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Estrogen-dependent facilitation on spinal reflex potentiation involves the Cdk5/ERK1/2/NR2B cascade in anesthetized rats

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¹Department of Physiology, ⁴Graduate Institute of Medicine, College of Medicine, ³Department of Obstetrics and Gynecology, Chung-Shan Medical University Hospital, Chung-Shan Medical University; ²Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University; ⁵Department of Physical Therapy, Graduate Institute of Rehabilitation Science, China Medical University; ⁶Department of Healthcare Administration, Asia University, Taichung; ⁷Medical Department, Saint Paul's Hospital, Taoyuan; and ⁸Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan

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Peng HY, Chen GD, Tung KC, Chien YW, Lai CY, Hsieh MC, Chiu CH, Cheng HL, Lee SD, Lin TB. Estrogen-dependent facilitation on spinal reflex potentiation involves the Cdk5/ERK1/2/NR2B cascade in anesthetized rats. *Am J Physiol Endocrinol Metab* 297: E416–E426, 2009. First published June 16, 2009; doi:10.1152/ajpendo.00129.2009.—Cyclin-dependent kinase-5 (Cdk5), a proline-directed serine/threonine kinase, may alter pain-related neuronal plasticity by regulating extracellular signal-related kinase-1/2 (ERK1/2) activation. This study investigated whether Cdk5-dependent ERK activation underlies the estrogen-elicited facilitation on the repetitive stimulation-induced spinal reflex potentiation (SRP) that is presumed to be involved in postinflammatory/neuropathic hyperalgesia and allodynia. Reflex activity of the external urethra sphincter electromyogram evoked by pelvic afferent nerve test stimulation (TS; 1 stimulation/30 s for 10 min) and repetitive stimulation (RS; 1 stimulation/1 s for 10 min) was recorded in anesthetized rats. TS evoked a baseline reflex activity, whereas RS produced SRP. Intrathecal (it) β -estradiol facilitated the repetitive stimulation-induced SRP that was reversed by pretreatment with the estrogen receptor antagonist ICI 162,780 (10 nM, 10 μ l it), Cdk5 inhibitor roscovitine (100 nM, 10 μ l it), ERK inhibitor (U-0126; 100 μ M, 10 μ l it) and *N*-methyl-D-aspartate (NMDA) NR2B subunit antagonist (Co-101244; 100 nM, 10 μ l it). Moreover, ER α (propylpyrazoletriol; 100 nM, 10 μ l it) and ER β (diarylpropionitrile; 100 μ M, 10 μ l it) agonists both facilitated the SRP, similar to results with a β -estradiol injection. In association with the facilitated RS-induced SRP, an intrathecal β -estradiol injection elicited ERK1/2 and NR2B subunit phosphorylation that were both reversed by intrathecal roscovitine and U-0126. These results indicated that the Cdk/ERK cascade, which is activated by ER α and ER β , may subsequently phosphorylate the NR2B subunit to develop NMDA-dependent postinflammatory hyperalgesia and allodynia to maintain the protective mechanisms of the body.

estradiol; cyclin-dependent kinase-5; NR2B; extracellular signal-related kinase; pelvic pain; spinal reflex potentiation; hyperalgesia

PAIN IS A PROTECTIVE MECHANISM required for the survival and the maintenance of the integrity of organism. However, sustained or chronic pain can result in secondary symptoms, such as anxiety and depression, that reduce life quality (30). Convincing evidence demonstrates that postinflammatory hyperalgesia and tactile allodynia are associated with the development of

activity-dependent hyperexcitability in the spinal cord (19, 91, 92), a process that shares common characteristics with glutamatergic *N*-methyl-D-aspartate (NMDA)-dependent neural plasticity in other brain areas (2, 27, 53, 76). Although specific molecules regulating nociception have been intensively investigated, the underlying molecular mechanisms remain undefined. So far, studies using genetic modification, antisense knock-down, and gene expression assays have identified several genes, whose expression levels are affected during pain sensation and/or are involved in modulation of pain (58). As a result, the list of proteins encoded by these genes has expanded gradually, and further investigation of their participation in pain pathway is required (64).

The extracellular signal-regulated kinase-1/2 (ERK1/2) pathway is a central cellular signaling pathway linking numerous extracellular signals to membrane receptors activated by extracellular stimuli (39, 81, 82, 84, 88, 94, 95). Glutamate-sensitive ERK phosphorylation is seen in brain areas (25, 41, 55, 68) and spinal dorsal horn neurons (33, 37, 40, 93). Electrophysiological studies have indicated the involvement of ERK in the induction of activity-dependent neural plasticity (8, 17, 20, 31, 38, 67, 83). In hippocampal long-term potentiation (LTP), it is accepted that the ERK pathway is activated by the calcium influx through both glutamatergic NMDA receptors and voltage-gated calcium channels which subsequently regulate gene expression crucial for LTP induction (31, 83, 85). In pain-related plasticity, ERK activation in nociceptive neurons increases various kinds of immediate early gene transcription to cause a transition from short-term adaptive processes to long-term hyperexcitability (19, 35, 57, 90) leading to the development of hyperalgesia and allodynia (1, 18, 54, 59, 61).

Cyclin-dependent kinase-5 (Cdk5) is a proline-directed serine/threonine kinase that plays essential roles in the development of the mammalian nervous system, including neuronal migration and neurite outgrowth (9, 29, 44, 60, 86), whereas Cdk5 deregulation by p25 has been implicated in degenerative neurological diseases such as Alzheimer's disease (66).

It has been shown that the expression of Cdk5 protein levels in dorsal root ganglion (DRG) neurons was altered in response to peripheral inflammation (64). Cdk5 knockout pups were unresponsive to noxious cutaneous pinch (24), implying that Cdk5 expression is closely related to nociceptive neurotransmission. Moreover, Cdk5 may alter pain-related neuronal plasticity (80) by regulating ERK1/2 activation through ERK phosphorylation at Thr²⁸⁶ in DRG neurons (65).

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At the lumbosacral spinal cord, our laboratory has recently demonstrated a novel form of neural plasticity, the spinal reflex potentiation (SRP) (48–50, 72), in which the reflex activity of the urethra could be sensitized by the activation of capsaicin-sensitive (75), mustard oil-sensitive (73), and transient receptor potential vanilloid subfamily member 1 (TRPV1)-sensitive (74) afferent fibers. Pharmacological investigations on SRP have demonstrated that, similar to other forms of neural plasticity, glutamatergic NMDA-dependent (11–14, 46, 47) ERK activation that subsequently phosphorylates the NMDA NR2B subunit (70–73), a subunit that is presumed to be essential for Ca^{2+} gating, to define the role of the NMDA receptor for dynamic synaptic plasticity (52, 69), underlies the induction of SRP. Although the physiological/pathophysiological relevance is still being sought for final proof, the induction of SRP has been linked to the development of neuropathic/postinflammatory visceral pain in the pelvic area, for it is characterized by pathological enhancement in urethra activity caused by the activation of nociceptive afferent fibers. (12, 14, 46, 47, 71).

