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# Sex Differences in $\mu$ -Opioid Receptor Expression in the Rat Midbrain Periaqueductal Gray Are Essential for Eliciting Sex Differences in Morphine Analgesia

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Opioid-based narcotics are the most widely prescribed therapeutic agent for the alleviation of persistent pain; however, it is becoming increasingly clear that morphine is significantly less potent in women compared with men. Morphine primarily binds to  $\mu$ -opioid receptors (MORs), and the periaqueductal gray (PAG) contains a dense population of MOR-expressing neurons. Via its descending projections to the rostral ventromedial medulla and the dorsal horn of the spinal cord, the PAG is considered an essential neural substrate for opioid-based analgesia. We hypothesized that MOR expression in the PAG was sexually dimorphic, and that these sex differences contribute to the observed sex differences in morphine potency. Using immunohistochemistry, we report that males had a significantly higher expression of MOR in the ventrolateral PAG compared with cycling females, whereas the lowest level of expression was observed in proestrus females. CFA-induced inflammatory pain produced thermal hyperalgesia in both males and females that was significantly reversed in males with a microinjection of morphine into the ventrolateral PAG; this effect was significantly greater than that observed in proestrus and estrus females. Selective lesions of MOR-expressing neurons in the ventrolateral PAG resulted in a significant reduction in the effects of systemic morphine in males only, and this reduction was positively correlated with the level of MOR expression in the ventrolateral PAG. Together, these results provide a mechanism for sex differences in morphine potency.

**Key words:** dermorphin-saporin; intra-vlPAG; estrous cycle; pain; inflammation; opiate

## Introduction

It is becoming increasingly clear that morphine is more potent in male compared with female rats, with similar, although not unequivocal, effects observed in humans (Cepeda et al., 2002; Cepeda and Carr, 2003; Miller and Ernst, 2004). Sex differences in morphine analgesia have been demonstrated in multiple preclinical studies using both acute and persistent orofacial (Okamoto et al., 2005), visceral (Ji et al., 2006; 2007) and somatic (Bartok and Craft, 1997; Cicero et al., 1997; Boyer et al., 1998; Kest et al., 1999; Barrett et al., 2001; Cook and Nickerson, 2005; Wang et al., 2006) pain models, with ED<sub>50</sub> values two times higher in female compared with male rats (Wang et al., 2006; Ji et al., 2007). Importantly, sex differences in opiate sensitivity are not due to the pharmacokinetics of morphine because no sex difference has been reported in serum or brain levels of morphine, and elimination and metabolic rates are comparable between sexes (Cicero et al., 1996; Craft et al., 1996; Cicero et al., 1997; Sarton et al., 2000). Rather, sex differences in morphine analgesia are likely

due to differences in opiate receptor density, binding and localization, as well as sex differences in the anatomy and physiology of opiate-responsive neural circuits (Loyd and Murphy, 2006, 2008; Loyd et al., 2007, 2008).

The midbrain periaqueductal gray (PAG) and its descending projections to the rostral ventromedial medulla (RVM) constitute an essential neural circuit for opioid-based analgesia (Basbaum et al., 1976, 1978; Fields and Basbaum, 1978; Basbaum and Fields, 1979; Behbehani and Fields, 1979; Shah and Dostrovsky, 1980; Abols and Basbaum, 1981; Beitz, 1985; Beitz and Shepard, 1985). Administration of  $\mu$ -opioid receptor (MOR) agonists into the PAG produces potent analgesia that is blocked by central or systemic administration of the opioid antagonist naloxone (Sato et al., 1983; Jensen and Yaksh, 1986; Bodnar et al., 1988). Similarly, direct administration of MOR antagonists into the PAG blocks the antinociceptive effects of systemic morphine (Wilcox et al., 1979; Ma and Han, 1991; Zhang et al., 1998) indicating that the PAG is an essential locus for exogenous opioid-mediated analgesia.

