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Sex-/Ovarian Steroid-Dependent Release of Endomorphin 2 from Spinal Cord[§]

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Abstract

Mu-opioid receptor (MOR) agonists have been shown to be more potent analgesics in male than female rodents. Regulation of spinal MOR-coupled antinociception by 17β-estradiol (estrogen, E_2) and progesterone (P) is also sexually dimorphic; pregnancy levels of E_2/P activate MOR-coupled analgesic pathways in male but not female rats. We hypothesized that the sexual dimorphic characteristics of MOR-coupled antinociception reflects sexual dimorphism in the regulation of the release from spinal cord of the endogenous MOR agonist, endomorphin 2 (EM2). Parameters of spinal EM2 release manifesting sexual dimorphism include its (1) magnitude: *in vitro* basal and K^+-evoked release of EM2 from spinal tissue of male rats is approximately 50% greater than that observed from spinal cord of females; (2) modulation by ovarian sex steroids: E_2/P treatment significantly enhanced K^+-evoked EM2 release from spinal tissue of males, but not females; (3) enhancement by opioid receptor blockade: naloxone enhanced stimulated EM2 release from spinal tissue of both males and females but it augmented basal release from spinal tissue of only males. Enhancement of EM2 release by naloxone reflects negative coupling of MOR to EM2 release and hence its modulation by negative feedback since only activation of MOR, not κ- or δ-opioid receptors, was able to inhibit evoked EM2 release. These data reveal that the EM2-MOR spinal analgesic system is more robust and 'higher gain' in male vs. female rodents. These findings could provide a mechanistic rubric for understanding the male female dichotomy in prevalence and intensity of chronic pain syndromes.

Introduction

Multiple observations suggest that spinal μ-opioid receptor- (MOR-) coupled analgesic pathways are sexually dimorphic. Males are more sensitive than are females to the antinociceptive properties of the potent MOR agonist, alfentanil (Cicero et al., 1997). Opioids with activity at MOR, including levorphanol, dezocine, buprenorphine, butorphanol, nalbuphine, and morphine are also generally more potent and/or effective in males using mechanical nociceptive stimuli (Barrett et al., 2002). Notably, sex-dependent differences in morphine antinociception have been reported despite the absence of sex-linked differences in morphine serum levels at the time of its peak MOR-mediated antinociceptive effects (Cicero et al., 1996), (Boyer et al., 1998; Cicero et al., 1996; Cook et al., 2000; Krzanowska and Bodnar, 1999; Peckham et al., 2005; Peckham and Traynor, 2006). This suggests that such differences most likely result from intrinsic sex-related divergence in the sensitivity of the CNS to MOR activation (Cicero et al., 1996).

Regulation of spinal MOR-coupled antinociception by ovarian sex steroids is also sexually dimorphic. In both rats and humans, gestation is accompanied by an opioid antinociception (Cogan and Spinnato, 1986; Gintzler, 1980) that is mediated via spinal opioid systems (Dawson-Basoa and Gintzler, 1998). In rats, the spinal pathways activated during gestation are also those that are activated by pregnancy levels of ovarian sex steroids (Dawson-Basoa and Gintzler, 1998). Interestingly, although ovarian sex steroids regulate spinal opioid systems in both male and female rats, the nature of the regulation differs (Dawson-Basoa and Gintzler, 1998; Liu and Gintzler, 2000). Ovariectomized female rats treated with pregnancy levels of estrogen (17-β-estradiol, E2) and progesterone (P) manifest an antinociception that is mediated by spinal κ- and δ-opioid receptors (KOR and DOR, respectively) (Dawson-Basoa and Gintzler, 1997; Dawson-Basoa and Gintzler, 1998; Medina et al., 1993). However, spinal MOR, not DOR

is a major component of the antinociception that results from analogous ovarian steroid treatment of orchidectomized male rats (Liu and Gintzler, 2000). This suggests that in male, but not in female rats, pregnancy levels of E_2/P activate MOR-coupled analgesic pathways.

Many endogenous opioid peptides, e.g., enkephalins, dynorphins, and endomorphins, can interact with and activate MOR. Of these, the endomorphins have the highest affinity and discrimination for MOR. Endormorphin 1 (EM1; Tyr-Pro-Trp-Phe-NH2) and endomorphin 2 (EM2; Tyr-Pro-Phe-Phe-NH2) have a 4,000- and 15,000-fold preference for MOR over DOR and KOR, respectively), as well as very high affinity for MOR (Ki ≈360 pM). This is consistent with their putative role as endogenous activators of MOR (Zadina et al., 1997). EM1 and EM2 also produce potent antinociception following intracerebroventricular (Soignier et al., 2000; Zadina et al., 1997) or intrathecal (i.t.) administration (Sakurada et al., 2000), the latter being abolished by the selective MOR antagonist beta-funaltrexamine (Sakurada et al., 2000). These observations underscore the relevance of endomorphins to endogenous MOR-coupled antinociceptive pathways.

The CNS distribution of endomorphins and their opioid receptor type selectivity profiles have been extensively studied, but elucidation of neural regulators of the release of endomorphins and in particular the sex-dependence of this regulation has not been assiduously pursued. Based on the reports noted above of sexual dimorphic MOR-mediated antinociception, we hypothesized that regulation of the release of EM from spinal tissue would also manifest sexual dimorphism. To test this hypothesis, we quantitatively compared basal and evoked release of EM2 from spinal tissue obtained from untreated and hormone-treated male and female rats, and the opioid receptor-coupled regulation thereof. Release of EM from spinal cord was studied since it was this region that mediated the previously reported (Liu and Gintzler, 2000) sex-dependent regulation by ovarian steroids of MOR antinociception. Attention was focused on

quantifying the release of EM2 (as opposed to EM1) since it is the predominant endomorphin species in spinal cord (Martin-Schild et al., 1999). Results demonstrate a significantly enhanced robustness of spinal EM2 release from spinal tissue of male vs. female rats and sexually dimorphic regulation by ovarian sex steroids. The relationship of these findings to sex-dependent differences in the modulation of and responsiveness to nociceptive stimuli is discussed.

Methods

Experimental Animals

Experiments employed male and female Sprague-Dawley rats (Charles River, Kingston, NY; 250-300 g, which were maintained in an approved controlled environment. Food and water were available ad libitum. Surgeries were performed under sodium pentobarbital anesthesia (40 mg/kg, i.p., Anpro Pharmaceutical). All experimental procedures were reviewed and approved by the Animal Care and Use Committee of SUNY Downstate Medical Center.

