Experimental Assessment

Different Analgesic Effects of Intrathecal Endomorphin-2 on Thermal Hyperalgesia and Evoked Inflammatory Pain in Ovariectomized Rats

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Hormone replacement remains one of the common therapies for menopause-related pain but is associated with risk of orofacial or back pain. Spinal endomorphin-2 (EM-2) is involved in varied pain and its release is steroid-dependent, but whether increasing spinal EM-2 can inhibit thermal hyperalgesia and inflammatory pain in ovariectomized (OVX) female rats, an animal model mimicking menopause, is not clear, nor is the potential involvement of spinal mu-opioid receptor (MOR). In the current study, we revealed that the temporal decrease of spinal EM-2 is accompanied with OVX-induced thermal hyperalgesia that was dose-dependently attenuated by intrathecal (IT) delivery of EM-2. The subcutaneous injection of formalin-induced inflammatory pain in OVX rats was exacerbated and IT delivery of EM-2 dose-dependently inhibited the inflammatory pain. However, the ED50 for IT delivery of EM-2 on thermal hyperalgesia is smaller than that on inflammatory pain in OVX rats, suggesting different contributions of the EM-2 system to these 2 pain modalities in OVX rats. IT pretreatment with MOR antagonist, beta-funaltrexamine (β-FNA), attenuated IT EM-2 analgesia on both thermal hyperalgesia and inflammatory pain in OVX rats. Furthermore, IT delivery of EM-2 did not affect the animals' locomotion or anxiety status. Our findings suggested that IT EM-2 might be a safer analgesia strategy than hormone replacement therapy in reducing risk of orofacial or back pain. However, a long-lasting form of EM-2 with less tolerance is needed to induce sustained analgesia.

Key words: analgesia, intrathecal, endomorphin, ovariectomize, rat

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Human (1-3) and laboratory animal (4,5) studies suggest that sex hormones (especially estrogen) are important determinants for sensitivity and response to pain. As one extremity of sex hormone decrease, menopause is suggested to be closely related with pain in women (6,7). By using a multiple logistic regression model that included age, a previous survey revealed that postmenopausal women were approximately twice as likely to report low back pain compared to premenopausal women, suggesting that menopause but not age is associated with low back pain (8). The treatment of pain in this population remains a clinical challenge because the feature of menopause pain varied very much (7) and the underlying mechanisms are far from being revealed.

To reveal the underlying mechanisms and develop efficient therapy for menopause pain, the ovariectomized (OVX) female rat model was commonly used to evaluate hormonal effects on pain and analgesia (9,10). However, the relationship between estrogen and hyperalgesia or exaggerated inflammatory pain is far from being revealed. OVX have been suggested

to induce both a decrease (11,12), and an increase (13) of thermal pain threshold revealed by tail flick latency (TFL) or paw withdrawal latency (PWL) to the nociceptive thermal stimuli. On the other hand, the potential exaggerating effect of OVX on inflammatory pain has not been elucidated. Previous studies reported that OVX has no effect on phase I and II inflammatory pain responses induced by 2% formalin (14), increases the phase I pain responses induced by 5% formalin (15), or significantly increases both phase pain responses induced by 10% formalin injection in rats (13,16).

Hormone replacement is one of the common therapies for menopause pain, however, it is closely associated with the risk of orofacial (17,18) or back pain (19). Thus, it is necessary to manipulate important molecules downstream to the sex hormone so as to reach satisfying analgesia with fewer side effects in menopausal women.

Endomorphin2 (EM-2) is released in an ovarian sex steroid-dependent way and predominant in the spinal dorsal horn where the pain information is first integrated (20). However, there still lacks experimental evidence on the potential effect of EM-2 on the hyperalgesia or the exacerbated inflammatory pain in OVX animals. We hypothesized that intrathecal (IT) administration of EM-2 can dose dependently inhibit the OVX-induced hyperalgesia as well as the exacerbated inflammatory pain in the OVX rats and designed the current experiments to test this hypothesis.

We first observed the temporal expression of spinal EM-2 in OVX rats. The effect of IT administered EM-2 on the thermal hyperalgesia and the biphasic inflammatory pain responses induced by subcutaneous injection of formalin in the OVX rats were evaluated. Furthermore, IT pretreatment with mu-opioid receptor (MOR) antagonist beta-funaltrexamine (β-FNA) was carried out to elucidate the possible involvement of MOR in EM-2 analgesia.

Methods

Animals and Surgery

Female Sprague-Dawley rats weighing 180 – 200 g were used. Animals were individually housed in breeding cages with free access to food and water, in a temperature (22 ± 1°C) and humidity (55 ± 10%) controlled room with a light-dark cycle (lights on at 07:00, off at 19:00).

OVX operation: OVX was carried out according to a previous report (21). Briefly, under deep anesthesia with sodium pentobarbital (40 mg/kg, i.p.), both flanks of a rat were shaved and swabbed. The skin was opened with a 5 mm incision along the midline just below the ribs, and a 10 mm incision was made through the muscle at –1.5 – 2 cm lateral to the midline. The ovary was pulled through the incision. The tissue between the oviduct and uterus was clamped with a hemostat and a ligature was placed just below the hemostat. The ovary was removed with scissors and the hemostats released. This procedure was repeated on the contralateral side. The muscle layer was suture-closed and the skin incision closed with 9 mm wound clips. In the sham-operated rats, the ovaries were exposed but not removed. All efforts were made to minimize animals' suffering and to reduce the number of animals used. All experimental procedures were reviewed and approved by the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, P. R. China).

IT catheterization: OVX animals were given a 2-week recovery prior to IT catheterization. The IT catheterization was performed as in our previous report (22). Briefly, under pentobarbital anesthesia (45 mg/kg, i.p.), a midline incision (3 cm) was made at the level of the thoracic vertebrae from the back of the rat. A guide cannula (20 gauge, 0.9 mm × 38 mm) was inserted into the IT space between the L5 and L6 vertebrae through the back skin incision. PE-10 tubing was inserted through the guide cannula. 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. Rats were allowed to recover for 3 to 5 days before behavioral testing. Only the animals that demonstrated no paralysis, normal walking, and complete paralysis of the tail and bilateral hind legs after IT administration of 2% lidocaine (10 μl) were used for the following experiments.

OVX and IT catheterized animals were randomly selected to inspect complete removal of gonads at the end of the experiments.

