergic NMDAR agonist; D-2-amino-5-phosphonovalerate (APV, 10 μM, 10 μl it, Sigma) (27), a glutamatergic NMDAR antagonist; ephrinB2-Fc chimera (5 μg/rat it, Sigma) (2), EphBR ligand; EphB1-Fc chimera (10 μg/rat it, Sigma) (2), EphB1 selective antagonist; EphB2-Fc chimera (10 μg/rat it, Sigma) (7), EphB2 selective antagonist; and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolol[3,4-d]pyrimidine (PP2, 50 μM, 10 μl it, Tocris) (27), a Src-family kinase inhibitor. In all cases, solvent solutions of identical volume to tested agents were dispensed to serve as the vehicle control.

Western Blotting

After the experimental procedures have been finished, animals were decapitated, and the dorsal half of the left spinal cord segments from L_6-S_2, the spinal level regulating urogenital system, was dissected because the left pelvic nerve was stimulation. Protein samples (20 μg) were separated on SDS-PAGE (8 and 12%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk and probed with antibodies against ephrinB2 (1:2,000, Santa Cruz), phosphorylated EphB1/2 (pEphB1/2, 1:2,000, Milipore), phosphorlated Src (1:1,000, Milipore), or phosphorylated tyr1336/tyr1472-NR2B (1:1,000 Milipore). The blots were incubated with horseradish peroxidase-conjugated antibody (1:2,000) for 1 h at room temperature and visualized with ECL solution (5 min) followed by film exposure (2 min). Densitometric analysis of the WB membranes was done with Science Lab 2003 (Fuji, Japan).

Experimental Protocols

Protocols for assessing effects of various kinds of reagents on reflex activity were as follows.

Protocol 1: Baseline reflex activity. Single shocks of TS (1 stimulation/30 s) were given through stimulation electrodes for 30 min.

Protocol 2: Agonist-induced reflex potentiation After equilibration (usually 30 min), vehicle solution, NMDA, or ephrinB2 was injected via the intrathecal catheter at 2 min before TS onset.

Protocol 3: Pharmacological antagonization. EphB1, EphB2, EphB1 and EphB2, APV, or PP2 was injected intrathecally 5 min before, and then ephrinB2 or NMDA was done 2 min before, TS stimulation onset.

Protocol 4: Effects of NMDA. In some studies, NMDA was intrathecally injected after PP2 (5 min before) and ephrinB2 (2 min before) at 1 min before stimulation onset.

Data Analysis

The reflex excitability was assayed by recording the EUSE resulting from the pelvic afferent nerve stimulations. We, therefore, counted the spike number within 1 min following each shock applied. The responses at 5, 10, 15, and 30 min were offline analyzed and were plotted as a line chart, and the evoked activity at 30 min was used to create the bar charts. The data from each specific time point were averaged from three evoked events in each animal, and then the data from all of the animals in this experiment were averaged in the statistical charts. For the data from the Western blot analysis, the density of specific bands was measured with a computer-assisted imaging analysis system (LAS-300; Fuji, Kanagawa, Japan). After normalized against corresponding loading control bands, the intensity of specific bands was expressed as percentage of loading control. Data were analyzed by using SigmaPlot 10.0 (Systat Software, San Jose, CA). All data in the text and Figs. 1–3 in this study are expressed as means ± SE. For serial measurements over time (i.e., spikes of EUSE and protein expression in response to capsaicin instillation at different time point), two-way repeated-measures ANOVA was used to assess changes in values before and after treatment. In other cases, two-way ANOVA was used to analyze data. In all cases, a post hoc Tukey test was used to compare means for groups when an adequate F ratio was achieved, and significance was assigned at a $P < 0.05$.

