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經 Estrogen 治療後大白鼠的逼尿肌與尿道及其支配神經之反應

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Estrogen Modulates the Spinal NMDA-Mediated Pelvic nerve-to-Urethra Reflex Plasticity in Anesthetized Rats

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Running title: estrogen-modulated reflex plasticity

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To investigate a gonadal steroid influence on glutamate-mediated micturition functions. We characterized the effects of estrogen on the pelvic nveve-to-urethra reflex (PUR) plasticity elicited by repetitive stimulation (RS, 1 Hz) in rat received sham operation (Sham), ovariectomy (OVX) and ovariectomy with daily supplement estrogen (OVX+E2; mg/kg). The magnitude of the RS-induced potentiation in PUR activity was significantly decreased in OVX compared with Sham and OVX+E2 group (± , ± and ± in Sham, OVX and OVX+E2, respectively), while no statistical significance was shown between Sham and OVX+E2 group. Intrathecal 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline (NBQX; 20 μM, 10 μl) attenuated and D-2-amino-5-phosphonoraleric acid (APV 100 μM, 10 μl) blocked the RS-induced potentiation in PUR activities in all the three group (from ± to ± , from ± to ± and from ± to ± in Sham, OVX and OVX+E2, respectively). L-glutamate (0.1 mM, 10 μl, *i.t.*) and N-methyl-D-aspartic acid (NMDA 0.1 mM, 10 μl, *i.t.*) elicited potentiation in PUR activities (from ± to ± , from ± to ± and from ± to ± in Sham, OVX and OVX+E2, respectively) like repetive stimulation did. The present data suggest that estrogen modulates the spinal cord NMDA-mediated PUR plasticity in female rats and may contribute to alterations in urinary dysfunction afer manopause.

Keyword: urinary bladder, voiding, menopause, NMDA, AMPA

Introduction

Urine storage is an important function of the urinary bladder. During the storage phase of a micturition cycle, impulses induced by bladder distension transmit centripetally onto dorsal horn neurons through the pelvic afferent fibers. After integration within the spinal cord, these impulses cause external urethral sphincter (EUS) contraction via the pudendal efferent nerve. This pelvic-to-urethral reflex (PUR) is essential for urine continence (De Groat and Yoshimura, 2001). The Glutamatergic-NMDA receptor is widely utilized in the spinal cord for primary afferent neurotransmission including the afferent fibers arising from the urinary bladder (Papka et al., 2001; Papka and Storey-Workley, 2002). Recent studies on reflex plasticity, using an intact spinal cords preparation, demonstrated a NMDA-mediated plasticity in pelvic to pudendal reflex induced by repetitive/tetanic peripheral inputs (Lin, 2003; 2004) as well as physiological bladder distension (Liao et al., 2005), indicating an important role for spinal NMDA neurotransmission in miturition functions.

Stress urinary incontinence is the leakage of urine during moments of increased abdominal pressure, such as when laughing or coughing. Pudendal nerve injury and resultant urethral sphincter denervation and atrophy contribute to the development of stress urinary incontinence [6]. However, the symptoms of stress urinary incontinence often do not

appear until menopause, suggesting that hormonal factors, such as estrogen deficiency, contribute to the cause of this common condition [7]. Estrogen is a gonadal steroid with pronounced tropic effects on many diverse populations of neurons throughout the peripheral and central nervous system. Both clinical and experimental evidences suggest a neurotherapeutic role for estrogen after neurological injury or disease, including the enhancement of regenerative properties of injured motor neurones [8,9]. Intracellular recordings showed that estradiol administration increased the synaptic excitation of pyramidal cell in CA1 area, suggesting that gonadal steroid levels impact the reflex plasticity. Pharmacological or surgical ablation of menses reportedly decreases the durtion of the excitatory post-synaptic potential (EPSP) in hippocampus (C53) (Prior et al., 1992; Mathias et al., 1994). Experimental studies also report that the synaptic plasticity in CA1 area is modulated across the menstrual cycle (C24,25) or by hormone replacement therapy (C55). More recently, Gazzaley et al. have demonstated that in the CA1 of estradiol treated rats pyramidal neuron harbor increased level of NMDAR1 mRNA and protein, finding that explain the greater sensitivity of these neurons to NMDA- but not to AMPA-mediated synaptic inputs (C55).