Ovariectomy and orchiectomy

Female rats were ovariectomized by parting the posterolateral abdominal muscle layers by blunt dissection to expose a thick fat deposit that contained the ovary. The ovarian bundles were tied off with 4-O silk sutures, excised, and removed from the body cavity (Wayneforth and Flecknell, 1992). The fascia and skin were separately closed with 5-O silk suture. For orchiectomy, making a single cutaneous incision through the scrotal sac and entering the peritoneal cavity exposed testes. The testicular bundles were ligated with 4-O silk suture and the testes removed. The cutaneous incision was closed with 5-O silk suture (Wayneforth and Flecknell, 1992). Ovarian sex steroid treatment was initiated at the time of ovariectomy and orchiectomy.

Ovarian Sex Steroid Administration

The pregnancy blood concentration profile of E_2 and P were simulated in non-pregnant, ovariectomized rats (hormone simulated pregnancy; HSP) and orchiectomized male rats via the subcutaneous implantation of Silastic tubing filled with either a solution of E_2 in sesame oil or crystalline P (Bridges, 1984). Day one of steroid hormone administration or its vehicle control was initiated at the time of ovariectomy or orchiectomy. Pregnancy-like levels of E_2 and P were achieved by changing the concentration of E_2 in the tubing (10 mm tubing/100 g bw) and by

altering the number of 45 mm P implants on days 5, 15 and 19 (see (Bridges, 1984; Bridges and Ronsheim, 1987) for details of implantation procedure and comparison with steroid plasma levels of physiological gestation).

Spinal Tissue Preparation

The spinal vertebral column was sectioned at the intervertebral spaces above vertebrae T-12 and L-1. The lumbar spinal cord contained within this segment (L-1 to L-5; 200-250 mg) was quickly expelled by injecting ice cold saline into the caudal end, minced using a McIliwain Tissue Chopper (Mickle Laboratory Engineering Co. U.K; 0.3 mm thickness), placed into a chamber (0.35 ml) and superfused (Brandel Superfusion System, Gaithersburg, MD). The Krebs' solution used for superfusion contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO4, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.1 mM dextrose, gelatin (saturated with 4 g/l) and gassed with a 95% O_2/5% CO_2 gas mixture. Additionally the Krebs' superfusate used to assess basal and stimulated EM2 release contained the protease inhibitors captopril (10 μM), thiorphan (0.3 μM), bestatin (10 μM) and) L-leucyl-L-leucine (2 mM) to protect peptides against the degradation resulting from the actions of the tissue proteases.

Superfusion Paradigm

The magnitude of stimulated EM2 release was determined by quantification of the rate of EM2 release into spinal superfusate that contained high potassium (K^+; 50 mM; the content of sodium was proportionally reduced to maintain osmolarity). High K^+-evoked release of EM2 was determined over a 3 min period (1.8 ml). This constituted the first cycle of release. Following stimulation with high K^+, a 15 min rest period ensued before re-determining basal and evoked release while in the presence of D-Pen^2,D-Pen^5-enkephalin (DPDPE, δ-opioid receptor agonist), U50,488H (10, 1000 nM; κ-opioid receptor agonist); sufentanil (1, 10, 100, 1000 nM; μ-

opioid receptor agonist); naloxone (1 μM) or nociceptin/ orphanin FQ (N/OFQ; 10, 100, 1000 nM). Responses to N/OFQ were determined with or without blockade of its receptor, ORL1, via 10 μM compound 15, a derivative of the ORL1 receptor antagonist J-113397 that does not contain a hydroxymethyl group on the piperidine ring) (Kawamoto et al., 1999). The effect of compound 15, alone, on basal and evoked release was separately determined. Determination of EM2 release in the presence of selected pharmacological agents constituted the second release cycle. In all cases, the magnitude of K^+-evoked release in the presence of drug (cycle 2) was compared with the magnitude of release observed in its absence (cycle 1). We subtracted release in the absence of K^+ (basal release; B), from the total release while in the presence of K^+ (stimulated release; S) in order to calculate the increment in evoked release (S-B). Drug-induced differences in S were calculated by subtracting the value obtained in cycle 2 from that obtained in cycle 1. A third cycle of release was obtained in which the conditions of cycle 1 was repeated In order to ensure that changes in basal and/or stimulated release of EM2 were attributable to the presence of drug, and not simply to the passage of time. Drug effects were always reversed following washout and re-equilibration (cycle 3).

Basal and stimulated superfusate were collected into pre-chilled tubes on ice. Superfusate containing basal release and evoked release were desalted and concentrated using reverse phase C-18 cartridges (Sep-Pak™; Waters Corp., Milford, MA). EM2 peptide eluted with 70% acetonitrile/0.1% trifluroacetic acid (TFA) was lyophilized to dryness and stored (4°C). Recovery of EM2 was quantitative (>95%). K^+-evoked release of EM2 could be elicited over three cycles, with a 30 min rest interval between exposures to high K^+, without observing a significant diminution in the magnitude of release (±10%)

Radioimmunoassay

EM2 was quantified in spinal superfusate using a radioimmunoassay (RIA) that employed a rabbit antibody (1:10,000) highly specific for this peptide (generously supplied by James Zadina). A standard curve (0.5-16 pg/assay tube) in which the percentage of inhibition of binding was plotted against the log concentration of unlabeled EM2 in the reaction tube was generated in each assay. Bovine serum albumin (0.1%) was included in the assay buffer to minimize nonspecific adherence to the tube surface. Samples were pre-incubated with anti-EM2 antibody for 48 h (4°C) after which 10,000-12,000 cpm ^{125}IEM2 (607 Ci/mmole; Peninsula Laboratories Inc., CA) was added and the reaction mixture incubated overnight (4^{o}C), Antibody-bound radioactivity was quantified using scintillation proximity. The reaction mixture was transferred to a 96 well plate that has been coated with sheep anti-rabbit antibody and impregnated with scintillant (Flashplate^{TM}, Perkin Elmer). Formation of a rabbit antibody, ^{125}IEM2 and sheep anti-rabbit-complex brings the ^{125}IEM2 into proximity with the scintillant-impregnated plate bottom, enabling quantification of the antibody-bound tracer. In contrast, non-antibody bound radioactivity is not brought into sufficient proximity with the scintillant to be detected thus does not confound quantification of antibody-bound radioactivity. Plates were counted by Microbeta Jet Counter (Perkin Elmer) after incubation for additional 12 hours at 4^{o}C. Values of experimental samples were calculated from the standard curve using the 'forecast function' of Microsoft Excel. The minimum detectable concentration ranged from 0.6 to 1.5 pg/assay well, which produced ~20% inhibition of maximum binding. A 50% reduction in binding was produced by 11-12 pg/assay well. Peptide concentrations were derived from RIA analyses of superfusate that produced between 20-75% inhibition of binding, the linear and sensitive portion of the standard curve. All standard and experimental samples were run in triplicate and duplicate, respectively.

