Animal Study

The Involvement of CaV1.2 in Estrogenic Modulation of Morphine Antinociception in Rats Under Uterine Cervix Pain

Li-Hong Sun, MD[1], Qi Xu, PhD[1], Lin Jin, MD[1], Li-Dan Jin, MD[2], Qing Chen, MD[1], Hui Wu, PhD[1], and Xin-Zhong Chen, PhD[1]

From: [1]Department of Anesthesia, Women's Hospital, Zhejiang University, School of Medicine, Hangzhou, China; [2]Department of Anesthesia, Women and Children Hospital, Linping Distinct, Hangzhou, China

Address Correspondence:
Xin-Zhong Chen, PhD
Department of Anesthesia,
Women's Hospital
Zhejiang University,
School of Medicine
Hangzhou, China
E-mail: chenxinz@zju.edu.cn

Disclaimer: Li-Hong Sun and Qi Xu contributed equally to this work This work was supported by grants from the National Natural Science Foundation of China (NSFC, No.81870868), and Natural Science Foundation of Zhejiang Province (No. LQ20C090008).

Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted manuscript.

Manuscript received: 03-23-2022
Revised manuscript received: 06-27-2022
Accepted for publication: 07-29-2022

Free full manuscript:
www.painphysicianjournal.com

Background: Morphine is one of the preferred drugs for the clinical treatment of pain. Both clinical and preclinical studies have reported sexual dimorphism in morphine analgesia. Different circulating levels of estrogen could be involved in sex differences in response to morphine analgesia. In our previous research, we found that capsaicin injection into the cervix of rats caused acute visceral pain that could be relieved by morphine. The role of estrogen in morphine analgesia in rats under uterine cervix pain and its underlying mechanisms remain to be explored.

Objectives: The present study aims to investigate the effect of estrogen on morphine analgesia and its underlying mechanism in rats under uterine cervix pain.

Study Design: Controlled animal study.

Setting: University laboratory.

Methods: First, we compared the analgesic effect of morphine in ovariectomized rats with uterine cervix pain with or without estrogen replacement. Then, the changes in the expression of opioid receptors and L-type voltage-gated calcium channels (L-type-VGCC, LTCC) at the spinal level were detected by real-time quantitative polymerase chain reaction. Finally, we investigated the effect of the manipulation of spinal LTCC (L-type CaV1.2 calcium channel, L-type CaV1.3 calcium channel) on the estrogen-mediated inhibition of morphine analgesia.

Results: Our study shows that morphine antinociception is diminished in rats with uterine cervix pain that are treated with estrogen. Estrogen treatment increases the expression of spinal CaV1.2 and CaV1.3, while only anti-CaV1.2 treatment impaired estrogenic suppression of morphine antinociception.

Limitations: More underlying mechanisms of the role of spinal CaV1.2 in modulating estrogen-mediated inhibition of morphine analgesia need to be explored in future research.

Conclusions: This is the first evidence that spinal CaV1.2 is involved in estrogenic modulation of morphine antinociception in rats under uterine cervix pain. Our results will provide new ideas and references for estrogen-related differential prescription of opioids.

Key words: Estrogen, morphine, pain, analgesia, LTCC, CaV1.2, uterine cervix, opioid

Pain Physician 2022: 25:E1241-E1250

Morphine, a classic opioid receptor agonist, is one of the most preferred drugs for the treatment of various types of pain (1). Numerous reports have shown that opioid antinociception manifests sexual dimorphism in both clinical and preclinical studies (2-4). The specific mechanisms of sex differences in response to opioid analgesia are not fully understood. Many associated factors have been reported, including neuroanatomical factors, sex hormones, psychological states, and social

and cultural aspects (2). Estrogen, predominantly comprised of estradiol and estriol, is considered one of the key arbiters of sexual dimorphism of opioid analgesic actions (5-7). The exact mechanism underlying estrogen-mediated modulation of opioid antinociception remains to be elucidated.

It is recognized that the pharmacological antinociception mechanism of morphine mainly involves stimulation of the μ-opioid receptor (MOR), a prototypical G-protein coupled receptor densely localized in the central nervous system. Stimulation of MOR depresses neuronal calcium current influx (Ca^2+i), suppresses excitatory synaptic transmission, and inhibits transmitter release (8). Electrophysiological experiments have found that 17β-estradiol (E2), the most common circulating form of estrogen in the body (9), can activate L-type calcium channels (LTCCs) and participates in the regulation of Ca^2+i in dorsal root ganglion (DRG) neurons (10). Voltage-gated calcium channels (VGCCs) are well known to act in response to changes in the membrane potential, thereby permitting Ca^2+ currents to flow through the plasma membrane, resulting in the upregulation of the intracellular calcium ion concentration (11).

The activity of LTCCs is closely linked to neuronal signal transduction by transmitting calcium-regulated signaling events to the nucleus (12). DRG and spinal cord neurons express CaV1.2 and CaV1.3, which are 2 representative members of the LTCC and are involved in the modulation of neuronal excitability and neurotransmitter release (13). LTCC blockers may potentiate the antinociceptive effects of opioids by modifying morphine metabolism (14). The specific mechanisms remain uncertain. The aims of the present research were to investigate the role of estrogen in modulating morphine analgesia in a uterine cervix pain model in rats and to explore whether spinal LTCC is involved in the underlying mechanisms.

Methods

Animals

Female adult Sprague-Dawley rats weighing 200 g–250 g were used in the current study. Each rat was fed in a single cage and kept under a 12 h/12 h light-dark cycle with a room temperature of 22°C ± 2°C. All animals had free access to food and water. The animals were allowed to habituate to the environmental conditions for more than one week before any experimental procedure. All rats were randomly assigned to different groups. The grouping and order of treatments were generated by a randomization sequence using the random number generator in Excel 2019 (Microsoft Corp.). All experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocol received approval from the Animal Experiment Ethics Review Committee of Zhejiang University (approval number: ZJU20200109). All efforts in this study were made to minimize the number and suffering of animals.

