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Study of morphine-induced dependence in gonadectomized male and female mice

Mahsa Sadeghi [1], Setareh Sianati [1], Dina Kalbasi Anaraki [1], Mehdi Ghasemi, Mehrak Javadi Paydar, Behrang Sharif, Shahram Ejtemaei Mehr *, Ahmad Reza Dehpour

Department of Pharmacology, School of Medicine, Medical Sciences/University of Tehran, P.O. Box: 13145-784, Tehran, Iran

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ABSTRACT

In this study we evaluated the effects of sex difference and also sex hormones on the naloxone-precipitated morphine withdrawal in both orchidectomized (ORC) male and ovariectomized (OVX) female mice. Morphine (50, 50 and 75 mg/kg/day for 4 days, s.c.) was administered to animals and at 5th day naloxone (4 mg/kg, i.p.)-precipitated morphine withdrawal signs, jumpings and the percentage of weight loss, were measured. There was no significant alteration in withdrawal jumpings between male and female mice, though weight loss was significantly higher in male ones. Jumpings was significantly lower in both OVX and ORC mice and percentage of weight loss was significantly higher in OVX mice than corresponding non-operated or sham animals. In OVX mice, E_2V (10 mg/kg, s.c.) increased number of jumpings and decreased percentage of weight loss. Progesterone (25 mg/kg, s.c.) had no effect on jumpings, whereas it decreased weight loss in OVX mice. Testosterone (2.5 mg/kg, s.c.) increased jumpings in ORC mice while it had no effect on percentage of weight loss. Our results demonstrated that sex hormones could play a role in the morphine withdrawal syndrome in both ORC male and OVX female mice.

1. Introduction

Animal studies have shown that males and females have different sensitivities to the effects of opioids. For example, male rats are more sensitive than females to the antinociceptive properties of morphine in several different pain models (Ali et al., 1995; Baamonde et al., 1989; Berkley, 1997; Cicero et al., 1996; Craft, 2003; Craft et al., 2004). Similar sex differences have been reported for morphine-induced sedation (Craft et al., 1999a; Stratmann and Craft, 1997), respiratory depression (Dahan and Kest, 2001; Kest et al., 1998; Pleym et al., 2003), urinary retention (Craft et al., 2000) and modulation of seizure susceptibility (Riazi et al., 2004) and locomotor activity (Craft et al., 2006; Li et al., 1990; Schnur and Barela, 1984). There is also several evidence demonstrating sex differences in the development of tolerance and dependence to opioids such as morphine (Craft et al., 2004; 1999b; Dahan et al., 2008). Additionally, it has been shown that sex differences in tolerance development could be explained by sex differences in morphine potency and when males and females receive the same mg/kg doses of morphine, males become more tolerant than females because they are more sensitive than females to the acute effects of morphine (Barrett et al., 2001). Sex hormones are probably the most investigated factors in the existing differences between males and females in response to opioids (Dahan et al., 2008). Some evidence exists for an interaction between sex hormones and opioid antinociceptive potency (Candido et al., 1992; Ji et al., 2007; Stoffel et al., 2003; Sumner et al., 2006). Moreover, the removal of sex steroids by gonadectomy affects the antinociceptive activity of morphine in some experimental paradigms (Banerjee et al., 1983; Bodnar et al., 1988; Cicero et al., 1996; Frye and Seliga, 2001; Negus and Mello, 2002; Romero et al., 1987).

