行政院國家科學委員會專題研究計畫 成果報告

性賀爾蒙對麻醉大白鼠骨盆-尿道反射增益現象之效應及對背角神經元細胞內訊息路徑的調節機轉(第 2 年)
研究成果報告(完整版)

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中文摘要：

探討雌激素如何調控細胞內的 PI3K 訊息，對於骨盆-尿道反射增益現象之影響。利用卵巢摘除的老鼠在皮下給予雌激素，探討對於尿道反射活性以及腰薦椎的脊髓背角神經組織蛋白質表現。比較生理食鹽水和皮下給予雌激素所造成的 NR2B 次單元依賴性尿道反射活性以及增加脊髓背角神經組織上的 pAkt 和 pNR2B 的表現量。此現象可以藉由椎管內給予雌激素接受器拮抗劑 ICI 182,780 (0.25 mg/kg, *i.t.*) 和 PI3K 抑制劑 LY294002 (50 mg/kg, *i.t.*) 所阻斷。在皮下給予雌激素六個小時後與生理食鹽水組作比較，利用免疫沉澱法觀察脊髓背角神經組織上的 pAkt 和 pNR2B 交互作用增加。初步結果指出雌激素可能活化 PI3K 訊息傳遞路徑造成 NR2B 次單元的磷酸化。此現象可能用來解釋骨盆腔疼痛，是藉由脊髓上的雌激素接受器α/雌激素接受器β 促進 NR2B 次單元依賴性反射敏感性所造成。

關鍵詞：骨盆腔疼痛，尿道，骨盆-尿道反射增益效應

ABSTRACT

To determine the role of 17β-estradiol (E2) and involvement of intracellular phosphatidylinositol-3-kinase (PI3K) signaling in pelvic-urethral reflex potentiation. we analyzed urethra reflex activity and protein expressions in lumbosacral (L6-S2) spinal dorsal horn in response to s.c. application of estrogen in ovariectomized female rats. When compared with vehicle solution, s.c. application of estrogen sensitized the N-methyl-D-aspartate receptor (NMDAR) NR2B subunit-dependent reflex activity

and increased expression levels of phosphorylated Akt (pAkt) and phosphorylated NR2B (pNR2B) in dorsal horn. This phenomenon was reversed both by intrathecal pretreatment with ICI 182,780 (0.25 mg/kg, *i.t.*) and LY294002 (50 mg/kg, *i.t.*). Immunoprecipitation of dorsal horn tissue revealed a protein-protein interaction between pAkt and pNR2B increases, six hours following the subcutaneous E2 when compared with vehicle injections. Results indicate E2 may activate the PI3K cascade, which subsequently phosphorylates the NR2B subunit, via spinal ERα/ERβ, to facilitate NMDA-dependent reflex sensitization, which is presumed to pelvic pain disorder.

Key words: pelvic pain syndrome, urethra, pelvic-urethral reflex potentiation

MATERIALS AND METHODS

Animal Preparations

Female Sprague Dawley rats (n=175; 210–275g) were used in this experiment. Animal care and experimental protocols were in accordance with the guidelines of the National Science Council of Taiwan. This study was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University. Animals were ovariectomized bilaterally via two small lumbar incisions under anesthesia with ethrane (Abbott Illinois, USA), and were tested 20–30 days after surgery. On experimental days, rats were anesthetized with intraperitoneal urethane (1.2 g/kg, i.p.). The occipital crest of the skull was exposed and the atlanto-occipital 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 T13 vertebral level (L6-S2 of the spinal cord) for Intrathecal injection. Fluid volume within the catheter was kept

constant at 10 μl in all experiments. In experiments that used intrathecal injection, a single 10 μl dose of drug solution was administered followed by a flush with 10 μl of artificial cerebrospinal fluid.

Pelvic-Urethra Reflex Activity Recording

The left pelvic nerve was carefully dissected from the surrounding tissue and mounted on a pair of stainless steel wire electrodes for stimulation. Oligo-/single unit spike action potentials of the external urethral sphincter electromyogram (EUSE) were continuously recorded by a pair of epoxy-coated, copper-wire electrodes and displayed on a recording system with a sampling rate of 20,000 Hz (MP30, Biopac, Santa Barbara, CA).

Subcutaneous injection of 17β-estradiol was done six hours before pelvic afferent nerve stimulation. In some experiments, ICI 182,780 or LY294002 was injected (0.25 and 50 mg/kg, i.p.) 30 min before estradiol injection to antagonize estradiol effects. Single shocks (pulse duration: 0.05 ms, 1/30 sec) (test stimulation: TS) were applied to the pelvic nerve through a pair of stimulation electrodes. At the beginning of each experiment, an intensity that caused a single spike action potential in the reflex activity was used to standardize baseline reflex activity. This intensity was used for stimulation throughout each experiment. Protocols for assessing effects of different reagents on reflex activity were as follows:

Protocol 1. Pelvic afferent nerve TS: Single electric shocks at a fixed suprathreshold strength repeated at 30 sec intervals for 10 min were applied to the left pelvic nerve through a pair of stimulation electrodes, six hours after subcutaneous estradiol injection. This frequency was chosen because it did not result in response facilitation.

Protocol 2. Agonist-induced reflex potentiation: After an equilibrium period (usually 10 minutes), NMDA (10 μM, 10 μL) was injected intrathecally 1 min before stimulation began. The TS was then applied to the pelvic afferent nerve to induce reflex potentiation. In some experiments, APV (10 μM, 10 μL) or Co-101244 (100 nM, 10 μL) was intrathecally administrated 10 min before NMDA injection to antagonize the effects of NMDA and the NR2B subunit, respectively.

Application of Drugs

The drugs used included 17β-estradiol (E2, estrogen agonist, 50 μg/kg s.c., Sigma), propylpyrazoletriol (PPT, ERα-preferring ligand, 10 mg/kg *i.t.*, Tocris), diarylpropionitrile (DPN, ERβ-preferring ligand, 10 mg/kg *i.t.*., Tocris), ICI 182,780 (ICI, nonselective ER antagonist, 0.25 mg/kg i.p., Tocris), LY294002 (LY, PI3K inhibitor, 50 mg/kg i.p., Tocris), N-methyl-D-aspartic acid (NMDA, selective glutamatergic NMDAR agonist, 10 μM, 10 μl i.t., Sigma), $_{D}$-2-amino-5-phosphonovalerate (APV, glutamatergic NMDA receptor antagonist; 10 μM, 10 μl i.t., Sigma), Co-101244 (Co, selective NMDA receptor NR2B subunit

antagonist, 100 nM, 10 μL i.t., Tocris). Doses were modified from previous studies and are summarized in Table 1.

Western Blotting

For the Western blot analysis, animals were decapitated after experimental procedures were finished. The dorsal halves of the spinal cord segments from L6-S2 on the left side (ipsilateral to the stimulated nerve) were dissected and the amounts of protein were quantified. Protein samples (20 μg) were separated on SDS-PAGE (12%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk and probed sequentially with antibodies against phosphorylated Akt (pAkt, 1:1000, Santa Cruz), total NR2B (tNR2B, 1:1000, Chemicon), phosphorylated NR2B (pNR2B, 1:1000, Chemicon) and β-actin (1:10000, Chemicon). Blots were incubated with HRP-conjugated antibody (1:10000, Santa Cruz) for one hour at room temperature. After visualization with ECL solution, protein levels were determined using LAS 3000 (Fuji, Japan). Densitometry of the blotted membranes was done using Science Lab 2003 (Fuji, Japan). Results were normalized against β-actin and are presented as mean ± SD.

Coimmunoprecipitation of pAkt with PSD95 and NR2B

Rabbit polyclonal pAkt antibody (5 μg; Santa Cruz) was incubated overnight at 4°C with the crude plasma membrane fraction (500 μg) extracted from the left

lumbosacral (L6-S2) dorsal horns of ovariectomized rats that received vehicle or estradiol. The 1:1 slurry protein agarose suspension (Millipore) added into that immuno-complex protein, and the mixture was incubated for 2–3 h at 4°C. Agarose beads were washed once with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 0.02% (w/v) sodium azide), twice with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer plus 300 mM NaCl, and three times with an immunoprecipitation buffer only. Binding proteins were eluted with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95°C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes electrophoretically, and detected using rabbit polyclonal anti-pAkt, mouse monoclonal anti-PSD-95 (Santa Cruze, USA), and rabbit polyclonal anti-NR2B (Millipore). Thirty micrograms of spinal cord plasma membrane fraction were loaded as a positive control (input).

Data Analysis

Electromyogram activity was recorded using a sampling rate of 5,000 samples/sec, with a conventional band-pass filter setting (30-3000 Hz). Spike number elicited by stimulation shocks was averaged using the mean spike numbers evoked by the last three stimulations. Comparisons across different stimulation parameters as well as all drug and vehicle treated groups were determined using one-way, repeated-measure analysis of variance, followed by a post-hoc test (Tukey test, SigmaStat 2.0; Systat Software Inc., San Jose, CA, USA). In all cases, a difference of $p<0.05$ was considered statistically significant.

Preliminary result

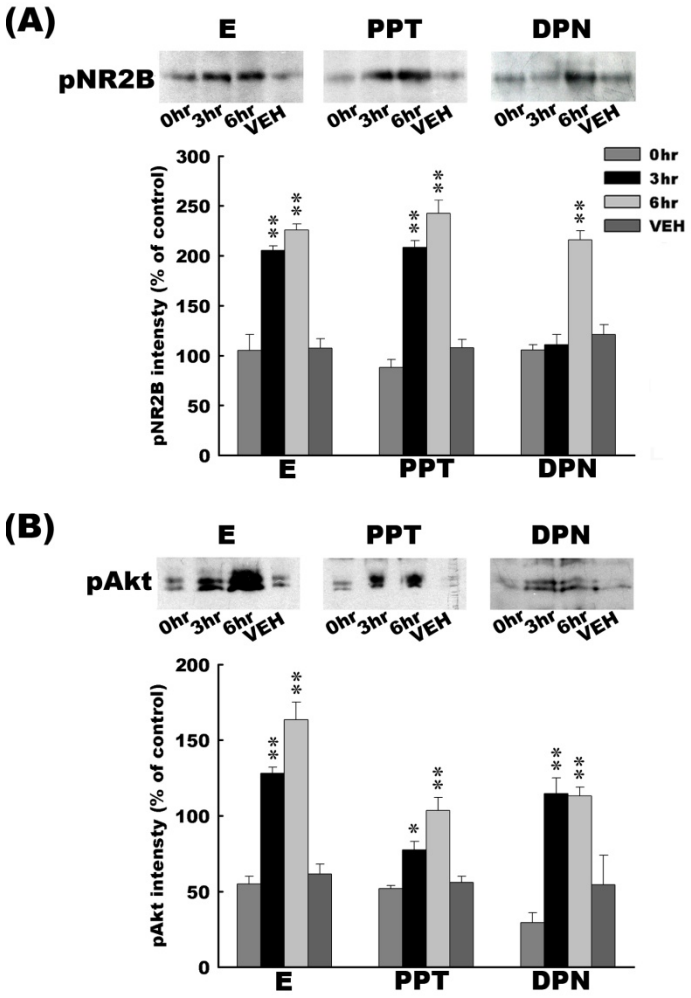

Figure 1. Effects of subcutaneous estradiol (E), propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) on expression levels of phosphorylated NR2B (pNR2B) and phosphorylated Akt (pAkt) in left lumbosacral (L6-S2) dorsal horn tissue of ovariectomized rats. Western blots show that at 0, 3, and 6 hours (hr) following injections, expression levels of (A) pNR2B and (B) pAkt were both increased in a

time-dependent manner by subcutaneous E, PPT, and DPN when compared with vehicle injections (VEH, * $p<0.05$, ** $p<0.01$ to VEH, n=4).