A number of chronic pain syndromes are more prevalent in women than in men (3, 87). Moreover, the severity of symptoms in women fluctuates with the menstrual cycle (16, 22, 23, 43), suggesting that gonadal steroid levels impact pain severity (36). It is well established that estrogen may modulate neural responses within the central nervous system (79). In addition to traditional genomic effects, 17β -estradiol may also activate the ERK pathway (39, 81, 82, 94, 95) and be involved in activity-dependent synaptic plasticity (15, 56, 83, 78). Electrophysiological evidence has demonstrated that estrogen might activate the ERK pathway, involving the rapid effect on LTP through phosphorylation of the NR2B subunits of NM $'$. We have demonstrated an estrous cycle-dependent facilitation of SRP induction (71). Moreover, ovariectomy attenuated the induction of SRP (48). However, the possibility that Cdk5 may mediate the estrogen-dependent modulations on the SRP through the ERK/NR2B pathway has yet been clarified.

Therefore, we investigate the role of the Cdk5-dependent ERK/NR2B pathway in the estrogen-elicited facilitation of SRP, which is presumed to be related to the development of hyperalgesia and allodynia using *in vivo* animal preparations in this study.

EXPERIMENTAL PROCEDURES

Animal preparations. Sixty-three adult female Wistar rats weighing 250–350 g were anesthetized with urethane (1.2 g/kg ip). Urethane was chosen because it lacks ganglionic blocking properties and allows neural inputs to/from the viscera to be maintained. The National Science Council of Taiwan approved the animal care and experimental protocols in this study. The trachea was intubated to keep the airway clear. A PE-50 catheter (Portex; Hythe, Kent, UK) was placed in the left femoral vein for administration of anesthetics when needed. A midline abdominal incision was made to expose the pelvic viscera. Both ureters were ligated distally and cut proximally to the sites of ligation. The proximal ends of the ureters drained freely within the abdominal cavity. A wide-bore cannula was inserted into the lumen of the urinary bladder at the apex of the bladder dome and was secured with cotton thread. The open end of the cannula drained freely to avoid urine accumulation within the urinary bladder, which could affect the reflex activity (Fig. 1A). Body temperature was kept at 36.5–37.0°C by infrared light and was monitored using a rectal thermometer. The rats were monitored for a corneal reflex and a response to noxious stimulation to the paw throughout the course of

the experiment. If responses were present, a supplementary dose of urethane (0.4 g/kg iv) was given through the venous catheter. When the experiments were completed, the animals were euthanized via an intravenous injection of potassium chloride saturation solution.

Intrathecal catheter. The occipital crest of the skull was exposed, and the atlantooccipital membrane was incised at the midline with the tip of an 18-gauge needle. A PE-10 catheter was inserted through the slit and passed caudally to the dorsal arachnoid space at the T13 vertebra level. The volume of fluid within the cannula was kept constant at 10 μ l in all experiments. A single 10- μ l volume of drug solution was administered followed by a 10- μ l flush of artificial cerebrospinal fluid. At the end of each experiment, the location of the injection site was marked by an injection of Alcian blue (10 μ l, 2%), and a laminectomy was performed to verify the location of the cannula tip. The volume of drug injected into the spinal cord in this experiment has been reported to spread from 0.5 to 1.5 mm from the site of injection, as described previously (13). The data obtained from animals whose cannula tip deviated by more than 0.5 mm from the upper and lower limits of the dorsal aspect of the arachnoid space along L6 to S2 were excluded from the statistical analysis.

Nerve dissection. The right pelvic nerve was carefully dissected from the surrounding tissues and was transected distally for stimulation, while the left pelvic nerve was kept intact (Fig. 1A). Then the central stump of the transected nerve was mounted on a pair of bipolar stainless steel wire electrodes for stimulation. Single shocks with pulse durations of 1 ms were applied to the pelvic afferent nerve from a stimulator (Grass S88, Cleveland, OH) through an isolation unit (Grass SIU 5B) and a constant current unit (Grass CCU1A). The stimulated nerve and the electrodes were bathed in a pool of warm paraffin oil (37°C) to prevent drying.

Electromyogram recording. Epoxy-coated copper wire electromyogram electrodes (50 μ m; M.T. Giken, Tokyo, Japan) were placed in the external urethra sphincter. The placement of the electrodes was performed using a 30-gauge needle with a hooked electromyogram electrode positioned at the tip (1.0–1.5 mm). The needle was inserted into the sphincter ~1–2 mm lateral to the urethra and then withdrawn, leaving the electromyogram wire embedded in the sphincter. The external urethral sphincter electromyogram (EUSE) activities were amplified 20,000-fold and filtered (high-frequency cut-off at 3,000 Hz and low at 30 Hz, respectively) by a preamplifier (Grass P511AC). Electromyogram activities were continuously displayed on an oscilloscope (TDS 3014; Tectronics, Wilsonville, OR) and a recording system with a sampling rate of 20,000 Hz (MP30; Biopac, Santa Barbara, CA).

Experimental protocols. The excitability of the reflex activities was assessed by recording the numbers of action potentials in the electromyogram under test stimulation or repetitive stimulation with/without the intrathecal application of the tested agents. At the beginning of all experiments, we manipulated the stimulation intensity from 10 to 15 V, and an electric intensity that caused a single spike action potential in the reflex activity was used to standardize the baseline reflex activity. This intensity was then used for stimulation throughout each experiment. The protocol for assessing the effects of electrical stimulation and different kinds of reagents on the reflex activity was as follows. 1) Test stimulation (TS): electric shocks at fixed suprathreshold strengths were repeated at intervals of 30 s (1 stimulation/30 s for 10 min). This frequency of stimulation was used for establishing a stable baseline reflex activity because it did not result in response facilitation. 2) Repetitive stimulation (RS): after the baseline reflex activity had been established by the test stimulation, RS (1 stimulation/1 s for 10 min) with the intensity identical to the TS was applied to the pelvic afferent nerve to induce reflex potentiation. 3) Estrogen agonists: after an equilibrium period (usually 30 min), β -estradiol or ER α and ER β agonists were injected via the intrathecal catheter 1 min before stimulation started, and then the RS was applied to the pelvic afferent nerve to induce reflex potentiation. 4) Cdk5, ERK, and NR2B antagonists: after another equilibrium period (30 min), the test agent