The chemical identity of EM2-like immunoreactivity was analyzed by combining high-pressure liquid chromatography fractionation (HPLC) with RIA detection. Spinal superfusate (from 2 lumbar spinal preparations) were collected under basal and stimulated conditions. The EM2 peptide contained therein was desalted and concentrated using reverse phase C-18 Sep-Pak^{TM} cartridges as described above. The 70% acetonitrile-0.1% TFA eluate was lyophilized to dryness, resuspended in 40 µl of 1% acetonitrile-0.1% TFA and centrifuged (500 x g for 5 min). Fractionation by HPLC was accomplished by applying supernatant (20 µl) or the same volume of standard peptide onto a 15 cm C-18 column (5 µm, Vydac). The column was eluted at a flow rate of I ml per min with a mobile phase containing 0.08% TFA throughout and a linear gradient of acetonitrile ranging from 4% at the start to 70% at 28.6 min. These chromatographic conditions permit authentic EM2 to be separated from other opioid peptides (methionine- and leucine-enkephalin, α-endorphin, β-endorphin, γ-endorphin, N-acetylated β-endorphin, dynorphin 1-17). HPLC eluates (1 ml fractions) were lyophilized to dryness and the content of EM2 was determined by RIA as described above. Approximately 89% of the EM2-like immunoreactivity that was contained in spinal tissue superfusate had a retention time comparable to that of standard EM2. The data shown have not been corrected for recovery.

Statistical Analyses

Factorial ANOVAs [General Linear Model (GLM)] were used to assess the effect of sex, release condition (basal, K^+-evoked), hormone treatment and drugs on EM2 release. Additionally, post hoc tests (least significant difference test) were used to further examine significant effects of the ANOVAs. Log-transformed data were used for all analyses in order to minimize the effect of any skew in the data set.

Drugs

Compound 15 was synthesized at SRI International (Menlo Park, CA) and provided to us by Larry Toll. D-Pen^{2}D-Pen^5 enkephalin, U50,488H, naloxone, nociceptin (Orphanin FQ) and sufentanil were provided by the National Institutes of Drug Abuse.

Results

In order to assess the interaction between sex, release condition, (basal, K^+-evoked) and hormone treatment, we performed a three-way ANOVA where the first factor of the ANOVA was release condition (basal K^+-evoked), the second factor was hormone treatment (untreated, E_2/P-treated) and the third factor was sex. Significant main effects and interactions were found for all factors. Most notably, a significant 3-way interaction was found between sex, hormone and release condition ($F_{1,129}$=15.9, $p<0.001$). The post hoc component of this analysis revealed that the rates of basal and K^+-evoked EM2 release from spinal tissue of male rats was significantly greater than from females (basal: 4.58+0.06 pg/6 min vs. 2.98+0.05 pg/6 min, respectively; K^+-evoked: 8.64±0.17 pg/6 min vs. 5.76±0.11 pg/6 min, respectively; $p<0.001$ for both comparisons) (Fig. 1, compare panels A and B). Additionally, the rate of K^+-evoked release, but not basal release, of EM2 from spinal cord of hormone-treated male rats was significantly greater ($p<0.001$) than that from control male rats (K^+-evoked: 8.64±0.17 pg/6 min vs. 10.53±0.27 pg/6 min; Fig.1A). However, in spinal tissue obtained from female rats, hormone condition affected neither basal nor evoked EM2 release ($p>0.3$ for both comparisons; Fig. 1B).

Sex dependency of opioid receptor-coupled regulation of EM2 release. We performed a 3-way ANOVA to examine the effects of naloxone (absence or presence), on the rate of EM2 release (basal, K^+-evoked) within the context of sex. The 3-way interaction was not significant

(p=0.87). However, main effects and all 2-way interactions were significant (p<0.02). Since the 2-way interaction between naloxone and release condition was significant and the 3-way interaction, which is the same analysis with the additional factor of sex, was not, the interaction between naloxone and release condition is not sex-dependent. This was examined in greater detail using post hoc tests (least significant difference test). Post hoc comparisons indicated that naloxone had no effect on basal release from spinal cord of females (p=0.115). In contrast, naloxone significantly enhanced the rate of basal release (~0.95 pg/6 min) from spinal cord of males (p<0.001) (Fig. 2A). Unexpectedly, the stimulated increment in the rate of EM2 release from spinal tissue of females was significantly enhanced by naloxone, as was also observed in spinal tissue of males (p<0.001 for both) (Fig. 2B). It should be noted that in spinal tissue obtained from males, the enhancement of the stimulated increment in the rate of EM2 release produced by naloxone was greater than its effect on basal release (3.7 pg/6 min vs. 0.95 pg/6 min; Fig. 2A). Thus, the ability of naloxone to enhance EM2 release is dependent on release condition in males as well as females.

We further investigated whether or not the magnitude of effect of naloxone on stimulated EM2 release from spinal tissue of males was influenced by hormone treatment. Once again, significant main effects of naloxone (($F_{1,6}$=754, p<0.001), and hormone treatment ($F_{1,6}$=1262, p<0.001) were observed. Most importantly, the interaction between hormone treatment and naloxone was significant ($F_{1,6}$=16.8, p=0.006). In other words, the ability of naloxone to enhance stimulated release of EM2 is influenced by the E_2/P state of male rats (4.6 pg/6 min vs. 8.2 pg/6 min) (Fig. 2C). In contrast, analogous assessment of the influence of E_2/P on naloxone enhancement of stimulated EM release from spinal cord of female rats indicated significant main effect only for naloxone ($F_{1,7}$=63.3, p<0.001). There was no significant interaction between naloxone and hormone treatment.