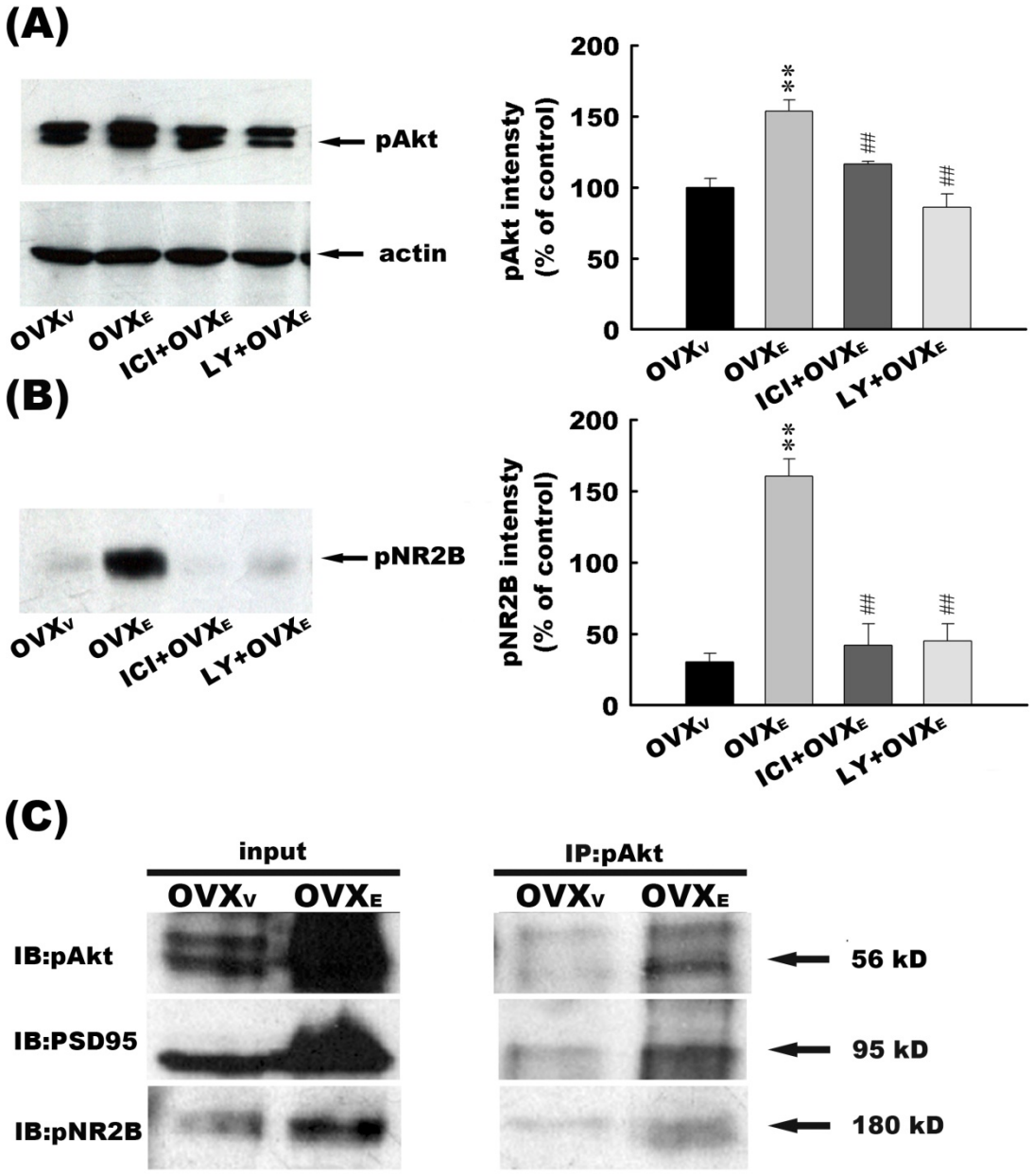

Figure 2. Effects of intrathecal ICI 182,780 and LY294002 pretreatments on estradiol-dependent upregulation of phosphorylated Akt (pAkt) and phosphorylated

NR2B (pNR2B) in left lumbosacral (L6-S2) dorsal horn tissue of ovariectomized rats. Six hours after subcutaneous estradiol injections (OVX_E), expression levels of (A) pAkt and (B) pNR2B both increased when compared with vehicle injections (OVX_V, ** p<0.01 to OVX_V, n=4). Pretreatments with ICI 182,780 (ICI+OVX_E) and LY294002 (LY+OVX_E), 30 min before estradiol, reversed the increases of pAkt and pNR2B expression caused by estradiol injections (## p<0.01 to OVX_V, n=4). (C) Co-immunoprecipitation analysis of left lumbosacral (L6-S2) dorsal horn tissue obtained from OVX_V and OVX_E animals. Immunoblotting (input) in the left column shows increases in expression levels of pAkt, PSD95, and pNR2B in OVX_E when compared to OVX_V rats. Immunoprecipitation blotting (IP) in the right column shows an increment of PSD95 and pNR2B immunoprecipitation with anti-pAkt antibody in crude membrane extract from the OVX_E but not the OVX_V group.

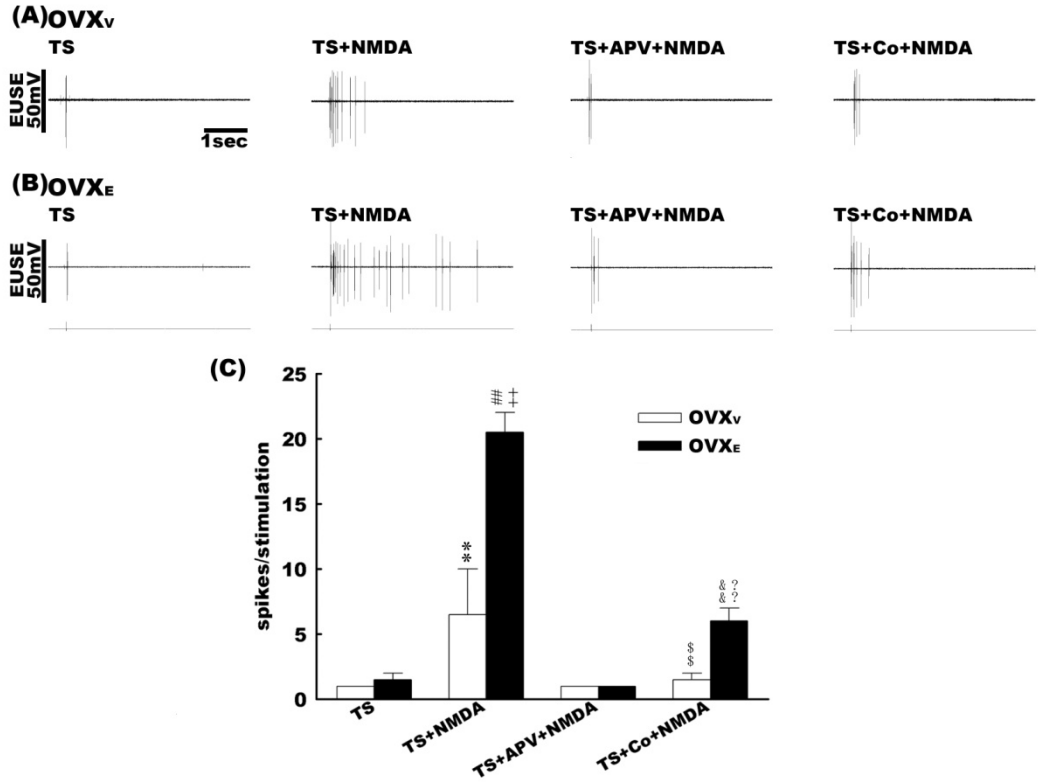

Figure 3. NMDA-induced reflex potentiation. In ovariectomized rats, which received subcutaneous vehicle (A OVX_V) and estradiol (B OVX_E) injections, test stimulation (TS, 1 stimulation/30 sec for 10 min) at 6 hours after injection evoked a constant baseline reflex activity with a single action potential in external urethra sphincter electromyogram (EUSE) activity in both groups. Intrathecal NMDA (TS+NMDA, 10 μM, 10 μL, 1 min before stimulation onset) induced reflex potentiation that persisted longer in OVX_E than OVX_V rats. Pretreatment with APV and Co-101244 (TS+APV+NMDA and TS+Co+NMDA, respectively; 10 μM and 100nM, 10 μL, 10 min before stimulation onset) abolished NMDA-induced reflex potentiation. (C)

Mean spike number evoked by each impulse counted 10 min following the TS onset in OVX_V (white bar) or OVX_E (black bar). No statistical significance was found in mean spike numbers evoked by TS between these groups (TS, $p>0.05$, n=7). Mean spike numbers evoked by TS in association with intrathecal NMDA injections were significantly increased in OVX_E compared with OVX_V (TS+NMDA, ** $p<0.01$ to OVX_V, n=7). Mean spike number increases caused by intrathecal NMDA were significantly reversed by pretreatment with APV and Co-101244 (TS+APV+NMDA and TS+Co+NMDA, respectively. ## $p<0.01$ to TS+NMDA, n=7).

