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Estrous cycle stage-dependent expression of acute tolerance to morphine analgesia in rats

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Abstract

Both baseline pain sensitivity and the response to antinociceptive treatment are sensitive to an animal's sex and estrous cycle stage. Sex differences are also observed in the development of antinociceptive tolerance induced by repetitive exposure to opiate drugs such as morphine. Conventional tolerance study protocols do not assess the impact of the estrous cycle stage. The present study aimed to compare the development of acute tolerance to morphine-induced antinociception in male and female (cycling and ovariectomized) Wistar rats using the tail-flick test. Acute tolerance was induced by two consecutive subcutaneous injections of morphine (10 mg/kg) or saline separated by an interval of 6 h. It was found that rats pretreated with morphine were tolerant to the second morphine dose. Tolerance was most pronounced in proestrous female rats and, to a lesser degree, in male rats. It was absent in ovariectomized rats as well as during the estrus, metestrus and diestrus phases. Thus, the estrous cycle exerts dramatic effects on the induction of acute tolerance to morphine-induced antinociception. These results suggest that pain management strategies can be optimized through the use of sex- and estrous cycle-specific techniques.

Keywords: Morphine; Antinociception; Tolerance; Sex; Estrous cycle

1. Introduction

There is a vast body of evidence indicating significant differences between the response of male and female subjects to a variety of experimental and clinical situations. It has been shown that there are sex differences in pain processing in both humans (Fillingim and Maixner, 1995; Unruh, 1996; Berkley, 1997) and laboratory animals (Mogil et al., 2000; Fillingim and Ness, 2000). Sex differences also affect the response to analgesic drugs, such as the μ-opioid receptor preferring agonist morphine, which are usually found to be more potent in intact male rats and mice than in female animals (Cicero et al., 1996; Boyer et al., 1998; Krzanowska and Bodnar, 1999; Cook et al., 2000; Craft and Bernal, 2001; Barrett et al., 2001; Terner et al., 2003; Kavaliers and Innes, 1987; Candido et al., 1992; Kest et al., 1999).

These differences are likely to result from the influence exerted by gonadal hormones, as suggested by studies where significant fluctuations in the responsiveness to noxious stimulation or opiate drug therapy were found in female rats in different stages of the estrous cycle (Fillingim and Ness, 2000; Mogil et al., 2000; Vincler et al., 2001; Stoffel et al., 2003).

Repeated administration of opiates such as morphine is accompanied by the development of tolerance. Several studies reported greater tolerance in male than in female rats, as assessed with the hot plate assay (Badillo-Martinez et al., 1984; Craft et al., 1999). Opposite results were detected when using mice and another acute thermal nociception test, the tail withdrawal test (Kest et al., 2000). Yet other studies argued against sex differences in the development of morphine analgesic tolerance in female and male rats (Kasson and George, 1984; Barrett et al., 2001).

There are at least two factors that are possibly responsible for this lack of consistency. First, the tolerance studies do not usually take into account the estrous cycle stage. In these experiments, morphine is given repeatedly over a number of days and it is difficult to assess the importance

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of the estrous cycle stage. Second, chronic morphine treatment per se can disrupt the estrous cycle of female rats (Siddiqui et al., 1997; Craft et al., 1999). Therefore, the design of the typical chronic morphine administration study makes it difficult to investigate the development of tolerance in female subjects. These problems can be avoided by using alternative designs where analgesic tolerance is observed after a single morphine injection.

The phenomenon of acute tolerance is often described as the recovery from antinociception induced by a single morphine injection occurring at a much faster rate than the decrease in morphine concentration in the brain (Kissin et al., 1991). In other words, some acute adaptive responses may limit the magnitude and duration of the antinociceptive effects of a drug. Whether these adaptive mechanisms are the same as in chronic tolerance or not, it is clear that they can be a target for both basic research and drug development. Given that acute morphine antinociception is differentially affected in male vs. female subjects, it would be particularly important to know how an individual's sex and the estrous cycle affect the expression of acute morphine tolerance.

The present experiments aimed to evaluate the development of acute tolerance to morphine analgesia in male and female rats. The experimental paradigm was based on earlier reports indicating that morphine loses at least some of its effects when injected within hours after the first dose (e.g., Hovav and Weinstock, 1987). The present study used a design in which four groups of animals were assessed in parallel. Two groups of rats received injections of morphine followed a few hours later by either saline or morphine injection. The other two groups received injections of saline followed by either saline or morphine injection. This design allowed comparison of nociceptive response in the non-drugged state (control treatment with two saline injections) after a single morphine injection (analgesia in nontolerant subjects) and after two morphine injections (i.e., in acutely tolerant subjects).

