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Original Article

Ovariectomy modulation of morphine analgesia of neuropathic pain is associated with the change of K^+-Cl^- cotransporter 2 protein level in spinal dorsal horn

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Abstract: Sex differences in opioid analgesia have been confirmed both in clinical and experimental studies. Gonadal hormones (estrogens in particular) have a great role in this process. However, the mechanisms that underlie these sex differences are not very clear. In this study, we used K^+-Cl^- cotransporter 2 (KCC2) as a molecule target to investigate the mechanism underlying the phenomenon. Sprague-Dawley rats were randomly assigned to ovariectomy (OVX)+morphine group, OVX+Saline group, sham surgery (OVX-sham)+morphine group and OVX-sham+saline group. All the rats received SNI surgery three weeks after ovariectomy. We used von Frey values as a sign of neuropathic pain. On PO day 14, 1 μg morphine or the vehicle saline was administered intrathecally *via* a PE-10 catheter formerly implanted. Hindpaw withdrawal threshold was determined before and 30, 60, 90, 120, 150, 180 min after drugs injection. The L4-L5 segments of the spinal cord were removed and immunoblotted for KCC2 protein at the time of 2 and 3 h after drugs administration. We find that ovariectomy can regulate the sensitivity to morphine analgesia of neuropathic pain and KCC2 protein level will change in the spinal dorsal horn.

Keywords: Ovariectomy, estrogen, neuropathic pain, hyperalgesia, morphine, KCC2

Introduction

Opioids are considered the gold standard for post-operative and many other forms of severe pain [1-3]. Although the efficacy of opioids for neuropathic pain remains controversial [4, 5], many experiments have indicated that intrathecal injection of morphine can produce remarkable antiallodynic effect on neuropathic pain. Clinical studies suggest that women require more morphine than men to achieve an identical degree of analgesia after surgical procedures [6]. These sex differences in morphine analgesia can be attributed to multiple factors, from hormones and genes to socio-culture and environment. Gonadal hormones, especially estrogens, have been indicated as the major determinants of the differences. A growing body of evidence suggests estrogens may modulate opioid receptor activity and therefore contribute to sex differences in opioid analgesia. In the spinal cord, many enkephalinergic neurons co-express estrogen receptor-alpha (ERα), suggesting that estrogen can modulate opioid activity [7]. Behavioral studies have also proved that estradiol (E2) dose- and time-dependently reduced morphine-induced antinociception compared with ovariectomized (OVX) rats [8].

Outwardly directed K^+-Cl^- cotransporter 2 (KCC2) is expressed in spinal cords and function to regulate intracellular Cl^- concentration [9, 10]. Decreased expression of KCC2 results in a shift of the transmembrane anion gradient such that GABA binding to post-synaptic GABA-A receptors no longer causes an influx of chloride ions and membrane hyperpolarization, but rather an outflow of chloride ions and subsequent depolarization [11]. A large amount of evidence suggests that changes of KCC2 expression play an important role in inflammatory and neuropathic pain [12-14]. Our hypothesis is that ovariectomy modulation of morphine analgesia in neuropathic pain is *via* changing the level of KCC2 protein in spinal dorsal horn.

Materials and methods

Animals

Female Sprague-Dawley rats weighing 180-200 g (180 to 200 g at the time of ovariectomy; approximately 250 to 280 g at the time of experiment) were used. Rats were placed in plastic cages with sawdust bedding and were maintained in a climate-controlled room under a 12 h-12 h light-dark cycle with food and water available ad libitum and were housed for a minimum of one-week prior to use. Particular efforts were made to minimize animal suffering and to reduce the number of animals used. All animal experiments were performed in accordance with the national legislation, and with the National Institutes of Health Guide regarding the care and use of animals for experimental procedures. Rats were randomly assigned to either sham operation (OVX-sham, n=13) or bilateral ovariectomy (OVX, n=13). On the first day of the research, ovariectomies were performed under sodium pentobarbital anesthesia (50 mg/kg i.p.) The ovaries were removed through bilateral incisions in the dorsal flank. For sham surgery, the ovaries were exposed but not removed. Ovariectomized animals were further divided into 2 groups: OVX given morphine (OVX+morphine, n=7), OVX given saline (OVX+saline, n=6). And the rats with sham operation were further divided into 2 groups too: OVX-sham given morphine (OVX-sham+ morphine, n=7), OVX given saline (OVX-sham +saline, n=6).

