Sexually Dimorphic Recruitment of Spinal Opioid Analgesic Pathways by the Spinal Application of Morphine

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ABSTRACT

Current evidence for sex-based nociception and antinociception, largely confined to behavioral measures of pain sensitivity, chronic pain syndromes, and analgesic efficacy, provides little mechanistic insights into biological substrates causally associated with sexual dimorphic pain experience. Spinal cord has been shown to be a central nervous system region in which regulation of opioid antinociceptive substrates manifest sexual dimorphism. This site was therefore chosen to explore whether or not differential mechanisms underlie comparable spinal opioid antinociception in male and female rodents. Intrathecal (i.t.) application of morphine to male and female rats produces a thermal antinociception equivalent in magnitude and temporal profile. Nevertheless, it results from the sex-based differential recruitment of spinal analgesic components. As expected, the spinal mu-opioid receptor is critical for i.t. morphine antinociception in both sexes. However, in females, but not males, activation by i.t. morphine of spinal kappa-opioid receptors is a prerequisite for spinal morphine antinociception. Furthermore, in females, but not males, i.t. application of antidyornorphin antibodies substantially attenuates the antinociception produced by i.t. morphine. This indicates that the antinociception that results from the i.t. application of morphine in females requires the functional recruitment of spinal dynorphin. Female-specific recruitment by i.t. morphine of a spinal dynorphin/kappa-opioid receptor pathway results from organizational consequences of ovarian sex steroids and not the absence of testicular hormones. These observations suggest that sexual dimorphic pain and analgesic mechanisms might be far more pervasive than commonly thought and underscore the imperative for including female as well as male subjects in all studies of pain and antinociception.

Sexual dimorphism in nociception and opioid antinociception has been well documented in humans (Ellermeier and Westphal, 1995; Unruh, 1996; Berkley, 1997; Fillingim et al., 1998; Walker and Carmody, 1998) and laboratory animals (Mogil et al., 1993; Coyle et al., 1995, 1996; Kayser et al., 1998; Mogil and Chanda, 2005). Previously, evidence for sex-based nociception and antinociception has been largely confined to behavioral measures revealing differential pain sensitivity, frequency and severity of chronic pain syndromes, and divergent analgesic efficacy of opioid receptor-type-selective agonists. Although underscoring the occurrence of sexual dimorphism in pain processing and its regulation, these studies provide little insight into biological substrates and neural organizational parameters that might underlie such sexual dimorphism.

The spinal cord has been shown to be a central nervous system region in which components of opioid analgesic pathways and their regulation manifest sexual dimorphism. For example, the density of the kappa-opioid receptor (KOR) and its distribution within axon terminals differs between the spinal cord of male and female rodents (Harris et al., 2004); spinal KOR density is significantly greater in estrous and proestrous female versus male rats (Chang et al., 2000). Furthermore, although the spinal cord of both male and female rodents contains opioid analgesic systems that can be activated by the same regimen of ovarian sex steroids, the mechanistic underpinnings of the resulting antinociception differ, i.e., KOR and delta-opioid receptor (DOR) in the female versus KOR and the mu-opioid receptor (MOR) in the male (Liu and Gintzler, 2000). Importantly, ovarian sex steroid antinociception in female and male rodents represents a situation in which sex-dependent endogenous opioid antinociception that is identical both in its temporal profile and magnitude still differs in their cellular mediators (Liu and Gintzler, 2000). This underscores the possibility that identical antinociceptive responsiveness to the exogenous administration of the same opioid might still result from sexual

ABBREVIATIONS: KOR, kappa-opioid receptor; DOR, delta-opioid receptor; MOR, mu-opioid receptor; TFL, tail-flick latency; beta-FNA, beta-funaltrexamine; nor-BNI, nor-binaltorphimine.
dimorphic processes. If indeed this is so, sex-dependent pain and analgesic mechanisms might be far more prevalent than commonly thought.

To investigate this possibility, we determined the opioid receptor profile mediating antinociceptive responsiveness to i.t. morphine. We selected morphine because it remains the most commonly employed opioid for the clinical management of pain and is capable of activating all three predominant types of opioid receptor, (i.e., MOR, KOR, and DOR). We chose to study the action of morphine on the spinal cord based on the previous demonstrations of the sexual dimorphic character of this central nervous system region (Liu and Gintzler, 2000; Gupta et al., 2007).