2. Methods

2.1. Animals

Adult male and female drug- and experimentally naive Wistar rats (240–260 g) were purchased from the State Breeding Farm "Rappolovo" (St. Petersburg, Russia). Animals were kept in groups of six with food (standard rodent lab chow; "Volosovo", St. Petersburg) and filtered tap water available ad libitum and maintained under standard laboratory conditions with controlled temperature (21 ± 1 °C) and humidity (60 ± 10%). All experiments were conducted during the light period of a 12/12-h day–night cycle (lights on at 08:00 a.m.). All tests were performed in accordance with the recommendations and policies of the US National Institutes of Health Guidelines for the Use of Animals. Experimental protocols were approved by the Pavlov Medical University Ethics Committee.

2.2. Drugs

Morphine hydrochloride ("Endocrinnyj Zavod", Moscow, Russia) was dissolved in physiological saline and injected subcutaneously in a volume of 1 ml/kg. Doses of morphine were calculated for the salt form.

2.3. Tail-flick test

A rat's tail (about 1 cm from the base) was exposed to a focused heat source (300-W white bulb). By withdrawing or removing the tail from the path of the stimulus and thereby exposing a photocell located in the apparatus ("Farmakolog", St. Petersburg, Russia) immediately below the tail, the rat could terminate the noxious stimulation and the reaction time was then recorded (Dewey et al., 1970). An animal that failed to respond before 10 s (cut-off time) was removed from the apparatus and assigned a latency of 10 s. Tail-flick latencies were measured twice for each subject—before and after drug treatment. Rats were returned to their home cages after each injection and/or tail-flick test.

2.4. Estrous cycle monitoring

Estrous cycle stage was monitored by analyzing the cell types in vaginal lavage. Vaginal smears in cycling and ovariectomized female rats were collected daily for at least eight consecutive days and classified by estimating the relative proportion of cornified, nucleated and leukocytic cells. The estrous cycle of a mature female rat lasts for about 4 days. This cycle can be divided into four identifiable stages: proestrus, estrus, metestrus, diestrus (Freeman, 1988). Only rats displaying stable 4-day estrous cycle patterns were used in the experiments described below.

2.5. Surgical procedure

Bilateral ovariectomy was performed under halothane anesthesia by removing the ovaries and the ovarian fat through a dorsal approach. Both vaginal cytology and postmortem evaluation of the presence of ovarian remnants were used to confirm the effectiveness of the surgical procedure. After a recovery period of 2 weeks, these rats were given injections of saline and/or morphine and subjected to tail-flick tests as described below.

2.6. Experimental procedure

Baseline measurements of tail-flick response were taken between 9 and 10 a.m. After that, animals were randomly assigned to four treatment groups. Separate groups of rats were treated subcutaneously with either 10 mg/kg of morphine (Morphine–Saline and Morphine–Morphine groups)

or saline (Saline–Saline and Saline–Morphine groups). Six hours later, rats received a second injection of either morphine (Saline–Morphine and Morphine–Morphine) or saline (Saline–Saline and Morphine–Saline). Our preliminary studies indicated that shorter between-injection intervals (e.g., 4 h) were less likely to reveal acute tolerance development because of the residual analgesic effects of the first morphine dose. It was also observed that lower doses of morphine could not be used because the magnitude of acute tolerance and, therefore, the power of the tests diminished.

The second tail-flick test was held 30 min after the second drug injection. See Table 1 for the group sizes.

In the first experiment, there was a total of 16 experimental groups representing each of the four stages of estrous cycle (proestrus, estrus, metestrus, diestrus). In the second experiment, four groups of male rats, two groups of ovariectomized and two groups of sham-operated proestrous female rats were used to evaluate the development of acute tolerance to morphine analgesia.

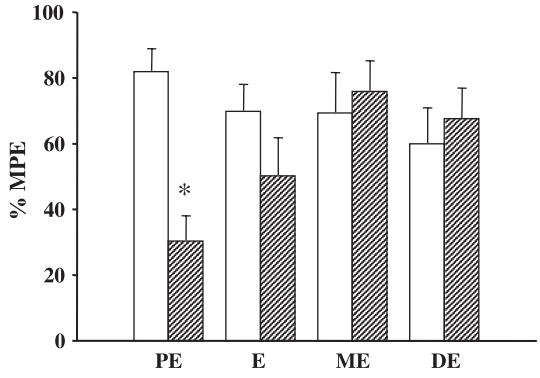

Fig. 1. Estrous cycle affects the development of acute tolerance to morphine analgesia in rats. Diestrus (DE), proestrus (PE), estrus (E), metestrus (ME). Rats received an injection of morphine (10 mg/kg) 6 h after an injection of either saline (Saline–Morphine; empty bars) or morphine (Morphine–Morphine; hatched bars). Data are expressed as means (± S.E.M.) %MPE. See text for details. *$P < 0.05$ (Tukey's test), compared to Saline–Morphine group. See Table 1 for sample sizes.