To examine the role of gonadal hormones on the PUR plasticity, and identify a locus of action, we tested the effects of ovariectomy and

estrogen replacement on responses electric stimulations. In addition, glutamatergic antagonists were tested to elucidate the possible neurotransmission.

METHODS

Animal Preparations.

Adult female Sprague Dawley rats (210–275 gm) were used in the present experiment. One group of rats was ovariectomized (OVX) and tested 10–30 d after the surgery. Another group was ovariectomized and was given daily subcutaneous injections of 10g of 17-beta-estradiol-3 benzoate dissolved in 100 ml of sesame oil (OVX+E2) at least 10 d after ovariectomy. Sham operations (Sham) performed in the other group to serve as control. On the days of experiments, rats were anesthetized with urethane (1.2 g/kg, i.p.). Urethane was chosen because it lacks ganglionic blocking properties and allows neural inputs to the viscera to be maintained. The animal care and experimental protocol were in accordance with the guidelines of the National Science Council of the Republic of China. All efforts were made to minimize both animal suffering and the number of animals used throughout the experiment. The trachea was intubated to keep the airway patent. A PE-50 catheter (Portex, Hythe, Kent, U.K.) was placed in the left femoral vein for administration of anesthetics when needed. The body temperature was kept at 36.5-37.0° C by an infrared light and was monitored using a rectal thermometer. The

rats were monitored for the corneal reflex and a response to noxious stimulation to the paw throughout the experiment. If either were present, a supplementary dose (0.4 g/kg, i.v.) of anesthetics was given through the venous catheter. At the end of the experiments, the animals were put down, under deep anesthesia, using an intravenous injection of potassium chloride saturation solution.

Surgical Preparations

A midline abdominal incision was made to expose the pelvic viscera. Both ureters were ligated distally and cut. The proximal ends of ureters were drained freely within the abdominal cavity. A wide-bore cannula, with a sidearm for the pressure measurement, was tied into the wall of the bladder at its apex of bladder dome. The open end of the cannula was drained freely to the abdominal cavity. In some experiments recording the intra-urethral pressure (IUP) two 4-0 silks sutures was placed around the bladder trigone and ligated. A wide-bore urethra cannula was inserted through the opening of urethra and connected to a pressure transducer and continuously recorded on the computer system (MP30, Biopac, Sata Babra, U.S.A.). The right pelvic nerve was dissected carefully from the surrounding tissue and was transected distally for stimulations.

Intrathecal catheter

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 L6 level of the spinal cord. The volume of fluid within the cannula was kept constant a 10 μl in all experiments. Single 10 μl volumes of drug solutions were administrated followed by a 10 μl flush of artificial cerebrospinal fluid (Izquierdo & Medina, 1995). At the end of experiment, a laminectomy was performed to verify the location of the cannula tip.

Laminectomy

Recording of electromyogram activity

Epoxy-coated copper wire (50 μm; M.T. Giken Co., Tokyo, Japan) electromyogram electrodes were placed in the external urinary sphincter. This was performed using a 30-gauge needle with a hooked electromyogram electrode positioned at the tip. The needle was inserted into the sphincter approximately 1-2 mm lateral to the urethra and then withdrawn, leaving the electromyogram wire embedded in the muscle. 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, Cleveland, OH, U.S.A.); then continuously displayed on an oscilloscope (Tectronics TDS 3014, Wilsonville, OR, U.S.A.) and the recording system (MP30, Biopac, Santa Barbara, U.S.A., Liao et al., 2002). The

stimulated nerve and the electrodes were bathed in a pool of warm paraffin oil (37°C) to prevent drying.