The results demonstrate a striking sexual dimorphism in the types of spinal opioid receptor that underlie analgesic responsiveness to the i.t. application of morphine. Furthermore, the data demonstrate that this sex-based dichotomy most probably results from organizational effects of gonadal hormones on the intersection of MOR-coupled spinal analgesic pathways with spinal KOR/dynorphin systems. These findings underscore the pervasiveness of sex-based pain and analgesic mechanisms and the imperative to include female subjects in all studies of acute and chronic pain.

**Materials and Methods**

**Animals and Housing**

Experiments employed adult Sprague-Dawley rats (Charles River, Kingston, NY; 250–275 g for females and 275–300 g for males) and neonatal rats (see below) that were maintained in an approved controlled environment. Food and water were available ad libitum. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of State University of New York Downstate.

**Orchidectomy and Ovariectomy in Adult Rats**

To determine whether the sex-related differences of i.t. morphine analgesia were the result of the acute (activation) effects of steroids, groups of adult male and female rats were sham-operated or castrated, concomitantly with i.t. cannulation, 1 week before nociceptive threshold determination. In brief, animals were anesthetized with sodium pentobarbital (40 mg/kg i.p.; Abbott Laboratories, North Chicago, IL) after pretreatment with atropine (0.85 mg/kg; IUX, NJ) was inserted through an incision in the atlanto-occipital membrane, slowly introduced into the spinal cord subarachnoid space (7.5 cm), and secured in place. The cephalic portion of the catheter was externalized through the skin above the skull area where it was relatively inaccessible to the paws. All animals appeared to be free of infection upon gross inspection. Motoric integrity was assessed in all groups using the righting reflex and the inclined plane test. Those exhibiting motor impairment following surgery were eliminated from the study.

**Implantation of Intrathecal Cannulae**

A permanent indwelling cannula was inserted into the lumbar spinal cord subarachnoid space as described originally (Yaksh and Rudy, 1976) and performed previously (Liu and Gintzler, 1999; Gintzler and Liu, 2000). In brief, animals were anesthetized as mentioned above. A saline-filled catheter (PE-10; Clay Adams, Parsippany, NJ) was inserted through an incision in the atlanto-occipital membrane, slowly introduced into the spinal cord subarachnoid space (7.5 cm), and secured in place. The cephalic portion of the catheter was externalized through the skin above the skull area where it was relatively inaccessible to the paws. All animals appeared to be free of infection upon gross inspection. Motoric integrity was assessed in all groups using the righting reflex and the inclined plane test. Those exhibiting motor impairment following surgery were eliminated from the study.

**Quantification of Acute Thermal Nociception**

Acute nociception was assessed by determining tail-flick latency (TFL) from a radiant heat source (Algesia Meter; IITC, Woodland Hills, CA). A 10-s cutoff was employed to prevent tissue damage. Thermal nociceptive thresholds were determined immediately before (basal TFL) and at various intervals after i.t. opioid administration.

**Intrathecal Administration of Drugs**

To determine sex-dependent recruitment of opioid receptor populations, both male and female rats were treated i.t. with morphine sulfate alone or following an 18-h pretreatment with either β-ent-funaltrexamine (β-FNA) (Ward et al., 1982) or nor-binaltorphimine (nor-BNI) (26 nmol each) (Portoghese et al., 1987; Takemori et al., 1988; Wongchanapai et al., 1998) (or their respective vehicles: saline for naltrindole and nor-BNI, water for β-FNA) or a 30-min pretreatment with either the DOR-selective antagonist naltrindole (Portoghese et al., 1988) or affinity-purified antidynorphin antibodies (200 ng) (or preadsorbed antibody). Preabsorbed antidynorphin antibody was obtained by overnight incubation (4°C) of affinity-purified antibodies (200 µg) with 30 µl of protein-A agarose (300–600 µG IgG binding capacity) followed by centrifugation to precipitate the IgG-protein A-agarose antibody complex. Each pharmacological agent was administered in 5 µl over a 60-s period to the subarachnoid space of the lumbar spinal cord via the permanent indwelling i.t. cannula. Complete delivery was insured by flushing the cannula with an additional 10 µl of saline. Thereafter, thermal nociceptive response thresholds were reetermined at various intervals and compared with predrug thresholds. Vehicle-treated control animals were always tested in parallel with experimental animals. No animal was used for determining responsiveness to more that one concentration of a single drug. Morphine- and opioid receptor-type-selective antagonists were generously supplied by the National Institute on Drug Abuse (Bethesda, MD). Affinity-purified antidynorphin antibodies were obtained from Neuromics (Edina, MN).

