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Persistent pain model reveals sex difference in morphine potency

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Sex differences in the analgesic and antinociceptive effects of higher efficacy, receptor-specific agonists have not been as consistent, and it is becoming increasingly clear that opioid receptor specificity, route, and dose of drug administration (13, 15, 16), genetic background (21, 37, 47, 58), hormonal status (56, 57), and type of analgesiometric test used (17) are critical factors in determining the pharmacodynamics of opioid analgesia. Surprisingly, although morphine is primarily prescribed for the alleviation of chronic pain, the majority of studies conducted to date that have examined the sexually dimorphic response of opiates have only used acute noxious stimuli, primarily the tail flick and hot plate tests. Thus potential sex difference in the response of centrally acting opiates in the alleviation of persistent pain has not been clearly established (12). Two lines of evidence suggest that persistent pain may have a differential influence on the analgesic and antinociceptive effects of opiates in males and in females. First, a number of studies in male rats have reported that the antihyperalgesic properties of opiates change in the presence of persistent inflammation, becoming more potent over time (26, 33, 54). Several mechanisms have been implicated, including changes in peripheral (55), spinal (18, 49, 50), and supraspinal (26, 27) opioidergic circuits. Second, persistent pain influences the ovulatory cycle and, therefore, the hormonal status of females. In particular, after induction of persistent inflammatory pain, female rats display irregular estrous cycles, spending the majority of time in diestrus (12). In female rats, this stage of estrus is associated with significantly lower levels of estradiol than in proestrus (12). In male rats, this stage of estrus is associated with significantly higher levels of testosterone than in proestrus (12). In female rats, this stage of estrus is associated with significantly lower levels of estradiol than in proestrus (12). In male rats, this stage of estrus is associated with significantly higher levels of testosterone than in proestrus (12).

MATERIALS AND METHODS

Animals. Gonadally intact Sprague-Dawley male and female rats (Zivic Miller), weighing between 250–300 g, were used in these studies. Rats were housed in same-sex pairs in Plexiglas cages with

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cardboard bedding. Males and females were housed in separate rooms and maintained on a 12:12-h light-dark cycle at constant ambient temperature (22°C, relative humidity 40–60%). Access to food and water was ad libitum except during testing. Body weight was monitored before testing and throughout the 21-day experimental session. To allow for acclimation to the environment, all animals were housed under the aforementioned conditions for 1 wk before the start of experimental testing. All experiments were conducted in strict compliance with the guidelines established by the International Association for the Study of Pain; all protocols were approved by the Institutional Animal Care and Use Committee at Georgia State University.

To characterize the estrous status of female rats, vaginal smears (using the saline lavage technique) were taken daily beginning 1 wk before testing and continuing to the end of the experimental session (using the saline lavage technique). Vaginal smears were conducted using the nonparametric Mann-Whitney U-test for the 30-, 45-, and 60-min time points, these values were averaged for derivation of half-maximal effective dose (ED50), defined as the dose of morphine that produced 50% of the maximum possible increase in PWL. ED50 determinations and 95% confidence intervals were determined by nonlinear regression analysis. Kruskal-Wallis ANOVA (KW) was used to assess for differences in %MPE as a function of time post-CFA. All values are reported as means ± SE; P < 0.05 was considered statistically significant. Where multiple comparisons were made, P values were adjusted accordingly using Bonferroni. The “antinociceptive” effects of morphine are in reference to saline-treated animals, whereas the term “antihyperalgesic” refers to the actions of morphine in CFA-treated animals (26).