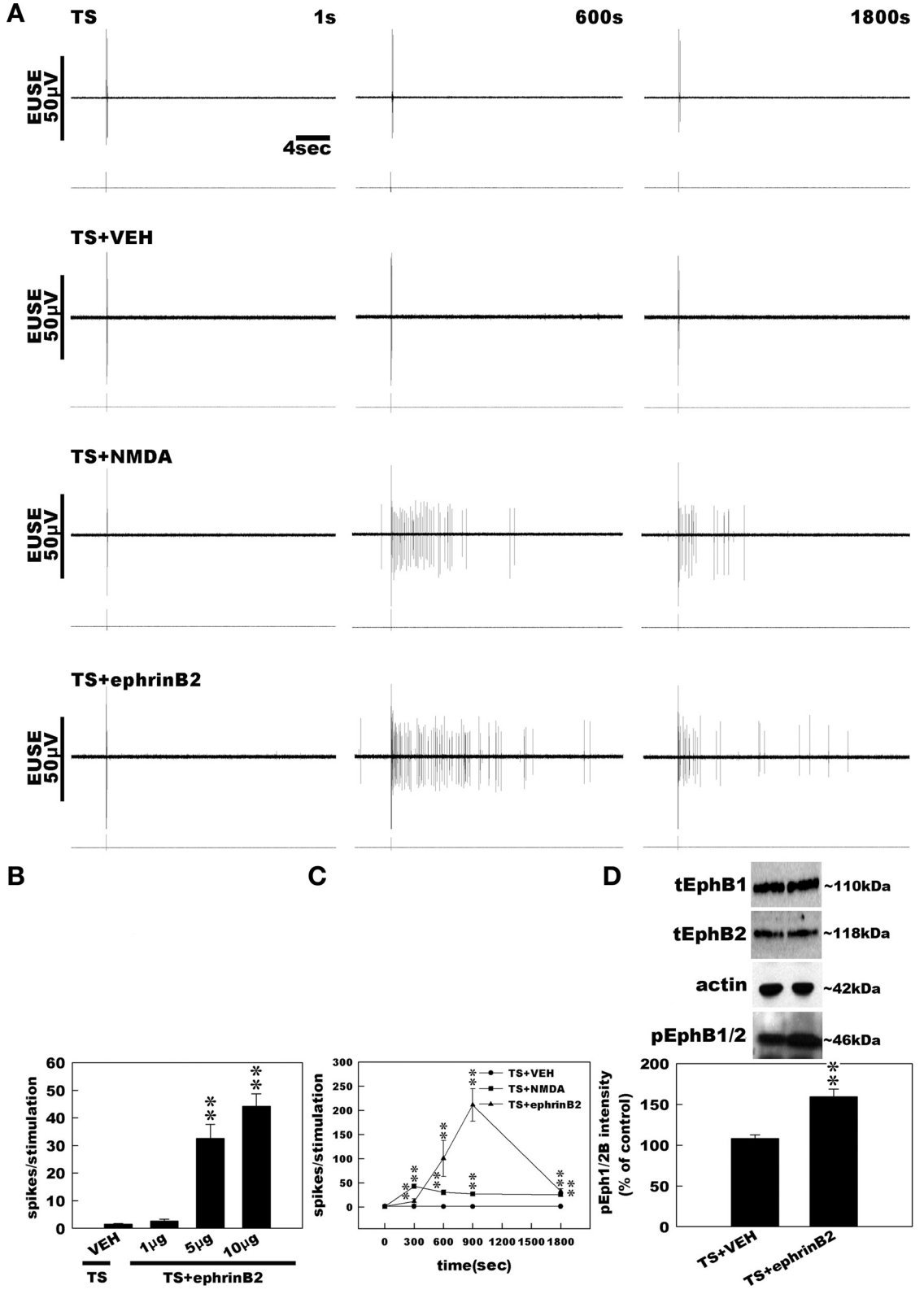
RESULTS

EphrinB2-Induced Reflex Potentiation

Initial experiments were performed in an attempt to establish baseline reflex activity and agonist-induced reflex potentiation in animal preparations. Pelvic afferent nerve TS (1 stimulation/30 s for 30 min) evoked baseline reflex activity with single

Fig. 1. Intrathecal ephrinB2 induces *N*-methyl-D-aspartic acid (NMDA)-dependent pelvic-urethra reflex potentiation. *A*: pelvic afferent nerve test stimulation (TS; 1 stimulation/30 s for 1,800 s) evoked baseline reflex activity with single action potentials in the external urethra sphincter electromyogram (EUSE). While intrathecal vehicle solution (TS + VEH) exhibited no effect on the evoked EUSE activity, intrathecal NMDA (TS + NMDA) and ephrinB2 (TS + ephrinB2) injections both produced reflex potentiation characterized by an elongated firing evoked by each pulse. Tracing shows reflex activity at 1,600 and 1,800 s following stimulation onset. *B*: compared with vehicle solution (VEH), intrathecal ephrinB2 administration (TS + ephrinB2) with concentrations of 1, 5, and 10 μg/rat dose-dependently increased the mean spike count evoked by TS. $^{**}P < 0.01$ to VEH, $n = 7$. *C*: vehicle solution exhibited no effect on the baseline reflex activity with single spike (TS + VEH). Intrathecal NMDA administration statistically increased the mean spike count evoked by TS during the stimulation period (TS + NMDA). $^{**}P < 0.01$ to TS + VEH, $n = 7$. Spinal ephrinB2 injection significantly increased the mean spike count that peaked at 15 min following TS onset and then gradually faded out toward baseline (TS + ephrinB2). $^{**}P < 0.01$ to TS + VEH, $n = 7$. *D*: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection statistically enhanced the expression of phosphorylated EphB1/2 (pEphB1/2), but not total EphB1 (tEphB1), tEphB2, or β-actin (EphB1, EphB2, and actin, respectively) in the lumbosacral (L_6-S_2) dorsal horn obtained from animals that received TS (TS + ephrinB2). $^{**}P < 0.01$ to TS + VEH, $n = 4$.