Intrathecal catheterization

The method for intrathecal catheterization is modified from that previously reported. Briefly, 14 days after the OVX or OVX-sham surgeries, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A 15-20 mm longitudinal incision was made through the skin right on the dorsal midline and 20-25 mm upper to a line between the two anterior iliac spines. With the L4 processus spinosus removed, a 20 gauge needle was used to prick a hole in ligamenta flava, and a PE-10 catheter (length, 19 cm) was inserted through the hole and between the L3 and L4 vertebra into the intrathecal space with 2 cm. The correct intrathecal localization was confirmed by a tail flick or a paw retraction. The catheter was tied in a loose knot and sutured on the back under the skin. The external end of the tube was passed subcutaneously and secured to the back of the neck. The external end of the tube was closed by plug. 5 days after the PE-10 tube was implanted, 10 μL lidocaine (2%) was administered to confirm whether the catheter was in the correct position. Lidocaine induces a transient paralysis of the hind paws when injected into the lumbar enlargement; if paralysis did not occur within 30 seconds, data from the rat were excluded from the study.

Spared nerve injury

Three weeks after OVX or OVX-sham surgeries, a SNI of the common sciatic nerve was performed on all rats according to the method described by Decosterd and Woolf [15]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for surgery, the common peroneal and tibial branches of the left sciatic nerve were ligated with 5.0 silk sutures and transected, leaving the sural nerve intact. A 2 mm portion of the nerve was removed. Muscle and skin were sutured in two distinct layers. Rats were used for experiments only when the left hind paw withdrawal threshold less than 1.0 g in response to von Frey filaments stimulation on PO day 4. In rats matching these criteria, it was considered that the mechanical allodynia was produced.

Drug administration

Two weeks after SNI, rats were gently under restraint, and a 10 μL microsyringe was inserted into the PE-10 catheter about 1 mm after the plug was removed. Drug dissolved in normal saline were then slowly infused into the tube within 60 s and the microsyringe was left in place for an additional 15 s to minimize the drug solution flowing back into the injector track. Drug used in this study was morphine (1 μg in 10 μl saline with 7 μL saline flush). The same volume of 0.9% saline was injected in control groups (OVX given saline and OVX-sham given saline).

Behavioral measures

In the present study, mechanical allodynia rather than thermal hyperalgesia was used as index of the nociceptive responses, because it was more constant in SNI model. The hind paw withdrawal threshold was tested blindly by an experimenter one day before SNI surgery, and the rats with von Frey values below 8.0 g were

Table 1. The rat hind paw withdrawal threshold responded to von Frey filaments at each time point (g, $\bar{X}\pm S_{\bar{X}}$)

Time	OVX group		OVX-sham group	
	OVX+M	OVX+S	OVX-sham+M	OOVX-sham+S
P.O.D.-1	15.00±0.00	14.17±0.83	15.000±0.00	15.00±0.00
P.O.D.4	0.35±0.08	0.45±0.13	0.31±0.08	0.35±0.07
P.O.D.14	0.17±0.04	0.16±0.05	0.20±0.05	0.17±0.05
Afterinjection 30 min	8.29±0.52	0.35±0.07*	9.29±1.08	0.31±0.07#
60 min	15.00±0.00	0.30±0.08*	15.00±0.00	0.21±0.06#
90 min	15.00±0.00	0.31±0.07*	14.29±0.71	0.39±0.08#
120 min	13.57±0.92	0.19±0.05*	10.14±0.88&	0.23±0.06#
150 min	11.25±1.25	0.31±0.15*	6.00±1.41&	0.24±0.08#
180 min	8.50±0.96	0.13±0.03*	3.50±0.50&	0.21±0.10#