Despite the aforementioned role of sex hormones in some of the properties of opioids, this issue has not been fully examined in the dependency to morphine. On the other hand, Reddy and Kulkarni (1997) demonstrated that chronic treatment of neurosteroids such as allopregnanolone, pregnenolone sulfate, dehydroepiandrosterone sulfate and progesterone during the induction of morphine dependency could affect the naloxone-precipitated withdrawal jumps. According to another observation, whereas chronic morphine decreased brain concentrations of pregnenolone, progesterone and pregnenolone sulfate, but not allopregnanolone, dihydroepiandrosterone and dihydroepiandosterone, naloxone-precipitated morphine withdrawal increased all of these steroid concentrations (Yan and Hou, 2004). Concas et al. (2006) has recently shown that acute treatment of morphine induced dose- and time-dependent increased in rat cerebrocortical and plasma concentrations of neurosteroids such as progesterone and pregnanolone. They also demonstrated that naloxone-precipitated morphine withdrawal also increased neurosteroid concentrations in rat brain and plasma. Another study by Ceccarelli et al. (2006) also revealed that opioid administration could alter the level of gonadal steroids such as estradiol and testosterone in both the central nervous system (CNS) and plasma of male rats. Considering the

* Corresponding author. Tel./fax: +98 21 6640 2569.
E-mail addresses: m82_ghasemi@yahoo.com (M. Ghasemi), ejtemam@tums.ac.ir (S.E. Mehr).
[1] The first three authors contributed equally to the study.

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interactions between sex hormones and opioid system, it is possible that sex hormones might be involved in the dependency to morphine and morphine withdrawal syndrome.

In the present study, we first examined the differences in the naloxone-precipitated morphine withdrawal between male and female mice, evaluating whether there is any sex difference in dependency to morphine. Then we examined the effect of gonadectomy in this manner. Effects of sex hormones on the morphine withdrawal in both ovariectomized female and orchidectomized male mice were further evaluated.

2. Materials and methods

2.1. Animals

Female and male NMRI mice (Pasteur Institute) weighing 25–30 g were used throughout the study. The animals were housed in a temperature-controlled room (24±1 °C) on a 12-h light/dark cycle with free access to food and water. All experiments were carried out in the same room between 08:00 to 16:00 to minimize diurnal variations. Separate groups of animals were used for each test. All procedures were carried out in accordance with the institutional animal care and use committee (Department of Pharmacology, School of Medicine, TUMS) guidelines for animal care and use. All of the animal studies were also approved by a group from the Ethics Committee of Tehran University of Medical Sciences (TUMS) and experiments were performed in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Either of male and female animals was divided into 3 main groups: unoperated control, sham-operated and operated (OVX in female and ORC in male mice). Each experimental group consisted of 10 animals.

2.2. Chemicals

The following drugs were used in the study: morphine sulfate (Tolid-daru, Tehran, Iran), estradiol valerate (E_2V) and progesterone (Sigma; Poole, UK), testosterone enanthate (Tolid-daru, Tehran, Iran), and naloxone hydrochloride (Tolid-daru, Tehran, Iran). Morphine and naloxone were dissolved in physiologic saline solution. Estradiol valerate (E_2V), progesterone and testosterone were dissolved in olive oil. Doses used were chosen based on previous published studies and pilot experiments (Bassol et al., 2000; Dambisya and Lee, 1996; Nakazawa et al., 2006; Quirarte et al., 2007; Shirwalkar et al., 2007; Zarrindast et al., 2002).

2.3. Ovariectomy (OVX) and orchidectomy (ORC)

Anesthesia was induced by intraperitoneally injection of 50 mg/kg ketamine (Alfasan, Woerden, Holland). In female mice, after the onset of anesthesia, the lumbar dorsum was shaved, and the exposed skin prepared for aseptic surgery (a 10% povidene–iodine scrub followed by a sterile saline wipe). Surgery was performed according to the method described by Eddy (1986) with modification (Riazi et al., 2004). In brief, skin was opened with a 1- to 2-cm incision in the midline on the lumbar vertebral line. About 1 cm to each flank, parovarian fatty tissue was identified and pulled out through a small incision. The exposed ovary and associated oviduct were removed. Hemostasis, if needed, was achieved by hemostat pressure for 1 to 2 min. Then the skin incision was sutured (5-0 nonabsorbable). In sham-operated animals, the parovarian fatty tissues and ovaries were just retracted and were replaced.

