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Contribution of Endogenous Spinal Endomorphin 2 to Intrathecal Opioid Antinociception in Rats Is Agonist-Dependent and Sexually Dimorphic

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Abstract

Interactions between exogenous and endogenous opioids are not commonly investigated as a basis for sexually dimorphic opioid analgesia. We investigated the influence of spinal endomorphin 2 (EM2), an endogenous mu-opioid receptor (MOR) ligand, on the spinal antinociception produced by intrathecally administered opioids. Activation of spinal MORs facilitated spinal EM2 release. This effect was sexually dimorphic, occurring in males but not females. Although activational effects of testosterone were required for opioid facilitation of spinal EM2 release in males, the absence of this facilitation in females resulted from neither insufficient levels of testosterone nor mitigating effects of estrogens. Strikingly, in males, the contribution of spinal EM2 to the analgesia produced by intrathecally applied MOR agonists depended on their analgesic efficacy relative to that of EM2. Spinal EM2 released by the higher efficacy MOR agonist sufentanil diminished sufentanil's analgesic effect, whereas EM2 released by the lower efficacy morphine had the opposite effect on spinal morphine antinociception. Understanding antithetical contributions of endogenous EM2 to intrathecal opioid antinociception not only enlightens the selection of opioid medications for pain management, but also helps explain variable sex-dependence of the antinociception produced by different opioids, facilitating the acceptance of sexually dimorphic antinociception as a basic tenet.

Perspective—The male-specific MOR-coupled enhancement of spinal EM2 release implies a parallel ability to harness endogenous EM2 antinociception. The inferred diminished ability of females to utilize the spinal EM2 antinociceptive system could contribute to their greater frequency and severity of chronic pain syndromes.

Keywords

endomorphin; sexual dimorphism; estrogen; testosterone; opioid

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Introduction

The growing pressure to provide evidence-based justification for new medicinal therapies underscores the imperative to better understand the sex divide in pain and to optimize pain management in both sexes. Epidemiological studies consistently demonstrate a greater prevalence among women than men of chronic pain disorders.²⁹ These include musculoskeletal pain,^{33,79,89,97} headaches and migraines,^{22,54,86,90} fibromyalgia,¹⁰⁰ and back pain.^{41,87} Furthermore, women exhibit higher self-reported severity of pain in clinical studies of osteoarthritis,⁴³ back pain,⁶ and pain following surgical procedures such as cholecystectomy,²⁴ colonoscopy,⁵⁰ and knee arthroplasty.⁷⁷ Moreover, multiple investigations of experimental pain have demonstrated that sexually dimorphic nociception and opioid antinociception are not idiosyncratic to a particular pain modality in either humans^{30,94} or laboratory animals.^{5,12,18,19,46,66,67} Although most studies document greater frequency and severity of pain among women than men, there are notable exceptions to this pattern in the epidemiological,^{25,93} clinical,^{53,80} and experimental^{8,72} literature. The underlying biological basis for this complexity remains unknown.

Sex differences are also observed in the effectiveness of opioid analgesics.^{28,29} There is a general consensus that mu-opioid receptor (MOR) agonists produce greater analgesia in males than females.^{3,7,13,14,16,42,48,73,74,96} However, human studies of sexual dimorphism in MOR-mediated antinociception are not without controversy, particularly with respect to morphine (a MOR-preferring agonist).^{2,9,31,32,38,44,45,81} Inconsistencies among human studies of sexually dimorphic analgesic responsiveness impede the development of a conceptual framework for understanding sex-dependent opioid analgesia, underscoring the utility of animal models. As an exemplar, in a study using rats, opioid antinociception qualitatively differed between males and females depending on a complex interaction between sex and pain type (and/or the body region receiving the nociceptive stimulus);⁵⁷ this could inform at least some of the diametrically opposed findings reported in humans.

In rats, MOR-coupled sequelae also frequently demonstrate sexual dimorphism. For example, acute systemic morphine activates more than twice the number of neurons in the periaqueductal gray-rostral ventromedial medulla of male rats than female rats,⁶⁰ and chronic systemic morphine upregulates mRNA encoding two MOR splice variants (MOR-1B2 and MOR-1C1) in the spinal cord of male but not female rats.⁹¹ Notably, although supraspinal/systemic morphine produces greater analgesia in male rats than female rats,^{3,7,13,14} effects of morphine on spinal antinociception are equianalgesic in males and females.⁵⁹ This difference underscores the complexity of sexually dimorphic opioid analgesia on both neuroanatomical and physiological levels (see Loyd and Murphy, 2014 for review⁶¹).