Note: Compared with OVX+M group at the same time point: *$P < 0.01$; compared with OVX-sham+M group at the same time point: #$P < 0.01$; compared with OVX+M group at the same time point: &$P < 0.01$.

obviated from subsequence procedures. And this threshold in response to mechanical stimulation (von Frey filaments) was measured again before and 30, 60, 90, 120, 150, 180 min after drug or saline administration on PO day 14. The rat was placed in transparent plastic box (280×250×210 mm) with a metal wire mesh floor that allowed full access to the paws from underneath. The plantar side of the paw ipsilateral to the surgery was stimulated with calibrated von Frey monofilaments. Monofilaments were perpendicularly applied to the glabrous skin with sufficient force to cause filament bending. The threshold was taken as the lowest force that evoked a brisk withdrawal response to two of ten repetitive stimuli, and no further monofilaments were applied. In the cases where continuous negative responses were observed all the way out to the end of the stimulus spectrum, values 15.0 g were assigned. 120 and 180 min after drug or saline injection, rats were deeply anesthetized by sodium pentobarbital, and the L4-L5 segments of the spinal cord were removed and immunoblotted for KCC2 protein [16].

Western blot

The collected ipsilateral spinal dorsal horn quadrants to SNI were homogenized in a protein extraction reagent and centrifuged at 14000 rpm for 20 min at 4°C. The protein extraction reagent contains inhibitors (to 1 ml protein extraction reagent, add 5 μl protease inhibitor cocktail, 5 μl PMSF, and 5 μl phosphatase cocktail; Kangchen). Total protein content in the homogenates was determined with a comercially available kit (Prod, #23236). The homogenates were boiled and the proteins separated by SDS-PAGE electrophoresis (10 μg of total protein per well), and transferred onto PVDF membranes (Millipore corporation). The membranes were placed in a blocking solution (Tris-buffered saline containing 0.1% Tween and 10% nonfat dry milk)at room temperature(22-25°C) for 2 h, and incubated overnight in primary antibodies to KCC2 (1:1000; Chemicon), or GAPDH (1:20000; Kang-chen) at room temperature for 2 h. The membranes were washed with Tris-buffered saline containing 0.1% Tween and incubation in appropriate peroxidase-conjugated anti-rabbit IgG (1:2000; Cell Signaling) for 1 h, and washing again, the proteins of interest were detected by chemiluminescence. Protein levels were estimated from optical density measurements of scanned images of the respective bands using Image Pro Plus 5.1.

Statistical analysis

All values were presented as mean±SEM. Differences in drug effects among groups were tested statistically by two-way repeated measures of analysis of variance (two-way RM ANOVA) with a post hoc multiple comparison (Bonferroni *t* test) for analysis of the differences in entire observation time or at each time point among different groups, and Mann-Whitney rank sum test was applied where necessary. In the Western blot study, one-way ANOVA followed by Levene's post hoc test was used to test the differences both within and between groups. A P value < 0.05 was considered to indicate statistical significance.