Reagents

Capsaicin was purchased from Aladdin Company (no.C107693). Estrogen pellets were purchased from Innovative Research of America (no.SE-121, 0.1 mg). Morphine sulfate was obtained from ShenYang First Pharmaceutical Company (Northeast Pharmaceutical Group). To specifically knockdown spinal CaV1.2/CaV1.3, small interfering ribonucleic acids (siRNAs) targeting different DNA sequences were designed and synthesized by the Company of Zorin Technology to target rat CaV1.2 and CaV1.3 based on the genomic sequence. For CaV1.2, the targeted nucleotide sequences were as follows: siRNA-1 (si-1): 5′-CCAGCUGAACAUACCCAAUTT-3′; siRNA-2 (si-2): 5′-GCAGUUUCGCUGUCUUCUATT-3′. siRNA-3 (si-3): 5′-GCAACCACGUCAGCUACUATT-3′. For CaV1.3, the targeted nucleotide sequences were as follows: siRNA-A (si-A): 5′-GAAGAUGAUUCUAAUUCAACA-3′; siRNA-B (si-B): 5′-GACGCAGAUUCAAGAUAUAGC-3′; siRNA-C (si-C): 5′-GCGUUGCUGUACAAAGCUATC-3′. A scrambled sequence was designed as a negative control: NC siRNA (NC-si).

Uterine Cervix Pain Model

The cervix pain model was established by injecting capsaicin into the cervix canal. For anesthesia induction, the rats inhaled 3% isoflurane (YiPin Pharmaceutical) in 100% oxygen from an anesthesia machine at a flow rate of one L/min. Then, the rats were transferred to a temperature-controlled surgery plate for maintained anesthesia with a continuous delivery of 0.5%–0.9% isoflurane from a nose cone. The rats were positioned on their back with a pelvic elevation of approximately 3 cm. We injected normal saline (NS), 40 μL, vehicle (saline-ethanol-Tween 80, in an 8:1:1 volume ratio, 40 μL), or 0.9 mg capsaicin dissolved in 40 μL vehicle into the rat's uterine cervix canal via a sterilized microsyringe with a blunt tip. The blunt tip of the syringe was

inserted approximately 2 cm into the vagina. Syringe injection was performed at a speed of 8-10 μL/min. The microsyringe was not removed until 10 minutes postinjection to avoid fluid flow. In our previous research, we injected dye into the uterine cervix; the staining of reproductive organs confirmed that reagent diffusion is limited within the cervix (15).

Behavioral Analysis

After cervix injection, the rat was placed in the testing chamber, which was equipped with a video camera to record behaviors. The video recording started at 5 minutes after injection to allow the rats to fully recover from anesthesia. Behavioral activity recording lasted for 90 minutes. An experiment technician who was blinded to the group allocation observed and recorded the time and frequency of behaviors, including licking, head-turning, hindlimb hyperextension, and humping of the back. Based on the method described in our previous research (15), we determined that licking and head-turning were the most relevant parameters for estimating uterine cervix pain scores by means of regression analysis between different doses of capsaicin and behaviors. Then, a multivariable linear regression equation was performed to quantify the uterine cervix pain behavior score within 90 minutes after capsaicin administration: pain score = 0.007 × time of licking + 0.02 × time of head-turning + 1.8.

Ovariectomy

First, the rat was anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Then, we gently placed the rat prone and prepared and disinfected the skin on the lower back. After making incisions in the flank below the costal margin on both sides, we entered the abdominal cavity vertically, bluntly separated the muscle tissues on both sides, and found the fallopian tubes and ovaries. The joint of the ovary and fallopian tube was ligated with No. 4 suture and both ovaries were removed. Finally, we sterilized, sutured, and closed the abdominal cavity layer by layer.

Estrogen Replacement

Estrogen replacement was performed on Day 7 postovariectomy by embedding estrogen sustained-release tablets under the skin. Briefly, after the rats were anesthetized with isoflurane, a tiny incision was made on the back of the rat's neck, where estrogen pellets (17β-estradiol, 0.1 mg) were implanted subcutaneously. The incision was carefully sterilized and sutured.

Estrogen Level Testing

For each rat, 0.5 mL of blood was taken from the orbital venous plexus. The blood was left to sit for 2 hours at room temperature. The blood sample was separated into layers and then centrifuged at 5,000 rpm for 10 minutes at -4°C to obtain the supernatant. The supernatant was stored at -20°C for later detection. The estrogen concentration was measured by high-performance liquid chromatography (Agilent 1290-AB 5500 MSD, Agilent Technologies).

In Vitro Transfection of siRNAs

Three CaV1.2-siRNAs (si-1, si-2, si-3), 3 CaV1.3-siRNAs (si-A, si-B, si-C), a negative control (NC si), and a reagent control (MOCK) were transfected into PC-12 cells. The PC-12 neuronal cell line (Shanghai Cell Bank of the Chinese Academy of Science) frozen in liquid nitrogen was quickly removed and resuscitated in a 37°C water bath. The cells were cultured in a 25 cm^2 cell culture flask with DMEM/F12 medium and then placed in a cell culture incubator. The medium was changed once a day. After the cell density reached 90%, the cells were digested with 0.25% trypsin for passage. Cells with stable growth were seeded in a 6-well plate, cultured stably overnight, and then prepared for transfection. According to the different groups, control or siRNA (10 μL) and Lipofectamine 2000 (10 μL) were mixed to form a transfection complex (according to the instructions of Lipofectamine 2000). RNA was extracted 24 hours after transfection, and real-time polymerase chain reaction (RT-PCR) was performed to detect the transfection efficiency.

Drug Injection

For intraperitoneal injection, morphine (dissolved with normal saline to a total volume of 0.5 mL) was injected into the abdomen with a 24G needle. For intrathecal injection, spinal cord puncture was made by a microsyringe (Hamilton) with a 30G needle between the L5 and L6 vertebrae of each rat while the rat was under inhalational anesthesia (2% isoflurane).

A sudden, slight flick of the tail indicated that the needle had entered the subarachnoid space. Reagents (CaV1.2 siRNA, CaV1.3 siRNA, and NC siRNA, 0.1 nmol/g, dissolved in 10 μL double distilled water [ddH_2O]) and reagent control (10 μL ddH_2O) were slowly and completely delivered into the spinal fluid in 30 seconds. The needle was held in place for an additional 10 seconds to prevent outflow. The time points and doses of drug administration were determined based on the results

of our preliminary experiments. Two rats exhibited signs of motor dysfunction and were excluded from the following experiments.