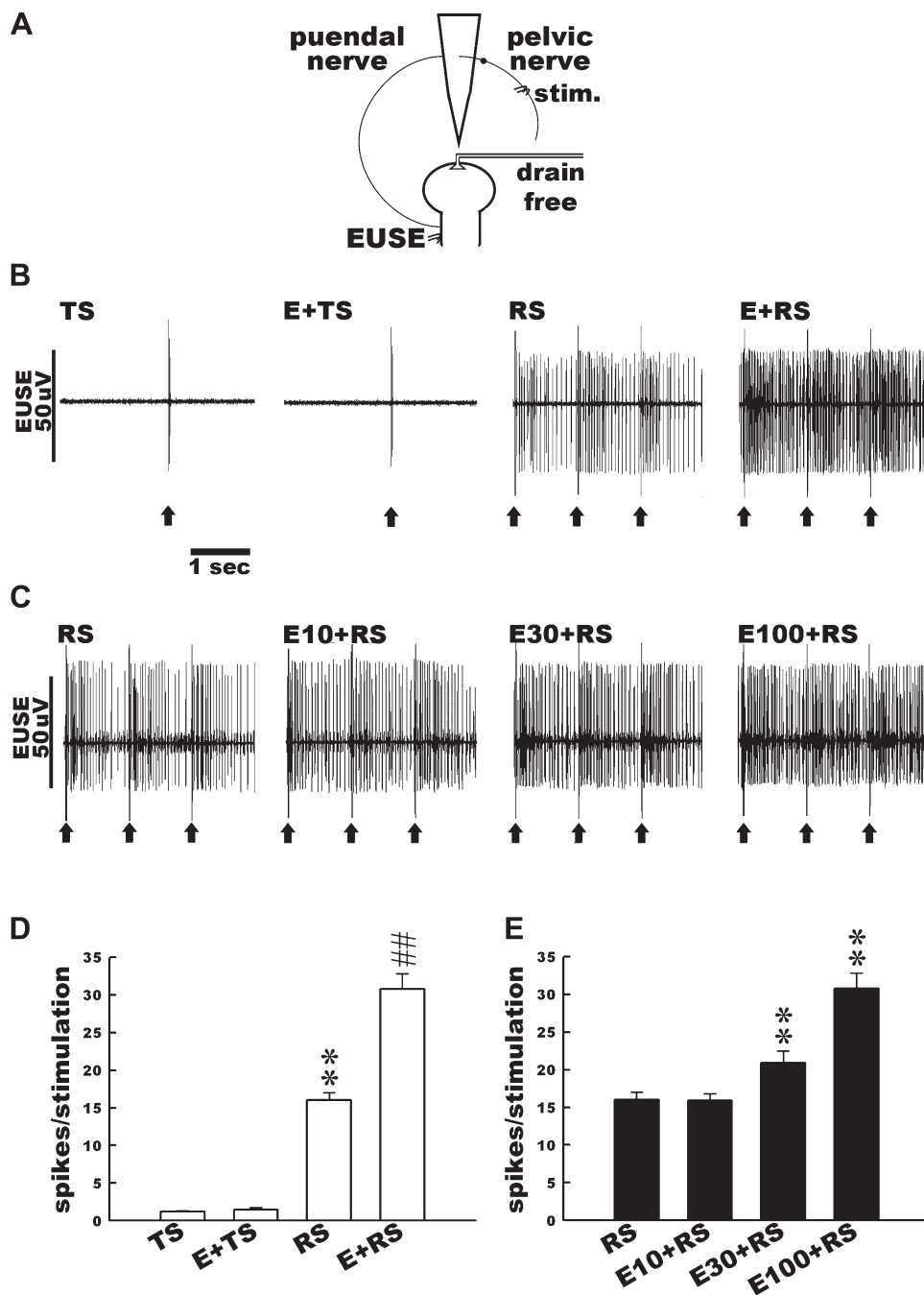


Fig. 1. Estrogen-dependent facilitation on spinal reflex potentiation. *A*: schematic diagram of the arrangement of the external urethra sphincter electromyogram (EUSE) recordings and pelvic afferent nerve stimulation (Stim). *B*: test stimulation evoked a baseline reflex activity with a single action potential (TS), whereas repetitive stimulation produced reflex potentiation (RS). Intrathecal β -estradiol (100 nM, 10 μ l) exhibited no effect on baseline reflex activity (E+TS), whereas it facilitated reflex potentiation (E+RS). *C*: intrathecal β -estradiol with a concentration of 10 nM (E10+RS) failed to have an effect, whereas concentrations of 30 nM (E30+RS) and 100 nM (E100RS) both facilitated reflex potentiation. Tracings show reflex activity at 10 min following stimulation onset in TS, E+TS, RS, and E+RS groups. *D*: summarized data showing mean number of action potentials caused by each stimulation averaged 10 min following stimulation onset in TS, E+TS, RS, and E+RS groups. Intrathecal β -estradiol significantly increased the mean spike number evoked by the RS but not by the TS (** and ##, $P < 0.01$, significantly different from TS and RS, respectively). *E*: summarized data showing that intrathecal β -estradiol with concentrations of 30 nM (E30+RS) and 100 nM (E100+RS) significantly increased mean spike numbers evoked by the RS compared with RS alone (** $P < 0.01$, significantly different from RS).

was injected 10 min before the stimulation started, and the β -estradiol and RS were applied as described in *protocol 3*.

Application of drugs. The drugs used included the following: water-soluble β -estradiol (ER agonist 10, 30, 100 nM, 10 μ l, Sigma), ICI 182,780 (ER antagonist, 10 μ M, 10 μ l, Tocris), propylpyrazoletriol (PPT; an ER α -selective ligand, 100 nM, 10 μ l, Tocris), diarylpropionitrile (DPN, an ER β -selective ligand, 100 nM, 10 μ l, Tocris), roscovitine (Cdk5 inhibitor, 100 nM, 10 μ l, Sigma), U-0126 (ERK inhibitor, 100 μ M, 10 μ l, Tocris), and 4-hydroxy-1-[2-(4-hydroxyphenoxy)ethyl]-4-(4-methylbenzyl)piperidine (Co-101244, a selective NR2B antagonist, 100 nM, 10 μ l, Tocris). β -Estradiol and Co-101244 were dissolved in artificial cerebrospinal fluid (ACSF), and PPT, DPN, roscovitine, and U-0126 were dissolved in DMSO and applied in a final DMSO concentration of less than 1%. ACSF or solvent solution

of identical volume to tested agents was dispensed intrathecally to serve as the vehicle.

Western blotting. Animals were decapitated after the experimental procedures had been finished. The dorsal half of the spinal cord segments from L6 to S2 ipsilateral to the stimulation site was dissected, and the amount of protein was quantitated. Protein samples (20 μ g) were separated on SDS-PAGE (8 and 12%) and transferred to a nitrocellulose membranes. Membranes were blocked in 5% nonfat milk and probed sequentially with antibodies against phosphorylated ERK (1:1,000), total ERK (1:1,000), phosphorylated NR2B (1:1,000), total NR2B (1:1,000), and antibody against β -actin (1:5,000). The blots were incubated with HRP-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

Western blot membranes was done with Science Lab 2003 (Fuji). Results were normalized against actin (in the case of total Cdk5, ERK, NR2A, and NR2B protein) or total ERK (in the case of phosphorylated Cdk5, phosphorylated ERK, phosphorylated NR2A, and phosphorylated NR2B) and are presented as means \pm SD.

siRNA of NMDA NR2B subunit. The protocol for siRNA of the NMDA NR2B subunit was adapted from our previous study (73). Briefly, the pooled negative control siRNA (c) or specific siRNA against NR2B (siNR2B) used in this study were carefully designed and subjected to a Blast-Search (National Center for Biotechnology Information Database) against the expressed sequence tag (EST) libraries to ensure that only one gene was being targeted. The 19-nucleotide duplex and two unpaired nucleotide overhangs of the 3' end were as follows: negative control 5'-UUCUCCGAACGUGU-CACGUTT-3', 3'-ACGUGACACGUUCGGAGAATT-5'; NR2B 5'-AGCUCGUUCCAAAAGAGCTT-3', 3'-UUUCGAGCAAGGGU-UUUCUTT-5'.