Determining the Stage of Estrous Cycle

The estrus cycle stage was determined using histology of vaginal smears. A predominance of small leukocytes indicates diestrous; while a predominance of large round nucleated cells indicates proestrous stage. During proestrus, when circulating estrogens are high, spinal EM-2 antinociception is robust and comparable in magnitude to that manifested by males according to a previous study (23), thus the proestrus female rats were selected as control in the current study.

Drug Administration

According to our pilot experiment, the significant thermal hyperalgeia was stabilized after 2 weeks of OVX and the expression of spinal EM-2 significantly reduced at 4 weeks after OVX, thus, all the behavioral observation was performed at 4 weeks after OVX. EM-2 and beta-funaltrexamine (β-FNA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The drugs were dissolved in vehicle (normal saline) to a total volume of 10 µl and were IT injected over 30 seconds, followed by a flush with 10 µl of vehicle. At 4 weeks after OVX operation, IT EM-2 (at doses of 2.5, 10, 25, or 50 µg in 10 µl saline) or β-FNA (500 ng, delivered at 25 minutes prior to EM-2) was delivered. The doses of EM-2 and β-FNA used in the current study were based on previous studies in that these doses produced maximal behavioral effects without causing behavioral toxicity (23-25).

Behavioral Observations

The OVX rats demonstrated both thermal hyperalgesia and tactile allodynia (unpublished data). We did not evaluate the effect of EM-2 on tactile allodynia in the current project because it takes time to measure thermal hyperalgesia and tactile allodynia and we could not measure both during the short time window (15 minutes). Thus, we assessed thermal hyperalgesia by measuring the PWL to nociceptive thermal stimuli.

PWL to nociceptive thermal stimuli

Thermal hyperalgesia was tested using a method that was modified from Hargreaves et al (26) and used in our previous study (27). The rats were habituated to an environment containing individual Perspex boxes on an elevated glass table with a portable radiant heat source under the table. The heat source was focused on the intra-plantar surface during testing. The PWL was defined as the time to withdraw the hind paw from the heat source, and 40 seconds was regarded as the cut-off point to avoid tissue damage.

Formalin test

The formalin test was performed according to one of our previous studies (28). After the rat's acclimation to the testing chamber for about 20 minutes, 50 µl of the 5% formalin solution (dissolved in saline) was subcutaneously (SC) injected into the plantar surface of the left hind paw using a microsyringe (Hamilton Co. NV, USA) attached to a 30-G needle. After formalin administration, the rats were returned to the observing cage and the video recordings were performed for 60 minutes, as described below.

A sound-attenuated clear Perspex testing cage (40 × 40 × 60 cm) was equipped with a reverse video camera to record video for offline analysis. A trained observer conducted the behavioral analysis of the video recordings to determine the pain responses induced by formalin. The observer was trained to provide a similar rating performance (at the 95% confidence limit) during the tests of different animals. The pain behaviors were manually recorded with a stopwatch by retrieving spontaneous flinching of the injected hindpaw from the recorded videos as an indicator of spontaneous pain responses (29).

Elevated plus maze (EPM) test

EPM test was done according to our previous reports (30-32). Briefly, animals were placed in an EPM made of black Plexiglas consisting of 2 opposite-facing open arms (OA, 50 × 10 cm), two opposite-facing closed arms (CA, 50 × 10 × 40 cm), and a central area (10 × 10 cm). The walls of the closed arm were made of black Plexiglas. The EPM was mounted on a base and elevated 50 cm above the floor. Rats were placed in the central area facing one open arm. Then, a 5-minute test session was started. The numbers of rats entering/climbing onto the open arms and closed arms were recorded by a video recorder, and later scored by 2 investigators blinded to the experiment. In addition, the amount of time the rats spent on each arm was recorded. An entry was defined as placing 4 paws onto the arm. The EPM relies on the animal's natural fear of open spaces, and the percent of time spent in OA (OA time %) and percent of OA entries (OA entries %) are believed to be measurements of general anxiety level. OA time % was calculated by dividing the time spent in the OA by the sum of the time spent in the open and closed arms. OA entries % was calculated by dividing the number of OA entries by the sum of the entries into both open and closed arms (Shanghai Mobiledatum Information Technology Co., Ltd, Shanghai China).

Open field (OF) test

OF test was done according to our previous reports (30-32). Rats were placed at the center of a cubic chamber (100 × 50 × 100 cm). The total distance that the animal traveled in 15 minutes was measured by an automated analyzing system (Shanghai Mobile Datum Information Technology Co., Ltd). This distance was used as a parameter for the rat's locomotion and the percentage of time spent in the center area (center time percentage) was used to evaluate anxiety levels.

All animals were habituated to the testing room for 20 minutes before the session started.

All behavioral tests were performed during 0800 – 1200, in a low illuminated sound-proof room based on a double-blind manner to reduce the possible bias introduced by fluctuation of hormonal levels, light effects, or observer.

Dose-effect curve and ED50 calculation

The dosages of IT EM-2 were transformed into logarithm dose and the non-line fit was performed so as to construct the dose-effect curve. Based on the dose-effect cure, the ED50s of IT EM-2 on analgesia were calculated. The reliability of ED50 calculated from a specific dose-effect curve can be evaluated by the slope factor returned by the GraphPad Prism version 5.01 for Windows (San Diego California USA, www.graphpad.com).

Immunohistochemistry

At 0, 4, and 8 weeks after OVX or sham operation, rats were anesthetized with an overdose of sodium pentobarbital and were perfused through the ascending aorta with 100 mL of normal saline, followed by 500 mL of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4-L5 spinal segments were immediately removed and placed into the same fixative for 2 – 4 hours (4°C), and then cryoprotected in 0.1 M PB (pH 7.4) containing 30% (w/v) sucrose for 24 hours at 4°C. Thirty-μm-thick transverse spinal sections were serially cut on a frozen microtome (Kryostat 1720; Leitz, Mannheim, Germany). All sections were serially collected into 6 dishes containing 0.01 M phosphate-buffered saline (PBS, pH 7.4). Each dish contained a set of serial sections that were processed for subsequent immunohistochemical staining.