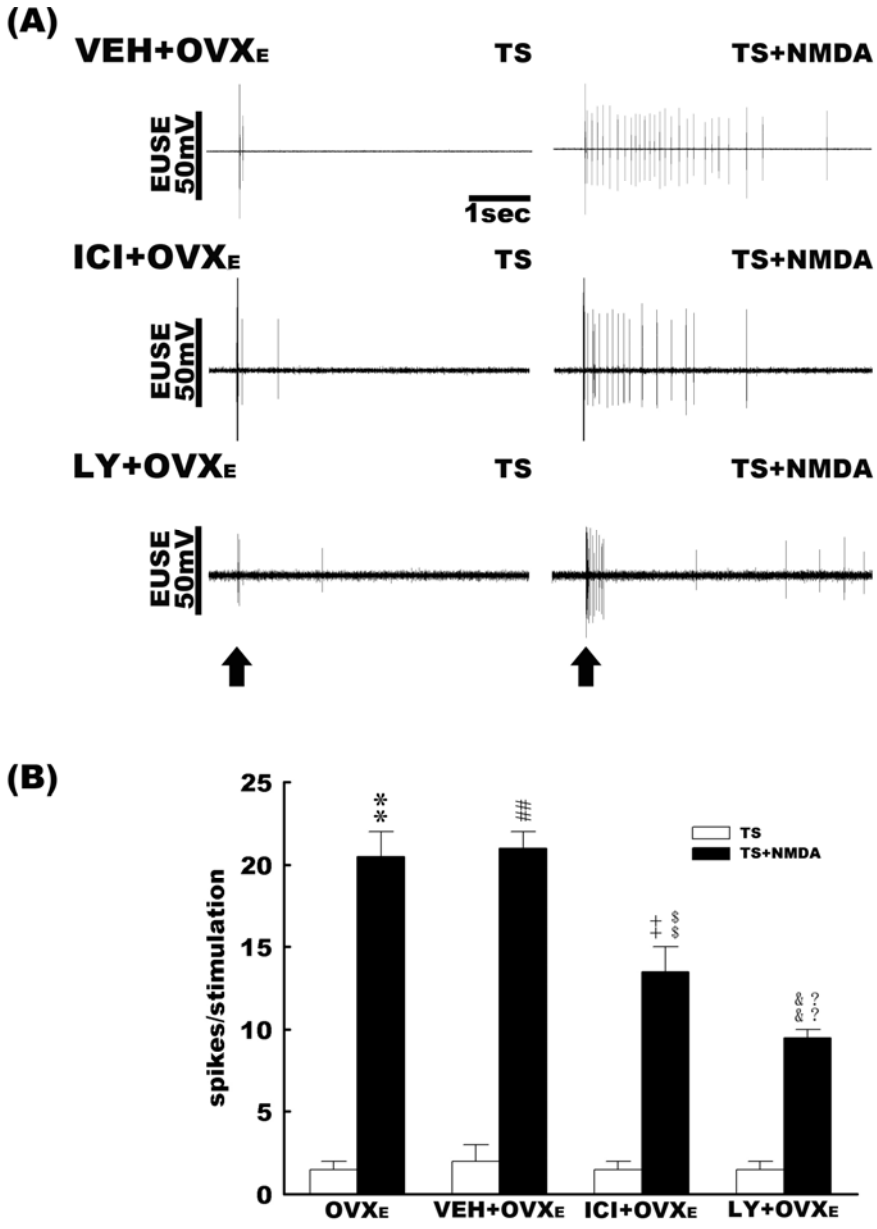

Figure 4. Effects of ICI 182,780 and LY294002 on estradiol-dependent facilitation of NMDA-induced reflex potentiation. (A) In ovariectomized rats that received subcutaneous estradiol (OVX_E), test stimulation (TS, 1 stimulation/30 sec for 10 min) 6 hours after injection evoked a baseline reflex activity with a single action potential in external urethra sphincter electromyogram (EUSE) activity. Intrathecal NMDA

(TS+NMDA, 10 μM, 10 μL), 1 min before stimulation onset, induced reflex potentiation in the same preparation. Pretreatment with intrathecal ICI 182,780 and LY294002 (ICI+OVX_E and LY+OVX_E, respectively), 30 min before estradiol injection, reversed NMDA-induced reflex potentiation. (B) Mean spike number evoked by TS (white bar) or TS with intrathecal NMDA (TS+NMDA, black bar) counted 10 min after stimulation onset in OVX_E rats and OVX_E in association with pretreatment with vehicle solution (VEH+OVX_E), ICI 182,780 (ICI+OVX_E) and LY294002 (LY+OVX_E). No statistically significant differences were found in mean spike numbers in those with TS with intrathecal NMDA (VEH+OVX_E) and without vehicle injections (OVX_E, $p>0.05$ to OVX_E, n=7), whereas mean spike numbers decreased significantly with ICI 182,780 and LY294002 pretreatments compared with those that received vehicle injections (VEH+OVX_E, ## $p<0.01$ to VEH+OVX_E, n=7).

一 、2009 年 International continence society 投稿文章

Estrogen modulates cross-organ sensitization between the colon and pelvic-urethra reflex

[a,b]Hsien-Yu Peng, [c]Gin-Den Chen, [a,b]Cheng-Yuan Lai, [b]Tzer-Bin Lin*

[a]Department of Physiology, Chung-Shan Medical University, Taichung, Taiwan
[b] Department of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan
[c]Department of Obstetrics and Gynecology, Chung-Shan Medical University Hospital,

Key words: irritable bowel syndrome, colitis, lower urinary tract, pelvic pain syndrome, central sensitization

Background

Visceral pain may not only arise from an injured/inflamed organ itself, but may also be referred from other diseased viscera. Bowel-to-LUT and LUT-to-bowel cross-organ sensitization have been recently demonstrated in rats. However, the detailed mechanism of the cross-organ sensitization needs further investigation to clarify the complicated interactions of afferent inputs involving central and peripheral neural sensitization. It is now presumed that the convergence of sensory fibers arising from adjacent pelvic structures or bifurcating afferent fibers might account for the peripheral mechanism for the induction of cross-organ sensitization. The induction of spinal reflex potentiation (SRP) has been postulated to be involved in the development of hypergesia/allodynia as shown in our previous study.

The NMDA-dependent Ca^{++} influx resulting in phosphorylation of the NR2B subunit underlies the induction of SRP. NR2B containing the NMDA receptor (NMDAR) plays important roles in neural plasticity induction and phosphorylation at NR2B tyrosine residues,

which was described as an important determinant for NMDA-mediated currents in our previous reports. In the spinal cord, gluatamatergic N-methyl-D-aspartate (NMDA)-dependent neurotransmission underlies the development of central sensitization. There is a large variation in the pain responses across stages of the estrous cycle implying that estrogen plays roles in the modulation on nociception. It has been demonstrated that the development of cross-organ sensitization is affected by levels of gonodal hormones, i.e. 17β-estradiol (E2) (1,2,3).

In this study, we tried to determine the role of E2 in the cross-organ sensitization between the colon and the pelvic-urethra reflex activity which is mediated by a signaling cascade activated by the estrogen receptor (ER)- phosphatidylinositol-3-kinase (PI3K) interaction at the dorsal lumbosacral spinal cord level.

Materials and Methods

One hundred and seventy-five female Sprague Dawley rats (210–275 g) were used in this experiment. The animal care and the experimental protocol were in accordance with the guidelines of the National Science Council of Taiwan. All efforts were made to minimize both animal suffering and the number of animals used throughout this study. Animals were ovariectomized and tested 20–30 days after the surgery. On the experimental days, rats were anesthetized with urethane intraperitoneally (1.2 g/kg, i.p.). The trachea was intubated to keep the airway patent. A PE-50 intra-colonic catheter was inserted into the descending colon (4 cm from the anus) for the dispensing of mustard or corn oil.

The occipital crest of the skull was exposed and the atlanto-occipital membrane was incised at the midline of the atlanto-occipital membrane with the tip of an 18-gauge needle. A PE-10 catheter was inserted through the slit and passed caudally to the vertebral level of T13 (around L6-S2 level of the spinal cord). The volume of fluid within the catheter was kept constant at 10 μl in all experiments. In experiments using intrathecal injections, a single 10 μl

injection of drug solution was administered followed by a flush with 10 µl of artificial cerebrospinal fluid.

The left pelvic nerve was carefully dissected from the surrounding tissue and was transected. The central stump of the transected nerve was mounted on a pair of stainless steel wire electrodes for stimulation.

The external urethral sphincter electromyogram (EUSE) activities were recorded and amplified 20,000-fold by a preamplifier (Grass P511AC, Cleveland, OH); then continuously displayed on an oscilloscope (Tectronics TDS 3014, Wilsonville, OR) and a recording system with a sampling rate of 20,000 Hz (MP30, Biopac, Santa Barbara, CA).

Results

The role of estrogen on the NMDAR NR2B-mediated spinal reflex potentiation was determined by recording the evoked potential of external urethra sphincter electromyogram activity in ovariectomized rats that received subcutaneous vehicle and estradiol injections. Initial experiments were performed in an attempt to establish a stable baseline reflex activity and the glutamergic NMDAR agonist-induced reflex potentiation in these animals. Single pulses of test simulation (TS, 1 stimulation/30 sec for 10 min) on the pelvic afferent nerve evoked a baseline reflex activity with a single action potential in both the vehicle and estradiol groups. Intrathecal NMDA (10 µM, 10 µL) injected 1 min before stimulation onset induced reflex potentiation characterized by an elongated firing in both groups, but the firing persisted longer in the rats that received E2 compared with those that received the vehicle injection. There was no statistical significance in the mean spike numbers evoked by the test stimulation between these groups (1.00±0.00 vs.1.68±0.31 spikes/stimulation, $P>0.05$, $N=7$). Whereas, the results from the test stimulation in association with the intrathecal NMDA injection increased significantly in the rats that received estradiol (20.87±1.85

spikes/stimulation) compared with those that received vehicle injections (6.48±3.41 spikes/stimulation, $P<0.01$, n=7).

We did a pretreatment with an NMDAR-selective antagonist to further ascertain the involvement of NMDAR in the induction of reflex potentiation. As expected, intrathecal pretreatment with a relatively excessive amount of APV (100 μM, 10 μL) 3 min before stimulation onset abolished the NMDA-induced reflex potentiation in both the ovariectomized rats that received the vehicle (OVX_V) and the E2 injections (OVX_E). The mean spike numbers evoked by the test stimulation in association with an intrathecal NMDA injection were significantly decreased by the pretreatment with APV (1.05±0.02 and 1.01±0.03 spikes/stimulation in OVX_V and OVX_E, respectively) and Co-101244 (a selective NMDA receptor NR2B subunit antagonist, 1.81±0.76 and 5.91±1.19 spikes/stimulation in OVX_V and OVX_E, respectively $P<0.01$ to NMDA, n=7). The selective NMDAR NR2B subunit antagonist, Co-101244, also blocked the NMDA-induced reflex potentiation. Intrathecal administration of a relatively excessive amount of Co-101244 (100 μM, 10 μL) 3 min before stimulation onset abolished the NMDA-induced reflex potentiation in both groups (the ovariectomized rats that received the vehicle and the E2 injections).

Mustard oil (MO) was instilled into the descending colon of ovariectomized rats 5 hours following subcutaneous vehicle or E2 injections. Mustard oil instillation induced sensitization on the evoked pelvic-urethra reflex activity. Moreover, the reflex sensitization caused by mustard oil instillation was higher in the rats that received E2 than in those that received the vehicle injections.

The expression levels of phosphorylated NR2B and total NR2B (pNR2B and tNR2B, respectively) of the left dorsal lumbosacral (L6-S2) spinal cord tissue from the ovariectomized rats that received the vehicle or subcutaneous estradiol injections (E2, 10 mg/kg) were obtained for Western blot analysis. The expression levels of phosphorylated NR2B and total NR2B (pNR2B and tNR2B, 226.7±8.1% and 232.5±8.7% of control at 6 hours, respectively)

increased in a time-dependent manner, when compared with the vehicle injection (VEH 107.6±9.1% and 45.0±5.4% of control in pNR2B and tNR2B, respectively).