Quantitative RT–PCR

At 2 hours after capsaicin or vehicle injection, tissue samples were collected from the lumbar and lower thoracic spinal cord and stored at -80°C. Total RNA was isolated from the sample using TRIzol reagent (Life Technologies). RT–PCR was performed using reagents and kits from Takara Company according to the manufacturer's instructions. Primer sets for CaV1.2 (Cacna1c, forward primer: GCATCACCAACTTCGACA; reverse primer: CAACTCATAGCCCATAGCG), CaV1.3 (Cacna1d, forward primer: GTCCATCGCCTCTCTGC; reverse primer: CGCTTGGTCTGTGTCTCAT), MOR (Oprm1, forward primer: CCTACCTAGTCCGCAGCA; reverse primer: AGTTGAGCCAGGAGCCA), and KOR (Oprk1, forward primer: GTCAGGGAAGATGTGGATG; reverse primer: ACAAAGGCAAAGACGAAGA) were used. β-actin was used as a housekeeping gene (forward primer: AGGTCGGTGTGAACGGATTTG, reverse primer: GGGGTCGTTGATGGCAACA).PCR amplification was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad). The efficiency of PCR was calculated from the slope of the standard curve. The slopes in our experiments were all found within the range of 90–110%. The relative expression of mRNA was normalized to the level of β-actin mRNA and was calculated using the ΔΔCt method.

Western Blots

At 2 hours after capsaicin or vehicle injection, tissue samples were collected from the lumbar and lower thoracic spinal cord and stored at -80°C. The tissues were homogenized in a lysis buffer. After centrifugation at 12,000 rpm at 4°C for 20 minutes, the supernatants were collected to determine the protein concentration with a BCA kit (Solarbio). Equivalent amounts of protein were separated on SDS PAGE Bis-Tris gels (Thermo Fisher Scientific). and transferred to polyvinylidene fluoride membranes (Millipore).

Then, the blots were blocked with blocking buffer (5% nonfat milk in Tris-buffered saline) for 2 hours at room temperature and incubated with primary antibodies overnight at 4°C. The primary antibodies were as follows: anti-CaV1.2 (cacna1c), 1:500 dilution, (Affinity Bioscience no.DF2267); anti-CaV1.3 (cacna1d), 1:500 dilution, (Affinity Bioscience no.DF10012); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1:1000 dilution, (Diagbio no.db106). The blots were washed and incubated with horseradish peroxidase-linked secondary antibodies (1:2000 dilution, Boster) for 2 hours at room temperature. Immunoblots were visualized on GeneSnap image acquisition software (Syngene). The density of the bands was quantified using Quantity One software (Bio-Rad). The intensity of each band was normalized to the corresponding GAPDH intensity.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). The sample size was calculated using PASS Version 11.0 (NCSS Statistical Software). Statistical analysis, including normal distribution tests of data, was performed using SPSS Statistics Version 22.0 (IBM Corporation). Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons tests. $P < 0.05$ was considered statistically significant.

Results

Estrogen results in a reduction in morphine antinociception in rats with uterine cervix pain. The behavior results showed that compared with the NS and vehicle treatments, capsaicin injection into the uterine cervix caused a significantly higher visceral pain score (VPS). Intraperitoneal administration of morphine obviously relieved capsaicin-induced visceral pain (Fig. 1A). As shown in Fig 1B, ovariectomized rats (OVX) had their serum E2 concentration reduced to an extremely low level, while estrogen replacement resulted in a stable, high E2 concentration in the rat serum (Fig. 1B).

Figure 1C shows that neither OVX nor the OVX combined with E2 treatment (OVX+E2) had an effect on the VPS. In both OVX and OVX+E2 rats, cervix administration of capsaicin caused a significant increase in VPS, which was suppressed by intraperitoneal treatment with morphine. However, compared with OVX rats with uterine cervix pain treated with morphine (OVX+Cap+Mor), E2 replacement resulted in a higher pain score after the same dose of morphine injection (OVX+E2+Cap+Mor), revealing that morphine antinociception is diminished in rats with higher E2 levels (Fig. 1C). We also observed the effect of intraperitoneal morphine with or without E2 treatment at different dose gradients. The dose–response curve showed a general right shift in E2-treated rats, demonstrating that the potency of morphine analgesia was reduced after estrogen treatment. There was a significant difference