The procedures for immunohistochemical staining of EM-2-immunoreactive (IR) structures were described in our previous reports (33-36). The sections in the first dish were mounted onto gelatin-coated glass slides and processed for Nissl staining. The sections in the second dish were incubated with rabbit antiserum against EM-2 (1:200; Chemicon, Temecula, CA, USA) in 0.01 M PBS containing 5% (v/v) normal donkey serum (NDS), 0.3% (v/v) Triton X-100, 0.05% (w/v) NaN3, and 0.25% (w/v) carrageenan (PBS-NDS, pH 7.4) at 4 °C. Forty-eight hours later, all sections of the second dish were incubated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) diluted in PBS-NDS for another 4 hours, and then with avidin–biotin–peroxidase complex (ABC) Elite Kit (1:100; Vector) in 0.01 M PBS (pH 7.4) for one hour. Between each step, the sections were completely washed with 0.01 M PBS. Finally, the sections were reacted with 0.05 M Tris–HCl buffer (pH 7.6) containing 0.04% diaminobenzidine tetrahydrochloride (DAB) (Dojin, Kumamoto, Japan) and 0.003% H2O2 to visualize EM-2-IR structures.

Experimental Design

Experiment 1 was designed to investigate the temporal alteration of spinal EM-2 after OVX. Eighteen female rats were randomly divided into 2 groups to receive OVX or sham operation, respectively. At 0 day, 4 weeks, or 8 weeks after operation, rats (3 for each time-point) were sacrificed for the semi-quantification of spinal EM-2 with immunohistochemistry.

Experiment 2 was designed to investigate the dose dependent analgesia of IT EM-2 on thermal hyperalgesia of OVX rats and the possible involvement of spinal MORs. Female rats at 4 weeks after OVX or sham operation were randomly divided into 6 groups: (1) OVX + Veh: OVX rats receiving IT Veh administration; (2) OVX + EM-2 2.5 μg: OVX rats receiving IT administration of 2.5 μg of EM-2; (3) OVX + EM-2 10 μg: OVX rats receiving IT administration of 10 μg of EM-2; (4) OVX + EM-2 25 μg: OVX rats receiving IT administration of 25 μg of EM-2; (5) OVX + EM-2 50 μg: OVX rats receiving IT administration of 50 μg of EM-2; (6) OVX +β-FNA + EM-2 50 μg: OVX rats receiving IT administration of 50 μg of EM-2, at 25 minutes prior to EM-2 administration, 500 ng of β-FNA was IT delivered. Six rats were assigned to each group.

Experiment 3 was designed to investigate the dose dependent analgesia of IT EM-2 on exacerbated inflammatory pain in OVX rats and the possible involvement of spinal MORs. SC injection with 50 μl of 5% formalin was performed right after IT delivery of EM-2. Female rats at 4 weeks after OVX or sham operation were divided into 7 groups: (1) Pro + Veh: proestrus female receiving IT Veh administration; (2) OVX + Veh: OVX rats receiving IT Veh administration; (3) OVX + EM-2 2.5 μg: OVX rats receiving IT administration of 2.5 μg of EM-2; (4) OVX + EM-2 10μg: OVX rats receiving IT administration of 10 μg of EM-2; (5) OVX + EM-2 25 μg: OVX rats receiving IT administration of 25 μg of EM-2; (6) OVX + EM-2 50 μg: OVX rats receiving IT administration of 50 μg of EM-2; (7) OVX +β-FNA + EM-2 50 μg: OVX rats receiving IT administration of 50 μg of EM-2, at 25 minutes prior to EM-2 administration, 500 ng of β-FNA was IT delivered. Six rats were assigned to each group.

Experiment 4 was designed to investigate the po-

tential influence of IT delivered EM-2 on the rats' locomotion and anxiety levels. Proestrus rats and OVX rats at 4 weeks after OVX were divided into 3 groups: (1) Pro + Veh: proestrus female receiving IT Veh administration; (1) OVX + Veh: OVX rats receiving IT Veh administration; (3) OVX + EM-2: OVX rats receiving IT administration of 50 μg of EM-2. All rats were naïve to pain response tests and were used for OF and EPM tests sequentially.

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). Thermal hyperalgesia data obtained during 2 hours recording of PWLs were pooled together for statistical analysis, and in the formalin test, data from the first or second phase were considered independently. The semi-quantified data of spinal EM-2 expression at each time point were compared using 2-tail paired student t-test. The AUCs of individual animals for thermal hyperalgesia, formalin pain response curves, as well as the data sets for OF or EPM tests were group pooled and one-way ANOVA with Bonferroni's multiple comparison tests were performed using GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego California USA, www.graphpad.com).

Results

Temporal Alterations of Spinal EM-2 Levels after OVX

To test our hypothesis that EM-2 is involved in the OVX-induced pain responses, we first tried to investigate the temporal expressions of spinal EM-2 at different time points in OVX and sham treated rats (Fig. 1). The spinal EM-2 expression was restricted to the superficial laminae and located mainly within terminals. OVX operation obviously decreased the spinal EM-2 levels, which reached the stable level until 4 weeks after OVX (Fig. 1A, B, and C). These temporal alterations could not be found in the sham operated rats (Fig. 1A', B', and C'). Semi-quantified analysis on these graphs revealed that the expression of EM-2 significantly decreased at 4 ($P < 0.01$, vs sham) and 8 ($P < 0.01$, vs sham) weeks after OVX. These data suggested that the decrease of spinal EM-2 might be one underlying pathophysiological mechanism for OVX-induced pain, thus, increasing the spinal EM-2 might be used as an analgesic strategy.

Effect of IT EM-2 on Thermal Pain Revealed with PWLs

Before OVX operation, the basal PWLs from 6 groups were similar (about 21 seconds) and the PWLs at 4 weeks after OVX were significantly decreased (about 10 seconds), suggesting the successful establishment of a OVX model. The IT delivery of EM-2 dose dependently increased the PWLs right after the EM-2 delivery and lasted for about 30 minutes (Fig. 2A). One-way ANOVA analysis of AUC for the temporal alterations of PWLs revealed significant group difference [Fig. 2B, between group factor: treatment, $F(5,35) = 18.97$, $P < 0.0001$]. Bonferroni's multiple comparison test revealed significant difference between 10 ($t = 3.286$, $P < 0.05$), 25 ($t = 5.347$, $P < 0.01$), or 50 ($t = 6.653$, $P < 0.01$) μg EM-2 treat-