**Data Analysis**

A general linear mixed model analysis of covariance was used to perform regression analysis to assess sex difference in antinocicep-
tive dose responsiveness to i.t. morphine. Comparison of the dose response slopes of male versus female rats was performed using contrast functions within the regression model. Repeated measures regression analysis and contrast functions therein were also utilized to assess the time course of i.t. morphine analgesia by comparing the slopes of the time-dependent response between conditions (i.e., sex and treatment).

**Results**

**Dose Responsiveness to i.t. Morphine in Males versus Females.** Intrathecal morphine produced a dose-dependent antinociception against thermal nociceptive stimuli applied to the tail. Dose responsiveness to i.t. morphine sulfate was assessed and compared for male versus female rats using regression analysis with sex and dose (0.3, 1, 3, and 5 μg) as factors. Main effects were found for dose ($F_{1,61} = 27.43, p < 0.001$). However, interactions between sex and dose were not significant ($F_{1,61} = 1.7, p = 0.2$). Dose responsiveness for male and female rats was calculated individually using contrast functions within the regression model. As expected, both male and female rats showed dose responsiveness to i.t. morphine ($p < 0.02$ for each; Fig. 1A). Comparison of slopes also showed no difference in dose responsiveness to i.t. morphine between male versus female rats. These results are consistent with an earlier report that, in contrast to systemic morphine sodium sulfate, the KOR component of i.t. morphine antinociception in adult female and males was not altered by orchiectomy (Fig. 2, male). Thus, acute gonadal ablation of sexually mature male and female rats using contrast functions within the repeated measures regression analysis revealed that the time course of i.t. morphine antinociception produced by i.t. morphine in female but not male rodents.

**Neither Orchietomy nor Ovariectomy of Adult Rats Alters Sex-Dependent Opioid Receptor Types Mediating Morphine Antinociception.** The relevance of activation of gonadal steroids to the sex-dependent recruitment of spinal KOR by i.t. morphine was assessed by determining the effect of adult gonadal ablation on the KOR component of i.t. morphine antinociception (Fig. 2). We examined the effect of KOR blockade on i.t. morphine antinociception in ovariectomized female and orchietomized male rats using contrast functions within the repeated measures regression analysis. In ovariectomized females, time-dependent slopes obtained in the presence versus absence of nor-BNI differed significantly ($p = 0.002$; Fig. 2, female). In males, however, this difference could not be detected ($p = 0.48$; Fig. 2, male). This reveals that a spinal KOR component is critical for the full manifestation of antinociception produced by i.t. morphine in female but not male rodents.

**Androgenization of Female Neonatal Pups Abolishes the KOR Component of Intrathecal Morphine Antinociception.** We assessed the relevance of organizational effects of gonadal sex steroids by determining the KOR component of i.t. morphine antinociception in adult female and male rats. Dose responsiveness to a single concentration of morphine. Each rat was used to determine analgesic effect from the TFL data at 30 min after i.t. application of morphine (A) and its attenuation by β-FNA (B) do not differ between males and females. Antinociception produced by the spinal application of morphine was quantified, in parallel, in untreated male and female rats and in rats that had been treated (18 h) i.t. with 20 nmol of β-FNA or vehicle before i.t. morphine (5 μg). Data shown in A were obtained and calculated from the TFL data at 30 min after i.t. application of morphine. Each rat was used to determine analgesic responsiveness to a single concentration of morphine. Maximum possible effect (percentage) = 100/[TFL(b)−TFL(a)]. Data are expressed as mean ± S.E.M., $n = 5$ to 15 for all groups. In males and females, i.t. morphine produced an equivalent antinociception that was abolished by MOR blockade. Mor, morphine.
male rats that had been either androgenized with a single injection of testosterone propionate or castrated, respectively, during neonatal day 1 (Fig. 3). In adult females that had been treated with vehicle during the neonatal period (or adult females that had not been manipulated during the neonatal period), i.t. pretreatment with nor-BNI substantially attenuated the antinociception produced by i.t. morphine. In contrast, however, in the neonatally androgenized female group, time-dependent slopes obtained in the presence versus absence of nor-BNI no longer differed (Fig. 3, female). Thus, organizational effects of ovarian sex steroids seem to be critical to the ability of i.t. morphine to recruit KOR. In the neonatally castrated group, time-dependent slopes obtained in the presence versus absence of nor-BNI did not differ (Fig. 3, male), as was observed in control males. Thus, the presence of ovarian sex steroids rather than the absence of testicular steroids appears to be critical for the dependence on spinal KOR ability of i.t. morphine antinociception in females.