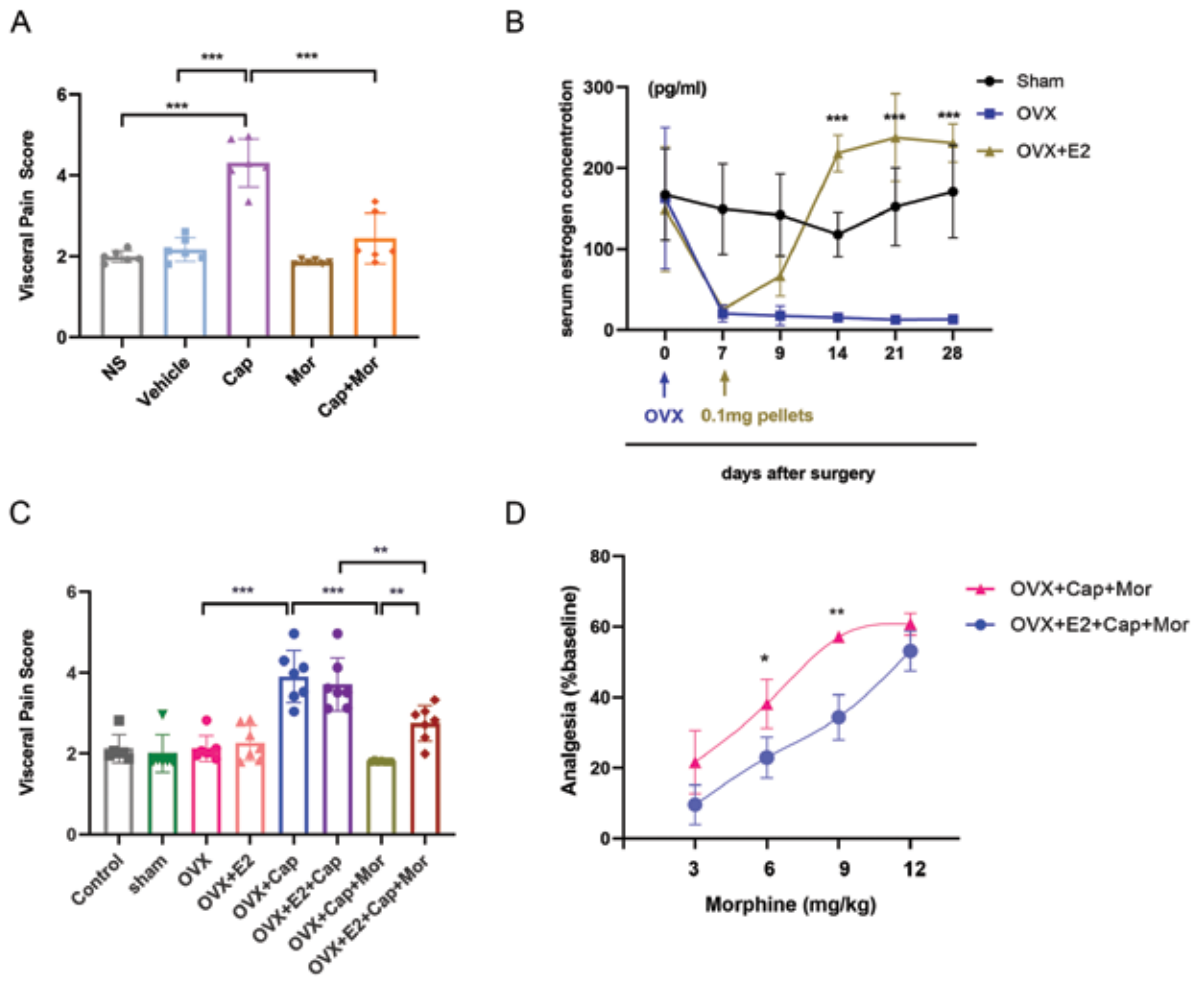

Fig 1. *Pain behavior results of rats with different treatments.*
*(A) Changes in the visceral pain score of rats in the NS (cervix injection of normal saline, 40 μL), vehicle (cervix injection of vehicle, 40 μL), Cap (cervix injection of capsaicin, 0.9 mg dissolved in 40 μL vehicle), Mor (intraperitoneal injection of morphine, 9 mg/kg), and Cap+Mor (cervix injection of capsaicin plus intraperitoneal morphine) groups. Morphine was administered intraperitoneally immediately after capsaicin injection.*** $P < 0.001$, $n = 6$ rats per group. (B) Fluctuations in serum estrogen levels in the sham (sham surgery corresponding to ovariectomy), OVX (ovariectomy), and OVX+E2 (OVX plus E2 replacement) groups. The blue arrow shows the day of OVX surgery, which was defined as Day 0. The brown arrow indicates the dose (0.1 mg) and time (Day 7 after OVX) of E2 pellet implantation.*** $P < 0.001$, $n = 4$ rats per group. (C) Changes in visceral pain scores in the control (untreated), sham (sham surgery corresponding to ovariectomy), OVX (ovariectomy), OVX+E2 (OVX plus E2 replacement), OVX+Cap (cervix injection of capsaicin in OVX rats), OVX+E2+Cap (cervix injection of capsaicin in OVX rats with E2 replacement), OVX+Cap+Mor (cervix injection of capsaicin in OVX rats treated by intraperitoneal morphine), and OVX+E2+Cap+Mor (cervix injection of capsaicin in OVX rats with E2 replacement treated by intraperitoneal morphine) groups. E2 replacement was performed on Day 7 after OVX. Capsaicin was injected into the uterine cervix on Day 21 after OVX. Morphine was administered intraperitoneally immediately after capsaicin injection.**$P < 0.01$,***$P < 0.001$, $n=6$ rats per group. (D) Dose–response curve between different intraperitoneal morphine doses (3 mg/kg, 6 mg/kg, 9 mg/kg, and 12 mg/kg) and the corresponding intensity of analgesia in the OVX+Cap+Mor and OVX+E2+Cap+Mor groups. The intensity of analgesia is displayed as a percentage, and the value is calculated as (VPS baseline-VPS after morphine)/VPS baseline. *$P < 0.05$, **$P < 0.01$, $n = 12$ rats per group (3 rats per dose gradient).*

in morphine analgesia between the OVX+Cap+Mor group and OVX+E2+Cap+Mor group at doses of 6 mg/kg and 9 mg/kg. (Fig. 1D).

Estrogen increased the expression of spinal CaV1.2 and CaV1.3. RT–PCR showed no difference in spinal MOR (oprm1) and KOR (oprk1) mRNA expression among the groups, demonstrating that estrogenic inhibition of morphine analgesia on uterine cervix pain is independent of the expression of opioid receptors in the spinal cord (Figs. 2A, 2B).

The expression of spinal CaV1.2 (Cacna1c) and CaV1.3 (Cacna1d) in rats subjected to different treatments was also examined. Compared with the OVX group, E2 replacement (OVX+E2 group) upregulated the mRNA levels of both spinal CaV1.2 and CaV1.3. Similarly, compared with capsaicin-treated OVX rats (OVX+Cap group), the mRNA levels of CaV1.2 and CaV1.3 in the spinal cord were significantly increased when E2 replacement was performed (OVX+E2+Cap group). Compared with rats treated with both mor-