The role of these receptors in the estrogen-dependent modulation on the spinal NMDAR NR2B subunit of the left dorsal lumbosacral (L6-S2) spinal cord tissue obtained from ovariectomized rats that received a subcutaneous application of propylpyrazoletriol (PPT, 10 mg/kg, an ERα preferring agonist) and diarylpropionitrile (DPN, 10 mg/kg, an ERβ preferring agonist) was evaluated by Western blot analysis. Parallel to estradiol, 6 hours following subcutaneous injections of propylpyrazoletriol and diarylpropionitrile, there were increments in expression levels of pNR2B (242.11±17.35% of control in PPT as well as 216.87±11.53% of control and DPN, respectively) and tNR2B (217.88±31.05% of control in PPT as well as l78.56±11.73% of control in DPN, respectively) when compared with the vehicle injections (pNR2B, 108.49±9.87% and 121.44±7.21% of control; tNR2B 62.37±9.13% and 59.82±14.80% of control in PPT and DPN, respectively).

Conclusion

We developed a novel model to demonstrate that acute colonic inflammation in rats enhances the reflex activity of the lower urinary tract. Our results show that estrogen may induce subsequent phosphorylation of the NMDAR NR2B subunit and result in modulation of the cross-organ sensitization of pelvic-urethra reflex activity caused by colon MO instillation. This result not only implies the role of estrogen in the regulation of nociception neurotransmission and provides unique insight into the pathogenesis of chronic pelvic pain syndrome but also offers the possibility for developing pharmacological strategies for therapeutics.

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Neuroactive steroids inhibit spinal reflex potentiation by selective enhancing specific spinal GABAA. Pain 2009 Feb 26.

二、出席 2009 年 International continence society 會議投稿回信證明

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Presentation Type: ORAL / POSTER

Authors: Peng H[1], Chen G[2], Lai C[3]

1. *Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung;,* ***2.*** *Department of Obstetrics and Gynecology, Chung Shan Medical University Hospital,* ***3.*** *Department of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan*

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Keywords:	pelvic pain syndrome, urethra, colon , irritable bowel syndrome

PI3k modulates estrogen-dependent facilitation of colon-to-urethra cross-organ reflex sensitization in ovariectomized female rats

[a,b,c]Hsien-Yu Peng, [d]Gin-Den Chen, [a,b]Cheng-Yuan Lai, [a]Ming-Chun Hsieh, [c]Hsao-Hsun Hsu, [b#]Kwong-Chung Tung, [a,d,f#]Tzer-Bin Lin*.

[a]*Department of Physiology, College of Medicine,* [d]*Department of Obstetrics and Gynecology,* [d]*Department of Urology, Chung-Shan Medical University Hospital, Chung-Shan Medical University, Taichung, Taiwan*
[b]*Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan*
[c]*Department of Surgery,* [f]*Graduate Institute of Biomedical Electronics and Bioinformatics, National Taiwan University, Taipei, Taiwan*

#Both authors contributed equally to this study.

Running Title: PI3k modulates cross-organ sensitization

Please send correspondence to:

Dr. Tzer-Bin Lin
Department of Physiology, College of Medicine
Chung-Shan Medical University
No. 110, Chang-Kuo North Rd. Section 1
Taichung, Taiwan 40201
Tel: +886-4-2473-0022-11652
Fax: +886-4-2473-9030
E-mail:tblin@csmu.edu.tw

Key words: pelvic pain syndrome, urethra, irritable bowel syndrome, colon, central sensitization

ABSTRACT

To determine the role of 17β-estradiol (E2) and involvement of intracellular phosphatidylinositol-3-kinase (PI3K) signaling in cross-organ sensitization between the descending colon and the urethra, we analyzed urethra reflex activity and protein expressions in lumbosacral (L6-S2) spinal dorsal horn in response to mustard oil (MO) instillation into the descending colon in ovariectomized female rats. When compared with vehicle solution, intracolonic MO sensitized the N-methyl-D-aspartate receptor (NMDAR) NR2B subunit-dependent reflex activity and increased expression levels of phosphorylated Akt (pAkt) and phosphorylated NR2B (pNR2B) in dorsal horn. Facilitation of reflex sensitization and increases in protein expressionS of pAkt and pNR2B in dorsal horn were induced after pretreatment with a subcutaneous injection of E2 (5 μg/kg), six hours ahead of time, when compared with vehicle solution. This phenomenon was reversed both by intrathecal pretreatment with ICI 182,780 (0.25 mg/kg, *i.t.*) and LY294002 (50 mg/kg, *i.t.*). Immunoprecipitation of dorsal horn tissue revealed a protein-protein interaction between pAkt and pNR2B increases, six hours following the subcutaneous E2 when compared with vehicle injections. Results indicate E2 may activate the PI3K cascade, which subsequently phosphorylates the NR2B subunit, via spinal ERα/ERβ, to facilitate NMDA-dependent cross-organ sensitization, which is presumed to underlie pelvic viscero-visceral referred pain.

Key words: pelvic pain syndrome, urethra, irritable bowel syndrome, colon, central sensitization

INTRODUCTION

Chronic abdominal and pelvic pain is common in women. Estimates suggest that more than 9 million women in the United States experience chronic pelvic pain [31]. Symptoms often localize to more than two pelvic organ systems, and considerable overlap occurs between symptoms in the lower urinary tract, reproductive organs and intestines [18, 28, 61, 78]. This phenomenon implies that pelvic pain may not only arise from an injured gynecological or urinary organ itself, but also be referred from other diseased viscera [20-22] a phenomenon called visero-visceral referred pain [8]. Bowel-to-urogenital tract and urogenital tract-to-bowel cross-organ sensitization have been recently suggested as possible mechanisms underlying viscero-visceral referred pain [59, 74]. However, the detailed mechanism is still obscure because it may involve complicated interactions in afferent neurons, known as central sensitization [81].

Glutamatergic N-methyl-D-aspartate (NMDA)-dependent neurotransmission is presumed involved in forms of spinal central sensitization [2, 23, 43, 62]. Phosphorylation of NR2B tyrosine residues has been noted an important determinant for NMDA-mediated currents [49] for this defines the role of NR2B-containing NMDA receptors (NMDARs) in pain-related neural plasticity [4, 40, 42, 46, 50, 58]. In the lumbosacral spinal cord, our laboratory recently demonstrated a novel form of

central sensitization, cross-organ sensitization, where instillation of mustard oil (MO) into the descending colon sensitized urethra reflex activity [56, 57]. Pharmacological investigation revealed that NMDA-dependent calcium ion influx resulted in phosphorylation of the NR2B subunit, which underlies cross-organ sensitization development [52-54]. Our laboratory also demonstrated that cross-organ sensitization induction is linked to development of viscero-visceral referred pain, for it is characterized by pathological enhancement of urethra activity, caused by activation of nociceptive afferent fibers arising from abdominal/pelvic viscera [12, 13, 37, 38, 55].

With gastrointestinal disorders, many female patients report worsening of symptoms in relationship to their menstrual cycles [6, 27]. Moreover, hormonal therapy is clinically efficacious in the treatment of chronic pelvic pain and functional bowel disease [47]. The severity of uterus-to-urethra cross-organ sensitization, which is suggested to underlie the development of viscero-visceral referred pain at the pelvic area, varies across the estrus cycle in rats [54] All these observations suggest that estrogen plays a role in modulation of nociception [7, 69]. In addition to genomic actions mediated by nucleus receptors, estrogen has nongenomic actions that lead to pathways that participate in acute responses via estrogen receptor (ER)-integrated activation of signal transduction cascades, including phosphatidylinositol-3 kinase (PI3K), which may be recruited through downstream activation of Akt [9, 19, 24, 29,

51, 65, 72, 75, 83, 87]. Gonadal hormones have been demonstrated as affecting induction of pain-related LTP and reflex potentiation at the spinal cord level [26, 35-36, 54-56]. However, the role of estrogen in cross-organ sensitization, a recently proposed form of spinal neural plasticity presumed to underlie viscero-visceral referred pain, has yet to be elucidated. We investigated the impact of estrogen in cross-organ sensitization caused by intracolonic MO-instillation. Moreover, the possibility that PI3K/Akt/NR2B signaling cascades may be downstream of ER and mediate estrogen-dependent modulation of cross-organ sensitization was also investigated.

MATERIALS AND METHODS

Animal Preparations

Female Sprague Dawley rats (n=175; 210–275g) were used in this experiment. Animal care and experimental protocols were in accordance with the guidelines of the National Science Council of Taiwan. This study was reviewed and approved by the Institutional Review Board of Chung-Shan Medical University. Animals were ovariectomized bilaterally via two small lumbar incisions under anesthesia with ethrane (Abbott Illinois, USA), and were tested 20–30 days after surgery. On experimental days, rats were anesthetized with intraperitoneal urethane (1.2 g/kg, i.p.). A PE-50 intracolonic catheter was inserted into the descending colon (4 cm from the anus) for the dispensing of mustard or corn oil. This catheter was held in place by taping the tubing to the tail. The occipital crest of the skull was exposed and the atlanto-occipital 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 T13 vertebral level (L6-S2 of the spinal cord) for Intrathecal injection. Fluid volume within the catheter was kept constant at 10 µl in all experiments. In experiments that used intrathecal injection, a single 10 µl dose of drug solution was administered followed by a flush with 10 µl of artificial cerebrospinal fluid.

Pelvic-Urethra Reflex Activity Recording

The left pelvic nerve was carefully dissected from the surrounding tissue and mounted on a pair of stainless steel wire electrodes for stimulation. Oligo-/single unit spike action potentials of the external urethral sphincter electromyogram (EUSE) were continuously recorded by a pair of epoxy-coated, copper-wire electrodes and displayed on a recording system with a sampling rate of 20,000 Hz (MP30, Biopac, Santa Barbara, CA).

Subcutaneous injection of 17β-estradiol was done six hours before pelvic afferent nerve stimulation. In some experiments, ICI 182,780 or LY294002 was injected (0.25 and 50 mg/kg, i.p.) 30 min before estradiol injection to antagonize estradiol effects. Single shocks (pulse duration: 0.05 ms, 1/30 sec) (test stimulation: TS) were applied to the pelvic nerve through a pair of stimulation electrodes. At the beginning of each experiment, an intensity that caused a single spike action potential in the reflex activity was used to standardize baseline reflex activity. This intensity was used for stimulation throughout each experiment. Protocols for assessing effects of different reagents on reflex activity were as follows:

Protocol 1. Pelvic afferent nerve TS: Single electric shocks at a fixed suprathreshold strength repeated at 30 sec intervals for 10 min were applied to the left pelvic nerve through a pair of stimulation electrodes, six hours after subcutaneous estradiol injection. This frequency was chosen because it did not result in response facilitation.