2.7. Data analysis

Tail-flick latencies were converted to percentage of maximal possible effect (%MPE). The individual rat %MPE values were calculated according to the formula: $(T_{EXP}\text{-}T_{BL}) \times 100/(10\text{-}T_{BL})$, where T_{EXP}—test tail-flick latency (s), T_{BL}—baseline latency (s).

Absolute values of tail-flick latencies and %MPE data were rank-transformed and then subjected to the distribution-free two- and three-way analyses of variance (ANOVA; General Linear Models procedure, SAS-STAT, SAS Institute, Cary, NC). Independent variables included (a) first injection (saline vs. morphine), (b) second injection (saline vs. morphine), and (c) estrous cycle stage. Whenever appropriate, the values of the tail-flick latencies were analyzed using baseline latencies as covariates. Tukey's test was used for post hoc between-group comparisons whenever indicated by ANOVA.

3. Results

Table 1 reveals absolute values of tail-flick response before and after saline/morphine treatment. There was no statistically significant effect of estrous cycle stage on baseline tail-flick response [$F(3,180) = 0.7$, n.s.]. Tail-flick latencies (mean ± S.E.M.) were 3.5 ± 0.1 for proestrus ($N = 54$), 3.5 ± 0.1 for estrus ($N = 44$), 3.7 ± 0.2 for metestrus ($N = 40$) and 3.4 ± 0.1 for diestrus ($N = 43$). However, when compared to the response of male (3.7 ± 0.1, $N = 48$)

Table 1
Tail-flick latencies (mean ± S.E.M., s) before and after treatment with morphine and/or saline

Group	Test	Female rats						Male rats
		DE	PE	E	ME	SHAM	OVX	
Saline–Saline	Before	3.7 ± 0.4	3.4 ± 0.3	3.1 ± 0.2	4.1 ± 0.4	n.t	n.t.	3.7 ± 0.3
	After	3.4 ± 0.3	3.0 ± 0.3	2.9 ± 0.2	4.0 ± 0.3			3.7 ± 0.3
	N	10	9	8	9			12
Saline–Morphine	Before	3.2 ± 0.2	3.9 ± 0.2	3.4 ± 0.4	3.2 ± 0.2	3.4 ± 0.2	4.6 ± 0.7	3.6 ± 0.3
	After	7.2 ± 0.7	8.8 ± 0.4	7.9 ± 0.5	8.0 ± 0.8	8.6 ± 0.3	8.8 ± 0.8	9.2 ± 0.4
	N	12	18	10	10	8	10	12
Morphine–Saline	Before	3.4 ± 0.2	3.3 ± 0.2	3.8 ± 0.3	3.9 ± 0.5	n.t.	n.t.	3.5 ± 0.2
	After	3.3 ± 0.3	3.1 ± 0.2	3.8 ± 0.3	3.9 ± 0.2			4.3 ± 0.3
	N	11	10	11	7			12
Morphine–Morphine	Before	3.2 ± 0.4	3.4 ± 0.3	3.4 ± 0.2	3.7 ± 0.2	3.5 ± 0.1	4.5 ± 0.3	3.9 ± 0.2
	After	7.7 ± 0.6	5.4 ± 0.6	6.6 ± 0.7	8.4 ± 0.6	6.3 ± 0.8	8.7 ± 0.8	7.5 ± 0.8
	N	10	17	15	14	8	10	12

Rats were injected with saline (Saline–Saline and Saline–Morphine) or morphine (Morphine–Saline and Morphine–Morphine), and 6 h later they were given a second injection of either saline (Saline–Saline and Morphine–Saline) or morphine (Saline–Morphine and Morphine–Morphine). Diestrus (DE); proestrus (PE); estrus (E); metestrus (ME); sham-operated female rats tested during the proestrus phase (SHAM); ovariectomized (OVX); not tested (n.t.); number of rats per group (*N*).

and cycling female rats (3.5 ± 0.07, $N = 181$), noxious stimulation appeared to produce longer response latencies in ovariectomized rats (4.1 ± 0.3, $N = 20$). This effect was confirmed by ANOVA [$F(2,248) = 3.7$, $P < 0.05$].