Chemically synthetic double-stranded (ds)RNAs against the negative control and the NR2B receptor were synthesized by MDBio (Taipei, Taiwan). Five micrograms of specific or nonspecific siRNAs was complexed with 0.45% of 100 mM PEI (St. Louis, MO). RNA-polymer complexes were allowed 10 min to form at room temperature before the injection. Of the final solution (5–7 μ l) was injected at the T13 vertebra level of adult rats once a day for 5 days. Animals were allowed 2 days to recover and then were used for reflex activity recording and immunoblotting processing. On the days of experiments, test stimulation and repetitive stimulation without (TS and RS, respectively) or with β -estradiol supplement (TS+E and RS+E, respectively) were applied to the animal, and the effects on the pelvic-urethra reflex activity were evaluated by counting the spike number evoked by the test stimulation to the pelvic afferent nerve.

Statistical analysis. Except for West blotting analysis, comparisons among the different stimulation paradigms as well as all the drug- and vehicle-treated groups were determined using one-way repeated-measures analysis of variance followed by the post hoc test (SigmaStat 2.0; Systat Software, San Jose, CA). In all cases, a difference of $P < 0.05$ was considered a statistically significant difference.

RESULTS

β -Estradiol facilitates reflex potentiation. Estrogen is a synaptic neuromodulator that has been implicated in pain-related neural plasticity in dorsal horn neurons (36, 48). To examine the impact of estrogen on SRP, we injected β -estradiol intrathecally to test its effects on the RS-induced SRP. Initial experiments were performed in an attempt to establish a stable baseline reflex activity and RS-induced SRP. As shown in Fig. 1B, a single pulse of the pelvic afferent nerve TS (1 stimulation/30 s) evoked a single action potential in the EUSE, whereas RS (1 stimulation/1 s) on the afferent pelvic nerve induced SRP characterized by an elongation in the evoked reflex activity. We next tested the effect of estrogen by intrathecally injecting β -estradiol 1 min before stimulation started. We found that intrathecal β -estradiol with a concentration of 100 nM exhibited no effect on the baseline reflex activity evoked by the test stimulation (E+TS), whereas it facilitated the RS-induced SRP (E+RS). Figure 1D summarized the mean spike number within 1 s after each pulse averaged at 10 min following stimulation onset evoked by the TS, RS, TS with β -estradiol injection (E+TS), and RS with β -estradiol injection (E+RS). Intrathecal β -estradiol significantly increased the mean spike number/stimulation evoked by the RS (E+RS, $P < 0.01$ to RS; $n = 7$). We then further tested the effect of various concentrations of β -estradiol on the RS-induced SRP. As shown in Fig. 1C, an intrathecal β -estradiol injection with a

concentration of 10 nM elicited no effect (E10+RS), whereas injection with a concentration of 30 nM facilitated the RS-induced SRP (E30+RS). Moreover, β -estradiol with a concentration of 100 nM exhibited a more pronounced facilitation of the SRP (E100+RS). Summarized data in Fig. 1E show that β -estradiol significantly increased the mean spike numbers/stimulation evoked by the RS when the concentrations were 30 and 100 nM (E30+RS and E100+RS, $P < 0.01$ to RS).

Involvement of ER. We next tested the involvement of the ER in the estrogen-dependent facilitation of SRP by the intrathecal application of ICI 182,780, a nonselective ER antagonist before β -estradiol injection. As shown in Fig. 2A, pretreatment with ICI 182,780 exhibited a slight but not significant inhibition on the RS-induced SRP (ICI+RS), whereas it dramatically reversed the facilitation of SRP caused by the β -estradiol injection (ICI+E+RS). The reversal effects of intrathecal ICI 182,780 on the estrogen-dependent facilitation of SRP are summarized in Fig. 2C. ICI 182,780 (ICI+E+RS) significantly decreased the spike number/stimulation evoked by the RS with a β -estradiol injection ($P < 0.01$ to E+RS; $n = 7$).

ER α and ER β mediate estradiol-elicited facilitation. Since evidence has shown that both ER α and ER β contribute to β -estradiol promotion of neuronal function and underlying mechanisms (94, 95), we evaluated the role of these two receptors in estrogen-dependent facilitation of SRP by the intrathecal application of PPT, a specific ER α agonist, and DPN, a specific ER β agonist, 1 min before stimulation started. Without affecting the baseline reflex activity evoked by the test stimulation (data not shown), as shown in Fig. 2B, intrathecal PPT and DPN injections both facilitated the RS-induced SRP. Moreover, PPT exhibited a more pronounced facilitation than DPN did. The effects of intrathecal PPT and DPN are summarized in Fig. 2D. Both PPT (PPT+RS) and DPN (DPN+RS) significantly increased the spike number/stimulation evoked by the RS ($P < 0.01$ to RS; $n = 7$).

Role of Cdk5. Recent studies have identified Cdk5 activity as an intracellular signaling involved in pain-related neural plasticity (64, 65). We tested the role of Cdk5 in the estrogen-dependent facilitation of SRP by intrathecal application of roscovitine, a Cdk5 antagonist, before the β -estradiol injection. As shown in Fig. 3A, pretreatment with roscovitine inhibited the RS-induced SRP. Moreover, the estradiol-elicited facilitation of SRP (E+RS, 100 nM, 10 μ l) was also blocked by roscovitine (ROS+E+RS). The reversal effects of intrathecal roscovitine on the estrogen-dependent facilitation are summarized in Fig. 3C. Roscovitine significantly decreased the mean spike number/stimulation evoked by the RS in association with the β -estradiol injection ($P < 0.01$ to E+RS; $n = 7$). Moreover, no statistical significance was shown between E + RS and ROS + E + RS groups.

ERK pathway. Cdk5 has been linked to neural plasticity-dependent hyperalgesia through the activation of ERK in nociceptive neurons (54, 59, 80). We tested the involvement of the ERK pathway in the Cdk5-dependent facilitation of SRP caused by β -estradiol by using U-0126, an ERK inhibitor, before the β -estradiol injection. As shown in Fig. 3B, pretreatment with U-0126 completely abolished the repetitive stimulation-induced SRP. Moreover, U-0126 (U0126+E+RS) reversed the facilitation of SRP caused by the β -estradiol injection (E+RS, 100 nM, 10 μ l). The reversal effects of intrathecal U-0126 on the estradiol-elicited facilitation of SRP are sum-