**Antinociception Produced in Female Rats by Intrathecal Sufentanil Does Not Involve a Spinal KOR Component.** We assessed the effect of spinal KOR blockade on the antinociception produced in female rats by i.t. application of the MOR-selective agonist sufentanil (0.6 nmol). This was performed to assess whether or not the activation of spinal MORs (in the absence of the direct activation of KOR) in females rats is sufficient to elicit a KOR component of the resulting spinal antinociception. Figure 4 illustrates that i.t. nor-BNI did not have any effect on the analgesic responsiveness to the spinal application of sufentanil.

**Spinal Dynorphin Is Required for i.t. Morphine Antinociception in Females but Not Males.** We investigated the effect of the i.t. application of antidynorphin antibodies on spinal morphine antinociception to probe the involvement in this process of spinal dynorphin (Fig. 5). A three-way repeated measures regression analysis of the time course of i.t. morphine antinociception revealed a significant three-way interaction for time, condition (absence or presence of antidynorphin antibodies), and sex ($F_{1,112} = 4.99, p < 0.03$). To localize the source of the interaction, we compared slopes of the morphine time course in the absence versus the presence of i.t. antidynorphin antibodies in male and female rats. In females, the i.t. application of affinity-purified antidynorphin antibodies, but not preadsorbed antibody, significantly altered the slopes of the morphine time course ($p = 0.002$; Fig. 4).

**Sex-Dependent Responsiveness to Spinal Morphine.** We investigated the dependence on spinal KOR ability of i.t. morphine antinociception in males and females. Antinociceptive responsiveness to i.t. morphine (5 μg) was quantified in untreated male and female rats and in rats subjected to ovariectomy or orchietomy, without or following (18 h) the i.t. application of the KOR blocker nor-BNI or vehicle. Data are expressed as mean ± S.E.M., $n = 5$ to 6 for all groups. In female, but not male, rats, blockade of spinal KOR substantially attenuated spinal morphine antinociception. Neither spinal morphine antinociception nor its modulation by KOR blockade was altered in adult females or males by gonadal ablation. Mor, morphine; OVX, ovariectomy; ORX, orchietomy.
The current study demonstrates the relevance of sexual dimorphism in pain processing systems and their modulation, even in the absence of sex-based differences in antinociceptive responsiveness to the same opioid. Sexual dimorphism in pain sensitivity and frequency of chronic pain syndromes has been extensively documented. Studies utilizing thermal (Fillingim et al., 1998), electrical (Walker and Carmody, 1998), and pressure (Ellermeier and Westphal, 1995) nociceptive stimuli have all revealed that recurrent pain, more severe levels of pain, and pain of longer duration are much more likely to be experienced in women than men (Unruh, 1996). Additionally, systemic and intracerebral administration of many opioid agonists, capable of activating MOR, the predominant opioid receptor type mediating opioid antinociception, produce a greater magnitude of antinociception in males than females (Krzanowska and Bodnar, 1999; Barrett et al., 2002). Our present study adds a new dimension to sex-based antinociception by demonstrating that equal analgesic behavioral effects resulting from the same i.t. concentration of morphine are mediated by different populations of spinal opioid receptors in females versus males. This underscores the prevalence of sex-based mechanisms subserving nociception and antinociception, which necessitates the need to study females as well as males in all pain-related studies.

Morphine is only weakly opioid receptor type selective; its affinity for MOR, DOR, and KOR is ~1.8, 160, and 47 nM, respectively (Mignat et al., 1995). This suggests that activation of all three types of opioid receptor can contribute to the antinociception produced by morphine. This notwithstanding, prior i.t. treatment of male rats with the MOR-selective antagonist, β-FNA, obliterated the elevation of TFL by i.t. morphine to thermal stimuli. This indicates that MOR is the predominant, if not exclusive, opioid receptor type mediating i.t. morphine antinociception. The abolishment by β-FNA of i.t. morphine antinociception is consistent with prior reports that the analgesic effects of systemic or i.c.v. administration of morphine are substantially attenuated in MOR knockout animals (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998). The salient involvement of KORs in the mediation of spinal morphine antinociception has only been reported in MOR knockout mice (Yamada et al., 2006) or following functional ablation of spinal MORs via i.t. administration of β-FNA (Takemori and Portoghese, 1987). These experiments, which utilized exclusively male rodents, are consistent with the current observation that the analgesia produced in male rats by i.t. morphine is not affected by spinal administration of the KOR-selective blocker nor-BNI.