Sexual dimorphism in rats also extends to utilization of the endogenous MOR ligand endomorphin (EM) 2 in spinal tissue. EMs are endogenous activators of MOR,¹⁰² having a higher affinity ($K_i = 360$ and 690 pM for EM1 and EM2 respectively) and selectivity for MOR than any other endogenous opioid. Intracerebroventricular or intrathecal (i.t.) administration of EM1 or EM2 in rats produces potent MOR-mediated antinociception.^{82,102}

Notably, the magnitude of *in vitro* basal and evoked EM2 release from spinal tissue of male rats is $\approx 50\%$ greater than that from spinal tissue of females,³⁶ a finding that is consistent with the enhanced activation of MOR by experimental pain in human males.¹⁰⁴ These data suggest that males and females differentially utilize the endogenous MOR/EM2 antinociceptive system.

One parameter of endogenous EM2 utilization that remains relatively unexplored is its relationship with intrathecally-administered opioids. We previously reported that sufentanil enhances *in vitro* K⁺-induced release of EM2, an effect that was observable only following *in vitro* pretreatment with pertussis toxin¹⁰ to eliminate MOR-mediated inhibition of K⁺-induced EM2 release.^{10,36} However, the contribution of endogenous EM2 to spinal antinociception resulting from intrathecally administered MOR agonists, and the mechanisms thereof, were not studied. Such knowledge could hold a key to harnessing endogenous opioids for pain management.

Accordingly, we investigated the ability of the highly specific MOR agonist sufentanil to elicit release of EM2 from spinal cords of male and female rats *in vivo*, as well as the contribution of this endogenously released EM2 to spinal opioid analgesia. We chose a MOR-selective agonist because MOR is the opioid receptor most abundantly expressed in the CNS and most frequently targeted for pain management. We selected EM2, not EM1, because EM2 is the predominant EM in spinal cord.⁶⁴ Results demonstrate MOR-mediated enhancement of EM2 release in males but not females, as well as sexually dimorphic regulation of this mechanism by gonadal sex steroids. The contribution of EM2 to the antinociception produced by i.t. MOR agonists was also found to be sexually dimorphic. These findings provide, in part, a physiological basis for sex differences in MOR-mediated opioid analgesia.

Materials and Methods

Animals and Housing

Experiments used Sprague-Dawley rats (Charles River Laboratories, Kingston, NY; 250–300 g) maintained in a controlled environment on a 12 hour light/dark cycle. Food and water were available *ad libitum*. All experimental procedures were reviewed and approved by our Institutional Animal Care and Use Committee.

Determination of Stage of Estrous Cycle

Estrous cycle stage was determined by histology of vaginal smears. Proestrus or diestrus showed a predominance of large round nucleated cells or small leukocytes, respectively. Potential disruptions of the estrous cycle resulting from surgery did not confound data interpretation because stage of cycle was defined by vaginal smear histology rather than predictions assuming regularity of cycling.

Orchiectomy and Ovariectomy in Adult Rats

To determine whether the sex-related differences in the effect of i.t. sufentanil on EM2 release were the result of the activational effects of steroids, groups of adult male and female

rats were gonadectomized either 1 or 2 weeks before measurement of EM2 release. In brief, animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Abbott Laboratories, North Chicago, IL) after pretreatment with atropine (0.85 mg/kg i.p.; IUX Animal Health, Inc., St. Joseph, MO). Orchiectomy was performed by the removal of the testes together with testicular fat and epididymis, and ovariectomy was performed by removal of the ovaries.⁶³ Activational effects of sex hormones have been reliably altered either 1 or 2 weeks following gonadectomy.^{11,23,26,85} In the current study, effects of ovariectomy did not differ between groups (1 week vs. 2 weeks). To underscore this point, we include data from both groups. The same experimental protocol (i.t. cannulation, i.t. treatment, *in vivo* perfusion, analysis of EM2 in the perfusate; see below for methods) was employed for both groups.

Androgenization of Female Adults

One group of adult female rats was ovariectomized, 1 week after which they received testosterone implants. These remained in place for an additional 1 week, at which time i.t. cannulation and perfusion (see below) were performed. This method, previously used to androgenize gonadectomized adult rats, achieves circulating testosterone levels comparable to those of intact adult male rats (11.7 ± 4.7 ng/ml).^{70,101} In brief, Silastic laboratory tubing (Dow Corning, Midland, MI) was used for testosterone implants, which consisted of two Silastic tubes (i.d. 0.20 cm, o.d. 0.32 cm, length 3 cm) packed with testosterone (Steraloids, Inc., Newport, RI) and sealed with A-100 Medical Silicone Adhesive (Factor II, Inc., Lakeside, AZ). Implants were submerged in 0.9% saline solution at 37°C for 24 hours, and then implanted subcutaneously 3 cm distal to the shoulder blade (under 2.5% isoflurane anesthesia).