Protocol 2. Agonist-induced reflex potentiation: After an equilibrium period (usually 10 minutes), NMDA (10 μM, 10 μL) was injected intrathecally 1 min before stimulation began. The TS was then applied to the pelvic afferent nerve to induce reflex potentiation. In some experiments, APV (10 μM, 10 μL) or Co-101244 (100 nM, 10 μL) was intrathecally administrated 10 min before NMDA injection to antagonize the effects of NMDA and the NR2B subunit, respectively.

Protocol 3. Cross-organ sensitization: Mustard oil (0.1 ml of 0.5%) was instilled into the lumen of the descending colon, five hours following estradiol injection (one hour before nerve stimulation onset) through the intracolonic catheter to induce acute colon irritation. Effects on pelvic-urethra reflex activity were evaluated by applying the TS to the pelvic nerve for 10 minutes at 1, 60, and 180 minutes after instillation.

Application of Drugs

The drugs used included 17β-estradiol (E2, estrogen agonist, 50 μg/kg s.c., Sigma), propylpyrazoletriol (PPT, ERα-preferring ligand, 10 mg/kg *i.t.*, Tocris), diarylpropionitrile (DPN, ERβ-preferring ligand, 10 mg/kg *i.t.*., Tocris), ICI 182,780 (ICI, nonselective ER antagonist, 0.25 mg/kg i.p., Tocris) [64], LY294002 (LY, PI3K inhibitor, 50 mg/kg i.p., Tocris) [66], N-methyl-D-aspartic acid (NMDA, selective glutamatergic NMDAR agonist, 10 μM, 10 μl i.t., Sigma), $_D$-2-amino-5-phosphonovalerate (APV, glutamatergic NMDA receptor antagonist; 10

μM, 10 μl i.t., Sigma), Co-101244 (Co, selective NMDA receptor NR2B subunit antagonist, 100 nM, 10 μL i.t., Tocris), allyl isothiocyanate (mustard oil, MO, pungent component that causes colitis, 0.1 ml of 0.5% diluted in corn oil, intracolonic, Sigma), corn oil (CO, control solution for mustard oil, 0.1 ml intracolonic, Sigma). Doses were modified from previous studies and are summarized in Table 1.

Western Blotting

For the Western blot analysis, animals were decapitated after experimental procedures were finished. The dorsal halves of the spinal cord segments from L6-S2 on the left side (ipsilateral to the stimulated nerve) were dissected and the amounts of protein were quantified. Protein samples (20 μg) were separated on SDS-PAGE (12%) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk and probed sequentially with antibodies against phosphorylated Akt (pAkt, 1:1000, Santa Cruz), total NR2B (tNR2B, 1:1000, Chemicon), phosphorylated NR2B (pNR2B, 1:1000, Chemicon) and β-actin (1:10000, Chemicon). Blots were incubated with HRP-conjugated antibody (1:10000, Santa Cruz) for one hour at room temperature. After visualization with ECL solution, protein levels were determined using LAS 3000 (Fuji, Japan). Densitometry of the blotted membranes was done using Science Lab 2003 (Fuji, Japan). Results were normalized againstβ-actin and are presented as mean ± SD.

Coimmunoprecipitation of pAkt with PSD95 and NR2B

Rabbit polyclonal pAkt antibody (5 µg; Santa Cruz) was incubated overnight at 4°C with the crude plasma membrane fraction (500 µg) extracted from the left lumbosacral (L6-S2) dorsal horns of ovariectomized rats that received vehicle or estradiol. The 1:1 slurry protein agarose suspension (Millipore) added into that immuno-complex protein, and the mixture was incubated for 2–3 h at 4°C. Agarose beads were washed once with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 0.02% (w/v) sodium azide), twice with 1% (vol/vol) Triton X-100 in an immunoprecipitation buffer plus 300 mM NaCl, and three times with an immunoprecipitation buffer only. Binding proteins were eluted with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 95°C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes electrophoretically, and detected using rabbit polyclonal anti-pAkt, mouse monoclonal anti-PSD-95 (Santa Cruze, USA), and rabbit polyclonal anti-NR2B (Millipore). Thirty micrograms of spinal cord plasma membrane fraction were loaded as a positive control (input).

Data Analysis

Electromyogram activity was recorded using a sampling rate of 5,000 samples/sec, with a conventional band-pass filter setting (30-3000 Hz). Spike number

elicited by stimulation shocks was averaged using the mean spike numbers evoked by the last three stimulations. Comparisons across different stimulation parameters as well as all drug and vehicle treated groups were determined using one-way, repeated-measure analysis of variance, followed by a post-hoc test (Tukey test, SigmaStat 2.0; Systat Software Inc., San Jose, CA, USA). In all cases, a difference of $p<0.05$ was considered statistically significant.

RESULTS

Estradiol effects on pNR2B expression

Using immunoblotting with an antibody against phosphorylated NMDAR NR2B subunit (pNR2B), we determined whether or not estrogen activates the NMDAR NR2B subunit at the spinal cord level by subcutaneously administering vehicle solution or estradiol (50 μg/kg) to ovariectomized rats. Moreover, because there is evidence demonstrated that estradiol can exert rapid effects on sexual behaviors [14], we measured the expression of phosphorylated NR2B protein at 0, 3, and 6 hours (hr) following injections. We observed that the expression levels of phosphorylated NR2B (Figure 1A pNR2B) in left lumbosacral (L6-S2) spinal dorsal horn tissue from rats that received subcutaneous estradiol injections (E) were remarkably increased in a time-dependent manner (105.17±22.41%, 205.33±7.91% and 226.73±8.12% of β-actin at 0, 3 and 6 hours, respectively, ** $p<0.01$ to 0 hr, n=4) when compared with those received vehicle injection (VEH 107.67±9.15% of β-actin at 6 hours).

ER α and ER β agonists

Evidence has shown that both ER α and ER β receptors contribute to estrogen-mediated promotion of neuronal functions and underlying mechanisms [85, 86]. We evaluated the role of these receptors in estradiol-dependent modulation of NMDAR NR2B subunits at the spinal cord level using Western blot analysis of left

lumbosacral (L6-S2) dorsal horn tissue obtained from ovariectomized rats that received subcutaneous application of propylpyrazoletriol (Figure 1A PPT, 10 mg/kg, ERα preferring agonist) or diarylpropionitrile (DPN, 10 mg/kg, ERβ preferring agonist). Consistent with estradiol effects, subcutaneous propylpyrazoletriol and diarylpropionitrile, at 0, 3, and 6 hours following injection, also exhibited time-dependent increases in expression levels of pNR2B (88.93±10.03%, 208.97± 8.12% and 242.11±17.35% of β-actin in PPT as well as 105.78±0.15%, 111.62± 12.35% and 216.87±11.53% of β-actin in DPN, respectively. ** p<0.01 to 0 hr, n=4) when compared with rats that received vehicle injections (108.49±9.87% and 121.44± 7.21% of β-actin in PPT and DPN, respectively). Moreover, no significant differences were noted in pNR2B expression between animals that received propylpyrazoletriol and diarylpropionitrile, at 6 hours after injection (242.11±17.35% and 216.87±11.53% of β-actin, respectively), indicating an equal contribution for these receptors in estradiol-dependent NMDAR NR2B subunit activation.

Akt phosphorylation

ER-dependent activation of the PI3K pathway mediates an acute estrogen response [29, 75]. We determined whether or not estradiol activates PI3K cascades to mediate NR2B activation in the spinal cord by immunoblotting the PI3K pathway using antibodies against phosphorylated Akt (pAkt), which is induced following PI3K

activation. As anticipated, phosphorylated Akt expression (Figure 1B pAkt) in the left lumbosacral (L6-S2) dorsal horn increased in a time-dependent manner at 0, 3, and 6 hours following subcutaneous estradiol (E, 5 μg/kg, 55.12±9.98%, 128.42±20.03% and l63.51.1±20.05% of β-actin at 0, 3, and 6 hours, respectively; ** p<0.01 to 0 hr, n=4), propylpyrazoletriol (PPT, 10 mg/kg, 52.36±4.71%, 77.51±23.35% and l03.92± 17.23% of β-actin at 0, 3, and 6 hours, respectively; * p<0.05 to 0 hr, n=4) and diarylpropionitrile (DPN, 10 mg/kg, 29.34±12.08%, 114.21±19.85% and l13.66± 11.30% of β-actin at 0, 3, and 6 hours, respectively; ** p<0.01 to 0 hr, n=4) injections when compared with those that received vehicle injections (52.36±4.71%, 56.72± 9.88% and 54.75±11.41% of β-actin in E, PPT and DPN, respectively). To further confirm that PI3K downstream the estrogen receptor to mediate estradiol-mediated NR2B phosphorylation, left lumbosacral (L6-S2) dorsal horn tissue was harvested from ovariectomized rats that received vehicle solution (OVX_V) or estradiol (OVX_E) injection for Western blot analysis, six hours following vehicle or estradiol injection. In the OVX_E group, expression levels of pAkt and pNR2B (Figure 2A&B, 153.66± 1.85% and 160.33±12.01% of β-actin, respectively; ** p<0.01 to OVX_V, n=4) were statistically higher than those for the OVX_V group (102.18±6.35% and 30.33±5.78% of control, respectively). We next pretreated OVX_E animals with ICI 182,780 (non-selective estrogen receptor antagonist, ICI+OVX_E) or LY294002 (PI3K inhibitor,

LY+OVX$_E$) 30 min before estradiol injection. We found that increases in pAkt and pNR2B caused by estradiol were successfully reversed by pretreatment with ICI 182,780 (116.33±1.85% and 42.00±14.97% of β-actin, respectively; ## p<0.01 to OVX$_E$, n=4) and LY294002 (86.12±9.45% and 45.00±11.78% of β-actin, respectively; ** p<0.01 to OVX$_E$, n=4).

Coprecipitation of pAkt with PSD and NR2B subunit

There is evidence to support that NMDAR NR2B subunit phosphorylation may be involved in the development of some of the phenomenon associated with neuropathic and post-inflammatory pain [1, 67, 68]. To clarify whether or not the Akt/PSD/NR2B cascade is involved in estrogen-mediated NR2B activation in the spinal cord, we examined pAkt interaction with PSD95 and NR2B subunits, first by using anti-pAkt antibodies to co-immunoprecipitate proteins from the crude membrane fraction extracted from left lumbosacral (L6-S2) dorsal horn tissue of the OVX$_V$ or OVX$_E$ groups. Immunoblotting showed an increase in pAkt, PSD95 and pNR2B expression in OVX$_E$, when compared with the OVX$_V$ group (Figure 2C). To further demonstrate that PSD95 and NR2B form a complex with pAkt in the dorsal horn, co-immunoprecipitation (IP: pAkt) was done with the anti-pAkt antibody. We found that PSD95 and NR2B were both pulled down by the anti-pAkt antibody in the crude membrane extract from the OVX$_E$ but not the OVX$_V$ group.