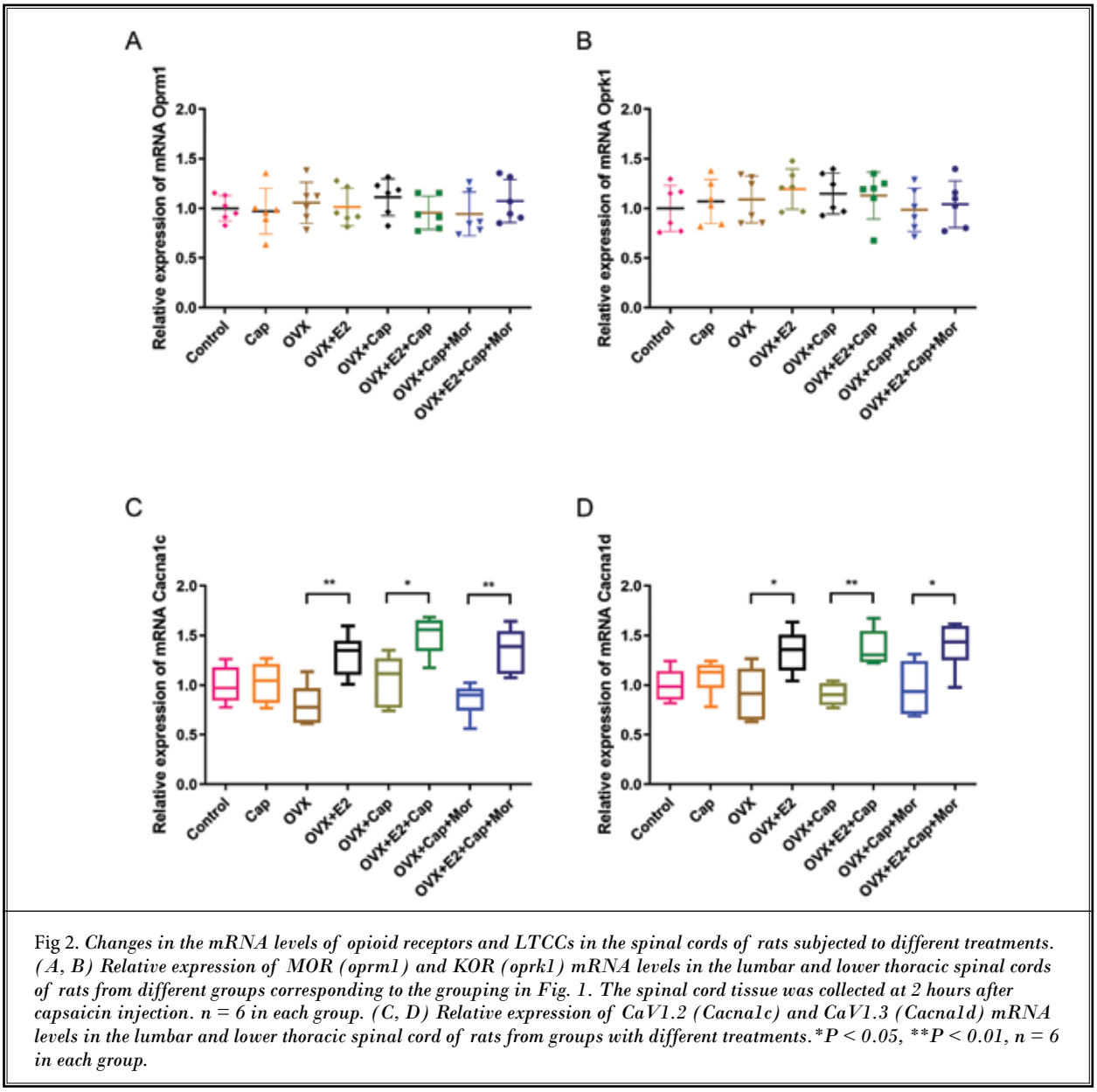

Fig 2. *Changes in the mRNA levels of opioid receptors and LTCCs in the spinal cords of rats subjected to different treatments. (A, B) Relative expression of MOR (oprm1) and KOR (oprk1) mRNA levels in the lumbar and lower thoracic spinal cords of rats from different groups corresponding to the grouping in Fig. 1. The spinal cord tissue was collected at 2 hours after capsaicin injection. n = 6 in each group. (C, D) Relative expression of CaV1.2 (Cacna1c) and CaV1.3 (Cacna1d) mRNA levels in the lumbar and lower thoracic spinal cord of rats from groups with different treatments.* $P < 0.05$, ** $P < 0.01$, $n = 6$ in each group.*

phine and capsaicin (OVX+Cap+Mor group), the spinal levels of CaV1.2 and CaV1.3 were also increased by E2 replacement (OVX+E2+Cap+Mor group) (Fig. 2C, 2D).

Manipulation of CaV1.2 in the spinal cord modulates morphine antinociception. The transfection efficiency of siRNAs targeting CaV1.2 and CaV1.3 was examined by RT–PCR. The results show that CaV1.2 mRNA was significantly inhibited in PC-12 cells treated with si-2 and si-3 (Fig. 3A), while CaV1.3 mRNA was largely reduced by si-A and si-C (Fig. 3B). By calculating the expression inhibition ratio, si-2 and si-A were selected for consecutive intrathecal administration. The specific experimental timeline is shown in Fig. 3C.

The in vivo knockdown effects of selected siRNAs were confirmed by Western blotting tests. The results show that compared with NC-si, si-2 treatment significantly blocks the protein expression of spinal CaV1.2 and has no effect on CaV1.3 expression. Si-A treatment significantly inhibits spinal CaV1.3 protein levels without affecting the expression of CaV1.2 (Fig. 3D). The behavioral results reveal that the E2-induced suppression of morphine antinociception is reversed by anti-CaV1.2 treatment rather than anti-CaV1.3 treatment. This indicates that although E2 treatment upregulates the spinal expression of both CaV1.2 and CaV1.3, only CaV1.2 is involved in the inner mechanism of altered morphine analgesia mediated by estrogen (Fig. 4).

Discussion

In the present study, we examined estrogenic modulation of morphine analgesia using a rat model of capsaicin-induced uterine cervix pain. The follow-