In males, after proper anesthesia, a small 10-mm median incision was made through the skin at the tip of the scrotum. The cremaster muscles were opened with a small 7-mm incision. The testicular fat pads on both sides were pulled through the incision by using a blunt forceps. A single ligature was placed around the vas deferens and the blood vessels on each side, and the testicles were removed. Then the muscle layer was closed by using two resorbable 5-0 sutures, and the skin, with nonresorbable 4-0 sutures. The operated-on animals were tested 10–12 days after surgery. The mortality rate was <5%.

2.4. Induction of morphine dependence

The animals were rendered dependent on morphine using the method described previously (Zarrindast et al., 2002) with some modification. Morphine sulfate was injected subcutaneously three times daily at 08.00, 11.00 and 16.00 h at doses of 50, 50 and 75 mg/kg, respectively. The higher dose at the third daily injection was aimed to minimize any overnight withdrawal. Morphine administration was carried out 4 days for all groups of mice. A dose of 100 mg/kg of morphine sulphate was also injected on the test (fifth) day (2 h before naloxone injection). Additionally, a separate group of experimental animals were administered saline (instead of morphine) as a control group for further investigation of the effect of sex hormones on naloxone-withdrawal signs.

2.5. Naloxone-precipitated withdrawal

Two hours after the last dose of morphine on the fifth day, signs of abstinence were precipitated by intraperitoneally injection of 4 mg/kg of naloxone. Immediately after the injection of naloxone, the animals were placed individually in a platform (40 cm long, 25 cm wide and 45 cm high) and then the number of jumpings (withdrawal signs) was observed continuously for 30 min. The percentage loss of body weight at 1 h after naloxone administration was also measured.

2.6. Treatments

In experiment 1, for assessing the effects of the ovarian sex hormones on the withdrawal signs in female mice, OVX animals received separately single dose of either E_2V (10 mg/kg, s.c.) 48 h before the injection of naloxone. Single administration of 10 mg/kg E_2V could provide continuous levels of circulating estradiol for an extended period in rodents (Quirarte et al., 2007). In addition, 2 mg doses of E_2V along with a progesterone-like drug, given monthly, are the doses used in birth control drugs touted as effective for use by women of underdeveloped countries with special reference to Latin America (Bassol et al., 2000). Progesterone (25 mg/kg, s.c.) was also injected to a separate group of sham and OVX mice daily for 4 days during the development of morphine dependence before administration of the first dose of morphine each day. Control animals received vehicle (olive oil).

In experiment 2, for assessing the effects of the male sex hormone testosterone on the withdrawal signs in male mice, ORC animals received separately single dose of testosterone enanthate (2.5 mg/kg, i.p.) 48 h before the injection of naloxone. Control animals received vehicle.

2.7. Statistical analysis

All data are shown as the means±S.E.M. of value for corresponding parameters. Statistical comparison between groups in each experiment was done with one- or two-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test. In a few cases in which only two groups were to be compared, Student's *t*-test was used. A *P* value less than 0.05 was considered the limit of significance.

3. Results

3.1. Sex differences in morphine withdrawal and effect of gonadectomy in this manner

Fig. 1 shows the effects of sex on the naloxone-precipitated withdrawal signs in intact mice and also effects of both ovariectomy