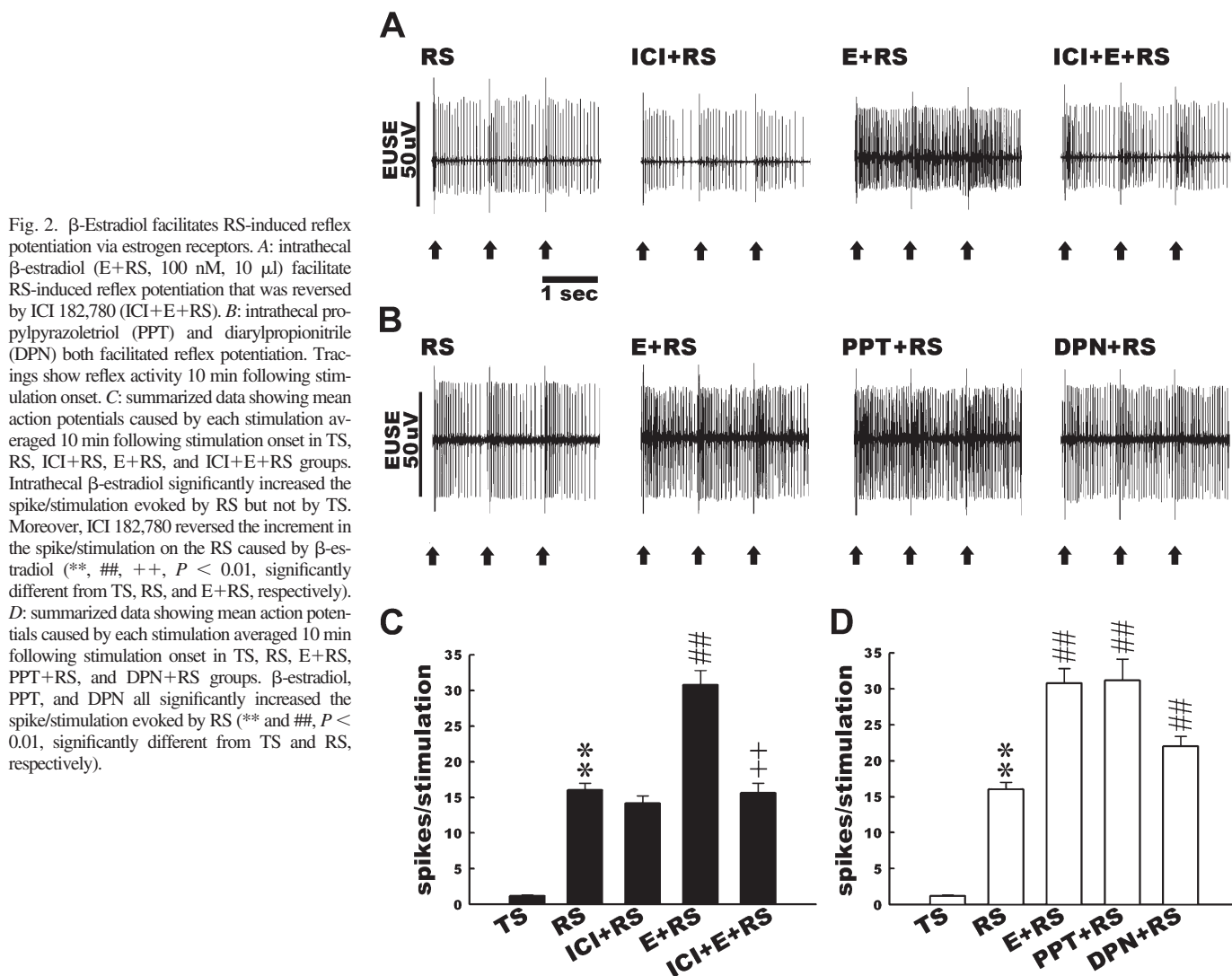


Fig. 2. β -Estradiol facilitates RS-induced reflex potentiation via estrogen receptors. *A*: intrathecal β -estradiol (E+RS, 100 nM, 10 μ l) facilitate RS-induced reflex potentiation that was reversed by ICI 182,780 (ICI+E+RS). *B*: intrathecal propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) both facilitated reflex potentiation. Tracings show reflex activity 10 min following stimulation onset. *C*: summarized data showing mean action potentials caused by each stimulation averaged 10 min following stimulation onset in TS, RS, ICI+RS, E+RS, and ICI+E+RS groups. Intrathecal β -estradiol significantly increased the spike/stimulation evoked by RS but not by TS. Moreover, ICI 182,780 reversed the increment in the spike/stimulation on the RS caused by β -estradiol (**, ##, ++, $P < 0.01$, significantly different from TS, RS, and E+RS, respectively). *D*: summarized data showing mean action potentials caused by each stimulation averaged 10 min following stimulation onset in TS, RS, E+RS, PPT+RS, and DPN+RS groups. β -estradiol, PPT, and DPN all significantly increased the spike/stimulation evoked by RS (** and ##, $P < 0.01$, significantly different from TS and RS, respectively).

marized in Fig. 3D. U-0126 (U0126+E+RS) significantly decreased the mean spike number/stimulation evoked by the RS with the β -estradiol injection ($P < 0.01$ to E+RS; $n = 7$). Moreover, no statistical significance was shown between E + RS and U0126 + E + RS groups. To further confirm that the ERK pathway downstream of the Cdk5 mediates estrogen-dependent facilitation of SRP, the spinal dorsal horns (L6-S2 level ipsilateral to the stimulated nerve) were harvested from rats that received β -estradiol (100 nM, 10 μ l), and performed RS without (E+RS) or with pretreatment of roscovitine (ROS+E+RS) or U-0126 (U0126+E+RS) at 10 min following stimulation onset. Western blot analysis (Fig. 4A) shows that, compared with RS, β -estradiol significantly increased the levels of phosphorylated ERK1/2 (p-ERK) without affecting the basal ERK level (ERK). In addition, roscovitine blocked the increment in the p-ERK1/2 level induced by the β -estradiol injection. The ERK activation induced by the TS, RS, and RS with β -estradiol injection without (E+RS) and pretreatment with roscovitine (ROS+E+RS) or U-0126 (U0126+E+RS) is summarized in o Fig. 4A, bottom ($n = 4$). Compared with the RS, β -estradiol significantly increased the p-ERK intensity ($P < 0.01$ to RS; $n = 4$). Moreover, pretreat-

ment with roscovitine and U-0126 both significantly decreased the pERK intensity caused by RS with β -estradiol ($P < 0.01$ to E+RS; $n = 4$).

Role of NR2B subunits. It has been reported that Cdk5 phosphorylates the NMDA NR2B subunit to exhibit its effects on neural plasticity (45). Electrophysiological evidence has demonstrated that inhibition of Cdk5 activity might regulate the NMDA NR2B subunit and thus prevent the induction of LTP (28). More recently, phosphorylation of tyrosine residues of the NR2B subunit has been described as an important determinant for NMDA receptor functions (42, 62). To clarify the role of the NR2B subunit in the estrogen-dependent facilitation of SRP, we tested the involvement of NR2B subunits in the Cdk5-dependent facilitation of SRP caused by estrogen. We pharmacologically antagonized NR2B1 using Co-101244, an NR2B subunit antagonist, before the β -estradiol injection. As shown in Fig. 4C, pretreatment with Co-101244 inhibited the RS-induced SRP. Moreover, the facilitation of SRP caused by the β -estradiol injection (E+RS, 100 nM, 10 μ l) was reversed by pretreatment with Co-101244 (Co+E+RS). The reversal effect of intrathecal Co-101244 is summarized in Fig. 4D. Intrathecal Co-101244 significantly decreased the mean