NMDA-induced reflex potentiation

The role of estrogen in NMDAR NR2B-mediated spinal reflex potentiation was determined using spike potentials in external urethra sphincter electromyogram (EUSE) activity evoked by 10 minutes of pelvic nerve TS in the OVX_V and OVX_E groups. Initial experiments were performed to establish a stable baseline reflex activity and NMDAR agonist-induced reflex potentiation in these animals. As shown in Figure 3, single TS pulses evoked a baseline reflex activity with a single action potential in both groups (TS, 1.00±0.00 and 1.68±0.31 spikes/stimulation in OVX_V and OVX_E, respectively). Intrathecal NMDA (TS+NMDA, 10 μM, 10 μL) at 1 min before stimulation onset induced reflex potentiation characterized by an elongated firing in these groups, but the firing persisted longer in the OVX_E (20.87±1.85 spikes/stimulation. ** p<0.01 to TS, ++ p<0.01 to OVX_V, n=7) when compared with OVX_V group (2.48±3.41 spikes/stimulation, ** p<0.01 to TS, n=7).

Glutamatergic NMDA and NR2B antagonists

To further ascertain the involvement of NMDAR in reflex potentiation induction, we carried out an intrathecal pretreatment using an NMDAR-selective antagonist, APV, 10 min before NMDA injection. As expected, (TS+APV+NMDA, 100 μM, 10 μL) it abolished the NMDA-induced reflex potentiation in the OVX_V and OVX_E

groups (Figure 3A, 1.05±0.02 and 1.01±0.03 spikes/stimulation in OVX_V and OVX_E, respectively. ## p<0.01 to TS+NMDA, n=7). Studies have revealed that the NMDAR NR2B subunit plays a crucial role in NMDA-dependent neural plasticity induction [4, 50]. We tested whether or not a selective NR2B subunit antagonist, Co-101244, also blocks NMDA-induced reflex potentiation. We found that it did (TS+Co+NMDA, 100 μM, 10 μL) in both the OVX_V and OVX_E groups (1.81±0.76 and 5.91±1.19 spikes/stimulation in OVX_V and OVX_E, respectively. ## p<0.01 to TS+NMDA, n=7).

Involvement of estrogen receptors

To determine ER-PI3K interaction involvement in estrogen-dependent facilitation of the NMDA-induced reflex potentiation, we investigated ER involvement by administering an intrathecal pretreatment using ICI 182,780, a non-selective ER antagonist, to OVX_E rats (Figure 4, ICI+ OVX_E). Pretreatment at 30 min before estradiol injection exhibited no effect on test stimulation-induced baseline reflex activity (TS, 1.78±0.82 spikes/stimulation), while it inhibited NMDA-induced reflex potentiation (TS+NMDA, 13.75 ± 1.23 spikes/stimulation, ## p<0.01 to VEH+OVX_E, n=7). We next determined the role of PI3K by intrathecal application of LY294002, a PI3K inhibitor, to OVX_E rats. Pretreatment at 30 min before estradiol injection caused no effect on test stimulation-induced baseline reflex activity (Figure 4) (TS, 1.62±0.75 spikes/stimulation), though it did attenuate NMDA-induced reflex

potentiation (TS+NMDA, 9.37±0.66 spikes/stimulation. ## p<0.01 to VEH+OVX_E, n=7).

Cross-organ sensitization

Mustard oil (MO) is a pungent compound that directly stimulates small-diameter sensory fibers [3]. It is used to study neuronal signaling in nociception [30, 41]. Intracolonic mustard oil instillation has been shown to induce NMDAR-dependent cross-organ sensitization of urethra reflex activity [54-56]. To investigate the role of estrogen in NMDA-dependent cross-organ sensitization between the colon and urethra, we instilled corn oil or mustard oil into the descending colon, five hours after subcutaneous vehicle (OVX_V) or estradiol injections (OVX_E). As shown in Figures 5A & B, in both groups, corn oil instillation into the colon exhibited no effect (TS+CO, 1.00±0.00 and 1.25±0.31 spikes/stimulation, in OVX_V and OVX_E, respectively), while mustard oil instillation induced sensitization of urethra reflex activity (TS+MO). The MO-elicited reflex sensitization was characterized by a higher firing rate and a longer discharge period in OVX_E (OVX_E, 577.25±81.14 spikes/stimulation, ** p<0.01 to TS+CO, ++p<0.01 to OVX_V, n=7) than OVX_V rats (OVX_v, 185.53±18.21 spikes/stimulation, ** p<0.01 to TS+CO, n=7). We next investigated the role of estrogen receptors and the involvement of PI3K in estrogen-dependent facilitation of cross-organ sensitization by pretreating the OVX_E

group with specific antagonists, 30 min before estradiol injection. Mustard oil-elicited cross-organ sensitization facilitation, caused by subcutaneous estradiol, was reversed by pretreatment with the ER antagonist, ICI 182,780 (Figure 5B TS+ICI+MO, 65.23± 15.11 spikes/stimulation, ## p<0.01 to MO, n=7), and the PI3K inhibitor, LY 294002 (TS+LY+MO, 15.23±1.48 spikes/stimulation, ## p<0.01 to MO, n=7).

To further clarify the role of Akt/NR2B in estrogen-dependent facilitation of cross-organ sensitization, lumbosacral (L6-S2) dorsal horn tissues ipsilateral to the stimulated nerve were harvested from the OVX_V and OVX_E animals, 60 min after corn or mustard oil instillation for immunoblotting. In both groups, expression levels of pAkt (Figure 6A, 95.33±7.42% and 143.66±6.43% of β-actin in OVX_V and OVX_E, respectively) and pNR2B (Figure 6B, 58.00±1.52% and 85.66±5.78 % of β-actin in OVX_V and OVX_E, respectively; ** p<0.01 to CO, n=4) were significantly increased by mustard oil when compared with corn oil (pAkt, 62.66±8,19% and 84.66±6.74% of β-actin, pNR2B 24.66±5.69% and 42.00±4.58% of β-actin in OVX_V and OVX_E, respectively). In addition, expression levels of pAkt and pNR2B were significantly higher in the OVX_E (OVX_E+TS) than the OVX_V group (OVX_V+TS). Pretreatment with ICI 182,780 reversed the MO-induced increase in pAkt and pNR2B expression in the OVX_E group (120.00±2.88% and 57.66±3.17% of β-actin, respectively. # p<0.05, ## p<0.01 to MO, n=4). LY294002 also exhibited a similar reversal of pAkt

and pNR2B expression (26.33±5.23% and 33.66±5.78% of β-actin in pAkt and pNR2B, respectively. ## p<0.01 to MO, n=4), like ICI 182,780.

DISCUSSION

Our results demonstrate that estradiol-dependent activation of PI3K, which is required for downstream activation of the ER-associated signaling pathway, pAkt, and subsequently phosphorylation of the NMDAR NR2B subunit, may modulate MO-elicited cross-organ sensitization between the descending colon and urethra reflex activity that is presumed to be pathophysiologically relevant to the development of viscero-visceral referred pain. This pathway may be related to the high concurrence of irritable bowel syndrome and chronic pelvic pain syndrome.

Cross-innervations of visceral organs in the pelvic cavity offer a complex sensory pathway within the spinal cord, presumed essential for physiological regulation and integration of sexual, bowel and bladder functions [6, 59, 77]. Such complicated communication in the nervous system may also underlie the pathophysiological mechanisms of viscero-visceral referred pain in the pelvic area; injury or inflammation in one pelvic organ may lead to modifications in the functions of others [10, 16, 17, 59, 71, 84]. In the present study, MO instillation into the descending colon demonstrates cross-organ sensitization of urethra activity. This cross-organ sensitization might, at least in part, mimic pathophysiological conditions that occur during acute colon irritation, therefore providing an animal model not only for pathophysiological mechanisms underlying the high concurrence of urological,

gynecological and gastrointestinal pain in the pelvic area but also for development of effective pharmacological strategies for pelvic pain.

A number of pain syndromes are more prevalent in women, including irritable bowel syndrome, fibromyalgia, and temporomandibular joint disorders [5, 73]. In many cases, the severity of pain fluctuates with the menstrual cycle [15, 32]. This suggests that gonadal steroid levels may be related to pain severity [26]. Estrogen is not only known to affect the urogenital system, but it also may modulate neural responses within the central nervous system [63]. Our previous research has shown that surgical ablation of menses attenuates repetitive stimulation-induced spinal reflex potentiation; hormone replacement therapy reverses such attenuation caused by castration [36]. In the current study, we studied the effects of gonodal hormone levels on reflex sensitization between the colon and urethra. In line with the well-established notion that estrogen facilitates neural responses to noxious stimulation, our results demonstrate estrogen-mediated, enhanced cross-organ sensitization in rats [5, 15, 32, 73]. These results extend the role of estrogen-dependent neural facilitation from amplifying pain-related responses, thus inducing hyperalgesia in a visceral organ itself, to participating in enhancement of cross-organ sensitization that might underlie viscero-visceral referred pain.

In hippocampal neurons, estrogen neural promoting effects through ERα and

ERβ receptors [85, 86]. Using receptor subtype-preferring ligands, we show that both ERα and ERβ receptor-preferring ligands upregulate expression levels of pAkt and pNR2B proteins in the lumbosacral dorsal horn. Moreover, no significant relative contribution for either receptor subtype was found. Both ERα and ERβ seem crucial for ongoing estrogen effects in the spinal cord. These results are in line with a recent study that investigated sex differences in nociceptive threshole that used genetic knock-out mice. Nociceptive threshold were significantly elevated in rats where ERα and ERβ receptors were both knocked-out, but not in wild-type animals or rats with knock-outs of either ERα or ERβ [33].

Non-genomic estrogen signaling was first proposed in the late 1970s when it was discovered that estrogen can bind receptor proteins located in the cell membrane and initiate a rapid generation of cAMP in endometrial cells [60]. Since then, nongenomic estrogen signaling has been linked to other aspects of neuroendocrinology, including GnRH secretion, electrophysiological responses in neurons, and reproductive behavior [75]. Akt is the principal downstream effector of PI3K, triggering several of its cellular effects, including cell growth, survival and the neuroprotective effects of estrogen in neurons [25, 45, 70]. By measuring protein expression levels in the lumbosacral dorsal horn, we demonstrate that subcutaneous estrogen, PPT and DPN injections phosphorylate Akt, a substrate of PI3K, and NR2B, the subunit defining the

electrophysiological characteristics of NMDAR. Estrogen may activate both the PI3k/Akt pathway and NR2B-containing NMDAR through ERα and ERβ. ICI 182,780 and LY 294002 both reversed the estrogen-dependent facilitation on NMDA-induced reflex potentiation. Finally, our coprecipitation results demonstrate that estrogen-induced protein-protein interactions between phosphorylated Akt and PSD95, as well as phosphorylated Akt and phosphorylated NR2B, indicating that protein interactions between pAkt and PSD95, downstream of PI3K, may lead to phosphorylation of the NMDA NR2B subunit. PI3K/Akt and subsequent NR2B phosphorylation mediated by PSD95 seem an essential intracellular cascade for estrogen-dependent facilitation of the NMDA-mediated neural plasticity that underlies pain response modulations, such as hypergesia and/or viscero-visceral referred pain. This proposal is consistent with a recent study that showed the spinal PSD95/NR2B pathway as having an important role in augmentation of reflex activity in a cross-talk manner [57].