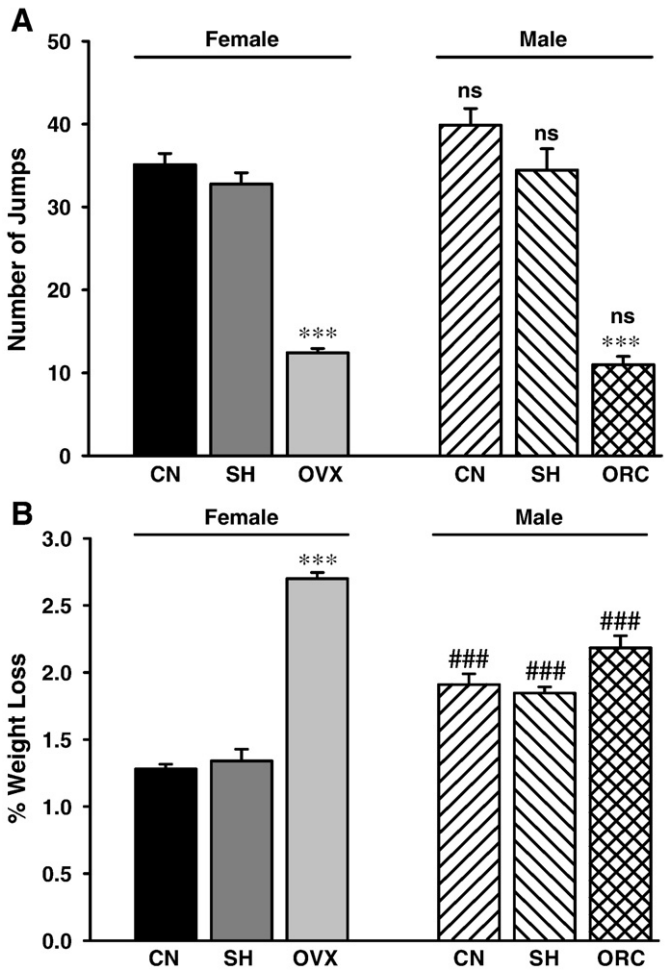

Fig. 1. Effects of sex differences and ovariectomy (OVX) and orchidectomy (ORC) on the induction of physical dependence on morphine in mice as assessed by naloxone (4 mg/kg, i.p.)-precipitated withdrawal (A) jumping and (B) weight loss. Data are expressed as mean±S.E.M. Each group consisted of 10 animals. ***$P<0.001$ compared with corresponding control (CN) or sham-operated (SH) mice. ns, non-significant and ###$P<0.001$ compared with corresponding group of female mice.

(OVX) and orchidectomy (ORC) on the withdrawal signs in this group of animals compared with corresponding control ones. Although the number of jumps was not significantly different between intact male and female mice (Student's *t*-test, $P>0.05$; Fig. 1A), the percentage of weight loss was significantly higher in male animals compared with female ones ($F_{3,36}=24.606$, $P<0.0001$; Fig. 1B).

One-way ANOVA revealed that compared with either control or sham female animals there was a significant decrease ($P<0.001$) in the number of jumps ($F_{2,27}=121.68$, $P<0.0001$; Fig. 1A) and increase ($P<0.001$) in the percentage of weight loss ($F_{2,27}=176.79$, $P<0.0001$; Fig. 1B) in the OVX female mice. Moreover, compared with intact or sham male mice the number of jumps was significantly ($P<0.001$) decreased in ORC male mice ($F_{2,27}=61.009$, $P<0.0001$; Fig. 1A). However, there was no significant difference in percentage of weight loss between all groups of male animals ($F_{2,27}=0.07576$, $P=0.9272$; Fig. 1B).

3.2. Effect of ovarian hormones on the development of morphine dependence in OVX female mice

Fig. 2 shows the effect of ovarian sex hormones, E_2V and progesterone, on the withdrawal signs in OVX mice. Two-way ANOVA revealed a significant effect of hormone treatment on the number of jumps in OVX mice ($F_{4,45}=55.035$, $P<0.0001$). Pretreatment with E_2V significantly ($P<0.001$) increased the number of jumps in OVX mice ($F_{3,36}=38.429$, $P<0.0001$). Progesterone had no effect on the number of jumps in all experimental groups (Fig. 2A). In addition, there was a significant reduction in the percentage of weight loss ($P<0.001$) in OVX mice treated with both E_2V ($F_{3,36}=141.16$, $P<0.0001$) and progesterone ($F_{3,36}=123.63$, $P<0.0001$; Fig. 2B).

Additionally, withdrawal signs after naloxone injection were not observed either in olive oil- or ovarian hormones (E_2V and progesterone)-treated OVX mice which was chronically treated with saline (instead of morphine; data not shown).