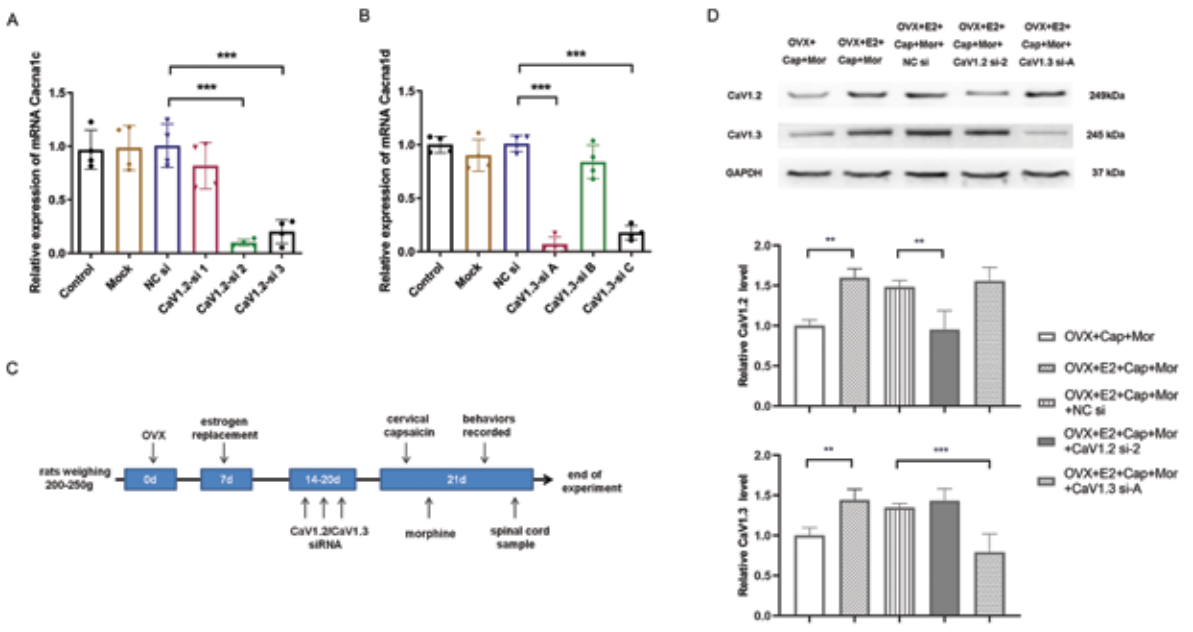

Fig. 3. *Examination of the transfection efficiency of siRNAs targeting CaV1.2 and CaV1.3 both in vitro and in vivo. (A, B) Relative expression of CaV1.2 (Cacna1c) and CaV1.3 (Cacna1d) mRNA levels in PC-12 cells in the control (nontreated), MOCK (reagent control), NC si (negative control siRNA), 3 CaV1.2-siRNAs (si-1,si-2, si-3), and 3 CaV1.3-siRNAs (si-A,si-B, si-C) groups. ***P < 0.001, n = 4 in each group. (C) Timeline of current experiments involving time points of siRNA treatment. The siRNAs were intrathecally administered consecutively on Days 14, 16, 18, and 20 after OVX. (D) Western blot showing the protein levels of spinal CaV1.2 and CaV1.3 in the OVX+Cap+Mor (cervix injection of capsaicin in OVX rats treated with morphine), OVX+E2+Cap+Mor (cervix injection of capsaicin in OVX rats with E2 replacement treated with morphine), OVX+E2+Cap+Mor+NC si, OVX+E2+Cap+Mor+CaV1.2 si-2, and OVX+E2+Cap+Mor+CaV1.3 si-A (cervix injection of capsaicin in OVX rats with E2 replacement treated with morphine plus intrathecal administration of negative control siRNA, CaV1.2 siRNA-2, and CaV1.3 siRNA-A) groups. The spinal cord tissue was collected at 2 hours after capsaicin injection. The lower histograms show the analysis of the density of relative CaV1.2 and CaV1.3 protein levels normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **P < 0.01, ***P < 0.001, n = 4 in each group.*

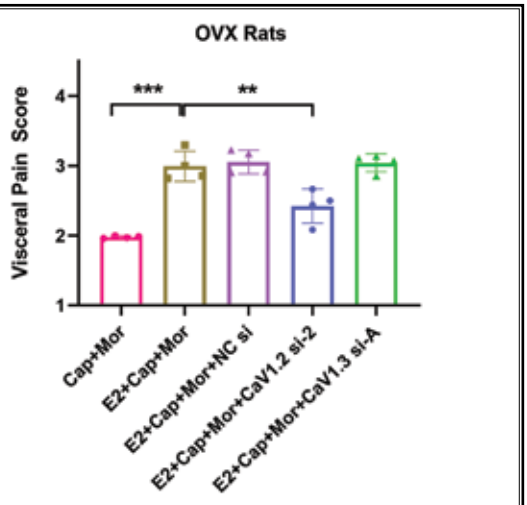

Fig. 4. *Manipulation of spinal CaV1.2 modulates morphine antinociception. Changes in the visceral pain score of OVX rats in different groups corresponding to the grouping in Fig. 3D. **P < 0.01, ***P < 0.001, n = 4 in each group.*

ing can be concluded from the results obtained in this study: 1) morphine antinociception is diminished by E2 treatment in rat uterine cervix pain; 2) estrogen increased the expression of spinal CaV1.2 and CaV1.3 in rat uterine cervix pain; and 3) anti-CaV1.2 treatment impaired estrogenic suppression of morphine antinociception in rats under uterine cervix pain.

Sex differences in opioid antinociception have long been recognized (16). Of the clinical studies that include sex as an independent variable affecting opioid use, the majority indicate a decreased analgesic efficacy of opioids in women (17,18). Morphine is a commonly adopted therapeutic drug for the clinical relief of cervix-related acute or chronic pain. Compared with males, females reportedly need a larger dose of morphine to achieve a similar analgesic effect (4). Preclinical studies have also demonstrated that female rats have an attenuated response to morphine compared with male rats (19). Estrogen has been found to play an essential role in the sexually dimorphic efficacy of opioid antinociception (20,21).

In this study, we focused exclusively on the effect of estrogen on sex-dependent morphine analgesic responsiveness and the related underlying mechanisms. To exclude the effect of other endogenous female hormones and different estrous cycle statuses, we used OVX female rats with or without estrogen replacement. Estrogen sustained-release tablets embedded in OVX rats produced a stable and high level of E2 in the serum. In addition, the current study adopted the capsaicin-induced uterine cervix pain model in rats to examine estrogenic influences on morphine analgesic responsiveness.