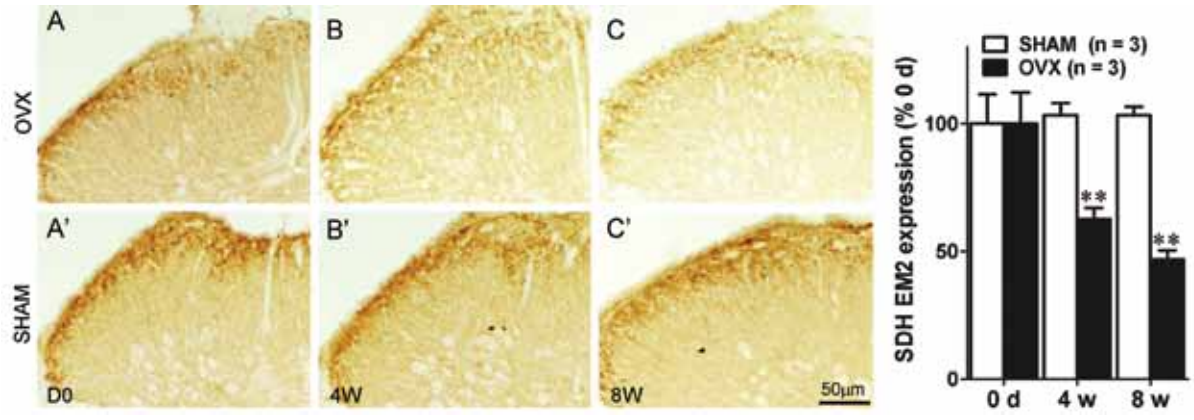

Fig. 1. *OVX decreased spinal expression of EM-2. The representative photographs of spinal dorsal horns (SDHs) from rats receiving OVX (A,B,C) or SHAM (A', B', C') operations at 0 (A, A'), 4 (B, B') or 8 (C, C') weeks. The semi-quantitative analyses of the EM-2 expression were presented in the histograph. **, P < 0.01 vs SHAM, n = 3 for each group at every time-point.*

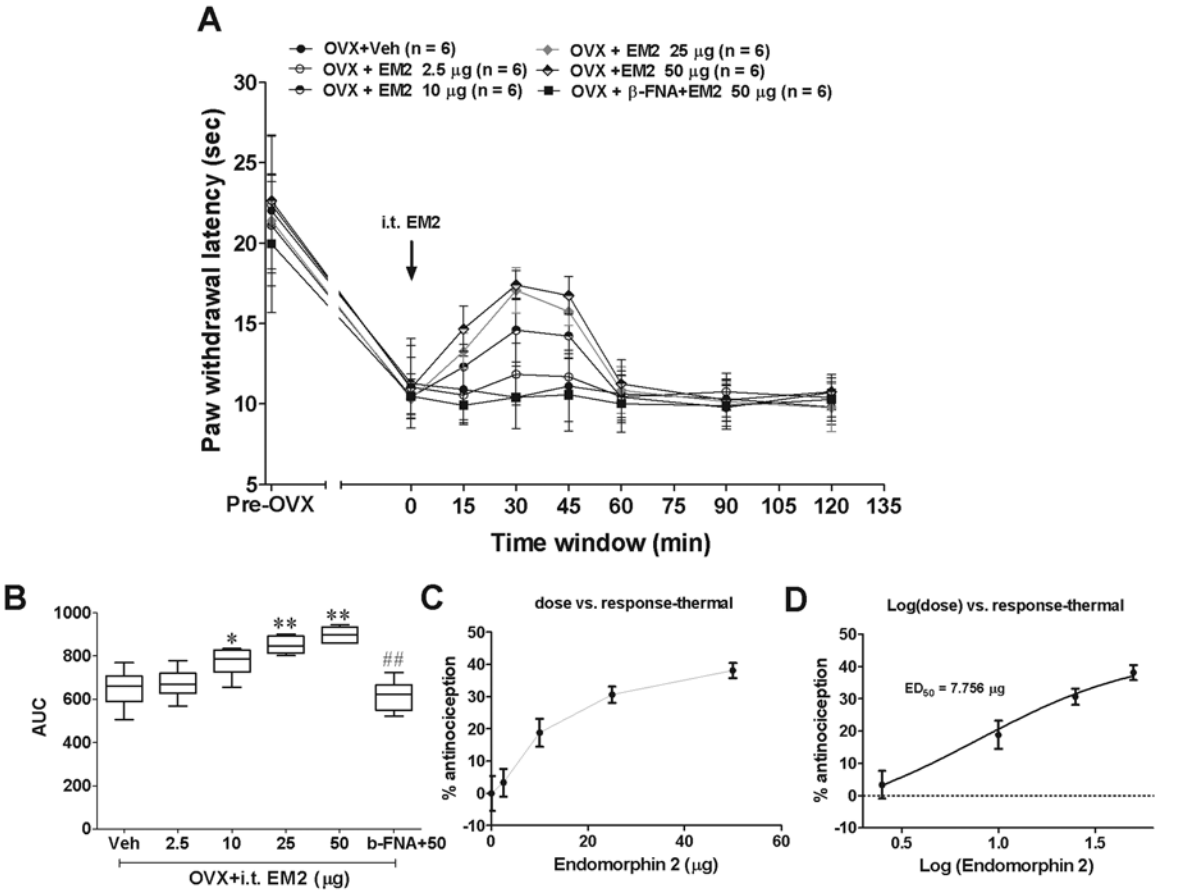

Fig. 2. *IT EM-2 dose-dependently inhibited OVX induced thermal hyperalgesia, which was reversed by IT pretreatment with EM-2 antagonist β-FNA. Paw withdrawal latency (PWL) to nociceptive thermal stimuli pre-OVX, at IT EM-2 administration and later on were measured at 15-minute intervals until 120 minutes after IT injection (A). The AUCs for different groups were calculated to perform statistical analysis on OVX-induced thermal hyperalgesia (B). The dose-effect (C) or log (dose)-effect (D) curves for the analgesic effects of IT EM-2 were shown to calculate the ED50 of analgesia effect of EM-2 on the OVX induced thermal hyperalgesia. Data were presented as mean ± SD from 6 rats in each group. *, $P < 0.05$, ** $P < 0.01$, vs OVX + Veh; ## $P < 0.01$, vs OVX + EM-2 50 μg.*

ed groups and Veh groups. There was no difference between 2.5 μg EM-2 treated groups and Veh groups (t = 0.5851, $P > 0.05$). The effect of IT EM-2 on thermal pain was calculated based on the log (dose) vs response curve (Fig. 2D) from the dose vs response curve (Fig. 2C). The ED50 of IT EM-2 on thermal hyperalgesia was 7.756 μg. Furthermore, pretreatment with selective MOR antagonist β-FNA completely abolished the analgesia effect of IT EM-2 at 50 μg, suggesting the involvement of spinal MOR in IT EM-2 mediated analgesia.