action potentials in the EUSE throughout the stimulation period (Fig. 1*A*, TS). Intrathecal administration of NMDA (10 μM, 10 μl, 2 min before TS onset, Fig. 1, *A* and *C*, TS + NMDA), but not vehicle solution (TS + VEH), produced reflex potentiation characterized by an elongated firing evoked by each pulse. We then tested whether spinal administration of ephrinB2 could also induce reflex potentiation or not. Electrophysiological recordings in Fig. 1*A* show the intrathecal ephinrB2 injection (5 μg/rat, 2 min before TS onset, TS + ephrinB2) provoked reflex potentiation, peaking at 15 min



following TS onset and then gradually faded out toward the baseline. We then injected ephrinB2 with concentrations of 1, 5, and 10 μg/rat. Compared with vehicle solution, intrathecal ephrinB2 administrations with these concentrations increased the mean spike count evoked by the TS in a dose-dependent manner (Fig. 1*B*).

Eephrin B2 Provokes Spinal EphB1/2 Phosphorylation

We then used immunoblotting to analyze whether ephrinB2 administration induces spinal EphB1/2 phosphorylation. When compared with vehicle solution (Fig. 1*D*, TS + VEH), the intrathecal ephrinB2 injection (5 μg/rat, TS + ephrinB2) statistically enhanced the expression level of pEphB1/2 (pEphB1/2 to EphB1 and EphB2) in the lumbosacral dorsal horn (L_6-S_2) of animals that received the TS, while levels of total EphB1, total EphB2, and β-actin (EphB1, EphB2, and actin, respectively) remained unchanged.

Involvement of EphB1 and EphB2 Receptors

The role of spinal EphB1 and EphB2 receptors in ephrinB2-dependent reflex potentiation was tested by administering selective Eph1B and Eph2B receptor antagonists, Eph1B-Fc and Eph2B-Fc, respectively. As described, intrathecal ephrinB2 (5 μg/rat, Fig. 2, *A* and *E*, TS + ephrinB2), but not vehicle solution (TS + VEH), induced reflex potentiation characterized by an elongated firing in animal preparations. Rather than vehicle solutions (data not shown), prior administration of EphB1-Fc (10 μg/rat it, 5 min before stimulation onset) and EphB2-Fc (10 μg/rat it, 5 min before stimulation onset) both prevented ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (TS + EphB1 + ephrinB2 and TS + EphB2 + ephrinB2, respectively), whereas neither EphB1-Fc nor EphB2-Fc affected the TS-evoked baseline reflex activity (Fig. 2, *A* and *D*, TS + Eph1B and TS + Eph2B, respectively). The participation of EphB1/2 in ephrinB2-dependent reflex potentiation was further investigated by immunoblotting analysis. Compared with vehicle solution (Fig. 3*A*, TS + VEH), intrathecal ephrinB2 (5 μg/rat, bar: ephrinB2, TS) significantly upregulated the expression of pEphB1/2 (pEphB2) in the lumbosacral dorsal horn without affecting the levels of total EphB1 and total EphB2 (EphB1 and EphB2, respectively). Prior EpB1-Fc and EphB2-Fc administrations prevented EphB1/2 phosphorylation caused by ephrinB2 by decreasing pEphB1/2 expression (TS + EphB1 and TS + EphB2, respectively).