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Modulation of K^+-evoked EM2 release by opioid receptor type-selective agonists. A three-way ANOVA was used to examine the effect of the MOR-selective agonist sufentanil on stimulated EM2 release from spinal tissue obtained from male and female rats. Factors were sex, drug and dosage. Significant main effects were found for sex ($F_{1,20}$=71.0, p<0.001), drug ($F_{1,20}$=52.9; p<0.001) and dosage ($F_{3,20}$=5.3; p<0.007). No significant drug X sex interactions were found ($F_{1,20}$=1.8; p=0.19). To formally analyze dose responsiveness of the inhibition by sufentanil of K^+-evoked EM2 release, we used GLM to perform regression and estimation of slopes for data obtained from spinal tissues of male and female rats. There was a linear dose response function, the slopes of which did not differ between spinal tissue of male and female animals (Fig. 3).

In order to assess the effect of DOR- and KOR-selective selective agonists on stimulated EM2 release, the ability of D-Pen^2D-Pen^5 enkephalin (DPDPE) and U50,488H, respectively, to inhibit EM2 release was investigated. For DPDPE, a 2-way ANOVA failed to reveal any significant main effects or interaction effects for drug and dosage in spinal tissue of male and female rats. Similar results were found for U50, 488H (data not shown).

N/OFQ enhances K^+-evoked EM2 release. A 3-way ANOVA revealed significant main effects for N/OFQ ($F_{1,15}$=104; p<0.001), and sex ($F_{1,15}$=60.8; p<0.001) but none for dosage, and none for the interaction between drug and sex. The enhancement of EM2 release by N/OFQ was not dose-dependent and did not differ between spinal tissue of male and female rats (Fig. 4). A 2-way ANOVA was subsequently performed to assess the effect of N/OFQ on basal and evoked EM2 release. Significant main effects were found for release condition (basal, K^+-evoked) ($F_{1,80}$=99.4; p<0.001) and drug (presence or absence) ($F_{1,80}$=6.65; p=0.012) but no interaction between drug and release condition was detected. However, post hoc analyses revealed that

basal release was not influenced by the presence of N/OFQ but N/OFQ did augment K^+-evoked EM2 release (≈30%; $p<0.005$).

Importantly, in the presence of compound 15, an ORL1 receptor antagonist, the enhancing effect of N/OFQ on K^+-evoked EM2 release was no longer observable. A 2-way ANOVA with N/OFQ (presence, absence) and compound 15 (presence, absence) was performed for the stimulated condition. The main effect for N/OFQ and compound 15 was not significant. However, a significant interaction effect between N/OFQ and compound 15 was observed ($F_{1,15}=7.19$; $p=0.017$) indicating that this ORL1 receptor antagonist modulated (abolished) the effect of N/OFQ (data not shown). An analogous ANOVA was performed to assess the effect of compound 15 on the release condition. The interaction between these two factors was not significant ($F_{1,20}=0.56$; $p=0.46$). Moreover, post hoc tests demonstrated no effect of compound 15 on either basal or evoked EM2 release.

Discussion

The present results demonstrate for the first time sexually dimorphic functionality of the spinal EM2 / MOR opioid analgesic pathway. Parameters of spinal EM2 release manifesting sexual dimorphism include its (1) magnitude (2) modulation by E_2/P and (3) enhancement by opioid receptor blockade. In the aggregate, these data indicate the presence in males of a more robust and dynamic endogenous MOR-coupled spinal antinociceptive system. This parallels the earlier reports of increased magnitudes of analgesia in male vs. female rodents that results from the exogenous activation of MOR.

The magnitude of *in vitro* basal and K^+-evoked release of EM2 from spinal tissue of male rats is approximately 50% greater than that observed from spinal cord of females. This would add to or synergize with enhanced analgesic consequences of MOR-coupled signaling in males vs. females that is reflected by the well-established greater antinociceptive potency of MOR agonists in male vs. female rodents (Barrett et al., 2002; Boyer et al., 1998; Cicero et al., 1996; Cicero et al., 1997; Cook et al., 2000; Krzanowska and Bodnar, 1999; Peckham et al., 2005; Peckham and Traynor, 2006). It remains to be established whether or not inferences from data derived from rat spinal tissue generalizes to men and women. This notwithstanding, it is tempting to speculate that sex-dependent differential activity of the spinal EM2/MOR-coupled opioid systems could be a contributory component of the greater prevalence and intensity of chronic pain syndromes in women vs. men. Clearly, additional studies will be required to validate this hypothesis but the current observations could provide a mechanistic framework for beginning to understand the sex-based dichotomy in the chronicity of painful experience in humans.

Sexual dimorphism in the frequency and severity of chronic pain has been very well documented in women. Women are more likely than men to experience recurrent pain, more severe levels of pain and pain of longer duration (Unruh, 1996). Such conclusions are supported using thermal (Fillingim et al., 1998), electrical (Walker and Carmody, 1998) and pressure (Ellermeier and Westphal, 1995) nociceptive stimuli. There is also evidence of sexually dimorphic pain and analgesic mechanisms in laboratory animals (Kayser et al., 1996; LaCroix-Fralish et al., 2005; Liu and Gintzler, 2000; Mogil and Chanda, 2005; Mogil et al., 1993). Current findings underscore the relevance of sexual dimorphic spinal release of EM2 to understanding sex-based pain and analgesic mechanisms on mechanistic levels.

The second notable outcome of this study is the paradoxical finding that the pregnancy blood profile of E_2/P significantly enhanced K^+-evoked EM2 release from spinal tissue of males, but not females. These results are consistent with and provide a neurochemical basis for the earlier demonstration by this laboratory (Dawson-Basoa and Gintzler, 1996; Liu and Gintzler, 2000) that although simulation of the pregnancy blood concentrations of E_2/P elicits an analogous spinal opioid antinociception in ovariectomized female and orchidectomized male rats, the mechanistic underpinnings of the antinociception are dependent on sex (Dawson-Basoa and Gintzler, 1998; Liu and Gintzler, 2000). The opioid antinociception elicited by pregnancy blood levels of E_2/P in ovariectomized female rats is mediated by spinal KOR and DOR; contributions of MOR cannot be detected (Dawson-Basoa and Gintzler, 1996). In contrast, MOR (together with KOR) mediates the antinociception manifest in orchidectomized males following analogous steroid treatment (Liu and Gintzler, 2000). The current demonstration of the ability of E_2/P to augment *in vitro* evoked release of spinal EM2 in males but not females is, most likely, a major determinant of the previously reported (Liu and Gintzler, 2000) sex-based differential activation by ovarian sex steroids of spinal MOR-coupled antinociception.