RESULTS

CFA-induced thermal hyperalgesia. No sex differences were noted in PWLs before CFA administration [male PWL, 7.30 ± 0.21 s (n = 54) vs. female PWL, 7.06 ± 0.18 s (n = 54); t = 0.873, df = 106, P > 0.05]. Intraplantar injection of CFA-induced a significant degree of inflammation and thermal hyperalgesia that was restricted to the injected paw. Twenty-four hours post-CFA administration, paw diameters for the injected paw increased on average from 5.79 to 10.57 mm. This percent increase in paw diameter was slightly greater in females (190 ± 2%) compared with males (176 ± 2%) (MWU, Z = −4.45, P < 0.01; Fig. 1). By contrast, no change in paw diameter was noted for either males or females 24 h after intraplantar injection of saline (data not shown). In both males and females, intraplantar CFA-induced a significant degree of thermal hyperalgesia, as reflected by a decrease in PWLs. In both males and females, PWLs decreased from 7.1 s to −3 s (Fig. 1). The percent change in PWL after CFA administration was slightly higher in females (−59 ± 1%) compared with males (−53 ± 2%). This small sex difference in %change in baseline was statistically significant (MWU, Z = −2.67, P < 0.18 s (54)). This small sex difference in %change in baseline was statistically significant (MWU, Z = −2.67, P < 0.18 s (54)).
No change in PWL was noted for the saline control group ($t = 0.17; df = 45; P > 0.05$); similarly, no significant differences were noted in PWLs for intraplantar saline-treated animals compared with uninjected controls ($t = 0.19, df = 24, P > 0.05$), indicating that intraplantar administration of saline had no effect on thermal sensory thresholds.

**Hormonal influences.** Estrous cycle status was determined for all female animals via vaginal lavage for a minimum of 1 wk before the onset of testing. There were no significant differences in any of the indices examined for diestrus 1 and diestrus 2, so these data are combined. Changes in hormone status had no significant impact on baseline PWLs ($F(2, 39) = 1.51, P > 0.05$). Similarly, no significant differences were noted in either the degree of edema, defined as a percent change in paw diameter produced by intraplantar administration of CFA ($KW, H = 1.54, df = 2, P > 0.05$) or in the degree of thermal hyperalgesia at 24 h post-CFA, defined as a decrease in PWL ($KW, H = 2.79, df = 2, P > 0.05$). Together, these data indicate that in females, changes in gonadal steroid levels had no impact on baseline nociceptive thresholds or in CFA-induced thermal hyperalgesia.

**Effect of morphine in 24 h CFA-treated rats.** Systemic administration of morphine (0.05–15.0 mg/kg sc) at 24 h post-CFA produced dose-dependent increases in PWL. There were no significant differences in %MPE for the 30, 45, and 60 min postmorphine measurements across all doses examined; therefore, these data are averaged. Significant sex differences were noted in the antihyperalgesic effect of morphine; at all doses tested, males consistently had a significantly higher %MPE compared with females ($P < 0.05$; Fig. 2). This sexually dimorphic effect was most evident at the higher doses of morphine. For example, administration of the 8.0 mg/kg dose produced an 80 ± 12% MPE in males; by contrast, in females, the %MPE was only 37 ± 11% (MWU; $P < 0.01$). Similar sexually dimorphic responses were noted for the 12.0 mg/kg dose, at which all males were at 100% MPE; in females, %MPE was 71 ± 13% (MWU; $P < 0.05$; Fig. 2). A maximum antihyperalgesic response in females was not noted until the 15 mg/kg dose; this dose of morphine was fatal to all males tested. An example of the sexually dimorphic effect of morphine as a function of time postinjection is shown in Fig. 3. ED$_{50}$ values, determined using sigmoidal dose-response function (variable slope) were 5.93 in males (95% confidence interval of 4.40–8.0) vs. 9.4 in females (95% confidence interval = 7.73–11.24) ($P < 0.01$). Unfortunately, the influence of gonadal steroids on morphine-induced antihyperalgesia could not be discerned in this study; at 24 h post-CFA, the majority of female animals stopped cycling (i.e., females were recorded to be in diestrus 1/2).

**Effect of morphine in saline-treated rats.** Morphine (4.0–12.0 mg/kg sc) also produced a significantly greater analgesic response in saline-treated males compared with females. As shown in Fig. 4, significant sex differences in the antinociceptive potency of morphine were noted at all doses examined. Administration of morphine at 8.0 or 12.0 mg/kg produced 100% MPE in all males tested; by contrast, %MPE in females was 37 ± 16% and 58 ± 12, respectively (MWU; $P < 0.01$).
mined at 24 h, 7 days, 14 days, and 21 days post-CFA or saline (n = 6/sex/days postinjection). No significant differences were noted in body weight for CFA vs. saline-injected animals [males, F(1,234) = 2.068, P > 0.05; females, F(1, 240) = 0.27, P > 0.05]. Figure 5A shows the time course for CFA-induced edema. Paw edema decreased as a function of time post-CFA injection from peak values observed at 24 h; at 7 days post-CFA, injected paw diameters were 139% of control paw diameter in both males and females. At 14 and 21 days post-CFA, paw diameters for the injected paw remained at ~131% of the uninjected paw, indicating persistent inflammation. There were no significant sex differences in paw diameter at 7, 14, and 21 days post-CFA (MWU, P > 0.05). No changes in left paw diameter were noted at any time point in CFA-treated animals, indicating that CFA-induced edema was limited to the injected paw and that at 21 days postinjection, the inflammation had not spread to the contralateral paw.