When injected 6 h prior to the test, morphine had no detectable effects on nociceptive response in any of the treatment conditions, in female or male rats (Table 1; group Saline–Saline vs. group Morphine–Saline). When injected 30 min prior to the test, morphine produced significant analgesia under all treatment conditions, in both female and male rats (group Saline–Saline vs. Saline–Morphine). Although it appeared that morphine produced longer tail-flick latencies in male rats (Table 1), ANOVA failed to confirm this observation [$F(5,71) = 1.8$, $P = 0.12$].

For cycling female rats, ANOVA indicated a significant main effect of the second morphine injection and significant interaction between the two morphine treatments [$F(1,180) = 307.2$, $P < 0.01$ and $F(1,180) = 4.3$, $P < 0.05$, respectively]. These effects are likely due to reduced morphine analgesia in the Morphine–Morphine group. In other words, rats pretreated with morphine appeared tolerant to the second morphine dose (group Saline–Morphine vs. Morphine–Morphine). This tolerance peaked in proestrus and was absent during metestrus and diestrus (Fig. 1). ANOVA confirmed a significant interaction between estrous cycle stage and the first morphine treatment [$F(3,180) = 2.9$, $P < 0.05$].

As shown in Fig. 2, acute tolerance was also observed in male rats [interaction between two morphine treatment factors: $F(1,47) = 8.4$, $P < 0.01$], albeit its magnitude was somewhat less than that in proestrous female rats. In contrast, ovariectomized rats demonstrated no tolerance to morphine analgesia [$F(1,19) = 0.0$, n.s.; Fig. 2]. Surgical manipulation itself had no effect on the induction of acute tolerance because sham-operated rats demonstrated significant tolerance when tested during proestrus [Table 1; $F(1,15) = 5.7$, $P < 0.05$].

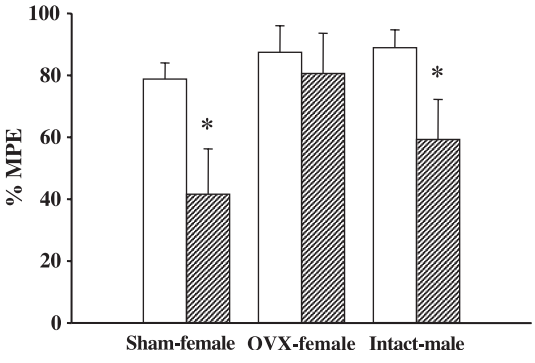

Fig. 2. Acute tolerance to morphine analgesia in male rats (MALE), ovariectomized (OVX) and sham-operated proestrous (SHAM) female rats. Rats received an injection of morphine (10 mg/kg) 6 h after an injection of either saline (Saline–Morphine; empty bars) or morphine (Morphine–Morphine; hatched bars). Data are expressed as means (± S.E.M.) %MPE. See text for details. *$P < 0.05$ (Tukey's test), compared to Saline–Morphine group. See Table 1 for sample sizes.

4. Discussion

The present study compared tail-flick reactivity in morphine-pretreated male and female rats. The main findings of this study can be summarized as follows. (a) Neither sex nor estrous cycle affected the baseline tail-flick latency. (b) There were negligible differences between male and female rats with respect to sensitivity to the antinociceptive effects of acute morphine administration. (c) Development of acute tolerance to morphine analgesia was profoundly affected by sex and estrous cycle stage.

As previously reviewed (e.g., Mogil et al., 2000), sex differences in sensitivity to noxious heat stimulation are less likely to be observed than when electric or chemical noxious stimuli are applied, especially when stronger stimuli are used (e.g., 54 vs. 50 °C hot plate; Stoffel et al., 2003). In fact, most of the earlier tail withdrawal studies (hot water or radiant heat tests) did not find significant differences between male and female rats including those of Wistar strain (Aloisi et al., 1994). Importantly, these studies typically did not include estrous cycle phase monitoring, and the results obtained for females at different cycle stages were pooled together (Aloisi et al., 1994; Boyer et al., 1998; Kanarek et al., 1998; Krzanowska and Bodnar, 1999). The present study explicitly focused on analyzing the impact of the estrous cycle stage; however, the results suggest that acute thermal nociceptive processing may not be affected by sex and estrous cycle stage. Nevertheless, it was observed that tail-flick latencies were significantly increased in ovariectomized females. Similar findings were reported in at least one previous study (e.g., see Table 2 in Kepler et al., 1989).