Results

Effects of ovariectomy on morphine analgesia of SNI-evoked allodynia

Before the drugs were administered, the baseline withdrawal thresholds had been determined using von Frey hairs, and there was no significant difference between each group, so

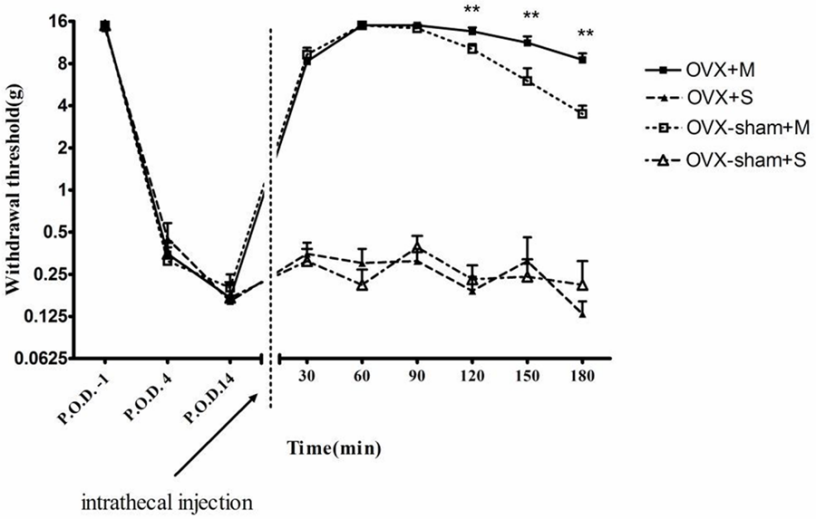

Figure 1. The effects of intrathecal 1 μg morphine on the withdrawal threshold to SNI in OVX and OVX-sham rats. $^{**}P < 0.01$ compared with OVX-sham+morphine group at those time points. (Post hoc Bonferroni *t* test). The withdrawal thresholds have significant difference between morphine-treated groups (OVX+morphine and OVX-sham+morphine group) and their vehicle control group (OVX+saline group or OVX-sham+saline group) after drug injection.

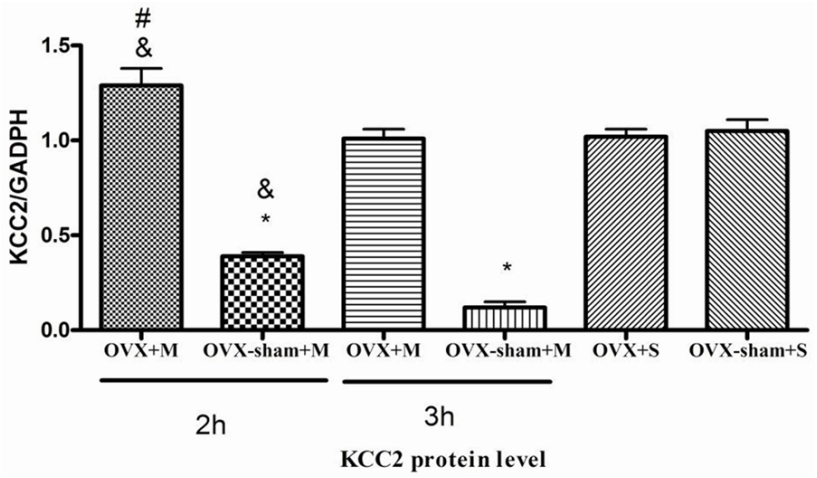

Figure 2. Ovariectomy enhances the analgesic potency of morphine associated with KCC2 protein level change in ipsilateral dorsal horn to SNI. $^{*}P < 0.01$ compared with OVX+morphine group, at 2 h and 3 h. $^{\&}P < 0.05$ compared with the some group. $^{\#}P < 0.05$ compared with OVX+S group. OVX+M means OVX+morphine group; OVX-sham+M means OVX-sham+morphine group; OVX+S means OVX+saline group; and OVX-sham+S means OVX-sham+saline group.

the baseline data were pooled for comparison. The von Frey values on PO day -1 (one day before SNI), PO day 4 and PO day 14 (time pre-injection) were 14.81±0.19 g, 0.36±0.04 g and 0.18±0.02 g respectively (**Table 1**).