There is precedent for the ability of E_2 and/or P to produce physiological effects in males. For example, P has been shown to enhance testicular secretion (Kalra and Kalra, 1980). Additionally, E_2 treatment has been shown to decease levels of several neurotransmitters in brain tissue consisting of medulla, pons, midbrain, thalamus and hypothalamus (Das and Chaudhuri, 1995). More recently, acute treatment with E_2 was shown to increase the concentration of mRNA encoding the serotonin$_{2A}$ receptor in the dorsal raphe nucleus as well as the density of serotonin$_{2A}$ receptor binding sites in numerous brain areas (Sumner and Fink, 1998). Moreover, consonant with current observations that E_2/P produces similar antinociception in males and females, sequential treatment with E_2/P followed by withdrawal of P has been shown to elevate mRNA encoding oxytocin in the paraventricular nucleus of ovariectomized female as well as orchidectomized male rats (Thomas and Amico, 1996).

The ability of opioid receptor blockade to enhance basal and stimulated release of EM2 indicates its negative modulation by an endogenous opioid(s). Notably, basal release from only spinal tissue of males was enhanced by naloxone. It is unlikely that this sex difference reflects sex-based anatomical differences in localization of MOR on EM2 perikarya and/or terminals since naloxone was able to enhance K^+-evoked release of EM2 from spinal cord of female as well as male rats. It is more probably that the magnitude of facilitation of EM2 release by naloxone is related to the magnitude of opioid receptor activation and thus the magnitude of the release of some endogenous opioid. The ability of MOR activation, (but not activation of DOR or KOR), to inhibit release of EM2 implies that EM2 release is subject to feed back inhibition, which is relieved by naloxone.

In this formulation, the magnitude of enhancement of EM2 release by naloxone would be proportional to the magnitude of the release of EM2 itself. This is supported by the observation that the magnitude of facilitation by naloxone of K^+-evoked release of EM2 from spinal tissue of

E_2/P-treated males is larger than from spinal tissue of untreated males, which in turn is larger than the enhancement of basal EM2 release. Thus, it is likely that sex-dependent differential regulation of basal EM2 release by naloxone results from the greater magnitude of that release from spinal cord of males vs. females (4.58 vs. 2.98 pg/6 min, respectively); basal spinal release of EM2 in females may not be sufficient to achieve threshold concentrations required for pre-synaptic inhibition of EM2 release.

Inhibition of EM2 release by negative feedback inhibition and the MOR agonist sufentanil is consistent with the colocalization of EM2 and MOR in fibers in superficial laminae of the lumbar dorsal horn (Abbadie et al., 2002). Interestingly, release from spinal tissue of females and males was equally sensitive to sufentanil inhibition, which did not exceed 50%. It is not clear whether or not this inhibitory ceiling results from the presence of an EM2 pool that is resistant to MOR-coupled inhibition or if it is a consequence of a restricted distribution of MOR on EM2 neurons. In contrast to MOR, activation of either spinal KOR (via U50,488H) or DOR (via DPDPE) did not produce detectable alteration of EM2 release indicating the unique importance of MOR to the regulation of EM2 release.

We previously demonstrated the ability of exogenous and endogenous N/OFQ to act as a negative modulator of the evoked release of both spinal dynorphin and methionine-enkephalin (Gupta and Gintzler, 2003; Gupta et al., 2001). Thus, we were surprised to observe that exogenous N/OFQ facilitated evoked EM2 release from spinal tissue of both male and female rats. The stimulatory effect of N/OFQ on EM2 release from spinal cord could contribute to antinociceptive effects of i.t. N/OFQ in rats reported earlier (Jhamandas et al., 1998) and could also suggest a mechanism for the reciprocal regulation of the release of endogenous spinal opioids. Divergent regulation of spinal opioid release via the same agent is not unprecedented. For example, the negative modulation of spinal dynorphin release by N/OFQ is neutralized

following E_2/P treatment (Gupta et al., 2001), whereas the ability of N/OFQ to inhibit methionine-enkephalin release persists following identical treatment (Gupta and Gintzler, 2003). The independent and divergent regulation of the release of spinal opioid peptides would maximize the ability to fine-tune the relative contribution of each to antinociceptive processes.

In contrast to the dose-dependent modulation of spinal dynorphin and methionine-enkephalin release by N/OFQ (Gupta and Gintzler, 2003; Gupta et al., 2001), its modulation of EM2 release appeared to be an all or none step function. It is possible that modulation of EM2 release by N/OFQ that is dose-dependent could be obscured by its steepness and narrow range. Alternatively, the inability to demonstrate dose-dependency of its modulation of EM2 release could result from its complex physiology, e.g., N/OFQ inhibits two distinct groups of neurons in the brain stem to cause either a hyperalgesia, via the removal of μ-opioid analgesia, or analgesia (Pan and Hirakawa, 2000). Complex actions of N/OFQ can also include modulation of multiple parameters of the same system. N/OFQ (i.t.) can enhance the release of spinal substance P (SP) and thereby promote nociception but, at higher i.t. concentrations, it can also act postsynaptically to inhibit the actions of SP (Inoue et al., 1999). The current experimental paradigm does not have sufficient resolving power to resolve opposing actions of N/OFQ on EM2 release mediated by discrete spinal anatomical sites.

In summary, spinal EM2 release exhibits sexual dimorphism in its magnitude and regulation by ovarian sex steroids. Notably, release from spinal tissue of males is much more robust than is the release from spinal cord of females. The ability of opioid receptor (MOR) blockade to enhance basal release from spinal tissue of males but not females indicates that in males, spinal EM2 release is also more dynamic and 'higher gain', i.e., spinal EM2 release in males can be enhanced by either stimulation or disinhibition or combinations of both. These findings

could provide a mechanistic framework for beginning to understand the male female dichotomy in prevalence and intensity of chronic pain syndromes.

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Footnotes

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Legends for Figures

Fig 1. The influence of sex on the magnitude of basal and evoked release of EM2 and its modulation by E_2/P. Lumbar spinal tissue was obtained from untreated and E_2/P-treated male (n=32 and 9 respectively; panel A) and female (n=55 and 32 respectively; panel B) animals and processed as described under Materials and Methods. Eluates (collected over two, 6 min periods, 3.6 ml each, for basal release and a 3 min period, 1.8 ml, for stimulated release) were desalted, lyophilized, and assayed by RIA for EM2 immunoreactivity. Each bar represents the mean ±S.E.M. (pg/6 min). Basal and evoked EM2 release were 50% higher in spinal tissue obtained from male vs. female rats. Enhancing effect of E_2/P treatment on evoked EM2 release was only observed in males.*=p<0.001 for basal and K^+-evoked EM2 release from spinal cord of male vs. female rats; ∉ = p<0.001 for K^+-evoked EM2 release from spinal cord of control male vs. hormone-treated male rats. HSP=hormone simulated pregnancy.