The ventrolateral PAG (vlPAG) contains a high density of MOR (Mansour et al., 1986, 1987; Kalyuzhny et al., 1996; Gutsstein et al., 1998; Commons et al., 1999, 2000; Wang and Wessendorf, 2002) and ~27–50% of PAG neurons projecting to the RVM express MOR (Commons et al., 2000; Wang and Wessendorf, 2002). Unfortunately, studies examining the distribution of MOR within the PAG–RVM circuit were conducted exclusively in males. The vlPAG is a critical site mediating the analgesic effects

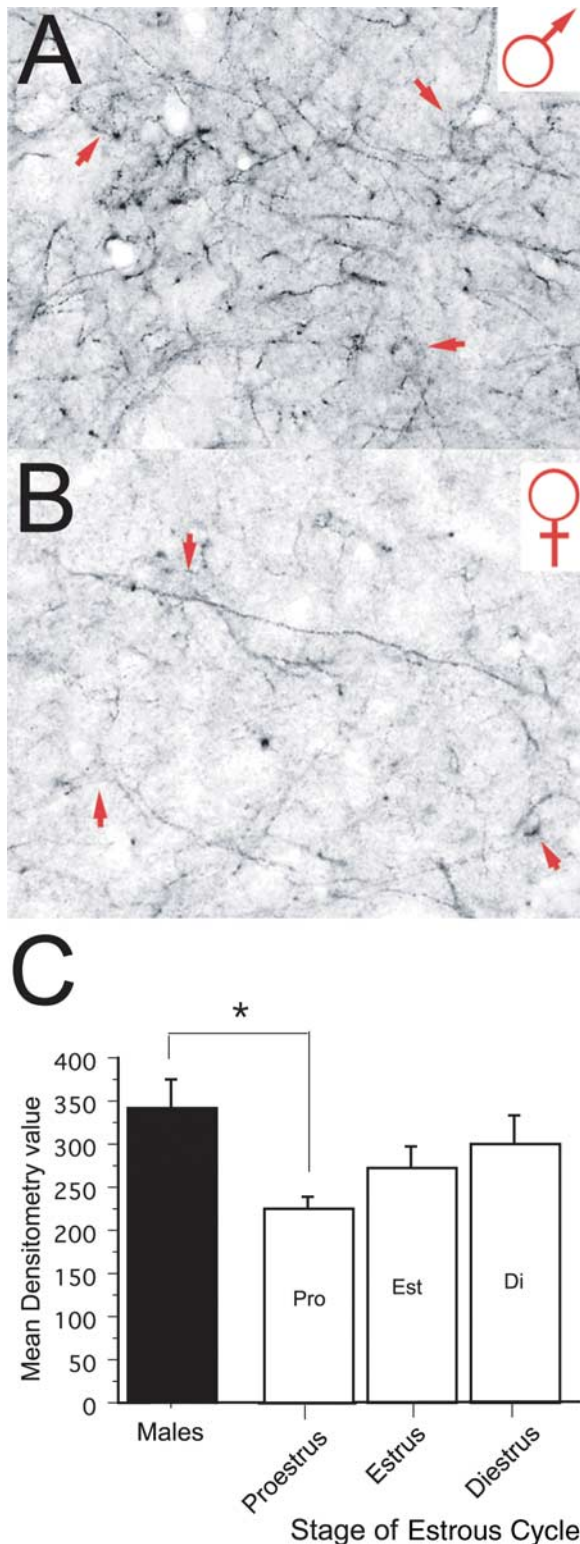