Experimental arrangement

The schematic arrangement of EUSE recordings as well as the pelvic afferent nerve fiber stimulation is shown in figure 1A. The protocol for assessing the PUR activities was as follows. Once the electrodes' positions were optimized, recording of EUSE activities began. An electric current of square wave pulses with pulse durations of 0.1 ms was applied from a stimulator (Grass S88, Cleveland, OH, U.S.A.) through a stimulus isolation unit (Grass SIU5B, Cleveland, OH, U.S.A.) and a constant current unit (Grass CCU1A, Cleveland, OH, U.S.A.). Single shocks were repeated at 30 sec intervals (referred to as test activity; TS), and given through a pair of stimulation electrodes. This frequency of stimulation was chosen for sampling data because it did not result in response facilitation. The intensity of stimulation was gradually increased from 0 to 30 V and a stimulus intensity that yielded a single spike action potential in the EUSE was usually chosen to standardize the baseline reflex activity. After the baseline period, a repetitive stimulation at 1 Hz that lasted for 30 min with intensity identical to the test stimulation (referred to as repetitive activity; RS) was applied to induce facilitation in reflex.

Application of drug

Drugs were administered by intrathecal injection with a solution of known drug concentrations. Drugs used were 2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline (NBQX; 20 μM, 10 μl, Sigma). $_{D}$-2-amino-5-phosphonoraleric acid (APV 100 μM, 10 μl, Sigma), Saline of identical volume to tested agents was dispensed intrathecally to serve as a vehicle. At the end of the experiment, the location of the injection site was marked by an injection of Alcian blue (10 μl, 2 %). The volume of drug injected into the spinal cord in this experiment has been reported to spread from 0.5-1.5 mm from the site of injection (Martinez & Derrick, 1996). Therefore, a cannula positioned more than 0.5 mm from the intended site of injection was not included in the statistical analysis. We use 30 rats in this study. In 5 out of these 30 rats, cannula tip deviated by more than 1 mm from the target structure and were excluded from statistical analysis.

Data analysis

All the data in the text and figures are mean value ± S.E.M. Statistical analysis of the data was performed by means of ANOVA. A value of less than 0.05 was accepted as significant.

RESULTS

The pelvic nerve-to-urethral reflex activities

Activities recorded from the EUSE, elicited by a single electric shock of the pelvic nerve obtained from rats received sham operation (Sham), ovariectomy (OVX) and ovariectomy with supplement estrogen (OVX+E2) are shown in figure 1B. The mean reflex times for the pelvic nerve afferent fiber stimulations to evoke an action potential in the EUSE were 53.13±6.26, 53.13±6.26 and 53.13.26 in the sham, OVX and OVX+E2 groups, respectively (n=25). No statistical differences were shown in the latencies of PUR induced by the electric stimulation between either two of these three groups (figure 1C, p=NS, n=25). The PUR activities elicited by the test stimulation (1/30 Hz for 30 min) in sham, OVX and OVX+E2 were shown in figure 1D. No statistical significance was shown in the spike numbers evoked by the test stimulation between either two of these three groups.

Changes in PUR activities induced by repetitive stimulation

The excitability of PUR was assayed by recording of action potentials of EUSE activities resulted from stimulations of the pelvic nerve with electrical shocks of single pulses derived at a frequency of 1/30 Hz. The EUSE activities varied little over 30 min of testing period. However, as shown in figure 2, in all the three groups including sham operation (A, Sham), ovariectomized (OVX) and ovariectomized with supplement estrogen (OVX+E2) potentiations in PUR activities were induced progressively when a repetitive stimulation (RS, at the same intensity as

the test stimulation, delivered at 1 Hz for 30 min) was applied to the pelvic nerve. The potentiations in PUR activities induced by the repetitive pelvic nerve stimulation in these thress groups are summarized in figure 2D. The RS-induced potentiation was significantly lower in the OVX than the Sham and OVX+E2 groups (53.13±6.26, 53.13±6.26 and 53.13±6.26 in Sham, OVX and OVX+E2 group, respectively, p=NS, n=25) while no statistical difference was shown between Sham and OVX+E2 groups.