Effect of IT EM-2 on the Inflammatory Pain Induced by SC Injection of Formalin

The SC injection of 5% formalin into the hindpaw produced biphasic pain behaviors as spontaneous flinching of the injected paw: The first transient phase lasted for the first 10 minutes post injection and was followed by the second prolonged phase. There was no difference in the phase of formalin-induced pain behaviors between groups of proestrous and OVX rats (Fig. 3A). The transient phase pain responses were not different between Pro + Veh and OVX + Veh groups (Fig. 3B, t = 1.743, $P > 0.05$), but the prolonged phase pain responses were significantly higher in the OVX + Veh group (Fig. 3B', t = 9.998, $P < 0.01$, vs Pre + Veh group). These data suggested that OVX exacerbate inflammatory pain in mammals.

The IT delivery of EM-2 dose dependently attenuated the formalin-induced inflammatory pain (Fig. 3A). One-way ANOVA analysis of AUC for transient phase

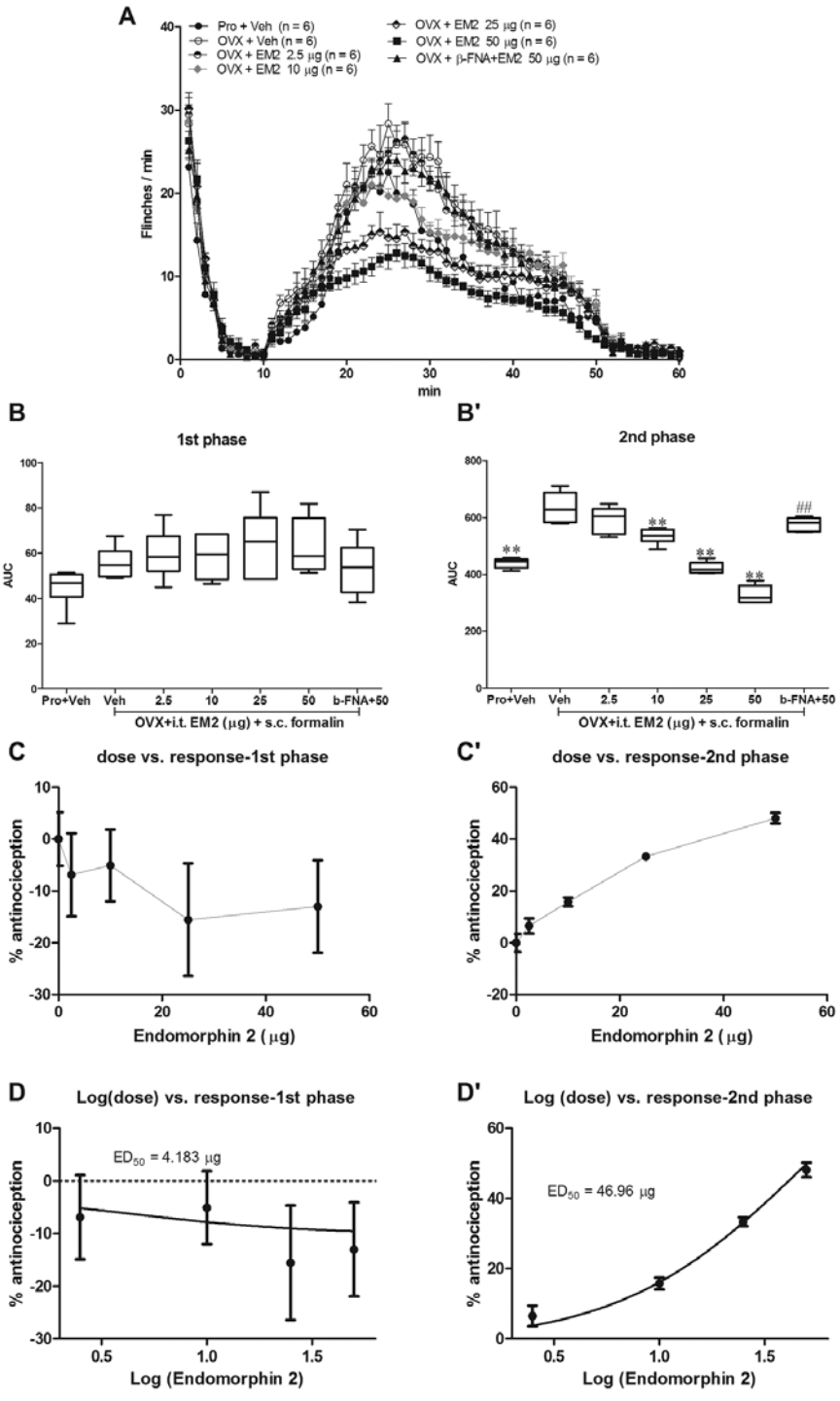

Fig. 3. *IT EM-2 dose-dependently inhibited formalin induced spontaneous flinching of the injected hind paw in proestrus or OVX rats receiving pretreatment with Veh or different dosages of EM-2. Spontaneous flinchings during 60 minutes after SC formalin injection from different groups were shown in A. The AUCs for different groups were calculated to perform statistical analysis on the first (B) and second (B') phases. The dose-effect or log (dose)-effect curves for EM-2's analgesic effects were shown in C and D (first phase) or C' and D' (second phase). Data were presented as mean ± SD from 6 rats in each group. ** P < 0.01, vs OVX + Veh; ## P < 0.01, vs OVX + EM-2 50 μg.*