F**ig 2.** Opioid receptor blockade enhances spinal release of EM2. Lumbar spinal tissue, obtained from male and female animals was processed, superfusate collected, and its content of EM2 determined by RIA as described under Materials and Methods. Basal (Panel A) and K^+-evoked (Panel B) of EM2 was obtained in the absence or presence of naloxone (1 μM). Each bar represents the mean ± S.E.M. (pg/6 min). Panel C illustrates that hormone treatment increases the naloxone-induced increment in K^+-evoked release from spinal cord of males but not females. In panels A and B, *= p<0.001 for the effect of naloxone on basal EM2 release from spinal tissue of males and on K^+-evoked release from spinal tissue of males and females. In panel C, *= p<0.001 for the comparison of the effect of naloxone on the increment in K^+-evoked rate of release of EM2 from spinal tissue of control male vs. hormone-treated males. The magnitude of enhancement of EM2 release produced

by naloxone depends on the release condition: naloxone enhancement of stimulated EM2 release from spinal tissue of steroid-treated males (8.2 pg/6 min) > effect on stimulated release from control males (4.6 pg/6 min) > effect on basal release (0.95 pg/6 min). NX=naloxone; HSP=hormone simulated pregnancy; (S-B)=difference between K^+-evoked and basal rates of release n=3-4.

Fig 3. Effect of MOR activation on K^+-evoked EM2 release. Lumbar spinal tissue was processed, superfusate collected, and its content of EM2 determined by RIA as described under Materials and Methods. Basal and K^+-evoked release of EM2, from spinal tissues of untreated male and female rats in the absence or presence of the indicated concentration of sufentanil. Each point represents the mean ± S.E.M. increment in the rate of EM2 release (pg/6 min). Sufentanil produced a dose-dependent inhibition of K^+-evoked release of EM2 that did not differ among spinal tissue obtained from male and female rats. (S-B)=difference between K^+-evoked and basal rates of release. n=3-4.

Fig. 4. N/OFQ enhances K^+-evoked release of EM2. Lumbar spinal tissue was processed, superfusate collected, and its content of EM2 determined by RIA as described under Materials and Methods. Basal and K^+-evoked release of EM2, was quantified from spinal tissue of untreated male and female rats in the absence or presence of the indicated concentration of N/OFQ. Each point represents the mean ± S.E.M. increment in EM2 release (pg/6 min). As shown in Fig. 1, K^+-evoked release of EM2 from spinal cord of males was greater than from spinal cord of females but both were similarly affected by N/OFQ. (S-B)=difference between K^+-evoked and basal rates of release. n=3-4.

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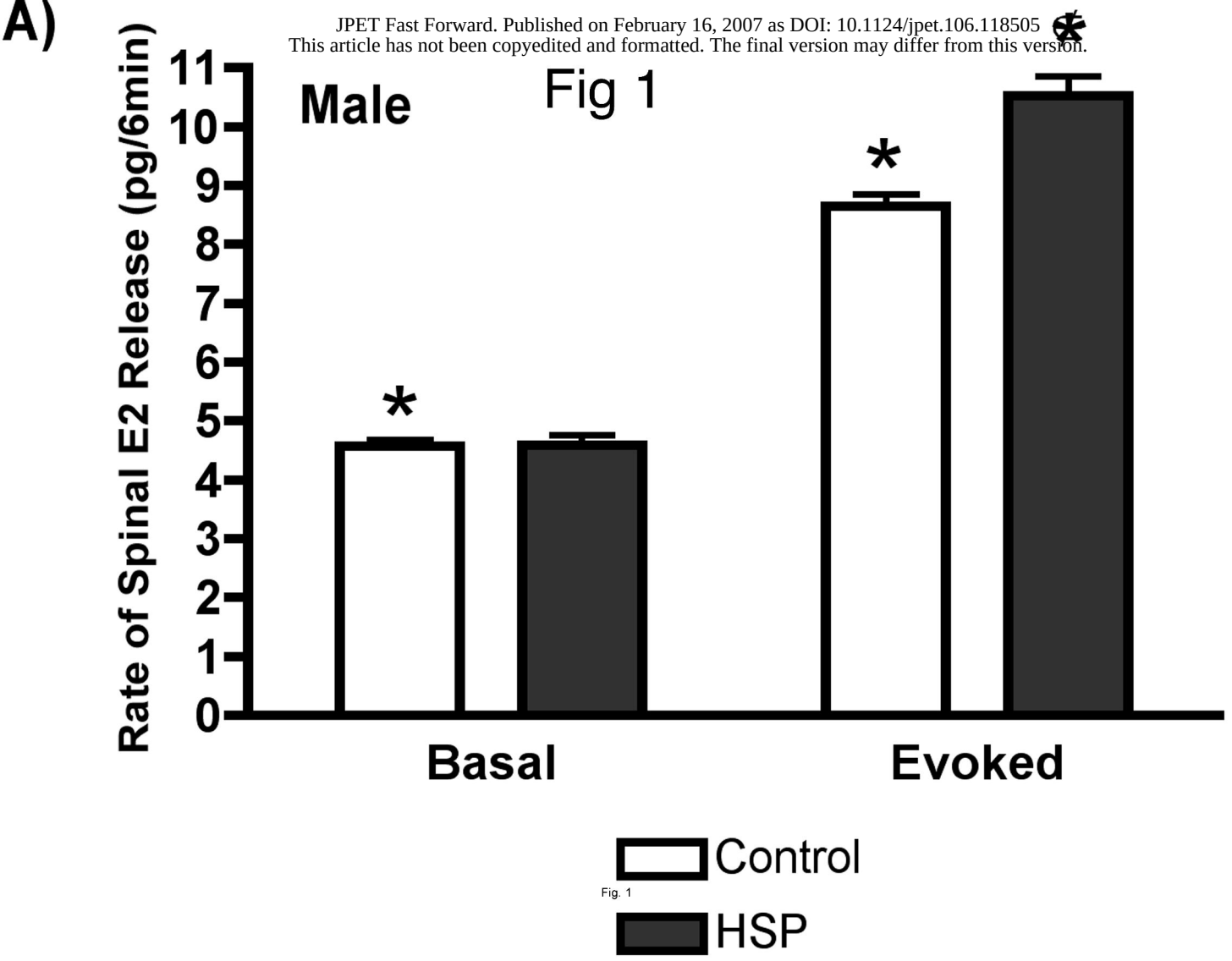