Androgenization of Female Pups

To determine whether the sex-related differences of i.t. sufentanil on EM2 release were the result of developmental (organizational) effects of steroids, female rats were subjected to gonadal ablation on neonatal day 1 as described previously.^{15,59} In brief, female pups were taken from their mother and put on a metal plate on top of ice. When they became motionless and pale (about 15 min), they received subcutaneous injections of testosterone propionate dissolved in sesame seed oil (500 µg in 30 µL). The pups were cleaned and put on a warming pad to recover (about 30 min) before being returned to their mother. Pups were weaned at 3 weeks of age. As adults (250–300 g), one week prior to testing, half the rats received Silastic implants filled with testosterone (described above), while the other half received empty Silastic implants.

Implantation of Intrathecal Cannulas

For measurement of acute thermal antinociception, a permanent indwelling cannula was inserted into the lumbar spinal cord subarachnoid space 1 week before experimentation as described previously.⁵⁹ In brief, animals were anesthetized as described above, and an 8.25 cm PE-10 catheter (Becton, Dickinson and Company, Franklin Lanes, NJ) was inserted through the atlanto-occipital membrane into the spinal subarachnoid space. The cephalic portion of the catheter was secured in place and externalized through the skin on the dorsal side of the neck, where it was relatively inaccessible to the paws. Upon gross inspection, all animals appeared to be free of infection. The righting reflex and the inclined plane test were

used to assess motoric integrity; any animals showing motor impairment following surgery were excluded. For *in vivo* perfusion of the spinal i.t. space, two PE-10 catheters (8.25 cm inflow and 6.75 cm outflow) were implanted immediately before perfusion.

Intrathecal Administration of Drugs

Drugs were administered over a 60-second period via i.t. cannula to the subarachnoid space of the lumbar spinal cord. Each drug was delivered in 5 to 10 μ L vehicle with an additional 10 μ L to flush the cannula. Responsiveness to nociceptive stimuli or EM2 release was determined at various intervals thereafter and compared with pre-drug values. Narcotics were obtained from NIDA, the androgen receptor antagonist flutamide was obtained from Sigma-Aldrich (St. Louis, MO), and affinity-purified anti-EM2 antibody was obtained from Neuromics (Edina, MN). This antibody has less than 3% cross-reactivity with EM1 and less than 0.01% cross-reactivity with Leu5-enkephalin, Met5-enkephalin, and β -endorphin. I.t. application of preadsorbed anti-EM2 antibody (negative control) failed to alter basal tail flick latency (TFL; described below) and spinal opioid antinociceptive responsiveness. We have reported that male and female rats do not differ in analgesic responsiveness to i.t. sufentanil⁵⁶ or i.t. morphine.⁵⁹ Thus, in the current study, the equivalent i.t. doses of sufentanil/morphine administered to males and females were equianalgesic.

Quantification of Acute Thermal Nociception

A radiant heat source (Analgesia Meter; IITC, Woodland Hills, CA) was used to assess thermal nociceptive threshold (i.e., TFL) immediately before and at various intervals after i.t. opioid administration. The radiant heat intensity used was identical for males and females and resulted in a basal TFL between 3 and 4 seconds. A 10-second cutoff was employed to prevent tissue damage. Male and female rats have been reported to differ in thermal pain sensitivity⁹² as well as cutaneous blood flow.^{17,51,52} Nevertheless, in the present study, basal TFL did not vary between males and females ($F_{7,54} = 0.70$; $p = 0.68$). Moreover, effects of opioids on thermal nociceptive thresholds were not compared between males and females. Instead, within-sex analysis was used to determine the effect of i.t. anti-EM2 antibody on spinal opioid analgesic responsiveness. Thus, sex-dependent thermal nociception and cutaneous blood flow are not confounding variables.

In Vivo Perfusion of the Spinal Intrathecal Space

The push-pull method was used to perfuse the i.t. space of intact, anesthetized rats as performed previously.⁵⁸ Two PE-10 catheters (8.25 cm inflow and 6.75 cm outflow) were inserted into the spinal subarachnoid space via the atlanto-occipital membrane under sodium pentobarbital anesthesia (50 mg/kg i.p.). The longer cannula extended to the middle of the lumbar enlargement; the shorter cannula extended to the caudal portion of the thoracic spinal cord. The i.t. space was perfused with Krebs-Ringer buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4 , 14 mM dextrose, 0.3 mM MgSO_4 , 25 mM NaHCO_3 , and 2.5 mM CaCl_2) pre-warmed to 37°C and containing protease inhibitors Phenanthroline (1 mM; Sigma-Aldrich, St. Louis, MO) and Actinonin (100 μ M; AG Scientific, San Diego, CA) to prevent EM2 degradation. The outflow tubing was placed on ice to cool the perfusate. The i.t. space was equilibrated with the perfusion medium for 10 min. Subsequently, four 10-min perfusate samples (5 μ L/min) were collected from each animal for quantification of EM2 release: two

before (for basal release) and two after i.t. opioid administration, with a 10-min resting period after each sample collection. Following the initial 10-min equilibration period, the basal rate of spinal EM2 release did not vary significantly over the period of i.t. perfusion. Therefore, EM2 release after drug treatment was compared against the basal rate of release immediately preceding it.