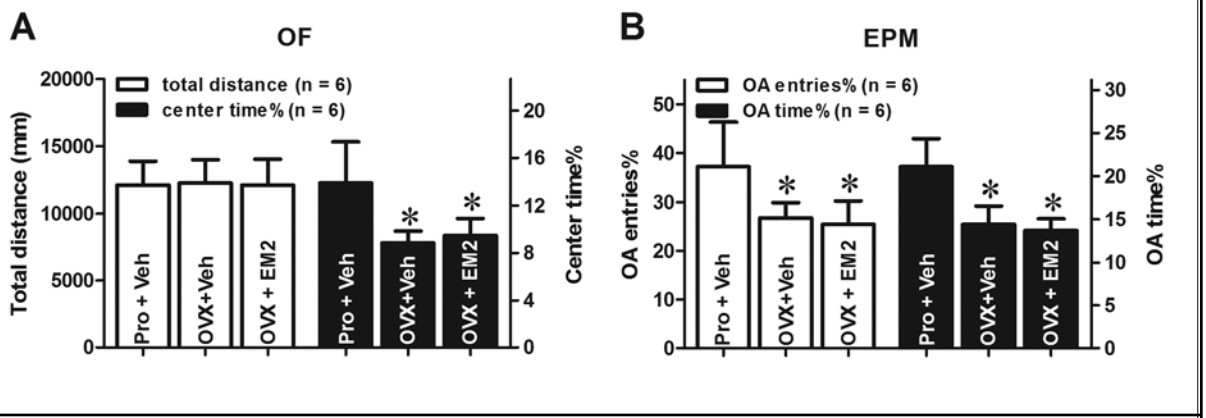

Fig. 4. *Maximal dosage of IT EM-2 treatment did not affect the locomotion, anxiety/depression level in the OVX rats. Total distance traveled and center time % in OF (A), OA entries % and OA time % in EPM (B) were used to detect the locomotion, anxiety/depression, respectively. Data were presented as mean ± SD from 6 rats in each group. * P < 0.05, vs Pro + Veh.*

pain responses revealed no significant group difference [Fig. 3B, between group factor: treatment, F(6,41) = 2.231, *P* = 0.063]. However, one-way ANOVA analysis of AUC for second phase pain responses revealed significant group difference [Fig. 3B', between group factor: treatment, F(6,41) = 63.81, *P* < 0.0001]. Bonferroni's multiple comparison test revealed significant difference between 10 (t = 5.149, *P* < 0.01), 25 (t = 10.87, *P* < 0.01), and 50 (t = 15.69, *P* < 0.01) μg EM-2 treated groups and Veh groups. There was no difference between 2.5 μg EM-2 treated groups and Veh groups (t = 2.133, *P* > 0.05). The effect of IT EM-2 on transient phase pain responses was calculated based on the log (dose) vs response curve (Fig. 3D) from the dose vs response curve (Fig. 3C). However, there was almost no analgesia effect of IT EM-2 on transient phase responses. The effect of IT EM-2 on second phase pain responses was calculated based on the log (dose) vs response curve (Fig. 3D) from the dose vs response curve (Fig. 3C). The ED50 of IT EM-2 on the second phase pain responses is 46.96 μg. Furthermore, pretreatment with β-FNA partially inhibited the analgesia effect of IT EM-2 at 50 μg, suggesting the involvement of spinal MOR in IT EM-2 mediated analgesia, the remaining unblocked analgesia might be mediated via kappa opioid receptor (KOR).

Effect of IT EM-2 on Locomotion and Anxiety Behaviors of OVX Rats

OVX is always accompanied with anxiety-like behaviors (37,38), and increasing the concentration of brain EM-2 renders anti-anxiety effects (39). We then asked whether IT delivery of EM-2 can affect the anxiety status of OVX rats, thereby complicating the explanation of analgesic effects. There was no significant group difference in the locomotion revealed by the total distance traveled in the 15 minutes recording time in OF [Fig. 4A; one way ANOVA (between-subjects factor: treatment) F(2,17) = 0.017, *P* = 0.984]. However, there was significant group difference in the percentage of center time in the OF test [Fig. 4A; one way ANOVA (between-subjects factor: treatment) F(2,17) = 8.976, *P* = 0.003], which indicated difference in their anxiety status. The significant difference in the anxiety status was also suggested by the OA entries % [Fig. 4B; one-way ANOVA (between-subjects factor: treatment) F(2,17) = 6.322, *P* = 0.010] and OA time % [Fig. 4B; one-way ANOVA (between-subjects factor: treatment) F(2,17) = 17.11, *P* < 0.01]. These differences in anxiety status were derived from the OVX treatment, but not IT EM-2 delivery.

Discussion

In the current study, we offered evidence that OVX-induced pain status is closely related with the spinal EM-2 decrease. Then we revealed that OVX both induces thermal hyperalgesia and exacerbates inflammatory pain responses. Based on these findings, we established the analgesia features of IT EM-2 on thermal hyperalgesia and inflammatory pain on OVX rats. The ED50 for thermal hyperalgesia is smaller than that for inflammatory pain responses, suggesting different responses to EM-2 for these 2 pain modalities.

Our study added information about OVX-induced pain to the current literature. Despite the fact that

menopausal women are more vulnerable to pain (6,7), the relationship between estrogen and nociceptive threshold remains inconclusive based on the current animal study. Our study suggested obvious hypersensitivity to noxious thermal stimuli, which is partly consistent with previous studies (11,12). However, the detailed explanation for the inconsistency is still lacking. The inconsistency might derive from the different OVX protocols, the time-window when the pain responses were tested, or how the pain responses were tested. For example, one study reported the increased hypersensitivity to noxious thermal stimuli, however, details about the OVX operation were not given and the pain tests were performed after 2 regular estrous cycles (about 8 days) (12) which is different from 4 weeks after OVX in the current study. Another study reported that estrogen had no effect on TFL to thermal stimuli (23), however, the estrogen levels were not fluctuating as much as in the condition of OVX. Future studies are needed to clarify the reason why different neuropharmacological studies on OVX could not reach the consolidated conclusion on the relationship between estrogen and pain.

Our study also added more evidence to the concept that OVX exacerbate inflammatory pain responses by using the conventional formalin model. The different influence of OVX on formalin pain seems to be highly dependent on the concentration of formalin used in the pain study. However, even using the same concentration of formalin, our findings that OVX increased the second phase pain responses are still different from a previous one (15). The possible explanation might be that we observed the formalin pain at 4 weeks after OVX, while the detailed time window was not given in that study (15). The underlying mechanism for inflammatory pain under different estrogen levels needs to be further investigated.

An interesting finding is that the ED50 of IT EM-2 on thermal hyperalgesia is smaller than that on inflammatory pain. Thermal hyperalgesia might be based on the long-lasting (molecular or structural) alterations within the spinal dorsal horn, which might be much more estrogen dependent and sensitive to EM-2. However, formalin-induced inflammatory pain, especially the second phase response, is the consequence of central sensitization that majorly involve the spinal cord neurons (40-42) or primary sensory neurons (43). A large amount of EM-2 is needed to reverse the drastic changes induced by OVX and formalin itself. For both thermal hyperalgesia and inflammatory pain, the efficacy of IT EM-2 lasted for less than 30 minutes, supporting the fast metabolizing of EM-2.