The present study failed to find significant differences between male and female rats in the antinociceptive effects of a 10 mg/kg dose of morphine (i.e., group Saline–Morphine). These results are consistent with some of the earlier reports that indicated little or no effect of sex on the response to acute morphine in thermal nociception tests in rats (Kasson and George, 1984; 54 °C hot plate data in Stoffel et al., 2003) but not with other studies where such differences were found (Cicero et al., 1996; Kanarek et al., 1998; Craft et al., 1999; 50 °C hot plate data in Stoffel et al., 2003). Because many of the latter studies did not monitor estrous cycle stage, such discrepancies could be explained by the effects of the estrous cycle on the response to morphine. Indeed, it was suggested that morphine antinociception is reduced during the late proestrous stage (Banerjee et al., 1983; Berglund and Simpkins, 1988). However, in the present study, the late proestrous phase was not distinguished and results failed to find any significant fluctuations in the magnitude of morphine-induced antinociception that could be attributed to the estrous cycle stage. It should be noted though that it is possible that sex differences in morphine antinociception may be revealed by testing a wider range of morphine challenge doses or using milder nociceptive stimulation (Stoffel et al., 2003).

The lack of effect of sex and estrous cycle stage on baseline tail-flick reactivity and acute morphine antinociception appear useful when comparing the development of acute tolerance to morphine-induced analgesia. In the present study, only male and proestrous female rats developed appreciable levels of acute tolerance. To the best of our knowledge, this is the first demonstration of the sex/estrous cycle-dependent expression of acute tolerance to morphine-induced antinociception.

Previous studies focused on the development of analgesic tolerance induced by repetitive administration of morphine and typically did not take the estrous cycle stage into account. Thus, for the reasons discussed above (see Introduction section), conventional tolerance study designs are not suited for the analysis of the effects of sex and estrous cycle on tolerance development. In contrast, procedures similar to those used in this study allow for a fairly rapid evaluation of tolerance-like changes in responsiveness to morphine. However, one should note that tolerance induced by acute vs. repeated (chronic) administration of opiates may have different mechanisms and, therefore, the results of the present study on acute tolerance may not be fully extrapolated to chronic tolerance.

It was found that acute tolerance was maximal in proestrous females and was completely reversed by ovariectomy. During proestrus, blood concentrations of estrogen, progesterone, luteinizing hormone and follicle-stimulating hormone are maximal (Butcher et al., 1974). Thus, it is tempting to speculate that these are among the hormonal factors that determine the expression of acute tolerance. Theoretically, this hypothesis could be tested directly by evaluating the effects of estrogens and other hormonal factors in ovariectomized rats. However, hormones, such as estrogens, may have significant effects on both baseline nociceptive response and morphine-induced antinociception (e.g., Ratka and Simpkins, 1991). Because of these effects, one could anticipate difficulties in analyzing the selective effects of estrogens on the induction and expression of acute tolerance to morphine.

Meanwhile, at least for estrogens, there are converging lines of evidence suggesting that high concentrations of estrogens may facilitate the development of opiate tolerance. For instance, estrogens elicit rapid and sustained activation of multiple members of the mitogen-activated protein (MAP) kinase cascade (Singh et al., 1999). Furthermore, both acute and prolonged exposure to morphine activate MAP kinases (Li and Chang, 1996; Ma et al., 2001). This pathway is required for μ-opioid receptor desensitization (Polakiewicz et al., 1998) and appears to be upregulated in morphine-tolerant rats (Ma et al., 2001). In addition, it is suggested that morphine-induced phosphorylation of MAP kinases may contribute to the development of analgesic tolerance at least in part through the increase in spinal calcitonin gene-related peptide (CGRP) and substance P levels in primary sensory afferents (Ma et al., 2001). Furthermore, there is direct evidence suggesting that estrogen treatment may increase plasma levels of CGRP in ovariectomized rats (Gangula et al., 2000). The plasma concentration of substance P is reported to correlate positively with plasma estrogen levels and to be maximal during proestrus (Duval et al., 1996). To sum up, activation of the MAP kinase cascade is one of the possible mechanisms through which hormonal factors may facilitate the effects of morphine, such as the induction of tolerance.

In conclusion, acute tolerance to morphine-induced antinociception was induced in both male and female rats. In female rats, acute tolerance was most pronounced during proestrus and was absent in ovariectomized rats. Thus, estrous cycle stage is critically important for the induction of acute tolerance to morphine. These results suggest that pain management strategies can be optimized through the use of sex- and estrous cycle-specific techniques.

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