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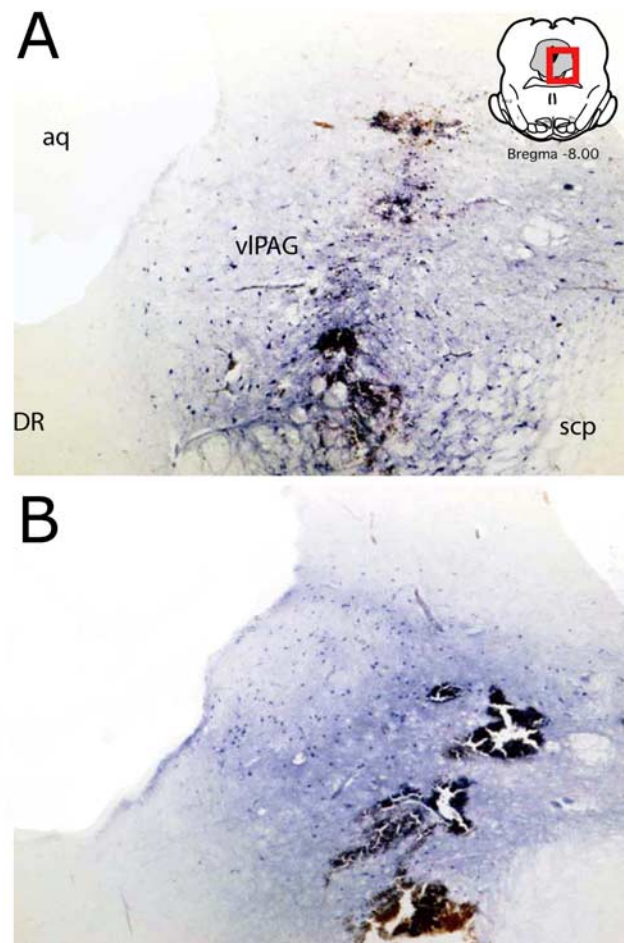
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**Figure 1.** *A, B*, Photomicrograph depicting  $\mu$ -opioid receptor immunoreactivity in the caudal ventrolateral PAG (bregma -8.00) in a male (*A*) and a female (*B*) rat. Arrows denote MOR-immunoreactive cell bodies. *C*, Mean densitometry value of  $\mu$ -opioid receptor immunoreactivity in male (closed bar) and proestrus, estrus, and diestrus females (open bars) averaged across the caudal PAG (bregma -7.04 to -8.30). \*Significant effect.