The involvement of MOR or other opioid receptors in the EM-2 induced analgesia on estrogen-related pain remains a hotly debated issue. In a previous study performed in normal cycles, researchers concluded that concomitant activation of dynorphin and KOR as well as MOR is involved in the EM-2 analgesia on acute physiological pain of women with high circulating estrogen (23). Another study reported that EM-2 plays a role in antinociception induced by spinal KOR activation, suggesting that the interaction between MOR and KOR contribute to EM-2 analgesia (44). However, both studies observed the analgesia effect of EM-2 on acute pain in the normal cycle female rats (23) and male rats (44); the receptor contribution to IT EM-2's analgesia has not been investigated. Our findings that MOR is involved in the analgesia of IT EM-2 on thermal hyperalgesia and inflammatory pain on OVX rats suggests that receptor mechanisms for pain under OVX are different than under normal conditions. Furthermore, our data suggested that MOR is involved in the EM-2 analgesia on the OVX-induced thermal hyperalgesia, while other opioid receptors might be involved in the EM-2 analgesia on the inflammatory pain responses on OVX rats because of the remaining pain responses after the IT delivery of β-FNA. However, which opioid receptor is involved in both procedures needs to be further elucidated.

The findings from the current study are promising for using EM-2 as a potent analgesic agent. However, the pain in the menopausal population is always chronic; it is very important to find a solution to make the EM-2 analgesia persistent before large sample clinical trials are done. A recent study suggested that cyclization of EM-2 is a promising strategy in the development of new long-lasting opioid analgesics but enhancing the blood-brain barrier permeability is another issue (45). In the current study, EM-2 analgesia is mostly mediated via MOR that is suggested to contribute to opioid tolerance, thus the tolerance of EM-2 should be taken into consideration in the future studies (46). There is still long way to go to enable EM-2 as a potent analgesic agent.

Conclusions

In summary, our study offered evidence that OVX induces long-lasting thermal hyperalgesia, spinal EM-2 decrease as well as exacerbated inflammatory pain responses. EM-2 can dose dependently attenuate the OVX-induced thermal hyperalgesia as well as the sec-

ond phase pain responses in the SC formalin induced pain among these OVX rats, mainly via spinal MOR signal pathway. Our findings may open a new analgesia strategy for menopause women in using a downstream molecule of estrogen so as to reduce the hormone-related side effects.

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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.

References

1. Teepker M, Peters M, Vedder H, Schepelmann K, Lautenbacher S. Menstrual variation in experimental pain: correlation with gonadal hormones. *Neuropsychobiology* 2010:61: 131-140.
2. Tousignant-Laflamme Y, Marchand S. Excitatory and inhibitory pain mechanisms during the menstrual cycle in healthy women. *Pain* 2009:146: 47-55.
3. Powell-Boone T, Ness TJ, Cannon R, Lloyd LK, Weigent DA, Fillingim RB. Menstrual cycle affects bladder pain sensation in subjects with interstitial cystitis. *The Journal of urology* 2005:174: 1832-1836.
4. Kramer PR, Bellinger LL. The effects of cycling levels of 17beta-estradiol and progesterone on the magnitude of temporomandibular joint-induced nociception. *Endocrinology* 2009:150: 3680-3689.
5. Ibironke GF, Aji KE. Pain threshold variations in female rats as a function of the estrus cycle. *Nigerian journal of physiological sciences : official publication of the Physiological Society of Nigeria* 2011:26: 67-70.
6. Shuster LT. The burden of pain with menopause. *Menopause* 2012:19: 494-495.
7. Meriggiola MC, Nanni M, Bachiocco V, Vodo S, Aloisi AM. Menopause affects pain depending on pain type and characteristics. *Menopause* 2012:19: 517-523.
8. Adera T, Deyo RA, Donatelle RJ. Premature menopause and low back pain. A population-based study. *Annals of epidemiology* 1994:4: 416-422.
9. Yang PY, Tang CC, Chang YC, Huang SY, Hsieh SP, Fan SS, Lee HP, Lin SC, Chen WF, Wen ZH, Jean YH. Effects of tibolone on osteoarthritis in ovariectomized rats: Association with nociceptive pain behaviour. *European journal of pain* 2013.
10. Zheng XF, Li B, Zhang YH, Yang YH, Meng XY, Jiang SD, Jiang LS. Blockade of substance P receptor attenuates osteoporotic pain, but not bone loss, in ovariectomized mice. *Menopause* 2013:20: 1074-1083.
11. Vishali N, Kamakshi K, Suresh S, Prakash S. Red clover Trifolium pratense (Linn.) isoflavones extract on the pain threshold of normal and ovariectomized rats--a long-term study. *Phytotherapy research : PTR* 2011:25: 53-58.
12. Lawson KP, Nag S, Thompson AD, Mokha SS. Sex-specificity and estrogen-dependence of kappa opioid receptor-mediated antinociception and antihyperalgesia. *Pain* 2010:151: 806-815.
13. Ceccarelli I, Fiorenzani P, Massafra C, Aloisi AM. Long-term ovariectomy changes formalin-induced licking in female rats: the role of estrogens. *Reproductive biology and endocrinology : RB&E* 2003:1: 24.
14. Gaumond I, Arsenault P, Marchand S. The role of sex hormones on formalin-induced nociceptive responses. *Brain research* 2002:958: 139-145.
15. Kuba T, Hunter D, Zhou L, Jenab S, Quinones-Jenab V. Endogenous gonadal hormones regulate females' behavioral responses to formalin through prostaglandin E2 release. *Ethnicity & disease* 2010:20: S1-55-59.
16. Aloisi AM, Albonetti ME, Carli G. Sex differences in the behavioural response to persistent pain in rats. *Neuroscience letters* 1994:179: 79-82.
17. LeResche L, Saunders K, Von Korff MR, Barlow W, Dworkin SF. Use of exogenous hormones and risk of temporomandibular disorder pain. *Pain* 1997:69: 153-160.
18. Wise EA, Riley JL, 3rd, Robinson ME. Clinical pain perception and hormone replacement therapy in postmenopausal women experiencing orofacial pain. *The Clinical journal of pain* 2000:16: 121-126.
19. Musgrave DS, Vogt MT, Nevitt MC, Cauley JA. Back problems among postmenopausal women taking estrogen replacement therapy: the study of osteoporotic fractures. *Spine* 2001:26: 1606-1612.
20. Gupta DS, von Gizycki H, Gintzler AR. Sex-/ovarian steroid-dependent release of endomorphin 2 from spinal cord. *The Journal of pharmacology and experimental therapeutics* 2007:321: 635-641.
21. Bobzean SA, Dennis TS, Perrotti LI. Acute estradiol treatment affects the expression of cocaine-induced conditioned place preference in ovariectomized female rats. *Brain research bulletin* 2014.
22. Chen L, Zhu L, Wang K, Wang W, Mei XP, Liu T, Zhang FX, Wang W, Chen T, Li YQ. Antinociceptive effect of prostatic acid phosphatase in a rat model of cancer-induced bone pain. *Pain physician* 2013:16: 533-546.
23. Liu NJ, Gintzler AR. Spinal endomorphin 2 antinociception and the mechanisms that produce it are both sex- and stage of estrus cycle-dependent in rats. *The journal of pain : official journal of the American Pain Society* 2013:14: 1522-1530.
24. Przewlocka B, Mika J, Labuz D, Toth G, Przewlocki R. Spinal analgesic action of endomorphins in acute, inflammatory and neuropathic pain in rats. *European journal of pharmacology* 1999:367: 189-196.
25. Horvath G, Szikszay M, Tomboly C, Benedek G. Antinociceptive effects of intrathecal endomorphin-1 and -2 in rats. *Life Sci* 1999:65: 2635-2641.
26. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal noci-