3.3. Effect of testosterone on the development of morphine dependence in ORC male mice

Fig. 3 shows the effect of testosterone on the withdrawal signs in ORC mice. Two-way ANOVA revealed a significant effect of male hormone on the number of jumps in the ORC mice ($F_{3,36}=41.582$, $P<0.0001$). There was a significant ($P<0.001$) increase in the number of jumps in the ORC mice treated with testosterone (Fig. 3A). Moreover, testosterone had no effect on the percentage of weight loss in ORC groups of male mice ($F_{3,36}=2.978$, $P=0.311$; Fig. 3B). Additionally, withdrawal signs after naloxone injection were not

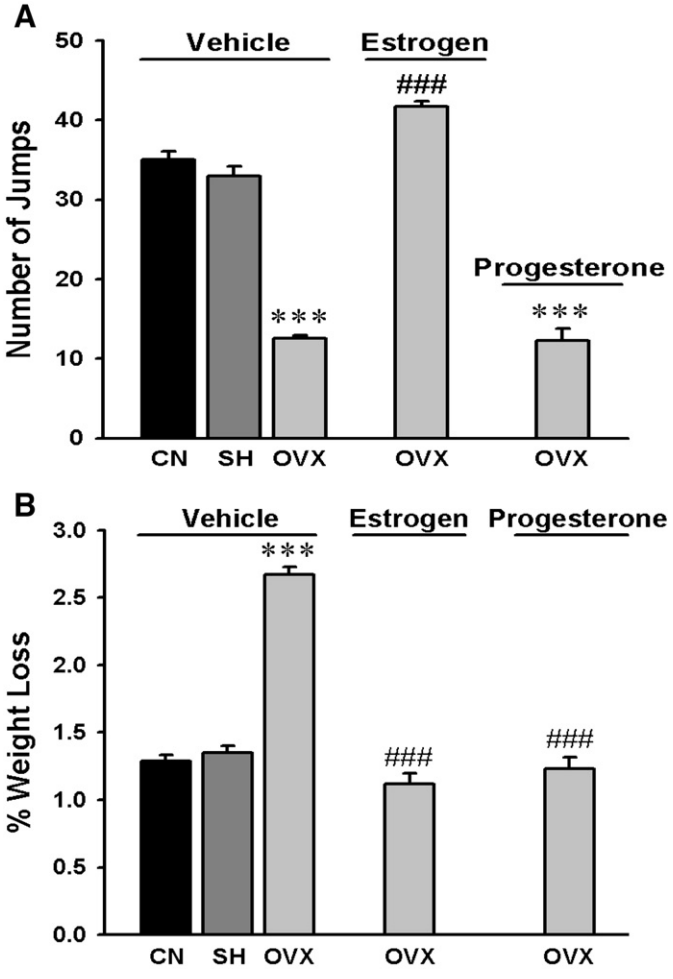

Fig. 2. Effects of treatment with ovarian hormones, estradiol valerate (E_2V; estrogen) and progesterone, on the induction of physical dependence on morphine in female mice as assessed by naloxone-precipitated withdrawal (A) jumping and (B) weight loss. Data are expressed as mean±S.E.M. Each group consisted of 10 animals. ***$P<0.001$ compared with vehicle-treated control (CN) or sham-operated (SH) mice. ###$P<0.001$ compared with corresponding vehicle-treated ovariectomized (OVX) group.