EM2 Competitive Radioimmunoassay

The content of EM2 in i.t. perfusate was quantified using a competitive radioimmunoassay (RIA) as described previously.³⁶ The anti-EM2 antibody used (generously supplied by Dr. James Zadina) is highly selective for EM2; it does not recognize EM1. ¹²⁵I-labeled EM2 (Phoenix Pharmaceuticals, Burlingame, CA) was used as tracer. Bovine serum albumin (0.1%; Sigma-Aldrich, St. Louis, MO) in the assay buffer minimized nonspecific adherence to the assay tube. Tube contents were transferred to plates coated with IgG and scintillant. Plates were counted with a MicroBeta plate reader (Perkin Elmer, Waltham, MA). A standard curve, plotted as percent inhibition of binding against the log concentration of unlabeled EM2 (0.9–28 fmol), was included with each assay and used to estimate EM2 quantities in perfusate samples using the “forecast” function in Excel (Microsoft, Redmond, WA).

Data Analysis

One-way repeated measures ANOVA was used to determine the effect of treatment at multiple time points after i.t. administration of drugs within each group. Tukey’s post-hoc test identified specific time points showing a significant effect. Two-way ANOVA was used to analyze interactions between sex/group and time after treatment. Bonferroni post-hoc tests identified differences between groups. One-way ANOVA was used to compare basal TFL between groups. $P < 0.05$ was considered significant. Data are expressed as mean $\pm$ SEM.

Results

Regulation of Spinal EM2 Release by i.t. Sufentanil Is MOR-Mediated, Sexually Dimorphic, and Estrous Cycle-Independent

The basal rate of EM2 release (before drug treatment) among males (3.71 ± 0.42 fmol/10 min; $n = 9$), diestrous females (2.47 ± 0.33 fmol/10 min; $n = 6$), and proestrous females (2.66 ± 0.44 fmol/10 min; $n = 8$) did not differ ($F_{2,20} = 2.70$; $p = 0.09$). Basal EM2 release also did not differ among males treated with sufentanil, naloxone, or sufentanil plus naloxone (described below; $F_{2,14} = 1.65$; $p = 0.23$, $n = 4-9$).

Based on the dose-effect relationship of i.t. sufentanil to generate spinal analgesia, we elected to study effects of 0.7 nmol sufentanil on spinal EM2 release because this dose produces near-maximal antinociception (TFL).⁵⁶ One-way ANOVA revealed a significant treatment effect of sufentanil in males ($F_{2,16} = 7.45$; $p < 0.01$): 30 to 40 min after the i.t. administration of sufentanil, EM2 release increased to 5.14 ± 0.56 fmol/10 min ($p < 0.01$), an increase of approximately 39% (Fig. 1). A lower dose of sufentanil (0.35 nmol) failed to enhance EM2 release ($F_{2,8} = 1.79$; $p = 0.23$, $n = 5$). Since an i.t. dose of 0.7 nmol sufentanil produces near-maximal antinociception,⁵⁶ effects of doses higher than 0.7 nmol on spinal

EM2 release would have dubious relevance to sufentanil analgesic responsiveness and were therefore not studied. In striking contrast to males, sufentanil (0.7 nmol) did not alter EM2 release in either diestrous ($F_{2,10} = 1.23$; $p = 0.33$) or proestrous females ($F_{2,14} = 0.01$; $p = 0.99$).

Although the opioid receptor antagonist naloxone (i.t., 100 nmol; Fig. 2) did not alter EM2 release in males ($F_{2,6} = 0.25$; $p = 0.78$, $n = 4$), naloxone pretreatment (30 min) prevented the facilitation of EM2 release by sufentanil, i.e., basal EM2 release did not differ from release in the presence of both sufentanil and naloxone ($F_{2,6} = 1.26$; $p = 0.35$, $n = 4$). Notably, the rate of EM2 release 30–40 min after treatment with naloxone plus sufentanil (3.44 ± 1.11 fmol/10 min) was indistinguishable from either EM2 release 30–40 min after treatment with naloxone alone (2.63 ± 0.73 fmol/10 min; $t_6 = 0.90$; $p = 0.40$) or basal EM2 release among males treated with sufentanil, naloxone, or both ($F_{3,18} = 0.86$; $p = 0.48$, $n = 4-9$). Naloxone is ≈ 100 -fold more selective for MOR than delta-opioid receptor.⁷⁵ Moreover, sufentanil is >200 -fold more selective for MOR than delta- or kappa-opioid receptor,¹⁰³ and MOR represents $\approx 90\%$ of the total opioid binding capacity in lumbosacral spinal cord laminae I and II.⁸³ Thus, it is highly likely that the mechanism by which sufentanil facilitates EM2 release in males is mediated predominantly, if not exclusively, by MOR.