Sensitization of neural activity may be the result of peripheral and/or central mechanisms. The convergence of sensory fibers, coming from adjacent pelvic structures or bifurcating afferent fibers, accounts for the peripheral mechanism [44]. Central integrations of neural activity at various levels, including the spinal cord, brain stem, thalamus and amygadala, have been suggested involved in central

mechanisms [11, 48, 61]. Since the cross-organ sensitization presented in this study is defined by peripheral actions (intracolonic MO instillation sensitize the urethra reflex activity), convergence of sensory afferent fibers or axon collaterals mediating the cross-organ sensitization cannot be excluded. On the other hand, along with the induction of cross-organ sensitization in this study, there were correlated increases of pAkt and pNR2B in the dorsal horn. Moreover, a parallel reversal of pAkt and pNR2B increases occurred, as did cross-organ sensitization, when ER and PI3K were pharmacologically antagonized at the spinal cord level. In contrast to studies showing peripheral mechanisms, the central mechanism in this study seemed to be in the spinal cord. This proposal is consistent with studies of the neurophysiological basis of CNS-mediated sensitization, based on animal models of chronic somatic pain, which have shown that following injury or inflammation, chronic somatic pain involves heightened activity of small diameter C-fiber neurons, inducing activation of NMDA receptors expressed in the dorsal horn, which increase their excitability and responsiveness [76, 79, 80, 82]. However, efforts should continue to clarify the mechanism involved in the induction of cross-organ sensitization.

In conclusion, we describe a new model where acute colon irritation enhances urethra reflex activity in ovariectomized rats. Our results further show that estrogen, via the ER/pAkt/PSD cascade, may induce subsequent phosphorylation of the

NMDAR NR2B subunit, resulting in modulation of cross-organ sensitization of urethra reflex activity caused by intracolonic MO instillation. This result not only demonstrates the role of estrogen in regulation of nociception neurotransmission, it provides unique insight into the pathogenesis of viscero-visceral referred pain syndrome. It also offers the possibility for developing pharmacological strategies for pelvic pain therapy.

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Legends

Figure 1. Effects of subcutaneous estradiol (E), propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) on expression levels of phosphorylated NR2B (pNR2B) and phosphorylated Akt (pAkt) in left lumbosacral (L6-S2) dorsal horn tissue of ovariectomized rats. Western blots show that at 0, 3, and 6 hours (hr) following injections, expression levels of (A) pNR2B and (B) pAkt were both increased in a time-dependent manner by subcutaneous E, PPT, and DPN when compared with vehicle injections (VEH, * $p<0.05$, ** $p<0.01$ to VEH, n=4).

Figure 2. Effects of intrathecal ICI 182,780 and LY294002 pretreatments on estradiol-dependent upregulation of phosphorylated Akt (pAkt) and phosphorylated NR2B (pNR2B) in left lumbosacral (L6-S2) dorsal horn tissue of ovariectomized rats. Six hours after subcutaneous estradiol injections (OVX_E), expression levels of (A) pAkt and (B) pNR2B both increased when compared with vehicle injections (OVX_V, ** $p<0.01$ to OVX_V, n=4). Pretreatments with ICI 182,780 (ICI+OVX_E) and LY294002 (LY+OVX_E), 30 min before estradiol, reversed the increases of pAkt and pNR2B expression caused by estradiol injections (## $p<0.01$ to OVX_V, n=4). (C) Co-immunoprecipitation analysis of left lumbosacral (L6-S2) dorsal horn tissue obtained from OVX_V and OVX_E animals. Immunoblotting (input) in the left column

shows increases in expression levels of pAkt, PSD95, and pNR2B in OVX_E when compared to OVX_V rats. Immunoprecipitation blotting (IP) in the right column shows an increment of PSD95 and pNR2B immunoprecipitation with anti-pAkt antibody in crude membrane extract from the OVX_E but not the OVX_V group.

Figure 3. NMDA-induced reflex potentiation. In ovariectomized rats, which received subcutaneous vehicle (A OVX_V) and estradiol (B OVX_E) injections, test stimulation (TS, 1 stimulation/30 sec for 10 min) at 6 hours after injection evoked a constant baseline reflex activity with a single action potential in external urethra sphincter electromyogram (EUSE) activity in both groups. Intrathecal NMDA (TS+NMDA, 10 μM, 10 μL, 1 min before stimulation onset) induced reflex potentiation that persisted longer in OVX_E than OVX_V rats. Pretreatment with APV and Co-101244 (TS+APV+NMDA and TS+Co+NMDA, respectively; 10 μM and 100nM, 10 μL, 10 min before stimulation onset) abolished NMDA-induced reflex potentiation. (C) Mean spike number evoked by each impulse counted 10 min following the TS onset in OVX_V (white bar) or OVX_E (black bar). No statistical significance was found in mean spike numbers evoked by TS between these groups (TS, $p>0.05$, n=7). Mean spike numbers evoked by TS in association with intrathecal NMDA injections were significantly increased in OVX_E compared with OVX_V (TS+NMDA, ** $p<0.01$ to

OVX_V, n=7). Mean spike number increases caused by intrathecal NMDA were significantly reversed by pretreatment with APV and Co-101244 (TS+APV+NMDA and TS+Co+NMDA, respectively. ## p<0.01 to TS+NMDA, n=7).

Figure 4. Effects of ICI 182,780 and LY294002 on estradiol-dependent facilitation of NMDA-induced reflex potentiation. (A) In ovariectomized rats that received subcutaneous estradiol (OVX_E), test stimulation (TS, 1 stimulation/30 sec for 10 min) 6 hours after injection evoked a baseline reflex activity with a single action potential in external urethra sphincter electromyogram (EUSE) activity. Intrathecal NMDA (TS+NMDA, 10 μM, 10 μL), 1 min before stimulation onset, induced reflex potentiation in the same preparation. Pretreatment with intrathecal ICI 182,780 and LY294002 (ICI+OVX_E and LY+OVX_E, respectively), 30 min before estradiol injection, reversed NMDA-induced reflex potentiation. (B) Mean spike number evoked by TS (white bar) or TS with intrathecal NMDA (TS+NMDA, black bar) counted 10 min after stimulation onset in OVX_E rats and OVX_E in association with pretreatment with vehicle solution (VEH+OVX_E), ICI 182,780 (ICI+OVX_E) and LY294002 (LY+OVX_E). No statistically significant differences were found in mean spike numbers in those with TS with intrathecal NMDA (VEH+OVX_E) and without vehicle injections (OVX_E, p>0.05 to OVX_E, n=7), whereas mean spike numbers

decreased significantly with ICI 182,780 and LY294002 pretreatments compared with those that received vehicle injections (VEH+OVX_E, ## p<0.01 to VEH+OVX_E, n=7).

Figure 5. Intracolonic mustard oil (MO) instillation induced cross-organ sensitization of urethra reflex activity. (A) and (B). When compared with corn oil (TS+CO), which exhibited no effect on baseline reflex activity evoked by test stimulation (TS), colon MO instillation (TS+MO) sensitized the reflex activity in both ovariectomized rats that received subcutaneous vehicle solution and estradiol injections (OVX_V and OVX_E, respectively) 60 and 180 min after instillation. Moreover, in OVX_E rats, the sensitized reflex activity was characterized by a longer firing rate and a longer discharge period. Intrathecal pretreatment with ICI 182,780 (TS+ICI+MO) and LY294002 (TS+LY+MO) both reversed the facilitation of cross-organ sensitization caused by estradiol. (C) There were no statistically significant differences in mean spike number between TS with corn oil (TS+CO) of OVX_V (white bars) and OVX_E (black bars) groups, while the mean spike number increased significantly in OVX_E compared with OVX_V when MO was used (TS+MO, ++ p<0.01 to OVX_V, n=7). (D) In OVX_E rats, colon MO instillation (MO) significantly increased mean spike numbers evoked by TS (OVX_E+TS) when compared with OVX_V rats (OVX_V+TS, ** p<0.01 to OVX_E+TS, n=7). The increase in mean spike number caused by estradiol

was significantly reduced by intrathecal pretreatments with ICI 182,780 and LY294002 (ICI+MO and LY+MO, respectively. ## p<0.01 to MO, n=7).

Figure 6. The role of estrogen receptors and PI3K signaling in estradiol-dependent facilitation of mustard oil-induced cross-organ sensitization. (A) and (B). Western blots showing expression levels of phosphorylated Akt (pAkt) and phosphorylated NR2B subunits (pNR2B) in protein samples from the lumbosacral (L6-S2) dorsal horn, ipsilateral to the stimulation site, obtained from ovariectomized rats that received vehicle (OVX_V) and estradiol (OVX_E) injections in response to intracolonic corn oil (CO) or mustard oil (MO) instillation. When compared with CO, MO instillation increased expression levels of pAkt and pNR2B in both groups (* p<0.05, ** p<0.01 to CO, n=4). The increase in protein expression was higher in OVX_E (OVX_E+TS, ++ p<0.01 to OVX_E+TS, n=4) than in the OVX_V group (OVX_V+TS). Intrathecal pretreatments with ICI 182,780 and LY294002 (TS+ICI+MO and TS+LY+MO, respectively) significantly reversed the increase in protein expression caused by estradiol (# p<0.05, ## p<0.01 to MO, n=7)

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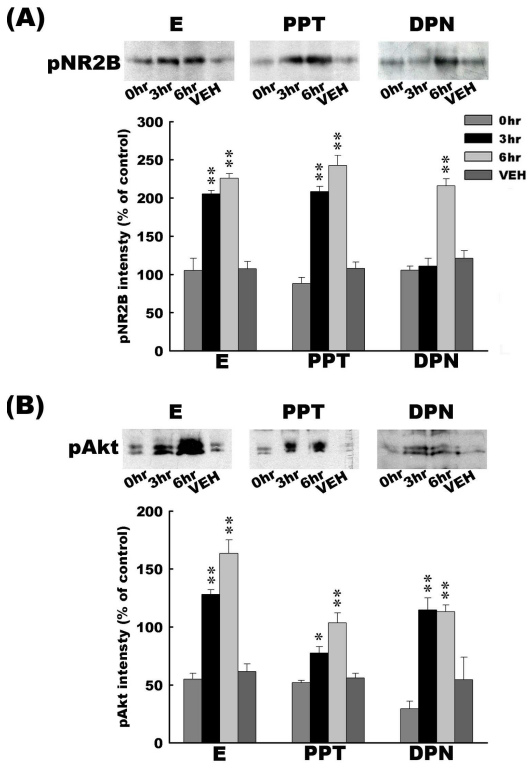
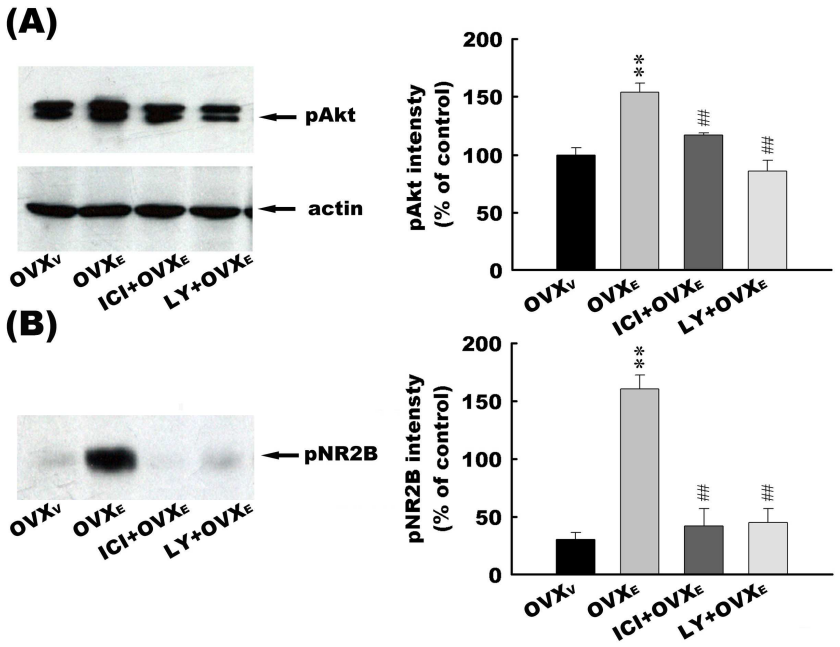
Table 1