of morphine, yet despite profound sex differences in morphine analgesia, surprisingly little is known about the distribution and function of MOR in females. The present studies tested the hypothesis that sex differences in MOR expression within the



**Figure 2.** Photomicrograph of an intra-PAG injection site in the caudal ventrolateral PAG (bregma -8.00) of a representative male (*A*) and female (*B*) rat. aq, Aqueduct; vipag, ventrolateral periaqueductal gray; DR, dorsal raphe; scp, superior cerebellar pedunculus.

vipag provide a mechanism underlying the sexually dimorphic effects of morphine. This hypothesis was tested in a series of anatomical and behavioral studies to establish the relationship between sex, antinociceptive potency of intra-PAG morphine and the density of MOR in the vipag.

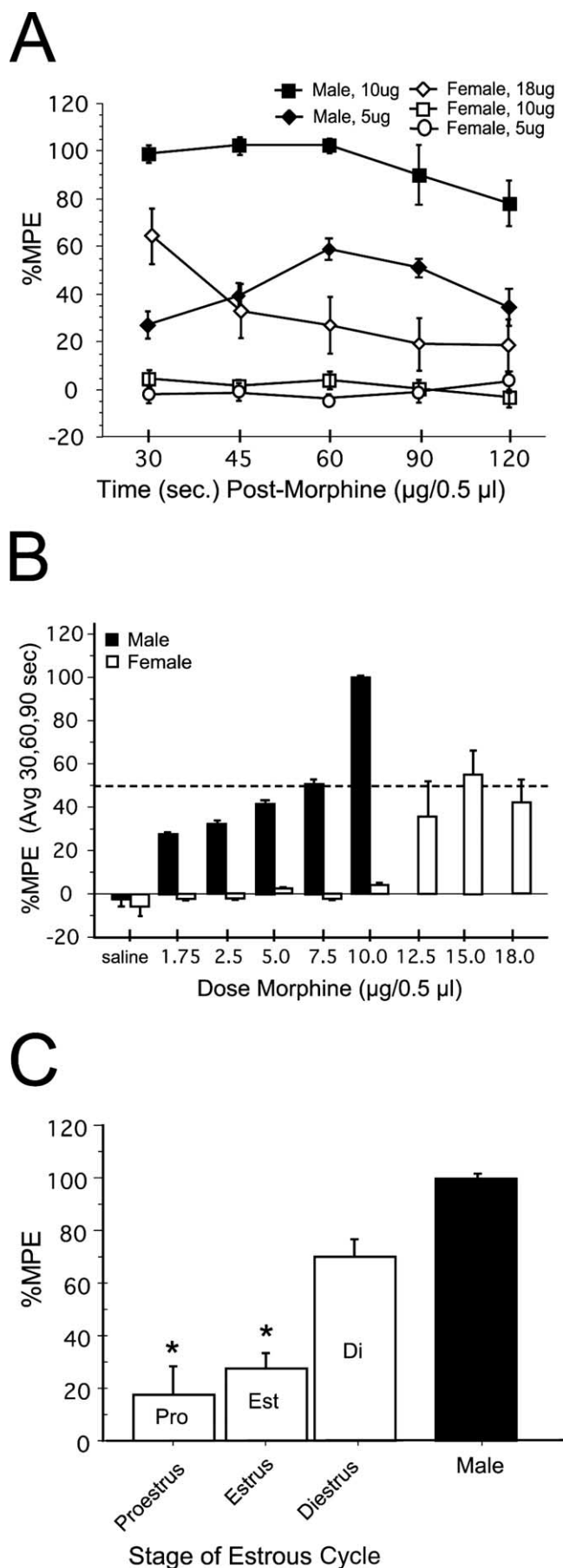
## Materials and Methods

### Subjects

Adult weight-matched (250–350 g) intact male and cycling female Sprague Dawley rats were used in these experiments (Zivic-Miller). Rats were housed in same-sex pairs on a 12:12 h light:dark cycle. Access to food and water was *ad libitum* throughout the experiment except during surgery. These studies were performed in compliance with the Institutional Animal Care and Use Committee at Georgia State University and the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) and the National Institutes of Health. All efforts were made to reduce the number of animals used in these experiments and to minimize any possible suffering by the animal.

### Vaginal cytology

Vaginal lavages were performed daily beginning 2 weeks before testing to confirm that all female rats were cycling normally and to keep daily records on the stages of their cycle in respect to experimental testing. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 was differentiated from diestrus 2 by the presence of leuko-



cytes. However, because there were no significant differences noted in either the anatomy or behavior of diestrus 1 and diestrus 2 animals, these data are pooled (diestrus). Rats that appeared between phases were noted as being in the more advanced stage.

*Intra-vPAG cannula implantation*

Intact males ( $n = 30$ ) and cycling females ( $n = 34$ ) were deeply anesthetized with a mixture of ketamine/xylazine/acepromazine (50, 3.3, 3.3 mg/kg, i.p.; Henry Shein). Guide cannulas (22 gauge; 5.0 mm; Plastics One) were lowered bilaterally into the ventrolateral PAG using the following coordinates (in mm): AP: +0.75 lambda; ML: 0.60; DV: -3.5. Cannula skull screws and dental acrylic were applied to secure placement. Cannulas were flushed every 48–72 h with 0.5 µl of saline over a 60 s period to acclimate the animals to the injection procedure and maintain cannula patency.