Involvement of NMDAR

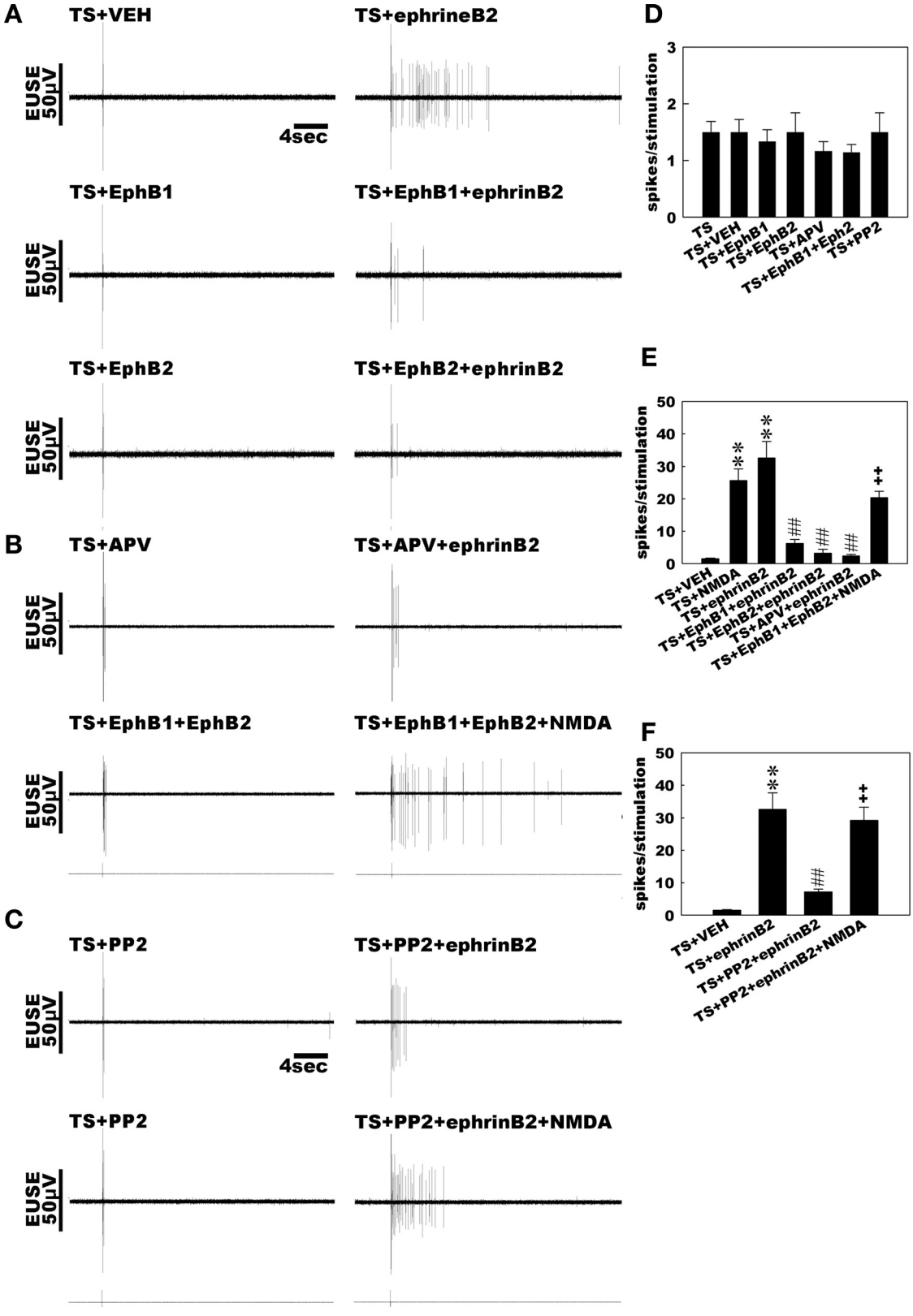
Then we investigated the role of the interactions between the EphB1/2 receptor and NMDAR in ephrinB2-dependent reflex potentiation. Compared with the animals that received the ephrinB2 injection (5 μg/rat, Fig. 2, TS + ephrinB2), the intrathecal APV (10 μM, 10 μl) injection prevented the ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (Fig. 2, *B* and *E*, TS + APV + ephrinB2). Furthermore, after both EphB1 and EphB2 had been blocked by the coadministration of selective antagonists, Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2), intrathecal NMDA injection (10 μM, 10 μl) provoked reflex potentiation characterized by an elongated firing in these preparations (TS + EphB1 + EphB2 + NMDA). On the other hand, immunoblotting showed that prior APV administration failed to prevent ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral dorsal horn (Fig. 3*A*, TS + APV).

Involvement of Src-Family Kinases

The role of Src-family kinases in ephrinB2-dependent reflex potentiation was studied using a selective antagonist, PP2. Electrophysiological recordings showed that, while pretreatment with PP2 (50 μM, 10 μl it) exhibited no effects on the TS-evoked baseline reflex activity (TS + PP2), it prevented ephrinB2-dependent reflex potentiation by decreasing the mean spike count evoked by the TS (Fig. 2, *B* and *F*, TS + PP2 + ephrinB2). Moreover, after ephrinB2-dependent reflex potentiation had been abolished by PP2, intrathecal NMDA reversed PP2-induced antagonization by increasing the mean spike count (TS + PP2 + ephrinB2 + NMDA). The role of Src-family kinases was further investigated using Western blotting analysis. Although ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral spinal cord was not antagonized by prior PP2 injection (Fig. 3*A*, TS + PP2), in the animals that received