Prior i.t. treatment of female rats with the MOR-selective antagonist, β-FNA, obliterated the elevation of TFL by i.t. morphine to thermal stimuli, as was observed in males. This reaffirms the preeminent role of MOR in mediating morphine antinociception. Strikingly, however, in female but not male rats, i.t. treatment with nor-BNI can also substantially attenuate i.t. morphine antinociception. Thus, activation of both MOR and KOR is a prerequisite for spinal morphine antinociception in female rats.

The contribution of spinal KORs to the observed morphine-mediated thermal antinociception in female rats could result from direct activation of KORs by morphine or via functional recruitment of spinal dynorphin systems. The prerequisite for recruiting spinal dynorphin was revealed by key studies demonstrating the ability of i.t. administered antidynorphin antibodies to markedly reduce or abolish the antinociceptive response produced by the spinal application of morphine. Antidynorphin antibodies can interact with des-tyr dynorphin, which interacts with NMDA and MC1R receptors but not KOR. Thus, the participation of des-tyr dynorphin in antinociception produced in females by i.t. morphine is not likely because it would not be consistent with the substantial attenuation of that antinociception by i.t. nor-BNI. Moreover, des-tyr dynorphin activates N-methyl-D-aspartate receptors, which produce hyperalgesia. Thus, one would predict that the antibody neutralization experiments would have revealed augmented antinociception were they removing des-tyr dynorphin from the i.t. space. Notably, the i.t. application of antidynorphin antibodies to male rats did not have any effect on the antinociception produced by i.t. morphine, consistent with the inability of the KOR blocker nor-BNI to attenuate the spinal morphine antinociception in males. At present, it is not clear whether i.t. morphine stimulates the release of spinal dynorphin, as has been reported for i.t. endomorphin (Mizoguchi et al., 2006), or if basal rates of dynorphin release are sufficient for the manifestation of i.t. morphine antinociception. It should be noted that we were not able to demonstrate morphine-induced release of dynorphin from minced superfused spinal tissue. However, the ability of morphine to stimulate spinal dynorphin release could require synaptic organization and excitatory inputs...
that were not retained in the mixed preparation. Additionally, or alternatively, any increment in synaptic dynorphin release produced by morphine, although physiologically relevant, could have been below the resolving power of the dynorphin radioimmunoassay employed.

Interestingly, the magnitude of thermal antinociception produced in female rats by the i.t. application of the highly selective MOR agonist sufentanil (0.6 nmol), which does not activate spinal KORs, is not reduced by prior treatment with nor-BNI. The ability of morphine to interact with and activate spinal KOR could be a contributory or permissive factor necessary for the emergence of the KOR component of spinal morphine antinociception. Thus, it seems unlikely that the antinociception produced in females by i.t. morphine results solely from the direct activation of spinal MORs, with KORs playing a permissive role in spinal antinociception. This is consistent with the observation that nor-BNI does not block spinal antinociception produced by morphine. Additionally, the manifestation of a KOR component of morphine- (but not sufentanil-) induced spinal antinociception could result from differences in their structure and/or physical/chemical properties.

The precise requirement(s) for the KOR component of i.t. morphine antinociception and the extent to which KOR mediation of spinal antinociception generalizes to other MOR agonists remains to be elucidated. This notwithstanding, the current study reveals a very complex interrelationship between spinal MOR and KOR analgesic pathways in female rats. Alternatively, the manifestation of a KOR component of morphine- (but not sufentanil-) induced spinal antinociception could result from differences in their structure and/or physical/chemical properties.

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The realization that sex-dependent recruitment of different populations of opioid receptor can underlie comparable pharmacologic antinociception in male and female rats underscores the imperative for including female as well as male subjects in all investigations of pain and antinociceptive mechanisms and their regulation. Clearly, implicit or explicit conclusions of common mechanistic underpinnings inferred from behavioral similarities between male and female subjects are fraught with ambiguity. Regardless of the degree to which the ability of i.t. morphine to recruit different spinal populations of opioid receptor generalizes to other opioid agonists, current findings reveal a heretofore unrecognized parameter of nociception and antinociception, attention to which could prove to have clinical utility.

References


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