*Lesions of µ-opioid receptor-expressing PAG neurons*

Intact males ( $n = 11$ ) and cycling females ( $n = 11$ ) were deeply anesthetized with a mixture of ketamine/xylazine/acepromazine (50, 3.3, 3.3 mg/kg, i.p.; Henry Shein). Dermorphin-saporin (DermSAP; MOR agonist-saporin cytotoxin conjugate; Advanced Targeting Systems) or blank-saporin (BlankSAP; nonsense peptide-saporin cytotoxin control conjugate; Advanced Targeting Systems) were freshly diluted from stock (DermSap 0.91 µg/µl, 46.9 µM; BlankSAP 1.5 µg/µl, 46.9 µM; 32 kDa) and stored on ice during experimental procedures. DermSAP ( $n = 6$  males;  $n = 6$  females) or BlankSAP ( $n = 5$  males;  $n = 5$  females) was injected into the ventrolateral PAG (coordinates in mm): AP: +0.75 lambda; ML: 0.60; DV: -3.5 using a 1 µl Hamilton syringe. DermSAP (3 pmol/400 nl; 7.5 µM) or BlankSAP (3 pmol/400 nl; 7.5 µM) was micro-injected over 30 s. This procedure was repeated on the contralateral side. Saporin has been previously shown to have no effect in the absence of conjugation (Porreca et al., 2001; Burgess et al., 2002; Vera-Portocarrero et al., 2006). In addition, dermorphin has been shown to have a high binding affinity selective for the MOR ( $K_i$  value of 0.7 nM) and that conjugation to saporin does not significantly alter its binding affinity ( $K_i$  value of 0.1 nM; Porreca et al., 2001). This technique has been previously shown to result in a significant attenuation of MOR expression in the RVM (Porreca et al., 2001) and the spinal cord (Kline and Wiley, 2008). Loss of MOR containing neurons was confirmed in the present study using both immunohistochemistry and autoradiography. Lack of complete cell loss due to the injection procedure was confirmed with immunocytochemistry for neuronal nuclei.

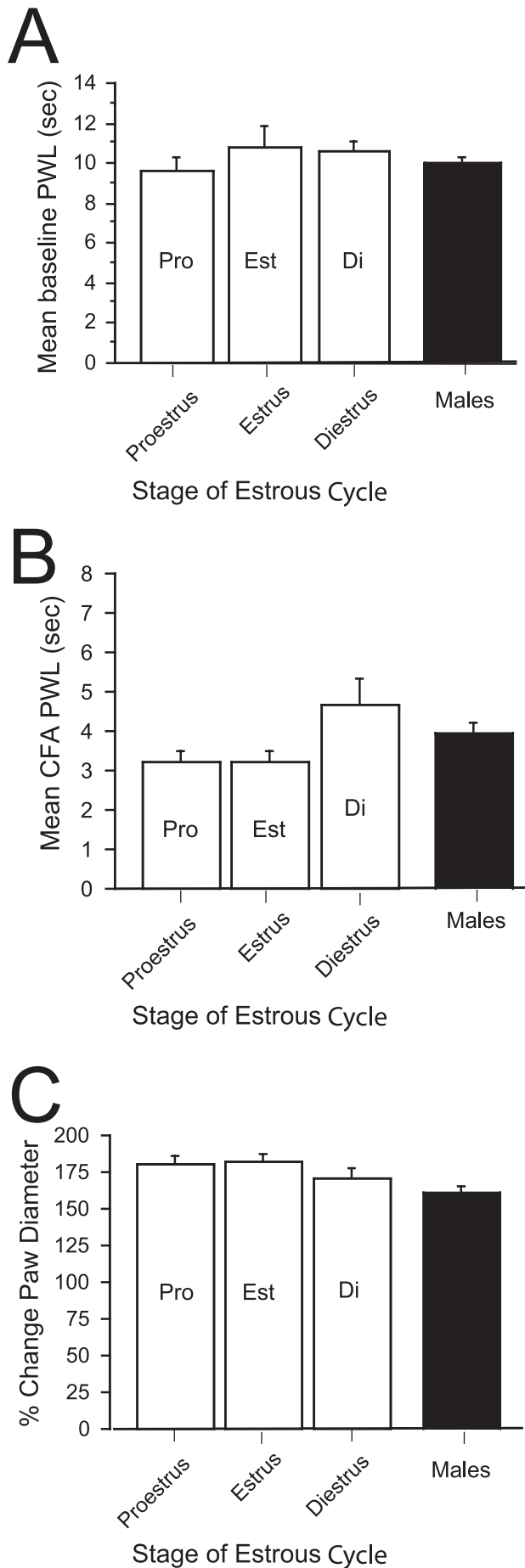
*Inflammatory hyperalgesia*

Persistent inflammation was induced by injection of complete Freund’s adjuvant (CFA; *Mycobacterium tuberculosis*; Sigma; 200 µl), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hind-paw. Paw diameters were determined using calibrated calipers applied midpoint across the dorsal to plantar surface of both hindpaws before and after induction of inflammation.