Influence of Gonadal Sex Hormones on MOR-Mediated Regulation of Spinal EM2 Release

We determined the contribution of testicular testosterone to MOR-mediated facilitation of spinal EM2 release in males by administering i.t. sufentanil to orchietomized males. Strikingly, facilitation of EM2 release by sufentanil was no longer manifest following orchietomy ($F_{2,10} = 0.40$; $p = 0.68$, $n = 6$), indicating that in males, facilitation of EM2 release by spinal MOR depends on activational effects of testicular testosterone. Consistent with this finding, i.t. 1-hour pretreatment with flutamide (60 μ g), a highly specific androgen receptor antagonist,^{68,69,71,84} also abolished the facilitation of EM2 release by sufentanil in males ($F_{2,6} = 0.35$; $p = 0.72$, $n = 4$).

To assess whether ovarian factors prevent MOR-mediated facilitation of EM2 release in naïve females, we measured the effect of sufentanil on EM2 release in ovariectomized females. One week following ovariectomy, i.t. sufentanil remained unable to enhance spinal EM2 release ($F_{2,4} = 1.62$; $p = 0.31$, $n = 3$). To ensure that circulating and tissue levels of estrogens were fully depleted, we repeated this experiment 2 weeks post-ovariectomy. This also failed to reveal an effect of sufentanil on EM2 release ($F_{2,6} = 0.18$; $p = 0.84$, $n = 4$). We further determined whether approximating male levels of circulating testosterone in females would reveal spinal MOR-mediated facilitation of EM2 release. Administration of chronic testosterone to ovariectomized females did not enable an effect of sufentanil on EM2 release ($F_{2,8} = 0.45$; $p = 0.66$, $n = 5$). Similarly, neonatal androgenization (via systemic testosterone) also failed to enable an effect of sufentanil on EM2 release in females ($F_{2,8} = 0.33$; $p = 0.73$, $n = 5$), even when followed by chronic testosterone in adulthood ($F_{2,8} = 0.68$; $p = 0.53$, $n = 5$). Importantly, using the current protocol, we and others have previously demonstrated that neonatal androgenization eliminates the spinal kappa-opioid receptor component of i.t. morphine analgesia⁵⁹ and eliminates sex differences in morphine analgesia elicited from the ventrolateral periaqueductal gray.⁴⁹ These earlier studies validate the ability of our neonatal

androgenization procedure to masculinize opioid responsiveness in females. Thus, the failure of neonatal androgenization to enable sufentanil facilitation of EM2 release was not idiosyncratic to our particular protocol.

Release of Spinal EM2 by I.t. MOR Agonists Is Pharmacologically Relevant to the Antinociception They Produce

To assess the contribution of spinal EM2 release to the antinociception produced by i.t. sufentanil, we investigated whether the analgesic action of i.t. sufentanil (0.7 nmol) could be reduced by neutralizing endogenously released EM2 via i.t. anti-EM2 antibody. Unexpectedly, i.t. anti-EM2 antibody (200–1000 ng) failed to alter the spinal sufentanil antinociception produced by this dose. In order to avoid the potential confound that 0.7 nmol i.t. sufentanil, which produces near maximum analgesia (TFL), masked contributions from spinal EM2, we repeated this experiment using 0.35 nmol sufentanil. This dose is near the ED_{50} for spinal sufentanil antinociception (unpublished data) and thus should be optimal for revealing hypothesized contributions from spinal EM2. Indeed, i.t. anti-EM2 antibody (330 ng) significantly altered the antinociception produced by 0.35 nmol sufentanil ($F_{1,56} = 7.13$; $p < 0.05$; Fig. 3A). However, paradoxically, anti-EM2 antibody approximately doubled peak TFL, rather than reducing it, as was hypothesized. Given that anti-EM2 antibody augments sufentanil antinociception, it is not surprising that the antibody had no effect on the near-maximal antinociception resulting from i.t. 0.7 nmol sufentanil.