Agonistism by NBQX and APV

As shown in figure 3A, intrathecal NBQX (RS+NBQX, 20 μM, 10 μL, bolus), the novel selective non-NMDA receptor antagonist (Sheardown et al. 1990), caused a reversible antagonism of the RS-induced potentiation in PUR activities in all the three groups (26.16±7.27, 26.16±7.27 and 1.72±0.31 spikes/stimulation in Sham, OVX and OVX+E2, respectively, $p<0.01$, n=18). On the other hand, APV (RS+APV, 100 μM, 10 μL, bolus), the selective NMDA receptor antagonist (Davies et al. 1981; 1989; Watkin & Evans, 1981), abolished the repetitive stimulation-induced potentiation in PUR activities in all the three groups (26.16±7.27, 26.16±7.27 and 1.72±0.31 spikes/stimulation in Sham, OVX and OVX+E2, respectively, $p<0.01$, n=18). Saline (10 μL, bolus) was also tested via *i.t.* injection, but showed no effects on the distension-induced

potentiation. Figure 3C summarized the effects of APV, and NBQX on the RS-induced potentiation in PUR activities in three groups.

Agonist-induced potentiation in PUR acitivities

As shown in figure 4A, intrathecal L-glutamate (100 μM, 10 μL, bolus; TS+Glu) caused a potentiation in PUR acitivities induced by the test stimulation (1/30 Hz) in all the three groups (26.16±7.27, 26.16±7.27 and 1.72±0.31 spikes/stimulation in Sham, OVX and OVX+E2, respectively, $p<0.01$, $n=18$). NMDA (100 μM, 10 μL, *i.t.,* bolus; TS+NMDA) also elicited a potentiation in PUR activities evoked by the test stimulation in the same preparations (26.16±7.27, 26.16±7.27 and 1.72±0.31 spikes/stimulation in Sham, OVX and OVX+E2, respectively, $p<0.01$, $n=18$).

Secondary changes in response plasticity

As shown in figure 5A, in Sham group, a PUR was induced by the test stimulation (1/30 Hz) and a contraction wave in intra-urethral pressure (IUP) was produced by the urethral sphincter contraction. The firing in EUSE was increased when the pelvic afferent fiber was stimulated by the repetitive stimulation. Furthermore, the duration of the contraction wave in IUP, secondary to the urethral sphincter contraction, increased in a parallel fashion, although the peak pressure remained unchanged. In the OVX group, RS also elicited a potentiation in PUR activities with a lower

firing frequency and the duration of contraction wave in IUP was also shorter than Sham group (figure 5B, OVX). In addition, daily supplement estrogen reversed the effects of OVX on PUR and IUP activities (figure 5C, OVX+E2).

Discussion

The results from the present *in vivo* electrophysiology experiments demonstrate that without affecting on the baseline reflex activities, estrogen modulates the neural plasticity of a subpopulation of urethra. In addition, the physiological functions secondarily to the plasticity, i.e., the contraction wave of urethra was also affected. These results provide possible mechanisms for the urodynamic changes observed in the clinical observations, which the sympatomes of stress urinary incontinence resulted from an insufficient urethral resistance often do not appear until menopause, i.e., suggest that gonadal hormones are important for the modulation of some aspects of micturition functions.

In the present study, intrathecal gluatamatergic antagonists blocked the estrogen-related PUR plasticity, providing a pharmacological basis for estrogen modulation of glutamate-mediated reflex plasticity at the spinal cord level.

It has been known for some time that neural plasticity depends on the available of glutamatergic NMDA receptors (C10) and coincidentally, estogen increases the numbers of NMDA type binding sites on the CA1

neuron (C52). Wooley et al., (C55) recently confirmed this finding, and proposed that this increase affinity may be explained by the greater concentrat9ion of NMDAR1 receptor protein, which is regulated by the level of estrogen. These changes in NMDA binding result in greater sensitivity to NMDA-dependent synaptic inputs. This effect was demonstrated in slice taken from estrogen-treated rats, in which response to AMPA-dependent synaptic inputs were blocked (C55; C). Because lumbosacral dorsal root ganglion neurons are immunoreactive for estrogen receptor-alpha/beta (McNeill et al., 1991; Sohrabji et al., 1994; Taleghany et al., 1999; Papka et al., 2001; Papka and Storey-Workley, 2002), in addition, immunohistochemistry studies investigating the spinal cord have shown estrogen receptors are expressed in dorsal horn neurons (Amandusson et al., 1995; Williams and Papka, 1996). Therefor, the simplest explanation of the present results would be that treatment with estrogen both induced the synaptic growth and increase availability of NMDA receptor, providing the grounds for facilitated potentiation of synaptic strength.