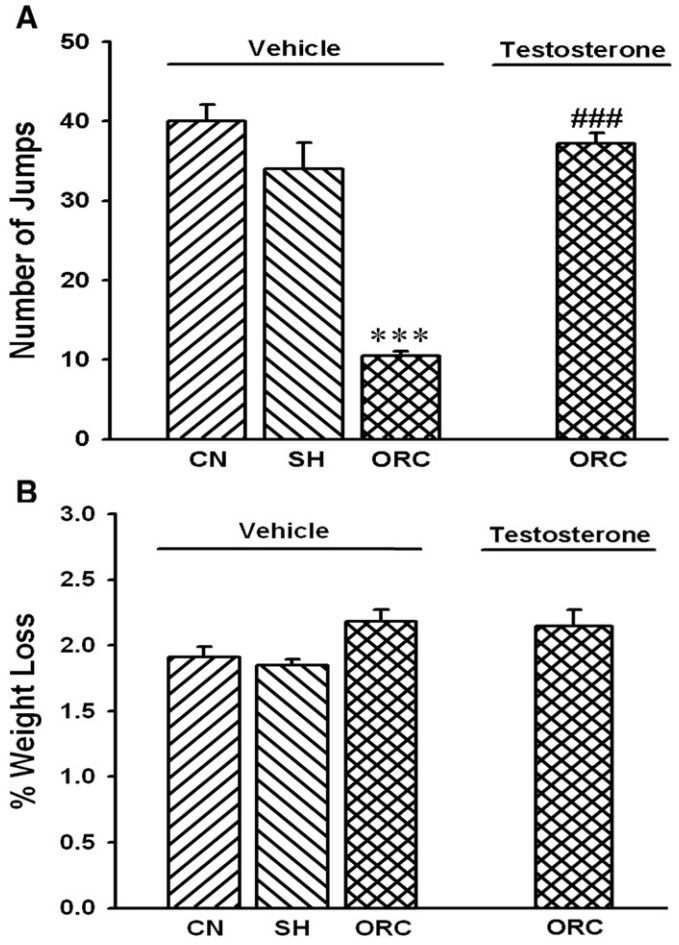

Fig. 3. Effects of treatment with the sex hormone testosterone on the induction of physical dependence on morphine in male mice as assessed by naloxone-precipitated withdrawal (A) jumping and (B) weight loss. Data are expressed as mean ± S.E.M. Each group consisted of 10 animals. ***$P<0.001$ compared with vehicle-treated control (CN) or sham-operated (SH) mice. ###$P<0.001$ compared with corresponding vehicle-treated orchidectomized (ORC) group.

observed either in olive oil- or testosterone-treated ORC mice which was chronically treated with saline (instead of morphine; data not shown).

4. Discussion

Morphine, the main pharmacologically active alkaloid of opium poppy, is a potent analgesic with known addictive properties. The repeated use of opiates induces adaptive changes in the CNS leading to the development of dependence. Withdrawal is typically observed following abrupt termination of morphine intake or precipitated by administration of a narcotic antagonist such as naloxone. In the present study we demonstrated that there was no significant alteration in some morphine withdrawal signs such as the number of jumping between male and female mice, though the percentage of weight loss was significantly higher in female mice than male ones. We also showed that the number of jumping was significantly lower in both OVX and ORC mice and the percentage of weight loss was significantly higher in OVX mice than the corresponding non-operated or sham animals. In OVX mice, E_2V increased the number of jumping and decreased the percentage of weight loss. Progesterone had no effect on the number of jumping, whereas it decreased the percentage of weight loss in OVX mice. Moreover, testosterone increased the number of jumping in ORC mice while it had not any effect on the percentage of weight loss in such animals.