Drug	Abbreviation	Concentration.	Route	Reference
17β-estradiol	E2	5 µg/kg	s.c.	[39]
Propylpyrazoletriol	PPT	10 mg/kg	s.c.	[39]
Diarylpropionitrile	DPN	10 mg/kg	s.c.	[39]
ICI 182,780	ICI	0.25 mg/kg	i.p.	[64]
LY294002	LY	50 mg/kg	i.p.	[66]
N-methyl-D-aspartic acid	NMDA	10 µM, 10 µl	i.t.	[56]
D-2-amino-5-phosphonovalerate	APV	10 µM, 10 µl	i.t.	[56]
Co-101244	Co	100 nM, 10 µl	i.t.	[56]
allyl isothiocyanate	Mustard oil, MO	0.1 ml of 0.5 %	intracolonic	[56]
Corn oil	CO	0.1 ml of 0.5 %	intracolonic	[56]



209x297mm (300 x 300 DPI)



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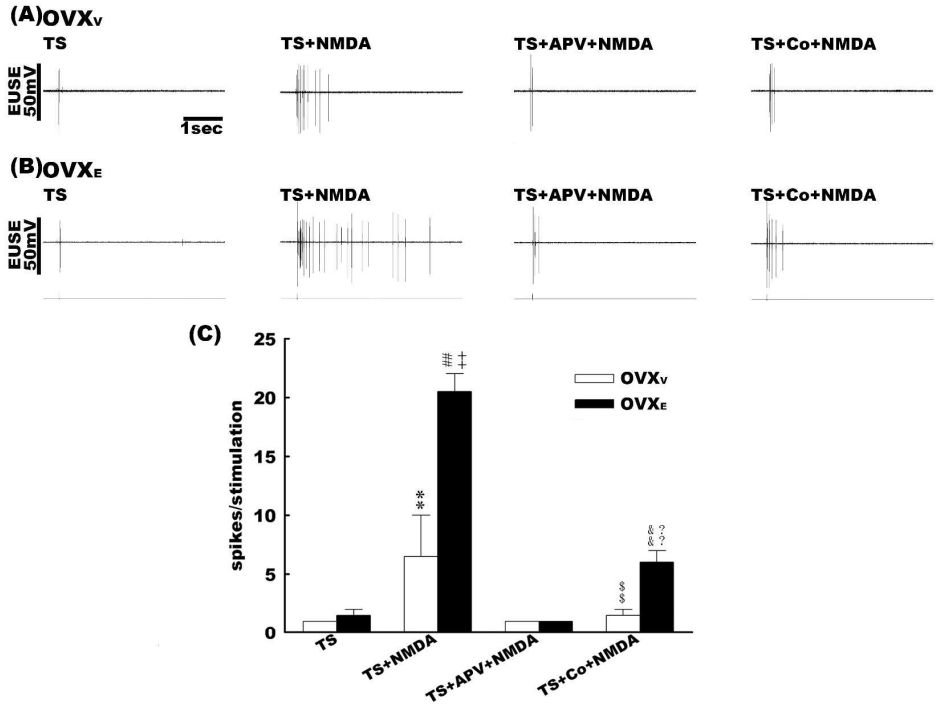

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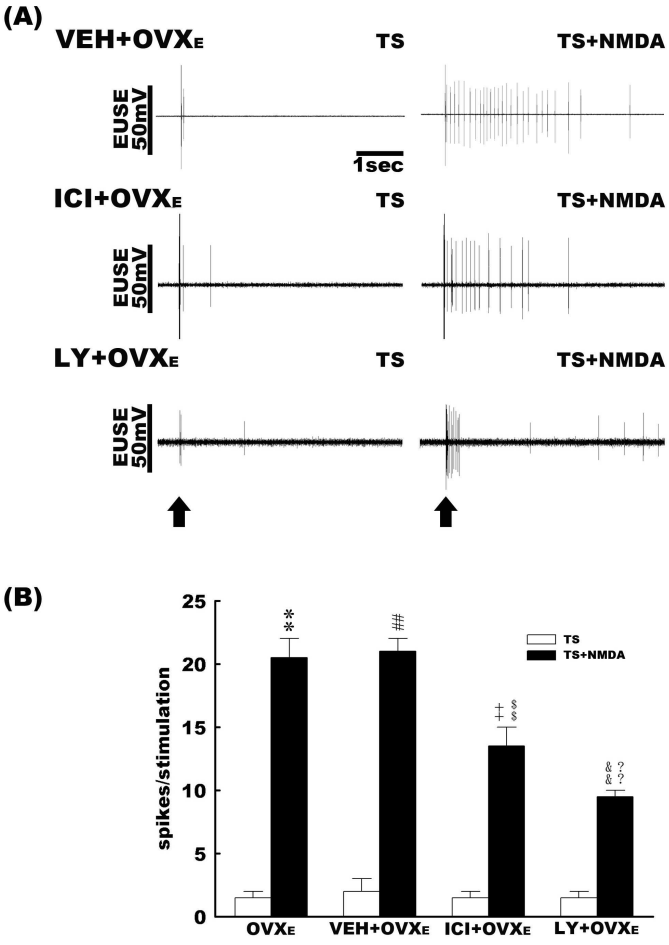

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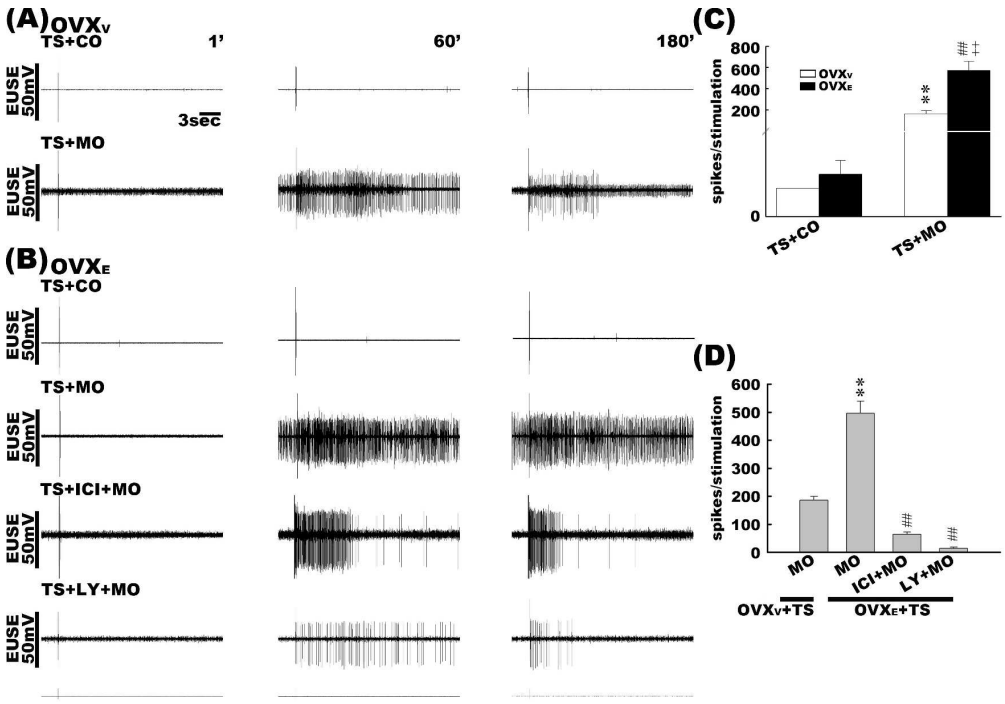
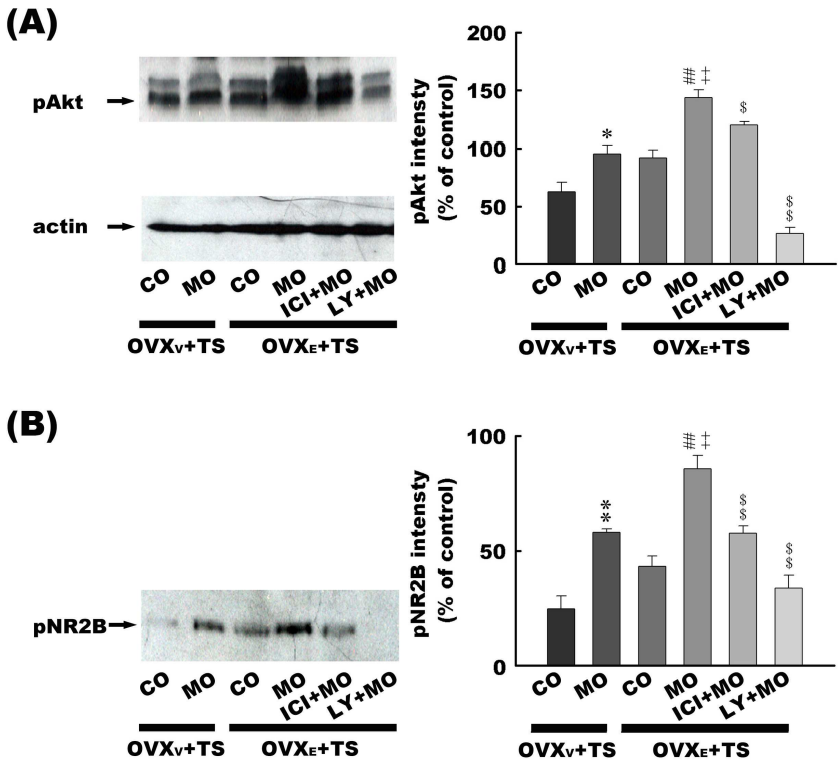


297x209mm (300 x 300 DPI)



209x297mm (300 x 300 DPI)