Determination of which of these mechanism(s) contribute to the present observations are currently underway. However, the result in the present study that estrogen-related the RS-induced plasticity in PUR reflex was attenuated by NBQX and blocked by APV, suggesting a glutamatergic NMDA-mediated plasticity at the spinal cord level was mediated by estrogen.

Wong and Moss (C53) found that E2 priming 2 days before obtaining the hippocampal slices, increased synaptic excitability by prolonging EPSP and inducing repetitive firing in response to Schaffer collateral stimulation in a small percentage of CA1 neurons recorded in vitro with intracellular electrodes. However, in a similar preparation Wooley et al. (C55) found no difference in the efficacy of synaptic input, unless the postsynaptic response had been striped of AMPA dependent components. In coincidence with these results, Warren et al. (C50) did not observe changes in basic excitability measured by I/O curves during the estrous cycle of chronically implanted rats. In our experiments baseline excitability remained unmodified after E2 administration as evidenced by a similar reflex latency in ovariectomized with sham operative rats

We may conclude that the amplitude of the baseline response of PUR was not affected by doses of E2 that increase the number of dendritic spines or augment the sensitivity to NMDA-mediated glutamate input. This

proposition is in agreement with recent findings of Tsien et al. 48 using mice genetically deprived of NMDA receptors in CA1. They found that fast responses of pyramidal neurons, presumably mediated by AMPA type receptors, were normal, as were I/O relations and paired-pulse facilitation, whereas NMDA dependent slow responses and LTP were eliminated in knockout mice.

In summary, the present electrophysiological studies clearly demonstrate that estrogen has a significant role in modulating urethra activity. The loss of gonadal hormones decreases sensitivity, and estrogen replacement leads to impairment in urethra functions. The decrease in the NMDA-mediated RS-induced PUR plasticity implicates decreased processing in the spinal cord micturition circuitry. These data further suggest that alterations in sensory processing in the spinal cord mediated by estrogen may underlie symptoms of SUI.

Figure Legends:

Figure 1. The pelvic nerve-to-urethra reflex activities. (A) A schematic arrangement of the external urethral sphincter electromyogram (EUSE) activities recording (Reco) and the pelvic afferent nerve stimulation (Stim). (B) An action potential in EUSE evoked by a single electric shock (indicated by a triangle at the bottom) at the pelvic afferent nerve in rats received sham operation (Sham), ovariectomy (OVX) and ovariectomy with supplement estrogen (OVX+E). (C) The latencies of pelvic nerve-to-urethra reflex in various groups. (D) The numbers of action potentials in EUSE evoked by test stimulation (1/30 Hz) in rats received sham operation (circle), ovariectomy (square) and ovariectomy with supplement estrogen (triangle) Abscissa: the spike numbers evoked by test stimulation (p=NS, n=7).

Figure 2. A potentiation in pelvic nerve-to-urethra reflex activities caused by repetitive stimulation (RS, 1 Hz, indicated by triangles at the bottom) at pelvic afferent nerve in rats received sham operation (A. Sham), ovariectomy (B. OVX) and ovariectomy with supplement estrogen (C. OVX+E). Left: the external urethral sphincter electromyogram (EUSE) activities evoked by the test stimulation (TS, 1/30 Hz, indicated by a triangle at the bottom). Middle: the EUSE activities evoked by the repetitive stimulation at the first 6 sec following stimulation onset. Right:

the EUSE activities evoked by repetitive stimulation at the last 6 sec of the stimulation period (30 min). Activities marked by *(s), #(s) and +(s) are investigated using a faster time-base at the lower trace. (D) Summarized data (mean±SEM) showing the potentiation in pelvic nerve-to-urethra reflex activities induced by the repetitive stimlulation (RS, 1 Hz, for 30 min) in rats received sham operation (Sham, circle), ovariectomy (OVX, square) and ovariectomy with supplement estrogen (OVX+E, tirangle). Abscissa: the spike numbers evoked by the repetitive stimulation (* $p<0.05$, ** $p<0.01$, n=7).