Fig. 2. EphB1 and EphB2 receptors are involved in ephrinB2-dependent pelvic-urethra reflex potentiation. *A*: while intrathecal vehicle solution (TS + VEH) exhibited no effect on the baseline EUSE activity evoked by pelvic afferent nerve TS (1 stimulation/30 s for 30 min), intrathecal ephrinB2 administration (TS + ephrinB2) produced reflex potentiation characterized by an elongated firing evoked by each pulse. Neither prior administration of EphB1-Fc (10 μg/rat it) nor EphB2-Fc (10 μg/rat it) affected the baseline reflex activity evoked by the TS (TS + Eph1B and TS + Eph2B, respectively), whereas both reagents prevented ephrinB2-dependent reflex potentiation (TS + EphB1 + ephrinB2 and TS + EphB2 + ephrinB2, respectively). *B*: intrathecal pretreatment with D-2-amino-5-phosphonovalerate (APV) (10 μM, 10 μl) displayed no effect on the TS-evoked baseline reflex activity (TS + APV), but prevented ephrinB2-dependent reflex potentiation (TS + APV + ephrinB2). Intrathecal NMDA injection provoked reflex potentiation (TS + EphB1 + EphB2 + NMDA), even though EphB1 and EphB2 receptors were both blocked by coadministration of Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2). *C*: while it exhibited no effects on the TS-evoked baseline reflex activity (TS + PP2), intrathecal pretreatment with PP2 (50 μM, 10 μl) prevented ephrinB2-dependent reflex potentiation (TS + PP2 + ephrinB2). The PP2-induced antagonization on ephrinB2-dependent reflex potentiation was reversed by spinal NMDA administration (TS + PP2 + ephrinB2 + NMDA). *D*: the mean spike count of EUSE activity evoked by pelvic afferent TS was not affected by all of the test agents, including vehicle solution (TS + VEH), Eph1B-Fc (TS + EphB1), EphB2-Fc (TS + EphB2), APV (TS + APV), PP2 (TS + PP2), as well as coadministration of Eph1B-Fc and Eph2B-Fc (TS + EphB1 + EphB2, all $P > 0.05$ to TS; $N = 7$). *E*: compared with vehicle solution (TS + VEH), intrathecal NMDA (TS + NMDA, $^{**}P < 0.01$ to TS+VEH, $n = 7$) and ephrinB2 (TS + ephrinB2, $^{**}P < 0.01$ to TS+VEH, $n = 7$) both significantly increased the mean spike count evoked by TS. The ephrinB2-dependent increment in spike count was reversed by prior administration of Eph1B-Fc (TS + EphB1, $\#\#P < 0.01$ to TS + ephrinB2, $n = 7$), EphB2-Fc (TS + EphB2, $\#\#P < 0.01$ to TS + ephrinB2, $n = 7$), and APV (TS + APV, $\#\#P < 0.01$ to TS + ephrinB2, $n = 7$). Spinal NMDA antagonized the reversal of the ephrinB2-dependent spike increment caused by coadministration of EphB1-Fc and EphB2-Fc (TS + EphB1 + EphB2 + NMDA, $^{++}P < 0.01$ to TS + ephrinB2, $n = 7$). *F*: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 (TS + ephrinB2, $^{**}P < 0.01$ to TS + VEH, $n = 7$) significantly increased the mean spike count evoked by TS. The ephrinB2-dependent increment in spike count was reversed by prior administration of PP2 (TS + PP2 + ephrinB2, $\#\#P < 0.01$ to TS + ephrinB2, $n = 7$). Spinal NMDA antagonized the reversal of ephrinB2-dependent spike increment caused by PP2 (TS + PP2 + NMDA, $^{++}P < 0.01$ to TS + ephrinB2, $n = 7$).

the TS, immunoblotting showed that the expression level of phosphorylated Src (Fig. 3*B*) but not total Src had increased significantly due to the intrathecal ephrinB2 administration (5 μg/rat, bar: ephrinB2 TS) compared with those given vehicle solution (TS + VEH). Pretreatment with EpB1-Fc, EphB2-Fc, and PP2 (TS + EphB1, TS + EphB2, and TS + PP2, respectively) but not APV (TS + APV) prevented ephrinB2-dependent Src phosphorylation in the lumbosacral dorsal horn.