*Behavioral testing*

Paw withdrawal latencies to a noxious thermal stimulus were determined using the Paw Thermal Stimulator (Univ. California San Diego) as previously described (Hargreaves et al., 1988; Wang et al., 2006). Briefly, for this test, the rat was placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. After a 1 h acclimation, a radiant beam of light was positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus was electronically recorded as the paw withdrawal latency (PWL). The intensity of the beam was set to

**Figure 3.** *A*, Percent maximal possible effect of intra-PAG morphine administration of 5 and 10 µg in male (closed symbols) and 5, 10, and 18 µg in female (open symbols) rats. *B*, Percent maximal possible effect of intra-PAG morphine administration averaged across the time points 30, 60, and 90 min in male (closed bars) and female (open bars) rats. *C*, Percent maximal possible effect averaged over the 12, 15, and 18 µg/0.5 µl doses of intra-PAG morphine in proestrus, estrus, and diestrus females (open bars) compared with intact male (closed bars) rats. \*Significant difference compared with males and diestrus females.



produce basal PWLs of ~10 s. A maximal PWL of 20 s was used to prevent excessive tissue damage due to repeated application of a noxious thermal stimulus.

#### Perfusion fixation

After experimental manipulations, animals were given a lethal dose of Nembutal (160 mg/kg, i.p.) and transcardially perfused with 200–250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately after removal of blood, 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polyscience) was perfused through the brain as a fixative. A final rinse with 200–250 ml of the sodium chloride/sodium nitrate solution was perfused through the brain to remove any residual acrolein. Brains were placed in a 30% sucrose solution and stored at 4°C.

#### Immunohistochemistry

Perfusion-fixed brains were sectioned into 25  $\mu$ m coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution (Watson et al., 1986) at -20°C. A 1:6 series through the rostrocaudal axis of each brain was processed for MOR1 or neuronal nuclei (NeuN) immunoreactivity using standard immunohistochemical techniques as previously described (Murphy and Hoffman, 2001). Briefly, sections were rinsed extensively in potassium PBS (KPBS) immediately followed by a 20 min incubation in 1% sodium borohydride. The tissue was then incubated in primary antibody solution rabbit anti-MOR1 (donated by Robert Elde, Ph.D., University of Minnesota, Minneapolis, MN; 1:70,000) or rabbit anti-MOR1 (Abcam; 1:50,000; lot 317653) or mouse anti-NeuN (Millipore Bioscience Research Reagents; 1:50,000; lot 23112968) in KPBS containing 1.0% Triton-X for 1 h at room temperature followed by 48 h at 4°C. Both MOR1 antibodies were prepared against the synthetic peptide (NHQLENLEAETAPLP) corresponding to amino acids 384–398 of rat MOR1 (Arvidsson et al., 1995; Starowicz et al., 2007). After rinsing with KPBS, the tissue was incubated for 1 h in biotinylated goat anti-rabbit IgG or anti-mouse (Jackson ImmunoResearch; 1:600), rinsed with KPBS and incubated for 1 h in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Laboratories). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), MOR1 or NeuN immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3'-diaminobenzidine solution (2 mg/10 ml) containing 0.08% hydrogen peroxide in sodium acetate buffer. After 15–30 min, tissue was rinsed in sodium acetate buffer followed by KPBS. Sections were then mounted out of saline onto gelatin-subbed slides, air-dried, and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in xylene and glass coverslipped using Permount.