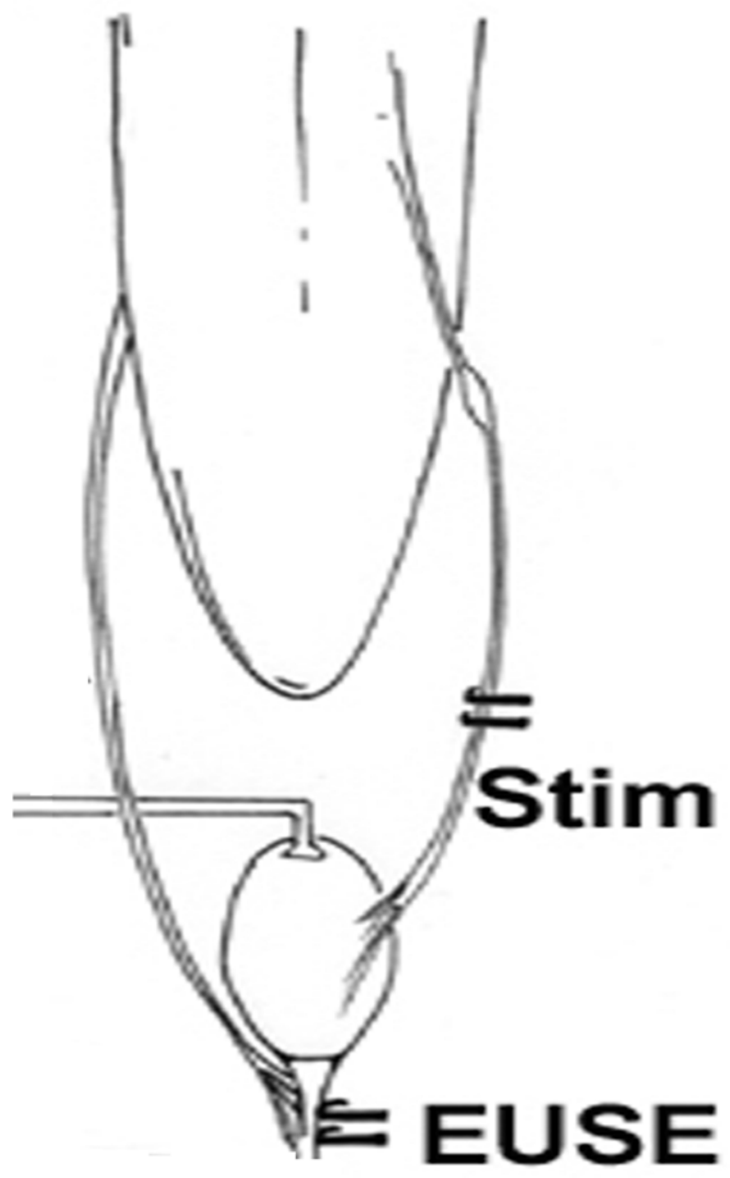
Figure 4. The antagonism of NBQX (A) and APV (B) on repetitive stimulation (RS, 1 Hz)-induced potentiaiton in pelvic nerve-to-urethra reflex activities. (A) Left: An action potential in external urethra sphincter (EUSE) activities was evoked by the test stimulation (TS, 1/30 Hz, indicated by a triangle at the bottom) at pelvic afferent nerve in rats received sham operation (Sham), ovariectomy (OVX) and ovariectomy with supplement estrogen (OVX+E). Middle: repetitive stimulation induced a potentiation in pelvic nerve-to-urethra reflex activities. Right: NBQX (20 μM, 10 μl, *i.t.* bolus) attenuated repetive stimulation-induced potentiation in pelvic nerve-to-urethra reflex activities. (B) APV (100 μM, 10 μl, *i.t.* bolus) abolished repetive stimulation-induced potentiation in pelvic nerve-to-urethra reflex activities in the same preparations. (C)

Summarized data (mean ±SEM) showing the effects of antagonism of NBQX and APV on the repetive stimulation-induced potentiation in pelvic nerve-to-urethra reflex activities. Abscissa: the spike numbers evoked by repetitive stimulation (* $p<0.05$, ** $p<0.01$, n=7).