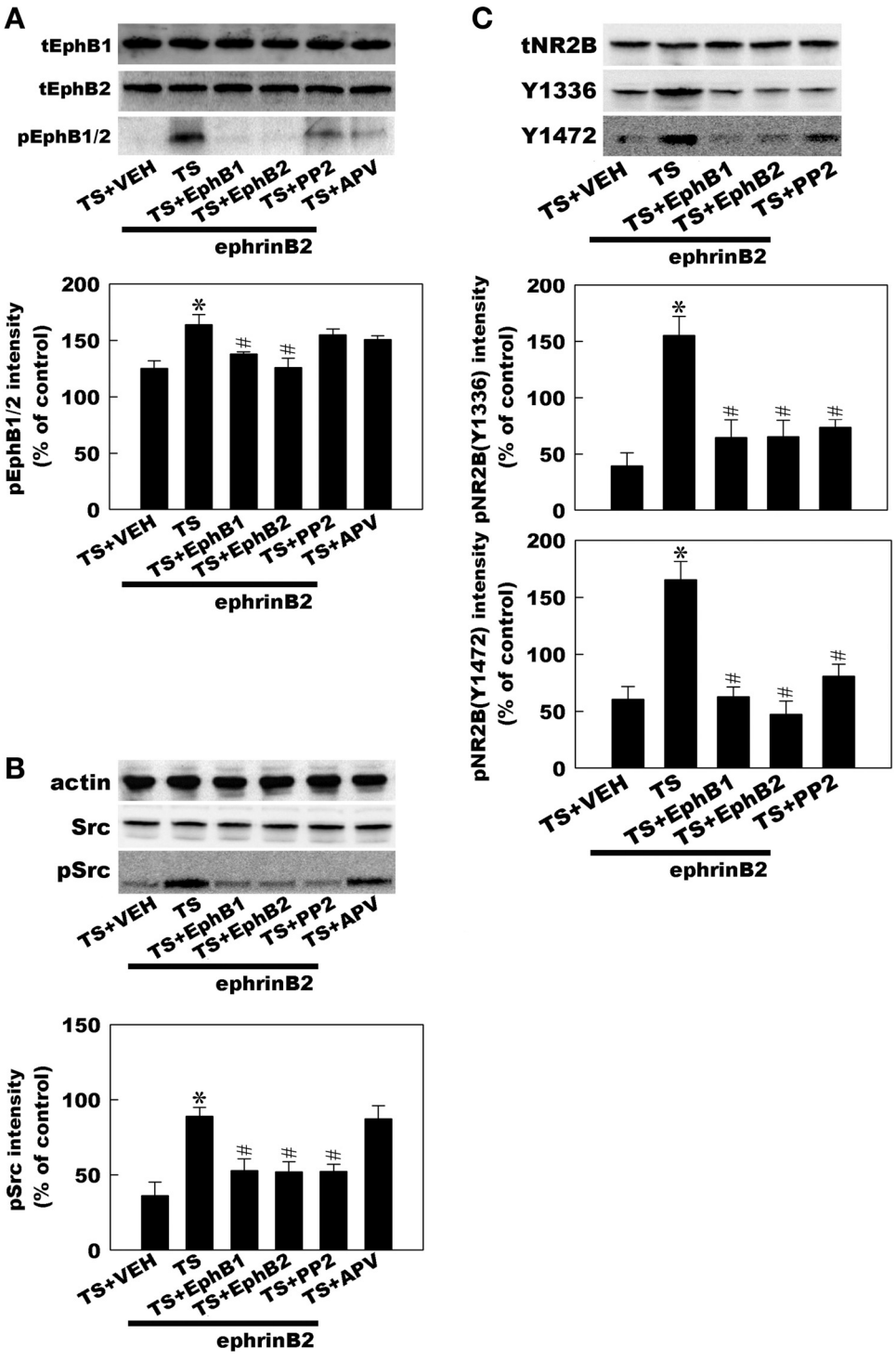


Fig. 3. Src-dependent NR2B phosphorylation mediates ephrinB2-dependent EphB1/2 phosphorylation in the lumbosacral (L_6-S_2) dorsal horn. *A*: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of pEphB1/2, but not tEphB1 and tEphB2, in the lumbosacral dorsal horn obtained from animals that received TS. $*P < 0.05$ to TS+VEH, $n = 4$. Prior administration of EpB1-Fc (TS + EphB1, $\#P < 0.05$ to TS, $n = 4$) and EphB2-Fc (TS + EphB2, $\#P < 0.05$ to TS, $n = 4$), but not PP2 (TS + PP2, $P > 0.05$ to TS, $n = 4$) and APV (TS + APV, $P > 0.05$ to TS, $n = 4$), prevented ephrinB2-dependent EphB1/2 phosphorylation. *B*: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of phosphorylated (pSrc) but not total Src in the lumbosacral dorsal horn obtained from animals that received TS. $*P < 0.05$ to TS + VEH, $n = 4$. Prior administration of EpB1-Fc (TS + EphB1, $\#P < 0.05$ to TS, $n = 4$), EphB2-Fc (TS + EphB2, $\#P < 0.05$ to TS, $n = 4$), and PP2 (TS + PP2, $\#P < 0.05$ to TS, $n = 4$), but not APV (TS + APV, $P > 0.05$ to TS, $n = 4$), prevented ephrinB2-dependent Src phosphorylation. *C*: compared with vehicle solution (TS + VEH), intrathecal ephrinB2 injection (bar: ephrinB2) statistically enhanced the expression of phosphorylated Y1336 and Y1472 residues of NR2B, but not total NR2B (tNR2B), in the lumbosacral dorsal horn obtained from animals that received TS. $*P < 0.05$ to TS + VEH, $n = 4$. Prior administration of EpB1-Fc (TS + EphB1, $\#P < 0.05$ to TS, $n = 4$), EphB2-Fc (TS + EphB2, $\#P < 0.05$ to TS, $n = 4$), and PP2 (TS + PP2, $P > 0.05$ to TS, $n = 4$) prevented ephrinB2-dependent EphB1/2 phosphorylation.

The Role of NMDAR NR2B Subunit

The involvement of Src-mediated phosphorylation of NR2B Y1336 and Y1472 tyrosine residues in ephrinB2-dependent reflex potentiation was explored using Western blotting analysis. Compared with intrathecal vehicle solution (Fig. 3*C*, TS + VEH), ephrinB2 enhanced the expression of phosphorylated Y1336 and phosphorylated Y1472 without affecting the level of total NR2B in the lumbosacral dorsal horn of animals that received the TS. All of the prior EpB1-Fc, EphB2-Fc, and PP2 treatments (TS + EphB1, TS + EphB2, and TS + PP2, respectively) prevented ephrinB2-dependent NR2B Y1336 and Y1472 phosphorylation in the lumbosacral spinal cord.

DISCUSSION

In this study, we found ephrinB2 administration in the lumbosacral spinal cord induced pelvic-urethra reflex potentiation with corroboration by EphB1/2, Src-family kinases, as well as phosphorylation of NR2B Y1336 and Y1472 tyrosine