The time course for CFA-induced thermal hyperalgesia is shown in Fig. 5B. In males, paw withdrawal latencies were significantly different from baseline at 1, 7, and 14 days post-CFA (P < 0.05). In females, paw withdrawal latencies were significantly shorter than baseline at all four time points (P < 0.05). No significant differences were noted in percent change in baseline for males vs. females at any of the time points examined (P > 0.05).

The effects of persistent pain on morphine-induced antihyperalgesia are shown in Fig. 6. In males, the %MPE induced by morphine (8 mg/kg) increased as a function of time post-CFA (KW, H = 13.12, P < 0.01). By contrast, in females, the duration of inflammation had no significant effect on morphine-induced antihyperalgesia (H = 2.67, df = 3, P > 0.05; Fig. 6). No differences in %MPE were noted for the 30-, 45-, and 60-min postmorphine time points, so these data are averaged in Fig. 6C. In females, the average %MPE after 8 mg/kg morphine increased from 45 ± 11% on day 1 post-CFA to ~59% on days 7 and 14, to 80 ± 7% at day 21 post-CFA. In males, the average %MPE increased from 69 ± 13% on day 1 post-CFA.
Sex Differences in Morphine Analgesia

Ranged from 14 to 100% at day 7; %MPE remained at 100% at 14 and 21 days post-CFA. No changes in the antinociceptive effects of morphine were noted in saline-treated animals (Fig. 7). In males, %MPE ranged from 90 ± 10% to 100 ± 0% (KW, H = 3.91, df = 3, P > 0.05), suggesting a ceiling effect; in females, %MPE ranged from 14 ± 4% to 49 ± 27% (KW, H = 4.44, df = 3, P > 0.05). The degree of antinociception produced by 8.0 mg/kg of morphine was significantly greater in males compared with females at all time points examined (MWU, P < 0.01).

Discussion

The results of these studies clearly demonstrate that morphine produces a differential degree of antihyperalgesia in response to thermal stimuli in a model of persistent inflammatory pain. At all doses tested, the antihyperalgesic effect of morphine in CFA-treated animals was significantly greater in males than in females. Similar results were noted in saline-treated animals, where morphine produced a significantly greater antinociceptive effect in males compared with females. The results of these studies also demonstrate that in males, the antihyperalgesic effects of morphine are significantly enhanced in the presence of persistent inflammation. In contrast, in females the presence of persistent inflammation had no influence on morphine potency. In the present study, sensory thresholds were assayed using a thermal noxious stimulus; however, as mechanical hypersensitivity and thermal hyperalgesia are genetically distinct (42), future studies using a mechanical stimulus are warranted.

The observed sex differences in the antihyperalgesic and antinociceptive effects of morphine are not due to sex differences in baseline sensory thresholds, CFA-induced edema, or in the degree of thermal hyperalgesia produced by intraplantar administration of CFA; rather, these results suggest that there is something inherently different between males and females in the mechanisms of morphine action in the presence of persistent inflammatory pain. Morphine’s actions are mediated primarily by the μ opioid receptor (46, 52), a member of the inhibitory G protein-coupled receptor superfamily. Kelly et al. (34) reported that in hypothalamic slices, estrogen rapidly uncouples the μ opioid receptor (MOR) from G-protein-gated inwardly rectifying potassium channels, thereby significantly decreasing MOR agonist-induced hyperpolarization (44). Estrogen has also been shown to result in MOR internalization in the rat hypothalamus, thereby limiting the amount of receptor available for exogenous ligand binding (20). These results parallel studies using acute pain assays, in which high estrogen levels were associated with decreased opioid potency (56). In the present study, the effects of estrogen on morphine potency in animals with persistent inflammatory pain could not be discerned; within 24 h of intraplantar CFA, most females were in diestrus and remained there for the duration of the study. Interestingly, during diestrus, plasma estrogen levels are low; therefore, the maintenance of our sex difference in opioid antihyperalgesia suggests that the levels of estrogen present during diestrus may be sufficient to influence morphine binding to the μ receptor and/or the subsequent signaling cascades. Alternatively, these results may also implicate additional mechanisms outside of the “activation” effects of gonadal steroids as contributing to the observed sex difference in morphine antihyperalgesia (43).