Several line of evidence has shown that morphine could produce similar effects – but of different magnitude and at different doses – in males and females on a variety of acute measures, including analgesia (Kest et al., 2000c) hypothermia (Kest et al., 2000a; Quock et al., 1985), activity (Craft et al., 2006; Li et al., 1990), respiration (Kest et al., 1998) and place conditioning (Cicero et al., 2003; Cicero et al., 2000). The consequences of chronic morphine treatment may also differ between sex, as suggested by the differential development of tolerance to its analgesic (Badillo-Martinez et al., 1984; Craft et al., 1999b; Kest et al., 2000b) and hypothermic (Kest et al., 2000a) effects in male and female rodents. Inspite of some reports regarding sex differences in the effects of morphine on several measures, there are scarce studies comparing males and females on morphine dependence. In mouse studies, sex differences in dependence were not observed after 3 or 7 days of morphine treatment (Blum et al., 1976; el-Kadi and Sharif, 1994; Kest et al., 2001). Kest et al. (2001) even demonstrated that sex difference in withdrawal jumping in mice was also not observed when morphine was treated via continuous subcutaneous infusion via an osmotic minipump (3.4 mg/kg/24 h). However, studies in rats report that a more severe spontaneous withdrawal syndrome is produced by chronic morphine injections or morphine pellet implantation in male rats than in females (Cicero et al., 2002). In another study, Papaleo and Contarino (2006) also reported that there is a sex-linked difference in the expression and duration of spontaneous somatic opiate withdrawal in mice. In their study, female and male C57BL/6J mice were treated with saline or increasing morphine doses (10–50 mg/kg or 20–100 mg/kg) during 6 consecutive days and tested for the spontaneous expression of somatic opiate withdrawal signs 8, 32, 56 and 80 h after last drug administration. In addition to sex- and morphine dose-linked differences in the spontaneous opiate withdrawal, it also showed that the severity and timing of expression of withdrawal signs varied as a function of both sex and morphine dose. Exposure to the opiate drug induced consistent levels of body weight loss in both female and male mice. However, whereas 20–100 mg/kg morphine-treated male mice displayed more body weight loss than 10–50 mg/kg morphine-treated male mice, the two morphine regimens produced similar levels of body weight loss in female mice, indicating a gender-linked resistance to this deleterious effect of opiate drugs. Treatment with 20–100 mg/kg morphine induced reliable levels of jumping behavior in both female and male mice; however, exposure to lower morphine doses (10–50 mg/kg) elicited this sign only in female mice. Additionally, the presence of jumping was observed earlier than other signs such as paw tremor and wet dog shake. Our present data also showed no significant sex difference in the naloxone-precipitated withdrawal jumpings in mice treated with morphine (50, 50 and 75 mg/kg) for 4 days, though the percentage of weight loss was higher in male animals than females. These discrepancies could be explained by the differences in the morphine regimen dose, the route of withdrawal signs and differences in mice strains used. It has been also reported that sex differences in the development of tolerance to the antinociceptive effects of opioids could be explained by sex differences in morphine potency and when males and females receive the same mg/kg doses of morphine, males become more tolerant than females because they are more sensitive than females to the acute effects of morphine; that is, males are receiving a functionally higher dose and thus would be expected to show more tolerance. In contrast, when males and females are given equi-analgesic doses, tolerance develops similarly (Barrett et al., 2001). This factor might explain sex differences in the weight loss found in the present study.

Moreover, we showed different effects of orchidectomy and testosterone replacement on the number of jumpings versus percentage of weight loss and also different effects of progesterone on the naloxone-precipitated jumpings versus weight loss in ORC mice. These discrepancies in results might be due to the sensitivity of the measured signs of withdrawal in rodents. Although among withdrawal behaviors in mice, jumping is widely considered the most

sensitive and reliable index of withdrawal intensity and is the most commonly used (Blum et al., 1976; el-Kadi and Sharif, 1994; Kest et al., 2001; Miyamoto and Takemori, 1993; Way et al., 1969), it should be noted that distinct neural substrates mediate various withdrawal symptoms (Brandao, 1993; Koob et al., 1992). Thus, findings using naloxone-precipitated jumping do not easily extrapolate to other indices of dependence such as percentage of weight loss. Therefore, future studies need to evaluate the effect of sex on other withdrawal signs such as diarrhea, lacrimation, etc. in mice.