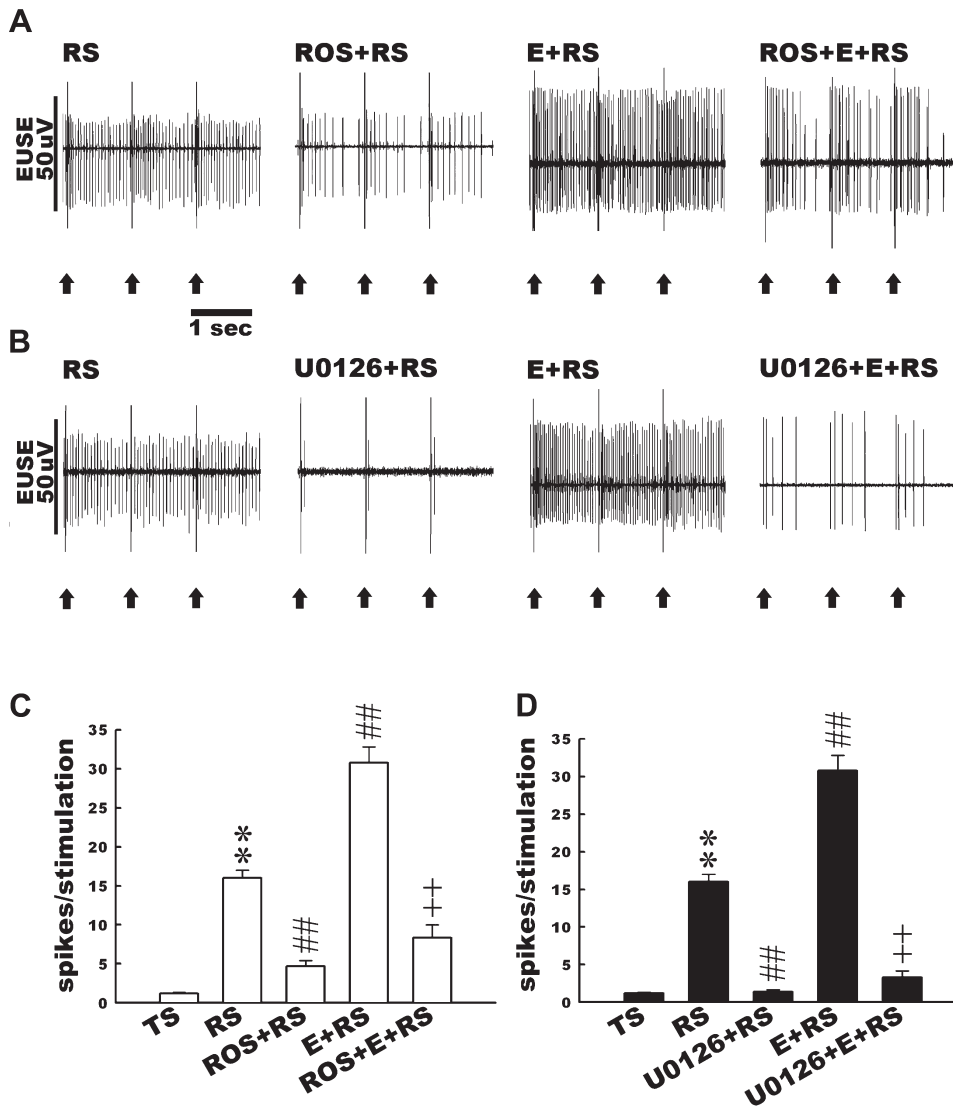


Fig. 3. Cyclin-dependent kinase-5 (Cdk5) and extracellular signal-related kinase (ERK) mediate estrogen-dependent facilitation on RS-induced reflex potentiation. **A**: intrathecal β -estradiol (E+RS) elicited facilitation on RS-induced reflex potentiation (RS) that was reversed by roscovitine (ROS+E+RS). **B**: intrathecal β -estradiol elicited facilitation on the reflex potentiation (E+RS) that was reversed by U-0126 (U0126+E+RS). Tracings show reflex activity 10 min following stimulation onset. **C**: summarized data showing mean action potentials caused by each stimulation averaged 10 min following stimulation onset in TS, RS, ROS+RS, E+RS, and ROS+E+RS groups. Intrathecal roscovitine significantly reversed the increase in spike/stimulation evoked by the RS caused by β -estradiol (**, ##, ++, $P < 0.01$, significantly different from TS, RS, and E+RS, respectively). **D**: summarized data showing mean action potentials caused by each stimulation averaged 10 min following stimulation onset in TS, RS, U0126+RS, E+RS, and U0126+E+RS groups. Intrathecal U-0126 significantly reversed the increase in spike/stimulation evoked by the RS caused by β -estradiol (**, ##, ++, $P < 0.01$, significantly different from TS, RS, and E+RS, respectively).

spike number/stimulation evoked by the RS with the β -estradiol injection ($P < 0.01$ to E+RS; $n = 7$). To further confirm the possibility that activation of the NMDA NR2B subunit downstream of the Cdk5/ERK pathway mediates estrogen-dependent facilitation of SRP, spinal dorsal horns (L6-S2 level ipsilateral to the stimulated nerve) were harvested from rats that received β -estradiol (100 nM, 10 μ l) and RS without (E+RS) or with pretreated roscovitine (ROS+E+RS) or U-0126 (U0126+E+RS) at 10 min following stimulation onset for Western blot analysis. As shown in Fig. 4B, compared with RS, β -estradiol significantly increased the levels of the phosphorylated NR2B subunit (p-NR2B) without affecting the basal protein level (NR2B). In addition, roscovitine and U-0126 both blocked the increment in the p-NR2B level induced by the β -estradiol injection. The NR2B intensity induced by the TS, RS, E+RS, ROS+E+RS or U0126+E+RS are summarized in Fig. 4B, bottom. Compared with RS, estradiol significantly increased the p-NR2B intensity ($P < 0.05$ to RS; $n = 4$). Moreover, pretreatments with roscovitine and U-0126 both significantly decreased the p-NR2B intensity caused by RS with β -estradiol ($P < 0.01$ to E+RS; $n = 4$).

siRNA. To further clarify the role of NR2B subunits in the estrogen-dependent facilitation of SRP, we next intrathecally injected β -estradiol to test its effect on the RS-induced SRP in negative controls (c) and siRNA of NR2B (siNR2B) rats. As shown in Fig. 5A, the expression level of the NR2B protein was decreased in the siNR2B animals compared with the control rats. An intrathecal β -estradiol injection facilitated the RS-induced SRP in both groups; however, the estradiol-elicited facilitation was attenuated in the siNR2B rats compared with the control animals (Fig. 5B). The reflex activity evoked by the pelvic afferent nerve RS without (RS) or with β -estradiol injection (E+RS) in control and siNR2B groups are summarized in Fig. 5C. The increment in spike number/stimulation caused by the β -estradiol injection (E+RS) was significantly reduced in the siNR2A rats ($P > 0.05$ to RS; $n = 7$).