residues. Pretreatment with EphBR selective antagonists prevented agonist-induced reflex potentiation and all protein phosphorylation. Intrathecal application of a Src-family kinase inhibitor blocked reflex potentiation, which occurred with a reversal in phosphorylation of Src kinase and NR2B tyrosine residues. Taken together, these results suggest that induction of pelvic-urethra reflex potentiation caused by spinal ephrinB2 administration may be, at least in part, due to the phosphorylation of specific tyrosine residues of the NMDAR NR2B subunit caused by a Src-family tyrosine kinase downstream of EphBR activation at the lumbosacral spinal cord level.

It has been shown that interactions between ephrinB2 and EphBRs in the spinal cord are required for the onset of somatic pain and hyperalgesia. Following nerve injury, the expression of ephrinB2 in dorsal root ganglion (DRG) neurons and the EphB1 receptor in the dorsal horn were both enhanced in a time-dependent manner corresponding to the development of thermal hyperalgesia in adult rats (36, 37). Knock-down of ephrinB2 using specific short interfering RNA decreased expression of ephrinB2 in the DRG, along with attenuation of mechanical allodynia caused by spinal nerve crushing (13). Intrathecal administration of ephrinB2-Fc chimera to Wistar rats induced behavioral evidence of thermal hyperalgesia (35). Conversely, pharmacological antagonization of EphB1 prevented nerve injury-induced dorsal horn neuron hyperexcitability and neuropathic pain (37). Not only in somatic pain caused by injury or inflammation, our study investigating cross talk between pelvic viscera has demonstrated that acute activation of transient receptor potential vanilloid 1 expressing nociceptive afferent fibers coming from the descending colon upregulated spinal ephrinB2 expression and simultaneously induced EphB1/2 phosphorylation in the lumbosacral dorsal horn (28). In analogy with this study, results showed spinal administration of ephrinB2 induced lumbosacral EphB1/2 phosphorylation in association with pelvic-urethra reflex potentiation, a phenomenon presumed to participate in the development of pain and/or hyperalgesia in the lower urinary tract. Pharmacological antagonization of EphB1 and EphB2 receptors prevented EphB1/2 phosphorylation and ephrinB2-dependent reflex potentiation. Together, these results suggest that the ephrinB2-EphBR interactions in the lumbosacral spinal cord are required for the onset of visceral pain in the pelvic area.