According to our data, gonadectomy (OVX or ORC) could exert a significant alteration in morphine withdrawal compared with either control or sham corresponding female or male animals. These results suggested a role for the sex hormones in the morphine withdrawal signs in both male and female mice. Thus, we further evaluated the effects of ovarian sex hormones, estrogen and progesterone, and testosterone on withdrawal in OVX and ORC mice, respectively. Our data interestingly demonstrated that hormone administration could alter the withdrawal measures of operated animals. However, there are some discrepancy between our results and previous studies. For instance, Reddy and Kulkarni (1997) have shown that concomitant chronic administration of neurosteroids, allopregnanolone (0.5 mg/kg), pregnenolone sulfate (2 and 5 mg/kg), dehydroepiandrosterone sulfate (2 and 5 mg/kg) or progesterone (1–10 mg/kg) followed by morphine (10 mg/kg, twice daily for 9 days) suppressed the naloxone-precipitated withdrawal jumps. It was also shown that acute treatment with these neurosteroids was not associated with any decrease in withdrawal jumping behavior in morphine-dependent mice (Reddy and Kulkarni, 1997). However, it is noteworthy that in their studies they administered neurosteroids to non-operated intact animals for a longer time than our present study in which morphine was administered for 4 days and there was a single administration of hormone in operated animals. Therefore, the differences in results might be due to the differences between the methods used in the studies.

There is also several line of evidence showing an interaction between opioid system and gonadal hormones in both male and female animals. Concas et al. (2006) have shown that acute morphine administration could increase the cerebrocortical and plasma concentrations of pregnenolone, progesterone, and allopregnanolone, which was abolished by adrenalectomy–orchiectomy. Spontaneous or naloxone-precipitated morphine withdrawal also increased neurosteroid concentrations in the brain and plasma (Concas et al., 2006). In addition, both acute and chronic morphine exposure decreases testosterone level in serum, brain and spinal cord (Abs et al., 2000; Amini and Ahmadiani, 2005; Barraclough and Sawyer, 1955; Cicero et al., 1976; Cui et al., 2004; Rajagopal et al., 2003; Yilmaz et al., 1999). Porcine basal androstenedione, testosterone and estradiol release were reduced by μ, δ and κ agonists (Kaminski et al., 2004). Additionally, it has been demonstrated that the level of sex hormones differ at different times after administration of opioids such as morphine (Ceccarelli et al., 2006). It has been also shown that dehydroepiandrosterone sulfate, a neuroactive steroid, prevents the development of morphine dependence and tolerance via c-*fos* expression linked to the extracellular signal-regulated protein kinase (Ren et al., 2004). On the other hand, Menard et al. (1995) reported that a 2 week exposure to testosterone compounds of rats significantly decreased the number of β-endorphin immunoreactive neurons in the rostral part of the arcuate nucleus and diminished the number of lightly to moderately stained neurons in this region. In contrast, Harlan et al. (2000) showed that β-endorphin levels were significantly increased, albeit in a different brain region, the paraventricular thalamic nucleus of pairs of rats subjected to testosterone compounds. Moreover, Vathy et al. (2000) showed that mid to late gestational morphine exposure differentially altered the influence of gonadal hormones on δ-opioid receptors in the rat frontal cortex, decreasing the sensitivity in females and increasing it in males. Taken our data together with previous studies into consideration, it could be concluded that sex steroids could be involved in the morphine dependency and withdrawal in both OVX and ORC mice. However, further studies are clearly needed to verify the exact mechanisms underlying this effect of sex hormones on morphine withdrawal.

In conclusion, the results of the present study showed that although there was no significant sex difference in the withdrawal jumpings, the percentage of weight loss was significantly higher in male mice than female ones. We also demonstrated that some morphine withdrawal signs were significantly altered in both OVX female and ORC male mice, and this alteration was prevented by administration of sex steroids in such animals. These results suggested a role for sex hormones in the dependency to morphine in both male and female mice. However, since in the present study we assessed only two withdrawal signs further studies evaluating more measures using different morphine dosing regimen in male and female animals are clearly needed to assess the exact effect of sex difference and sex hormones on the morphine dependence. Using different species such as rats it would be better to evaluate the morphine withdrawal signs in different phases of estrous cycle in female animals compared with male animals. Moreover, the use of supra-physiologic doses of ovarian sex hormones rather that physiological ones was a limitation of the present study which warrants more studies using physiologic doses of ovarian hormones.

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