The uterine cervix is one of the most innervated parts of the female reproductive tract, with cervix dilatation constituting the main source of labor pain and cervix inflammation accounting for the visceral pain caused by mechanical, chemical, or infectious injury of the cervix (22, 23). Capsaicin is a well-known activator of the transient receptor potential vanilloid 1 (TRPV1). It has been widely employed to induce a stinging and burning pain sensation and hyperalgesia in animals (24). Injection of capsaicin into the cervix produces visceral pain via chemical inflammation. This visceral pain model induced by capsaicin injection into the cervix was confirmed to be stable in our previous research. Capsaicin injection into the uterine cervix significantly increased neuron activities in the lumbar and lower thoracic spinal cord (15), which is consistent with the anatomy of the uterine cervix being innervated by hypogastric and pelvic nerves (25). The behavioral results reveal that estrogen did not change the degree of capsaicin-induced visceral pain but might inhibit the efficiency of morphine analgesia.

It is well known that morphine predominantly exerts analgesic effects by binding to MOR. Some studies have reported that activation of spinal KOR is also an important component of spinal morphine analgesia in females (20,26). To determine whether estrogen-mediated differences in morphine analgesia were due to altered basal expression of opioid receptors, we measured the levels of MOR and KOR mRNA in the lumbar and lower thoracic spinal cord in each group. Our results revealed no difference in spinal MOR or KOR expression between the capsaicin-treated groups with or without E2 replacement, indicating that the observed inhibition of morphine analgesia is not initiated by a change in the expression of spinal opioid receptors. Thus, it is highly possible that the altered potency of morphine antinociception can be induced by the intersection of estrogenic signaling and the physiological mechanism triggered by the activation of opioid receptors.

Opioid antinociceptive cellular actions mainly include suppressing the function of calcium channels and reducing Ca^{2}+i (27).This indicates that reagents that influence Ca^{2}+i can modulate opioid analgesia. The activation of opioid receptors ultimately causes

a decrease in Ca^2+i and inhibits transmitter release, whereas increased expression or activation of LTCCs increases the influx of this cation and evokes transmitter release (8,28,29). E2 was reported to participate in the regulation of Ca^2+i by activating LTCCs, which open in a depolarized state induced by noxious stimulation (12). Therefore, we speculated that E2-modulated LTCC activation might be involved in the diminished morphine antinociception.

To test our hypothesis, we examined the mRNA expression of spinal LTCC in rats under uterine cervix pain with or without E2 replacement. RT–PCR analysis showed that capsaicin injection or OVX had no effect on the mRNA levels of either spinal CaV1.2 or CaV1.3. However, both CaV1.2 and CaV1.3 at the spinal cord were elevated after E2 replacement. To identify whether the upregulation of spinal CaV1.2 and CaV1.3 expression is related to E2-induced impairment of morphine antinociception in cervix pain, we observed the effect of spinal knockdown of CaV1.2/CaV1.3 on morphine antinociception. The result showed that knockdown of spinal CaV1.2, rather than CaV1.3, impaired E2-induced suppression of morphine antinociception. This observation suggests that spinal CaV1.2 is involved in estrogenic modulation of morphine antinociception.

The specific mechanism underlying estrogenic modulation of spinal LTCC is unknown. Initial reports have shown that estrogen exerts dramatic effects on CaMKII, a Ca^2+-activated enzyme, in the central nervous system (30). Calmodulin (CaM) is the major intracellular calcium ion binding protein and has been implicated in diversified neuronal functions. CaMKII acts as a modulator of excitation-transcription coupling in neurons and is known as a key regulator of CaV1.2 through its phosphorylation of CaV1.2 at its C-terminus (31,32). Ca^2+/CaM/CaMKII signaling might play a major role in estrogen modulation of spinal CaV1.2. Future investigations are needed to elucidate the upstream and downstream mechanisms of estrogenic modulation of central CaV1.2 regarding morphine antinociception.

While the majority of preclinical data indicates that estrogen exerts an attenuated response to morphine, some behavioral studies report no such difference (33,34). The contradictory findings might be due to differences in rat or mouse strains, type and route of analgesic drugs administered, the duration of pain (acute or chronic), and the modality of pain examined (peripheral or visceral, neuropathic or inflammatory) (35,36).

Collectively, the present study highlights the pivotal role of CaV1.2 in estrogenic modulation of morphine analgesia. We believe that the results obtained in this study will help to explain the observed clinical and preclinical sex differences in visceral hypersensitivity and suggest a potential target for mediating sexually dimorphic nociception. From a public health perspective, the outcome of this study will have a substantial effect on increasing our knowledge of estrogen-related differential prescription of opioids. When treating female patients with cervix pain who have high estrogen levels, administering LTCC blockers together with opioids appears to be a useful option.

Conclusion

In conclusion, using behavioral, pharmacological, and biological approaches, our study is the first to report that estrogen contributes to the decline of morphine analgesia in rats with uterine cervix pain, and that the upregulation of spinal CaV1.2 plays a vital role in estrogenic inhibition of morphine antinociception. More basic studies and clinical trials are warranted to explore the deeper modulations of sex differences in opioid needs.

Acknowledgements

We are extremely grateful to Professor Xiao-Ming Zhang (Zhejiang University School of Medicine, Zhejiang, China) for his technological guidance in the experiments.

Authors' Contributions

Li-Hong Sun and Qi Xu designed the study, conducted the behavioral experiments, performed operations and drug injections on animals, and drafted the manuscript. Lin Jin participated in the in vitro experiments. Li-Dan Jin and Qing Chen carried out the polymerase chain reaction and western blots experiments. Hui Wu participated in data collection and performed the statistical analyses. Xin-Zhong Chen participated in study designing and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