The unanticipated enhancement of spinal sufentanil antinociception by i.t. anti-EM2 antibody could result from the antibody's ability to effectively remove EM2, which has a much lower efficacy at MOR than sufentanil.^{1,27} As a partial agonist, EM2 competes with sufentanil for MOR activation. Thus, EM2 plus sufentanil would produce less antinociception than sufentanil itself. We investigated this possibility by determining the effect of i.t. anti-EM2 antibody on the spinal antinociception produced by i.t. morphine. Since the efficacy of morphine is similar to that of EM2 for MOR G protein activation³⁹ (which correlates with antinociceptive efficacy⁶²), eliminating the EM2 released by i.t. morphine (via anti-EM2 antibody) should effectively lower the aggregate concentration of MOR agonist, and therefore reduce spinal morphine antinociception. As predicted, i.t. anti-EM2 antibody significantly reduced the antinociception resulting from i.t. morphine (7.5 nmol), approximately halving peak antinociception ($F_{1,88} = 5.18$; $p < 0.05$; Fig. 3B).

Since i.t. sufentanil does not alter spinal EM2 release in females, i.t. anti-EM2 antibody (200–1000 ng) was not expected to alter their antinociceptive responsiveness to i.t. sufentanil. This expectation was confirmed in both diestrous ($F_{1,42} = 0.001$; $p = 0.98$; Fig. 3A) and proestrous females ($F_{1,32} = 0.027$; $p = 0.87$; data not shown). I.t. anti-EM2 antibody similarly failed to alter i.t. morphine antinociception in either diestrous ($F_{1,68} = 0.22$; $p = 0.65$; Fig. 3B) or proestrous females ($F_{1,40} = 0.85$; $p = 0.38$; data not shown). Thus, the influence of spinal EM2 on the antinociception produced by MOR agonists is not only agonist-dependent but also sexually dimorphic.

Discussion

Major findings of the present study include: (1) activation of spinal MOR augments endogenous spinal EM2 release in males but not females, irrespective of estrous cycle stage; (2) MOR-mediated facilitation of EM2 release in males depends on the activational effects of testicular (circulating) testosterone via spinal androgen receptors; (3) the absence of MOR-mediated facilitation of EM2 release in females does not result from activational or organizational effects of ovarian steroids; (4) activational effects of testosterone are not sufficient for spinal MOR-mediated facilitation of spinal EM2 release to be manifest in females; (5) the EM2 released by i.t. sufentanil or i.t. morphine influences the spinal antinociception produced by these drugs in males but not females.

The validity of formulations derived from these findings is bolstered by an experimental approach that cross-validates behavioral and neurochemical data. Sexually dimorphic recruitment and regulation of spinal MOR/EM2 antinociception underscore that MOR analgesics differentially activate spinal antinociceptive systems in male and female rats. It remains to be determined whether the sex-based ability of MOR agonists to augment EM2 release and the consequences thereof on analgesic responsiveness extend to humans. If so, this could represent a new dimension of sex-based opioid antinociception in men vs. women, thereby enlightening the selection of MOR agonists in a clinical context.

Augmentation of EM2 release by i.t. sufentanil suggests the presence of a positive feedback mechanism in the male spinal cord that could be activated by endogenous EM2 itself. Such a feed-forward mechanism would enhance the ability of males to harness the EM2 system in response to a noxious stimulus, thereby enhancing physiological antinociception. This could contribute to the higher pain tolerance often observed in males vs. females. Conversely, the failure of i.t. sufentanil to release spinal EM2 in females suggests their compromised ability to physiologically utilize the endogenous spinal MOR/EM2 antinociceptive system when confronted by noxious stimuli. This, combined with estrous cycle-dependent fluctuations in EM2-mediated antinociception reported previously,⁵⁶ could be causally associated with the greater severity and frequency of some chronic pain syndromes in women (e.g., irritable bowel syndrome, interstitial cystitis, generalized pelvic pain), as well as sex differences in the effectiveness of opioid analgesia.^{28,29} Interestingly, compromised endogenous pain inhibition is associated with the increased pain sensitivity characteristic of irritable bowel syndrome and temporomandibular joint dysfunction.⁴⁷ Future pharmacological therapies to increase activity of the MOR/EM2 system could prove useful for pain management in women.