ception in cutaneous hyperalgesia. *Pain* 1988:32: 77-88.

27. Wang W, Wu SX, Wang YY, Liu XY, Li YQ. 5-hydroxytryptamine1A receptor is involved in the bee venom induced inflammatory pain. *Pain* 2003:106: 135-142.
28. Sun YH, Dong YL, Wang YT, Zhao GL, Lu GJ, Yang J, Wu SX, Gu ZX, Wang W. Synergistic analgesia of duloxetine and celecoxib in the mouse formalin test: a combination analysis. *PloS one* 2013:8: e76603.
29. Bai L, Wang W, Dong YL, Huang J, Wang XY, Wang LY, Li YQ, Wu SX. Attenuation of mouse somatic and emotional inflammatory pain by hydralazine through scavenging acrolein and inhibiting neuronal activation. *Pain physician* 2012:15: 311-326.
30. Wang W, Liu Y, Zheng H, Wang HN, Jin X, Chen YC, Zheng LN, Luo XX, Tan QR. A modified single-prolonged stress model for post-traumatic stress disorder. *Neuroscience letters* 2008:441: 237-241.
31. Liu JL, Li M, Dang XR, Wang ZH, Rao ZR, Wu SX, Li YQ, Wang W. A NMDA receptor antagonist, MK-801 impairs consolidating extinction of auditory conditioned fear responses in a Pavlovian model. *PloS one* 2009:4: e7548.
32. Xia L, Zhai M, Wang L, Miao D, Zhu X, Wang W. FGF2 blocks PTSD symptoms via an astrocyte-based mechanism. *Behavioural brain research* 2013:256: 472-480.
33. Chen T, Hui R, Dong YX, Li YQ, Mizuno N. Endomorphin 1- and endomorphin 2-like immunoreactive neurons in the hypothalamus send axons to the parabrachial nucleus in the rat. *Neuroscience letters* 2004:357: 139-142.
34. Chen T, Hui R, Wang XL, Zhang T, Dong YX, Li YQ. Origins of endomorphin-immunoreactive fibers and terminals in different columns of the periaqueductal gray in the rat. *The Journal of comparative neurology* 2008:509: 72-87.
35. Zhu C, Hui R, Chen T, Zuo ZF, Wang W, Gao CJ, Zhang T, Wang YY, Li H, Wu SX, Li YQ. Origins of endomorphin-2 immunopositive fibers and terminals in the rat medullary dorsal horn. *Brain research* 2011:1410: 38-47.
36. Hui R, Chen T, Li YQ. The reciprocal connections of endomorphin 1- and endomorphin 2-containing neurons between the hypothalamus and nucleus tractus solitarii in the rat. *Neuroscience* 2006:138: 171-181.
37. De Jesus-Burgos M, Torres-Llenza V, Perez-Acevedo NL. Activation of amygdalar metabotropic glutamate receptors modulates anxiety, and risk assessment behaviors in ovariectomized estradiol-treated female rats. *Pharmacology, biochemistry, and behavior* 2012:101: 369-378.
38. Ho YJ, Tai SY, Pawlak CR, Wang AL, Cheng CW, Hsieh MH. Behavioral and IL-2 responses to diosgenin in ovariectomized rats. *The Chinese journal of physiology* 2012:55: 91-100.
39. Asakawa A, Inui A, Momose K, Ueno N, Fujino MA, Kasuga M. Endomorphins have orexigenic and anxiolytic activities in mice. *Neuroreport* 1998:9: 2265-2267.
40. Huang J, Chang JY, Woodward DJ, Baccala LA, Han JS, Wang JY, Luo F. Dynamic neuronal responses in cortical and thalamic areas during different phases of formalin test in rats. *Exp Neurol* 2006:200: 124-134.
41. Chen HS, Li MM, Shi J, Chen J. Supraspinal contribution to development of both tonic nociception and referred mirror hyperalgesia: a comparative study between formalin test and bee venom test in the rat. *Anesthesiology* 2003:98: 1231-1236.
42. Taylor BK, Basbaum AI. Early antinociception delays edema but does not reduce the magnitude of persistent pain in the formalin test. *The journal of pain : official journal of the American Pain Society* 2000:1: 218-228.
43. McRoberts JA, Ennes HS, Marvizon JC, Fanselow MS, Mayer EA, Vissel B. Selective knockdown of NMDA receptors in primary afferent neurons decreases pain during phase 2 of the formalin test. *Neuroscience* 2011:172: 474-482.
44. Shimoyama M, Toyama S, Tagaito Y, Shimoyama N. Endogenous endomorphin-2 contributes to spinal k-opioid antinociception. *Pharmacology* 2012:89: 145-148.
45. Fichna J, Perlikowska R, Wyrebska A, Gach K, Piekielna J, do-Rego JC, Toth G, Kluczyk A, Janecki T, Janecka A. Effect of 2',6'-dimethyl-L-tyrosine (Dmt) on pharmacological activity of cyclic endomorphin-2 and morphiceptin analogs. *Bioorganic & medicinal chemistry* 2011:19: 6977-6981.
46. Li G, Ma F, Gu Y, Huang LY. Analgesic tolerance of opioid agonists in mutant mu-opioid receptors expressed in sensory neurons following intrathecal plasmid gene delivery. *Molecular pain* 2013:9: 63.