DISCUSSION

Estrogen is a gonadal steroid with pronounced tropic effects on many diverse populations of neurons throughout the peripheral and central nervous systems. In the present study, intra-

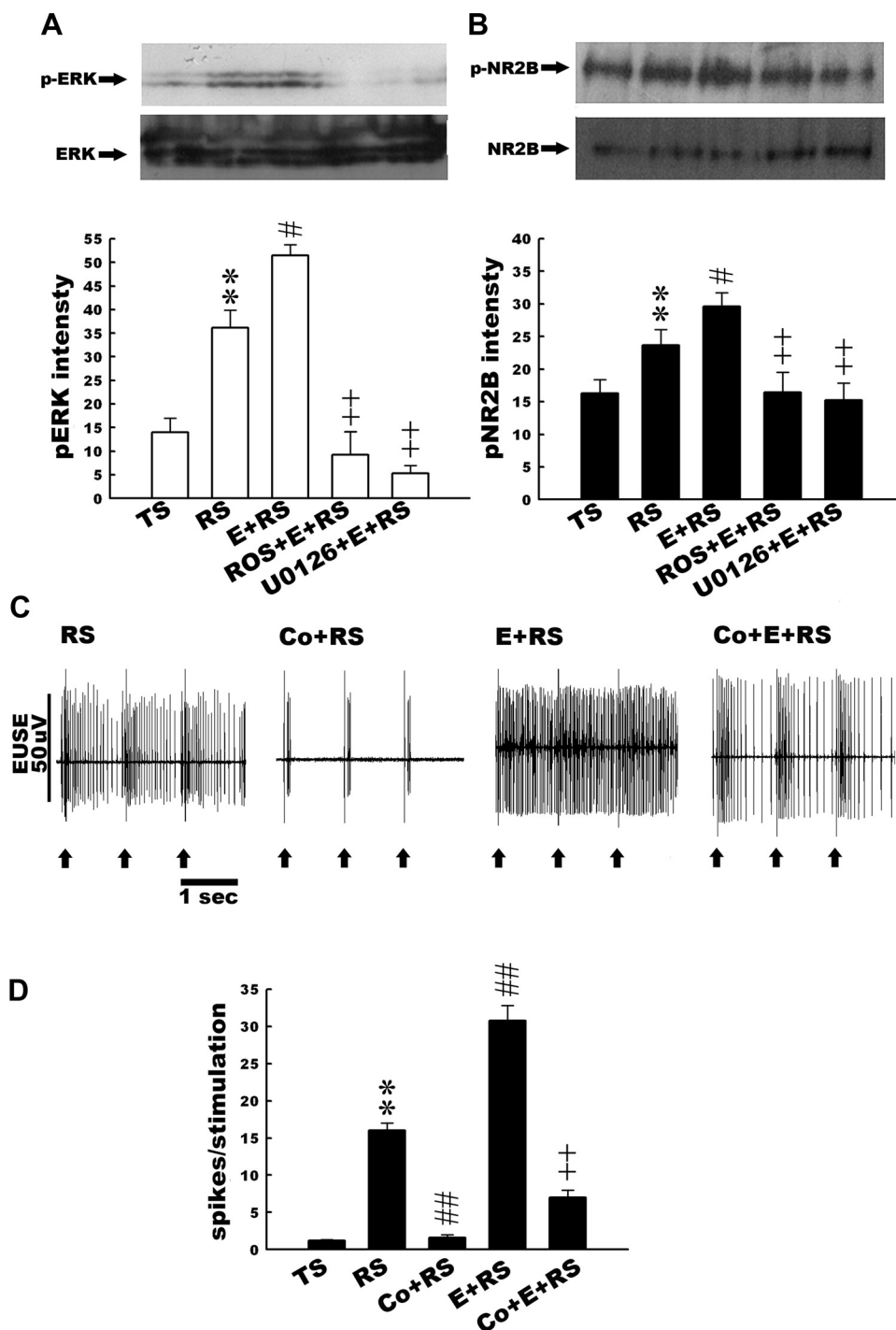


Fig. 4. Western blotting analysis of phosphorylated ERK 1/2 and NR2B subunit. **A**: representative Western blot showing total (ERK) and phosphorylated ERK (p-ERK) expression in the lumbosacral (L6-S2) spinal dorsal horn samples ipsilateral to the stimulation site obtained from rats receiving TS, RS, RS with β -estradiol injection (E+RS), and RS with β -estradiol injection associated with pretreated roscovitine (ROS+E+RS) or U-0126 (U0126+E+RS). **Bottom**: summarized data demonstrating that, compared with RS alone, β -estradiol significantly increased the levels of p-ERK immunoreactivity caused by RS. Pretreatment with roscovitine and U-0126 both reversed the increment in immunoreactivity caused by β -estradiol (**, ##, ++, $P < 0.01$ to TS, RS, and E+RS, respectively; $n = 4$). **B**: representative Western blot showing total (NR2B) and p-NR2B expression in the lumbosacral spinal dorsal horn sample ipsilateral to the stimulation site obtained from TS, RS, E+RS, ROS+E+RS, and U0126+E+RS groups. **Bottom**: summarized data demonstrating that, compared with RS alone, β -estradiol significantly increased the levels of p-NR2B immunoreactivity caused by RS (E+RS). Pretreatment of roscovitine (ROS+E+RS) and pretreated U-0126 (U0126+E+RS) both reversed the increment in immunoreactivity caused by β -estradiol (**, ##, ++, $P < 0.01$ to TS, RS, and E+RS, respectively; $n = 4$). **C**: Intrathecal β -estradiol (E+RS) elicited facilitation on RS-induced reflex potentiation (RS) that was reversed by Co-101244 (Co+E+RS). Tracings show reflex activity at 10 min following stimulation onset. **D**: summarized data showing mean action potentials caused by each stimulation averaged at 10 min following stimulation onset in TS, RS, Co+RS, E+RS, and Co+E+RS groups. Intrathecal Co-101244 significantly reversed the increase in spike/stimulation evoked by the RS caused by β -estradiol (**, ##, ++, $P < 0.01$, significantly different from TS, RS, and E+RS, respectively).

theal β -estradiol injections exhibited facilitation on the repetitive stimulation-induced SRP, a novel form of activity-dependent reflex plasticity, in a dose-dependent manner. This result correlates with investigations on LTP, the well-known tetanic-induced neural plasticity, demonstrating that estradiol administration increases the synaptic excitation of pyramidal cells in the hippocampal CA1 area and suggests that the gonadal steroid impacts reflex plasticity (94, 95). In vitro studies investigating the calcium dynamics related to the LTP induction at hippocampal CA1 area have shown that both membrane ER α and ER β contribute to estrogen-elicited promotion of

neuronal plasticity and underlying mechanisms (94, 95). This result was parallel to the data presented in this study that, similar to β -estradiol, both selective ER α and ER β agonists produced facilitation on the repetitive stimulation-induced SRP, implying the involvement of ER α and ER β receptors in the estradiol-elicited facilitation of SRP.