References

1. Cooper TE, Chen J, Wiffen PJ, et al. Morphine for chronic neuropathic pain in adults. *Cochrane Database Syst Rev* 2017; 5: CD011669.
2. Nasser AS, Afify EA. Sex differences in pain and opioid mediated antinociception: Modulatory role of gonadal hormones. *Life Sci* 2019; 237:116926.
3. Bartley EJ, Fillingim RB. Sex differences in pain: A brief review of clinical and experimental findings. *Br J Anaesth* 2013; 111:52-58.
4. Cepeda MS, Carr DB. Women experience more pain and require more morphine than men to achieve a similar degree of analgesia. *Anesth Analg* 2003; 97:1464-1468.
5. Mogil JS. Qualitative sex differences in pain processing: Emerging evidence of a biased literature. *Nat Rev Neurosci* 2020; 21:353-365.
6. Sandner-Kiesling A, Eisenach JC. Estrogen reduces efficacy of mu- but not kappa-opioid agonist inhibition in response to uterine cervical distension., *Anesthesiology* 2002; 96:375-379.
7. Fullerton EF, Doyle HH, Murphy AZ. Impact of sex on pain and opioid analgesia: A review. *Curr Opin Behav Sci* 2018; 23:183-190.
8. Al-Hasani R, Bruchas MR. Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 2011; 115:1363-1381.
9. Eid RS, Gobinath AR, Galea LAM. Sex differences in depression: Insights from clinical and preclinical studies. *Prog Neurobiol* 2019; 176:86-102.
10. Chaban V. Visceral pain modulation in female primary afferent sensory neurons. *Curr Trends Neurol* 2015; 9:111-114.
11. Xu L, Sun L, Xie L, et al. Advances in L-type calcium channel structures, functions and molecular modeling. *Curr Med Chem* 2021; 28:514-524.
12. Lee S. Pharmacological inhibition of voltage-gated Ca(2+) channels for chronic pain relief. *Curr Neuropharmacol* 2013; 11:606-620.
13. Dobremez E, Bouali-Benazzouz R, Fossat P, et al. Distribution and regulation of L-type calcium channels in deep dorsal horn neurons after sciatic nerve injury in rats. *Eur J Neurosci* 2005; 21:3321-3333.
14. Shimizu N, Kishioka S, Maeda T, et al. Role of pharmacokinetic effects in the potentiation of morphine analgesia by L-type calcium channel blockers in mice. *J Pharmacol Sci* 2004; 94:240-245.
15. Xu Q, Sun L, Zhang W, et al. A novel visceral pain model of uterine cervix inflammation in rat. *Eur J Pharmacol* 2021; 900:174080.
16. Pisanu C, Franconi F, Gessa GL, et al. Sex differences in the response to opioids for pain relief: A systematic review and meta-analysis, *Pharmacol Res* 2019; 148:104447.
17. Aubrun F, Salvi N, Coriat P, Riou B. Sex- and age-related differences in morphine requirements for postoperative pain relief. *Anesthesiology* 2005; 103:156-160.
18. Mogil JS, Bailey AL. Sex and gender differences in pain and analgesia. *Prog Brain Res* 2010; 186:141-157.
19. Larauche M, Mulak A, Kim YS, et al. Visceral analgesia induced by acute and repeated water avoidance stress in rats: Sex difference in opioid involvement. *Neurogastroenterol Motil* 2012; 24:1031-e547.
20. Gintzler AR, Storman E, MLiu NJ. Estrogens as arbiters of sex-specific and reproductive cycle-dependent opioid analgesic mechanisms. *Vitam Horm* 2019; 111:227-246.
21. Ji Y, Murphy AZ, Traub RJ. Estrogen modulation of morphine analgesia of visceral pain in female rats is supraspinally and peripherally mediated. *J Pain* 2007; 8:494-502.
22. Arendt-Nielsen L, Madsen H, Jarrell J, Gregersen H, Drewes AM. Pain evoked by distension of the uterine cervix in women with dysmenorrhea: Evidence for central sensitization. *Acta Obstet Gynecol Scand* 2014; 93:741-748.
23. Schiff L, Wegienka G, Sangha R, Eisenstein D. Is cervix removal associated with patient-centered outcomes of pain, dyspareunia, well-being and satisfaction after laparoscopic hysterectomy? *Arch Gynecol Obstet* 2015; 291:371-376.
24. Ortiz-Renteria M, Juarez-Contreras R, Gonzalez-Ramirez R, et al. TRPV1 channels and the progesterone receptor Sig-1R interact to regulate pain. *Proc Natl Acad Sci U S A* 2018; 115:E1657-E1666.
25. Berkley KJ, Robbins A, Sato Y. Functional differences between afferent fibers in the hypogastric and pelvic nerves innervating female reproductive organs in the rat. *J Neurophysiol* 1993; 69:533-544.
26. Liu N J, von Gizycki H, Gintzler AR. Sexually dimorphic recruitment of spinal opioid analgesic pathways by the spinal application of morphine. *J Pharmacol Exp Ther* 2007; 322:654-660.
27. Andrade A, Denome S, Jiang YQ, Marangoudakis S, Lipscombe D. Opioid inhibition of N-type Ca2+ channels and spinal analgesia couple to alternative splicing. *Nat Neurosci* 2010; 13:1249-1256.
28. Sarne Y, Fields A, Keren O, Gafni M. Stimulatory effects of opioids on transmitter release and possible cellular mechanisms: Overview and original results. *Neurochem Res* 1996; 21:1353-1361.
29. Hansen PB. New role of P/Q-type voltage-gated calcium channels: From transmitter release to contraction of renal vasculature. *J Cardiovasc Pharmacol* 2015; 65:406-411.
30. Zhen X, Goswami S, Abdali SA, Frankfurt M, Friedman E. Estrogen-modulated frontal cortical CaMKII activity and behavioral supersensitization induced by prolonged cocaine treatment in female rats. *Psychopharmacology (Berl)* 2007; 191:323-331.
31. Fu Y, Westenbroek RE, Yu FH, et al. Deletion of the distal C terminus of CaV1.2 channels leads to loss of beta-adrenergic regulation and heart failure in vivo. *J Biol Chem* 2011; 286:12617-12626.
32. Li J, Wang S, Zhang J, et al. The CaMKII phosphorylation site Thr1604 in the CaV1.2 channel is involved in pathological myocardial hypertrophy in rats. *Channels (Austin)* 2020; 14:151-162.
33. Ali BH, Sharif SI, Elkadi A. Sex differences and the effect of gonadectomy on morphine-induced antinociception and dependence in rats and mice. *Clin Exp Pharmacol Physiol* 1995; 22:342-344.
34. Kepler KL, Standifer KM, Paul D, et al. Gender effects and central opioid analgesia. *Pain* 1991; 45:87-94.
35. Averitt DL, Eidson LN, Doyle HH Murphy AZ. Neuronal and glial factors contributing to sex differences in opioid modulation of pain. *Neuropsychopharmacology* 2019; 44:155-165.
36. Mogil JS. Sex differences in pain and pain inhibition: Multiple explanations of a controversial phenomenon. *Nat Rev Neurosci* 2012; 13:859-866.