The effects of i.t. anti-EM2 antibodies on the spinal antinociception produced by i.t. sufentanil and morphine not only underscore that endogenous spinal EM2 contributes to the antinociceptive action of these drugs, but also reveal that the direction of this contribution is opioid agonist-dependent. The variable nature of the influence of endogenous spinal EM2 on the antinociception produced by i.t. MOR agonists could explain variability among MOR agonists in the sex-dependence of the analgesia they produce. For example, in males, the negative contribution of endogenous spinal EM2 to the analgesia produced by higher efficacy MOR analgesics (e.g., sufentanil) would be expected to mitigate what might

otherwise be more robust antinociception in males vs. females. In contrast, sex-dependent differences in spinal analgesic responsiveness to MOR analgesics of comparable efficacy to EM2 should not be obscured by contributions of endogenous spinal EM2. Rather, such contributions should bolster manifestation of sex-dependent differences. This would explain why high efficacy MOR-selective opioid analgesics such as sufentanil, DAMGO [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin, and fentanyl produce comparable antinociception in male vs. female rats,^{4,21,56,65,88} whereas the sexual dimorphism of lower efficacy MOR-preferring analgesics such as hydromorphone and hydrocodone is considerably more robust.⁷⁴ Notably, Cook et al.¹⁶ directly compared sexually dimorphic antinociception among opioids with high vs. low efficacy at MOR, using a consistently employed nociceptive paradigm (rat tail thermal nociception). They definitively established an inverse relationship between agonist efficacy and the magnitude of sexual dimorphism in analgesic responsiveness. Current findings provide a physiological context for understanding their observations. The variable contribution of EM2 to opioid antinociception could also explain, at least in part, why some opioid analgesics are equally effective in men vs. women while others are not.^{20,28,29}

Diffusion of EM2 from spinal tissue into i.t. perfusate would result in a substantial dilution of EM2 content. Thus, the observed sufentanil-induced increment in EM2 released into i.t. perfusate is undoubtedly an underestimate of the magnitude of change in synaptic EM2. However, irrespective of its magnitude, the increment in synaptic EM2 resulting from i.t. sufentanil or morphine is functionally relevant, as evidenced by the ability of endogenous EM2 to influence opioid analgesic responsiveness (Fig. 3). These considerations are underscored by the ability of anti-EM2 antibody to enhance the spinal antinociception produced by 0.35 nmol sufentanil despite the inability of current methods to quantify a corresponding increment in EM2 release.

The current demonstration that i.t. sufentanil augmented spinal EM2 release contrasts with our previous finding that MOR-mediated modulation of neurotransmitter release and intracellular signaling *in vitro* is bimodal, with stimulatory (G_s-mediated) and inhibitory (G_i-mediated) effects occurring at low and high concentrations of MOR agonist, respectively.^{10,34,36,95,99} Multiple factors could account for the current ability of a seemingly high dose of i.t. sufentanil to facilitate EM2 release: (1) An i.t. dose that produces maximum analgesia (e.g., 0.7 nmol) does not necessarily produce high synaptic concentrations of sufentanil at spinal MORs, given the need for tissue diffusion. (2) Given that pentobarbital activates/inhibits specific G proteins in some cell types,^{35,40,78} it is possible that under *in vivo* conditions and/or the influence of anesthesia, facilitative (G_s-mediated) effects of MOR predominate, such that a high dose of i.t. sufentanil augments EM2 release. (3) A predominance of facilitative vs. inhibitory effects of MOR *in vivo* could result from the absence of high K⁺, which was present in *in vitro* experiments, and which could influence neuronal state-dependent effects of sufentanil.⁹⁸

Interestingly, in contrast to our previous finding *in vitro* that naloxone increases EM2 release from spinal tissue of male rats,³⁶ naloxone itself failed to augment *in vivo* EM2 release. The suggested ongoing negative feedback regulation of spinal EM2 release observed *in vitro*, but not *in vivo*, could be a consequence of the higher basal EM2 release *in vitro* (≈1.3 fmol/min) vs. *in vivo* (≈0.4 fmol/min), resulting in greater feedback inhibition *in vitro* and thus a larger

effect of naloxone. Differential basal EM2 release *in vivo* vs. *in vitro* could result from depressant effects of the anesthetic in the former and the absence of descending inhibition and/or afferent inputs in the latter.

Sex steroids exert long-term “organizational” effects throughout post-natal development (which can be eliminated by gonadal ablation at birth), as well as “activational” effects via systemic circulation (which can be eliminated by either gonadectomy in adulthood or acute blockade of corresponding hormone receptors).^{37,76} We assessed whether the ability (or inability) of MOR to facilitate EM2 release results from effects of testosterone in males and estrogens in females.

Orchiectomy eliminated the effect of sufentanil in adult males, demonstrating that MOR-mediated facilitation of EM2 release requires activational effects of testosterone. Pretreatment (1 hour) of intact adult males with the androgen receptor antagonist flutamide also eliminated the effect of sufentanil on EM2 release. The rapid onset of effects of blocking spinal androgen receptors indicates that testosterone acts, at least in part, acutely to enable MOR-mediated facilitation of spinal EM2 release in males. The cellular and molecular mechanisms by which testosterone influences spinal EM2 release remain to be elucidated.