Intrathecal injection of 1 μg morphine remarkable increased the paw withdrawal threshold in both OVX+morphine group and OVX-sham+morphine group across times, compared with their corresponding vehicle control group (OVX+saline group or OVX-sham+saline group), and there was significant difference of the paw withdrawal threshold between this two groups 120, 150 and 180 min after morphine microinjection. Whereas, there was no significant difference of the paw withdrawal threshold between OVX+saline group and OVX-sham+saline group across times (**Figure 1**).

Western blot of KCC2 in spinal dorsal horn

At the time of 2 h and 3 h after morphine injection, the expression of KCC2 protein in OVX-sham+morphine group was significantly lower than that in OVX+morphine group. And the expression of KCC2 protein was remarkably decreased at 3 h after morphine injection, compared with 2 h within each morphine-treated group. Whereas there was no statistical difference of KCC2 protein level between OVX+saline and OVX-sham+saline group (**Figure 2**).

Discussion

The present study demonstrates that the analgesic effect of intrathecal injection of morphine in a neuropathic pain model is gonadal hormones (estrogens in particular) dependent. Furthermore, our experiment suggests that this phenomenon is mediated by changing KCC2 expression in the spinal dorsal horn.

Sex differences in pain have been confirmed both in clinical and experimental studies [17-

20]. Gonadal hormones, especially the female gonadal hormones, have been indicated as the major determinants of the differences. Many previous studies have indicated ovariectomy could decrease nociception to noxious stimulation, compared with intact female rats, and estradiol replacement in OVX rats could reverse it [21]. Furthermore, previous studies have shown a sex difference in the analgesic potency of morphine to nociceptive stimuli and estradiol had a tendency to decrease opioid antinociception, suggesting a modulatory effect of estradiol on the analgesic potency of morphine [22]. Our current study reveals that compared with the intact female rats, ovariectomy can enhance the analgesic potency of morphine at the time of effect fadeaway in SNI, a neuropathic pain model, on PO day 14. However, the baseline response to innocuous mechanical von Frey hair stimulation has no statistical difference between OVX and intact female rats across times.

The current study also examined the role of KCC2 in modulating morphine's efficacy on chronic neuropathic pain following SNI in OVX and OVX-sham rats. Many experiments have shown that reduced expression of KCC2 protein contributed to the reverse of GABA-A inhibitory to nociception. Moreover, inhibition of KCC2 activity *in vivo* reduces mechanical and thermal nociceptive thresholds in control, and uninjured animals [23]. All of this evidence suggests KCC2 plays an important role in neuronal excitability and pain signaling. Immunoblotting showed a significant down-regulation of KCC2 protein in spinal dorsal horn 2 h and 3 h after intrathecal injection of morphine in intact female rats, compared with OVX rats. And the expression of KCC2 protein had a significant difference within each morphine-treated group at the same time. This was in line with our behavioral result. However, the paw withdrawal threshold of morphine-treated intact female rats was much higher than it of saline-treated intact female rats, but we have seen the converse results in western blot of KCC2 protein. The reason underlying this phenomenon was quite confusing. It looked as if the difference of KCC2 level was not matching the behavioral manifestation very much. Furthermore, a recent study reported that a decrease in expression of KCC2 in the spinal dorsal horn accompanied the initial stage of formalin-evoked hyperalgesia [24]. And another experiment show that the expression level of KCC2 would recover 7 days after CCI (a neuropathic pain model similar to SNI) [25]. In consideration of our experiment condition (rats were administered with drugs and killed 14 days after SNI), We surmise KCC2 may be only an important early step in the transition from normal to pathophysiological processing, The rapid down-regulation of KCC2 could quickly cause a loss of the all important GABA inhibition before slower but more permanent changes in the overall circuitry can take place. And it will come back to the normal level after the early stage of pain process. To further address the phenomenon, we need more investigations to find out the underlying mechanism.

In conclusion, our findings suggest that ovariectomy enhances the analgesic potency of morphine by modulating the expression level of KCC2 protein in ipsilateral dorsal horn to SNI.

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Disclosure of conflict of interest

None.

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