Figure 4. The angonist-induced potentiaiton in pelvic nerve-to-urethra reflex activities. (A) Left: An action potential in external urethra sphincter (EUSE) activities was evoked by the test stimulation (TS, 1/30 Hz, indicated by a triangle at the bottom) at pelvic afferent nerve in rats received sham operation (Sham), ovariectomy (OVX) and ovariectomy with supplement estrogen (OVX+E). Right: glutamate (TS+Glu, 100 μM, 10 μl, *i.t.* bolus) potentiated the external urethral sphinter actitivities evoked by the test stimulation. (B) NMDA (TS+NMDA, 100 μM, 10 μl, *i.t.* bolus) potentiated the pelvic nerve-to-urethra reflex actitivities in the same preparations. (C) Summarized data (mean ±SEM) showing the effects of glutamate and APV on the pelvic nerve-to-urethra reflex activities evoked by test stimlulation. Abscissa: the spike numbers evoked by repetitive stimulation (* $p<0.05$, ** $p<0.01$, n=7).

Figure 5. Changes in intro-urethral pressure (IUP) resulting from the repetitive stimulation (RS, 1 Hz)-induced potentiation in pelvic nerve-to-urethra reflex activities. (A) Left: An action potential in external

urethra sphincter (EUSE) activities in associated with a contraction was in IUP wave were evoked by the test stimulation (TS, 1/30 Hz, indicated by a triangle at the bottom) at pelvic afferent nverv inrats received sham operation (A. Sham), ovariectomy (B. OVX) and ovariectomy with supplement estrogen (C. OVX+E). Right: the repetitive stimulation induced a potentiation in EUSE activities caused several successive contraction waves following the first one. (B) Repetitive stimulation-induced potentiation in EUSE activities and the numbers of the contraction waves was reduced in rat received ovariectomy. (C) Estrogen supplement reversed the effects of ovarietomy on the repetitive stimulation-induced potentiation on EUSE activities and IUP contraction waves.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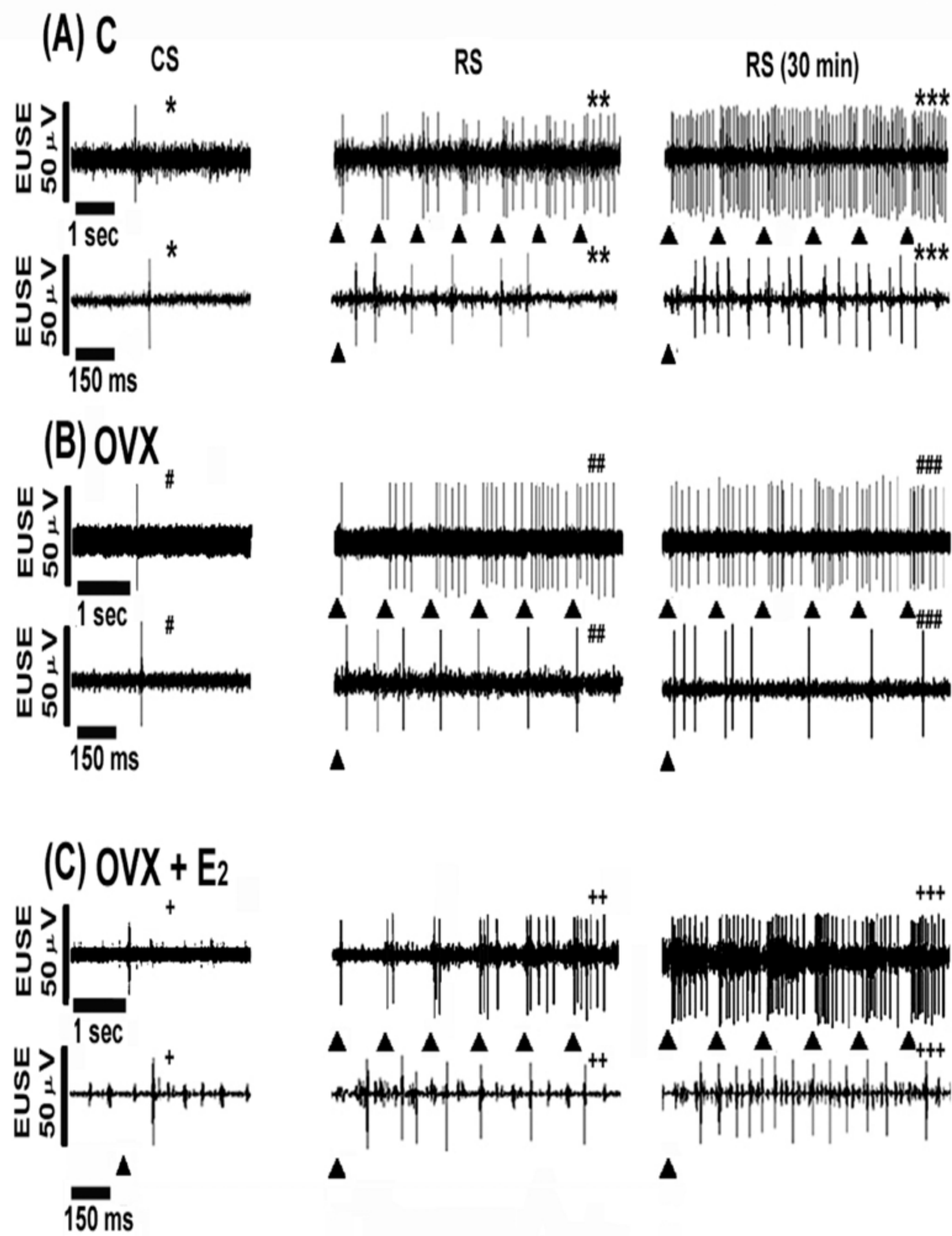