In adult females, the failure of MOR activation to alter EM2 release persisted following ovariectomy, and is therefore independent of presumptive restrictive consequences of activational effects of circulating ovarian sex steroids and/or other ovarian factors. Ovariectomy plus chronic testosterone also failed to unmask a facilitative effect of sufentanil on spinal EM2 release, eliminating the possibility that females possess the cellular machinery to augment EM2 release via MOR, but lack the testosterone to mobilize this mechanism. Lastly, androgenization of female pups (via a bolus injection of testosterone resulting in gonadal ablation) followed by chronic testosterone in adulthood also failed to enable sufentanil facilitation of spinal EM2 release in females. Collectively, these results suggest that the molecular and cellular elements required for MOR-mediated facilitation of spinal EM2 release are hard-wired and presumably sex-linked.

The present study demonstrates sexual dimorphism in the mechanisms regulating spinal EM2 release as well as their hormonal activation. In males, testosterone is a permissive factor, enabling MOR-mediated facilitation of spinal EM2 release, whereas in females, spinal MOR is functionally disconnected from spinal EM2 release, independent of both estrogens and testosterone. These findings bolster previous evidence that steroidal regulation of the MOR/EM2 system is sexually dimorphic. For instance, pregnancy levels of progesterone and estradiol activate MOR-coupled antinociception in orchiectomized males but not ovariectomized females,⁵⁵ and EM2 antinociception is constant in males but fluctuates with circulating estrogen levels (i.e., the estrous cycle) in females.⁵⁶

Despite the evidence for sexual dimorphism in nociception and opioid antinociception, sex-specific pharmacotherapies for acute and chronic pain remain elusive. One impediment to improving pain management is that sex differences in nociception and opioid antinociception across human and animal studies are often antithetical. The present

demonstration of agonist-dependent and sexually dimorphic contributions of endogenous EM2 to spinal opioid antinociception in rats provides a conceptual framework for investigating the biology of sex-based differences in pain and opioid analgesia in humans. This could enlighten the selection of opioid medications for pain management. Furthermore, the current study identifies a compromised functionality in females (attenuated ability to harness spinal EM2 antinociception), which could serve as a novel pharmacological target for pain management in women.

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- We studied contributions of spinal endomorphin to opioid analgesia in rats.
- Mu-opioids enhance spinal endomorphin release in males but not females.
- Released endomorphin reduces analgesia of high efficacy mu-opioids.
- Findings explain variable sex-dependence of opioid antinociception.
- Findings could inform the selection of opioid analgesics in humans.

Figure 1.

Enhancement of spinal EM2 release by sufentanil was sexually dimorphic. Spinal EM2 release increased 30–40 min after i.t. sufentanil in males but not diestrus or proestrus females. The spinal i.t. space was perfused at 5 μ L/min using the push-pull method as described in *Materials and Methods*. Drugs were administered via the inflow spinal cannula. The EM2 content of i.t. perfusate was quantified using a competitive radioimmunoassay. Data are expressed as fmol EM2 per 10-min period. * $p < 0.01$ for EM2 release in males 30–40 min after i.t. administration of sufentanil. $n = 6-9$.

Figure 2.

Facilitation of spinal EM2 release in males by sufentanil was MOR-mediated. The opioid antagonist naloxone (NX) did not itself alter EM2 release but prevented facilitation of EM2 release by sufentanil (SUF). Data are expressed as fmol EM2 per 10-min period. The basal rate of EM2 release did not differ significantly among groups ($F_{2,14} = 1.65$; $p = 0.23$). * $p < 0.01$ for EM2 release vs. basal in males 30–40 min after i.t. administration of sufentanil. $n = 4-9$.

Figure 3.

I.t. application of anti-EM2 antibody revealed agonist-dependent contributions of spinal EM2 to the antinociception produced by either i.t. sufentanil or i.t. morphine in males but not females. Since data obtained in diestrous and proestrous females did not differ, data is shown for only diestrous females. Thirty minutes following the i.t. application of anti-EM2 antibody (330 ng) or its preadsorbed control, i.t. sufentanil (0.35 nmol) or morphine (7.5 nmol) was injected and antinociception quantified at various time points. I.t. application of anti-EM2 antibody failed to alter spinal sufentanil or morphine antinociception in diestrous females (left panels, A and B). In contrast, in males, i.t. anti-EM2 antibody, but not its preadsorbed control, substantially increased spinal antinociception of sufentanil but significantly attenuated the spinal antinociception of morphine (right panels, A and B). Data are expressed as mean $\pm$ S.E.M. $n = 4-5$ and $9-12$ for sufentanil- and morphine-treated groups, respectively.