Fig. 1

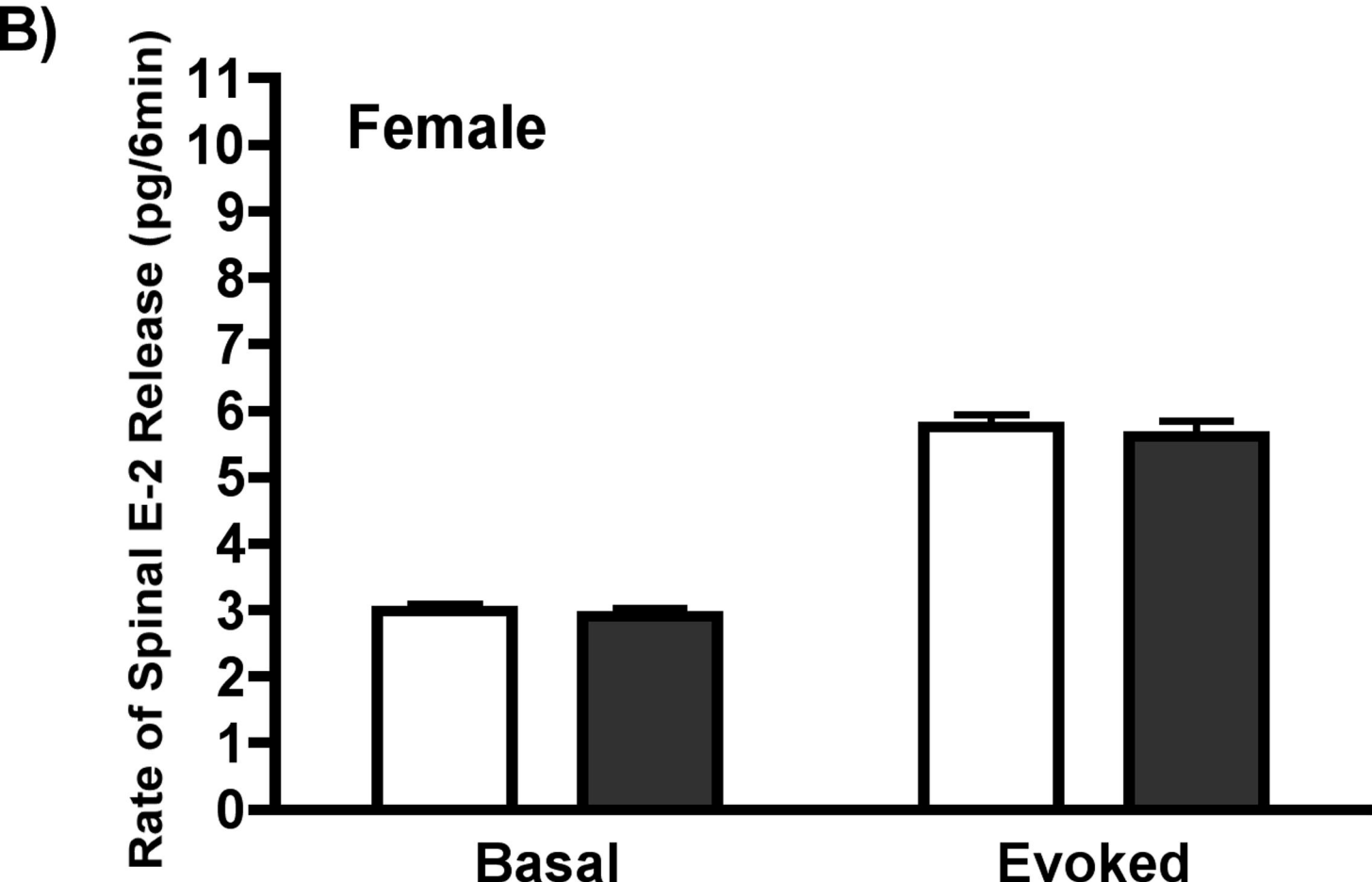
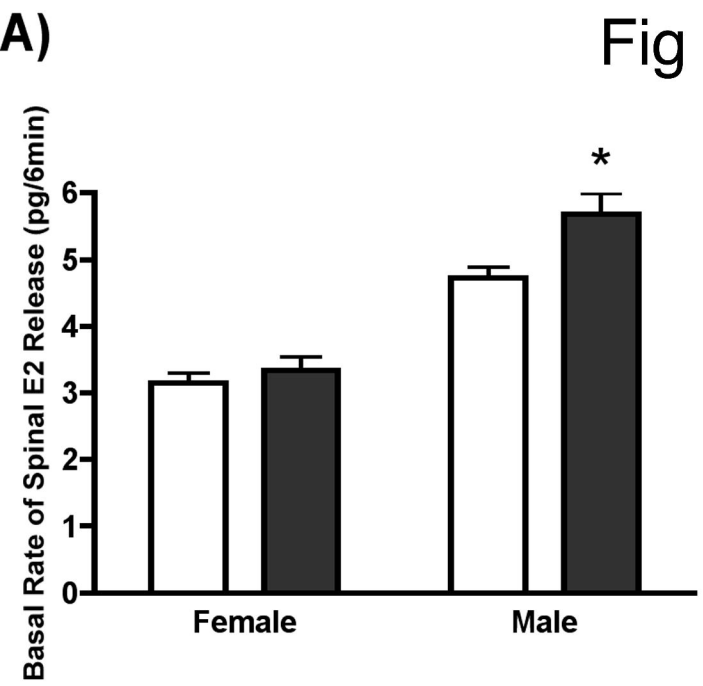
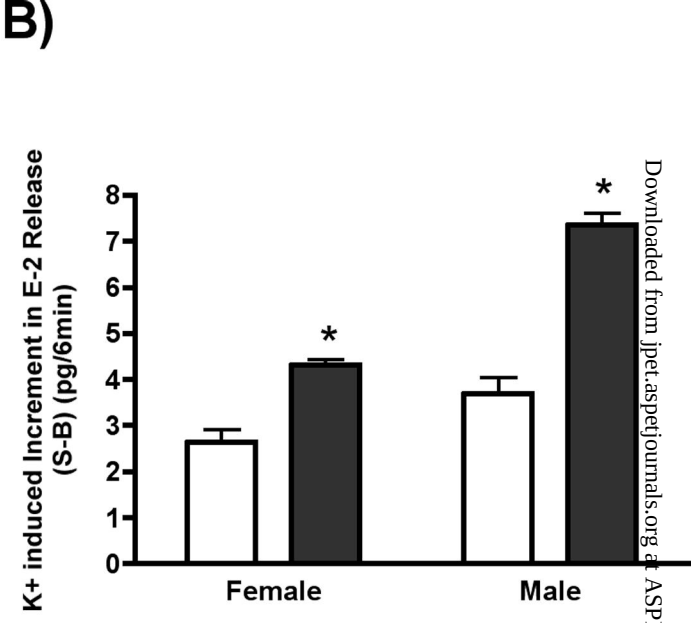
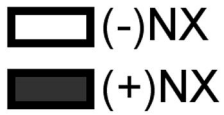