Although morphine has been shown to act at a variety of central and peripheral sites, several lines of evidence suggest that the midbrain PAG is a primary site of action for systemic morphine. Lesions of the PAG, or intra-PAG administration of MOR antagonists, significantly attenuate the antinociceptive effects of systemic morphine (22, 41). There are no direct projections from the PAG to the dorsal horn of the spinal cord, the site of primary afferent termination. Rather, PAG efferents terminate directly onto spinally projecting neurons within the rostral ventromedial medulla (RVM), and this PAG-RVM-spinal cord circuit has been shown to be a primary circuit for both endogenous pain modulation and opioid-based analgesia (2–4). Recent anatomical studies in the rat have demonstrated that the PAG-RVM circuit is sexually dimorphic both in its anatomical organization, as well as in its activation during persistent inflammatory pain (43). In addition, systemic morphine preferentially suppresses the activation of this circuit in males, but not females. More recently, we have reported significant sex differences in MOR protein levels within the caudal ventrolateral PAG, with intact males having 11% higher expression levels than diestrus females (19). Together, these data suggest that the PAG and its descending projections to the RVM and spinal cord may provide the anatomical substrate for the observed sex differences in the effects of morphine.

The results of the present study demonstrating a significant sex difference in morphine potency are consistent with previous studies using acute pain models in which morphine was injected either systemically (1, 11, 31, 32, 36, 48), or directly into the RVM (6) or PAG (39, 40). The results of animal studies parallel recent findings in humans, indicating that females require 30% more morphine than males to achieve a comparable level of analgesia for relief of postsurgical pain (9). Recently, using the CFA adjuvant-induced arthritis model, Cook and Nickerson (12) reported that the antihyperalgesic effects of morphine, as well as the μ agonists oxycodone and butorphanol, were significantly greater in males compared with females. Similar to the present results, the authors also reported an increase in morphine potency as a function of postarthritic time in males, with no corresponding change in ED50 values in vehicle-treated animals or CFA-treated females. Interestingly, these investigators also reported that female rats ceased to cycle normally after CFA administration, suggesting that chronic inflammation interferes with the normal estrous cycle.
in females. These results together suggest serious implications for the reproductive health of women suffering from chronic pain conditions.

In males, the antihyperalgesic effects of morphine, assessed using a thermal stimulus, were potentiated as a function of time postinflammatory pain. No change in morphine antinociception was noted in saline-treated animals, suggesting that the observed increase in morphine potency was due to the presence of persistent inflammation. Similar to the results obtained in the present study, Hurley and Hammond (27), also using a thermal stimulus, reported a significant increase in the antihyperalgesic potency of the μ agonist DAMGO, administered directly into the RVM, as a function of time postinflammation. More importantly, they reported that the antinociceptive potency of DAMGO, determined for the contralateral, uninflamed paw, was also progressively enhanced as a function of time postinjury, with the magnitude of enhancement paralleling the chronicity of the inflammation. Together with the present findings, this suggests that in males, persistent inflammation alters the responsiveness of opioid-sensitive CNS circuits to exogenous opioid administration. No significant change in opioid potency was observed in females as a function of time post-CFA. However, a slight increase in the antihyperalgesic effect of morphine was observed at 21 days post-CFA, suggesting that a leftward shift in morphine potency may have been observed given a longer inflammatory time.

Several mechanisms have been proposed to account for persistent inflammation-induced changes in opioid potency, including upregulation of glutamate receptors in the RVM (24, 25), increased MOR expression and second messenger coupling in the lumbar dorsal root ganglia (51, 60), and increased MOR expression and second messenger coupling at another.

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