The expression of Cdk5 protein levels in DRG neurons has been shown to be altered in response to peripheral inflammation (64). Recent studies investigating pain signals by use of gene knockout mice have identified Cdk5 activity as an intracellular signal involved in the induction of pain-related neural

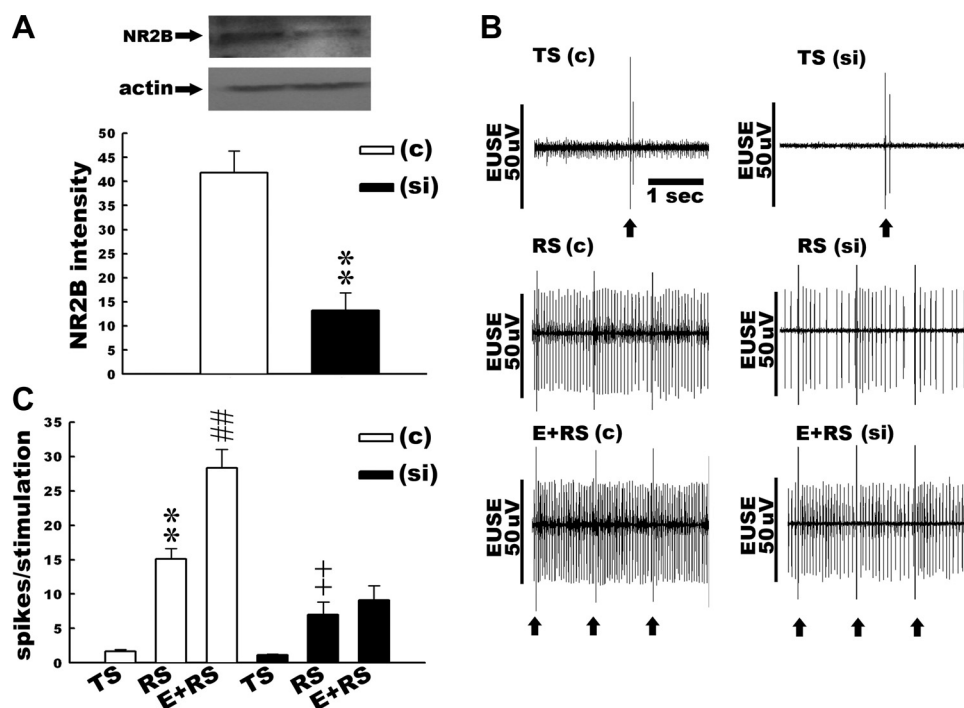


Fig. 5. Attenuation of estrogen-dependent facilitation on RS-induced reflex potentiation in siRNA of NR2B rats. A: representative Western blot showing β -actin (actin) and total NR2B (NR2B) in negative controls (c) and NR2B si-RNA (siNR2B) rats. (B) Test stimulation (TS) evoked a baseline reflex activity with a single action potential, whereas repetitive stimulation produced reflex potentiation (RS) in both control (c) and NR2B si-RNA (si) rats. Intrathecal β -estradiol (E+RS) elicited facilitation on reflex potentiation in both control (c) and NR2B si-RNA (si) rats. However, the facilitation was attenuated in si-RNA rat. Tracings show the reflex activity 10 min following the stimulation onset. (C) Summarized data showing the mean action potentials caused by each stimulation averaged within 10 min following stimulation onset in TS, RS and E+RS groups. Intrathecal β -estradiol significantly increased the spike/stimulation evoked by the RS but not by the TS (** and ## $P < 0.01$, significantly different from TS and RS, respectively).

plasticity (64, 65, 80) by regulating ERK1/2 activation through ERK phosphorylation at Thr²⁸⁶ in DRG neurons (65). In the present study, as indicated by the induction of NMDA-dependent LTP in the CA1 neurons (45), intrathecal pretreatment with roscovitine reversed the estrogen-dependent facilitation of SRP. This result implies that Cdk5 might be involved in the modulation of the induction of activity-dependent neural plasticity caused by estrogen, not only in the brain area but also at the spinal cord level. On the other hand, pretreatment with the ERK inhibitor U-0126 also reversed estradiol-elicited facilitation of the SRP. Western blot analysis showed that β -estradiol significantly increased the levels of ERK1/2 phosphorylation, which was reversed by roscovitine, indicating that ERK1/2 phosphorylation downstream of Cdk5 participates in the estradiol-elicited facilitation of SRP. This result correlates with investigations on nociception, which have demonstrated that Cdk5 might modulate activity-dependent neural plasticity, which underlies postinflammatory or neuropathic pain by regulating the phosphorylation of ERK at the Thr²⁸⁶ residue (65, 80).

It has been shown that increased phosphorylation of the NMDA NR2B subunits, which defines the properties essential for synaptic plasticity of NMDA receptors (7, 52, 69), is accompanied by the induction of activity-dependent neural plasticity (77). More recently, phosphorylation at tyrosine residues of NR2B has been described as an important determinant for NMDAR functions (42, 62). On the other hand, electrophysiological studies on hippocampal brain slices have demonstrated that Cdk5 phosphorylated the NMDA NR2B subunit, affecting NMDA-regulated synaptic activity (45, 51). Behavior studies using intact animals have shown that the enhanced tyrosine phosphorylation of the NMDA NR2B subunit contributes to nociceptive activity-induced spinal plasticity and the development of behavioral hyperalgesia and allodynia (26). In the present study, pharmacological blockage

using a selective NR2B antagonist, Co-101244, abolished the estradiol-elicited facilitation of SRP. Moreover, the results of Western blotting demonstrated that β -estradiol elicited an increment in phosphorylated NR2B expression that was blocked both by Cdk5 and ERK antagonists. Furthermore, in siNR2B rats, the estradiol-elicited facilitation was attenuated compared with the negative control group. All these results indicate that ERK phosphorylation caused by Cdk5 activation may activate NMDA NR2B subunit via phosphorylating the tyrosine residues of NR2B to underlie the estradiol-elicited facilitation of SRP.

In addition to exhibiting genomic effects by binding to cytoplasmic receptors, estrogen may also bind to membrane receptors to induce physiological functions (39, 81, 82). Evidence has shown that E₂-BSA, a membrane-bound estrogen receptor agonist, displays a maximal physiological effect at 5–10 min following administration (10). Considering that the latency for the intrathecal β -estradiol injection to facilitate SRP in this study, which was too short to activate cytoplasmic estrogen receptors, was within this range, we suggested that the membrane estrogen receptors mediated the estradiol-mediated facilitation of the SRP presented in this study. However, since the tested agents should penetrate across the pia from the arachnoid space to exhibit their effects on the neurons of the spinal cord when they were applied intrathecally, we failed to verify this hypothesis by using E₂-BSA, the membrane-bound estrogen agonist, as it is characterized by a low permeability to the membrane. Therefore, the precise site for the β -estradiol to elicit facilitation of the SRP needs further investigation for elucidation.

The dynamic regulation of reflex strength by ongoing neural activities is one of the fundamental components of the functions in the central nervous system (6). Activity-dependent reflex potentiation occurring at the spinal cord level has been presumed to mediate the postinflammatory or neurogenic hy-

peralgesia and allodynia (32–34, 89). In this study, we have demonstrated a role for Cdk5 in the regulation of ERK/NR2B activity during the nongenomic estradiol-elicited facilitation of the SRP, a novel form of spinal mediated neural plasticity that was suggested to be related to the development of hyperalgesia. These findings support the potential use of selective Cdk5 modulators to alleviate pain.

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