Fig 2







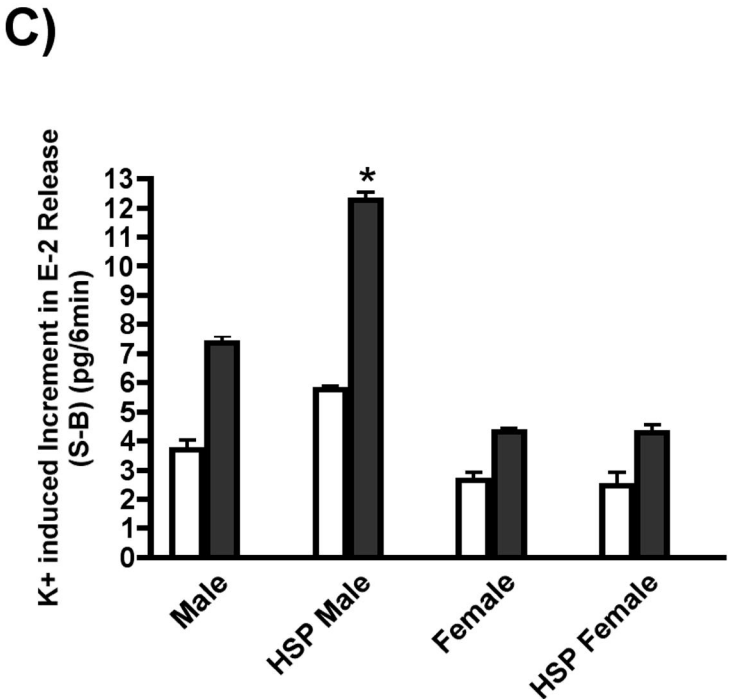


Fig 3

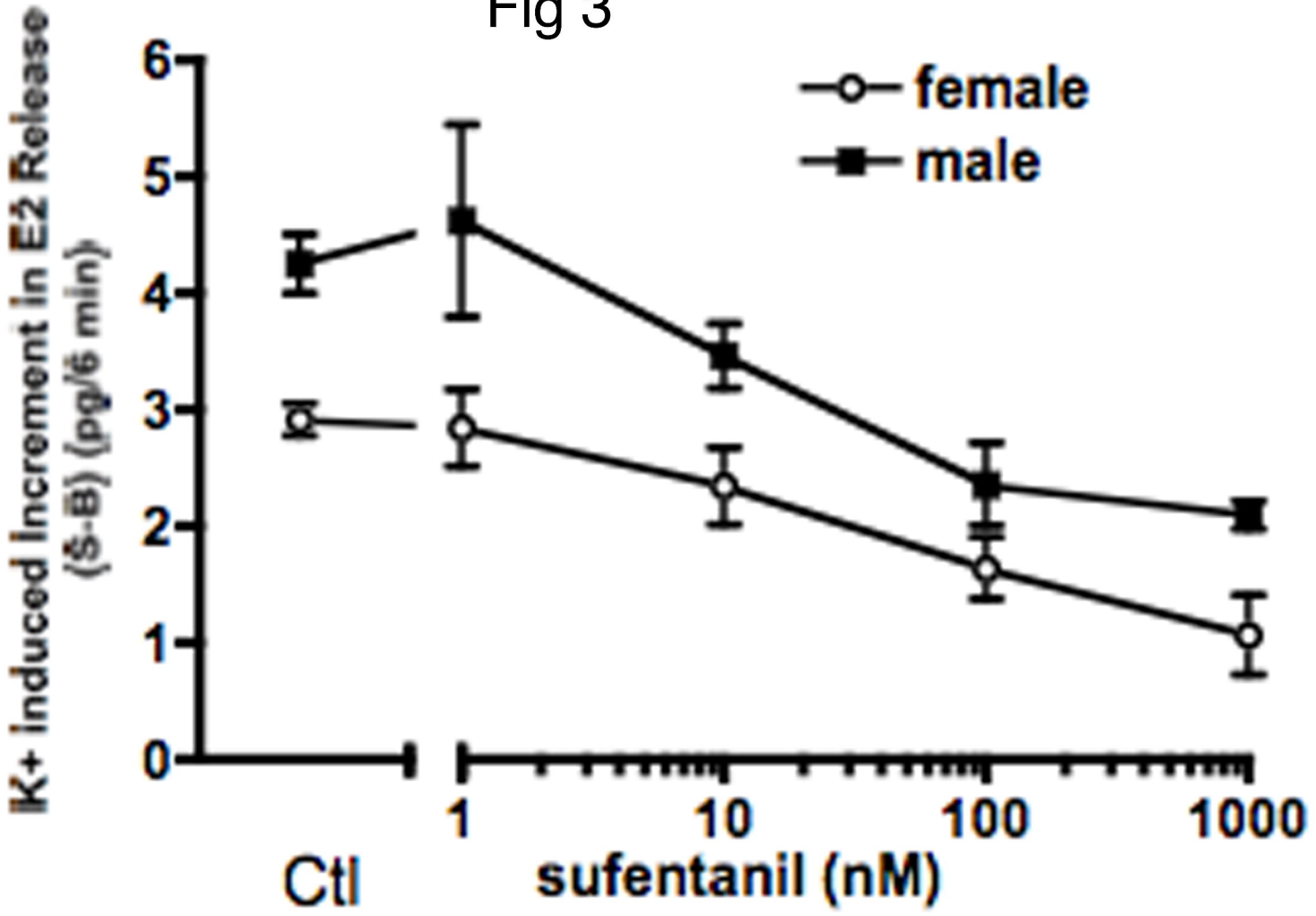

female
male
K+ Induced Increment in E2 Release
(S-B) (pg/6 min)
0
1
2
3
4
5
6
Ctl
1
10
100
1000
sufentanil (nM)

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Fig 4