In the spinal dorsal horn, NMDARs integrate primary afferent inputs and provide mechanisms to amplify nociceptive signals, leading to the development of neuropathic and/or postinflammatory pain (6, 19, 40). Although the exact role of specific NMDAR subunits is still unclear, functional NMDARs are mostly heteromeric complexes composed of NR1/NR2 subunits in the mammalian central nervous system (21). The NR1 subunit is obligatory for NMDARs, whereas NR2 subunits are essential for calcium ion gating (34), which demonstrates the functional diversity of NMDARs (7, 11). An immunohistochemical study investigating the development of neuropathic pain has demonstrated that, in the superficial lamina of the dorsal horn, NR2B phosphorylation at Tyr1472 is crucial for the development of hyperalgesia (9). Using antibody labeling of specific tyrosine residues, our data in the present study showed that the spinal ephrinB2 injection induced NR2B phosphorylation at residues Tyr1336 and Tyr1472, along with urethra reflex potentiation. We propose a possible role of NMDAR NR2B phosphorylation plays a role in the induction of visceral pain. In further support of this proposal, a recent study showed NMDAR NR2B phosphorylation in the lumbosacral spinal cord is essential for cross-organ sensitization caused by acute viscera irritation (28). However, our data were obtained from antibody-specific experiments, but there are no other findings to corroborate our results. Further studies are needed to clarify the role of phosphorylation in particular tyrosine residues in spinal reflex potentiation. Moreover, besides Tyr1336 and Tyr1472, there are 23 tyrosine residues in the carboxyl tail of the NR2B subunit that could be phosphorylated (1). Due to the limitations of this study, it could not be elucidated whether there are other tyrosine residues involved in ephrinB2-dependent reflex potentiation. Through Src-family kinase-dependent modulations of NMDAR, the interaction between EphBR tyrosine kinases and their ephrinB ligands has been shown to play a crucial role in pain processing at the spinal cord level (2, 35). Intrathecal administration of exogenous ephrinB2, which activates EphBR, induced behavioral thermal hyperalgesia, and increments in the expression of phosphorylated Src-EphBR complex were both counteracted by pretreatment with NMDAR antagonist (35). Conversely, an Src-family inhibitor has been shown to reverse ephrinB2-induced thermal hyperalgesia and NR2B phosphorylation in the spinal dorsal horn (2). These results are consistent with our finding that intrathecal ephrinB2 induced reflex potentiation in association with Src-family kinases and NMDAR NR2B phosphorylation in the lumbosacral spinal cord. Pharmacological blockage of Src-family kinase activity prevented reflex potentiation and NR2B phosphorylation, indicating NMDAR NR2B phosphorylation downstream of Src-family kinase activation mediated pain-related spinal neural plasticity. However, Src-family kinases may target NMDAR subunits other than NR2B (23); therefore, the role of other NMDAR subunit(s), e.g., NR2A, in ephrinB2-dependent reflex potentiation, cannot be ruled out.

Studies have demonstrated that an intrathecal EphBR activator can induce thermal hyperalgesia, and mechanical allodynia has been correlated with a reduced long-term spinal potentiation threshold between nociceptive C afferent fibers and dorsal horn neurons in naive animals. Conversely, pharmacological blocking of EphBR using EphB-Fc prevents nerve injury-induced thermohyperalgesia and mechanical allodynia and reverses enhanced long-term potentiation. This suggests that ephrinB2/EphB effects on neuropathic pain depend on regulation of both neuronal excitability and spinal synaptic plasticity (37). In clinical scenarios, syndromes of patients being treated, such as irritable bowel syndrome and chronic pelvic pain, are usually chronic conditions. In the present study, we demonstrated the onset of a spinal EphBR-dependent reflex potentiation 10 min after an ephrinB2 injection, which lasted for at least 30 min. Although this study offers an animal model to investigate the spinal neural mechanism underlying viscero-visceral pain, it is limited by insult acuity and the subsequent measurement interval. On the other hand, behavior studies that investigated neuropathic pain caused by sciatic nerve ligation showed time-dependent upregulation of EphrinB and EphBR in DRG and dorsal horn neurons, which peaks at 7 days and lasts for 21 days after nerve ligation (2, 36). Further studies are needed to determine whether the ephrinB2-dependent reflex potentiation plays a role in the induction of acute injury/inflammation pain and eventually contributes to the

maintenance of a pain condition or the development of hyperalgesia/allodynia by establishing pathological neural plasticity.

In conclusion, our findings add to the understanding of the ephrin/EphBR system, which is an important player in spinal reflex potentiation, acting as a modulator of NMDAR in the adult spinal cord in vivo. These results help bring to light the mechanisms underlying reflex potentiation in the spinal cord, which is considered essential to the induction of visceral pain.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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