Figure 2

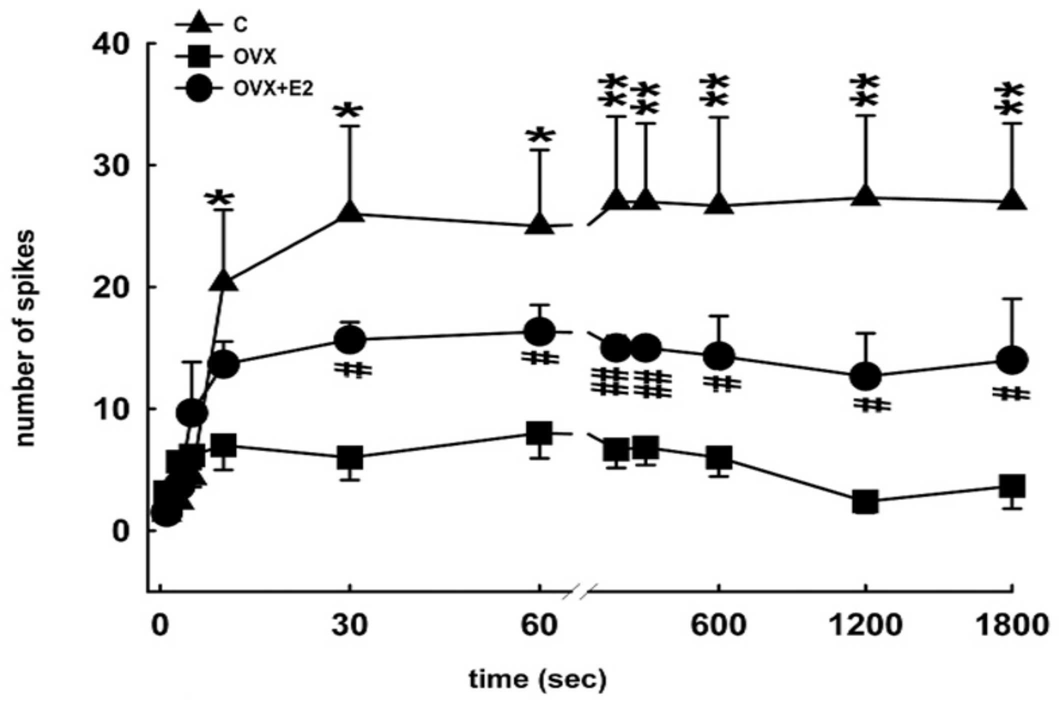

figure 3