#### Receptor autoradiography

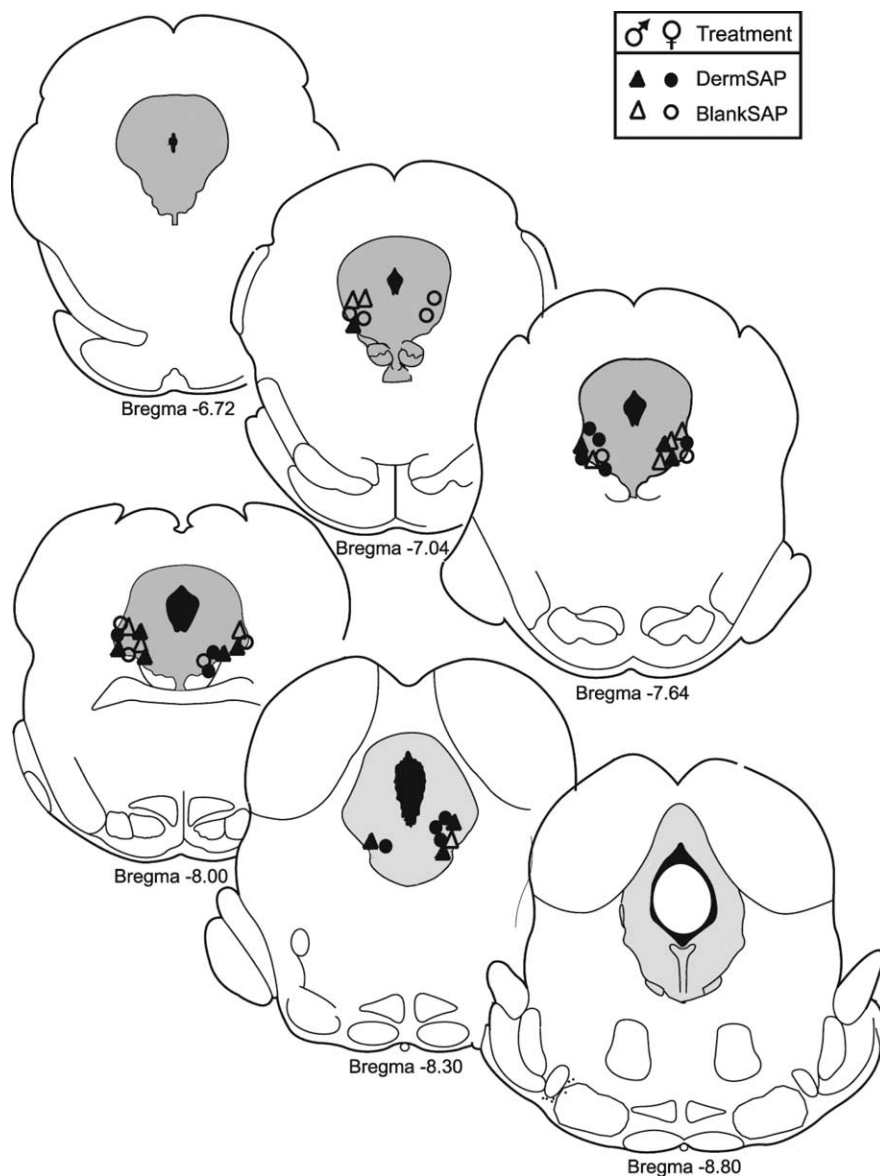
Dermorphin-saporin- ( $n = 4$  males;  $n = 4$  females) and blank-saporin- ( $n = 4$  males;  $n = 4$  females) treated animals were rapidly decapitated. Brains were removed rapidly, flash frozen in 2-methylbutane and stored at -80°C. Fresh frozen tissue was cut in a 1:4 series of 20  $\mu$ m coronal sections at -20°C with a Leica CM3050S cryostat, immediately mounted onto glass slides and stored at -80°C. Slides were dried and fixed in 4% paraformaldehyde followed by rinses in 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl. Slides were then placed in a tracer buffer containing tritiated DAMGO (1 nM; GE Healthcare) for 60 min followed by a series of rinses in 50 mM Tris buffer, pH 7.4, containing MgCl<sub>2</sub>. Tissue was allowed to dry and placed on autoradiographic film for 5 weeks at which point films were developed with a FujiFilm BAS 5000.

#### Densitometry

For immunohistochemistry data, 12-bit grayscale images were captured using a QImaging Retiga EXi CCD camera and IPLab Image Analysis

←

**Figure 4.** *A*, Mean paw withdrawal latency in seconds to a thermal stimulus in proestrus, estrus, and diestrus female (open bars) compared with male (closed bars) rats. *B*, Mean paw withdrawal latency in seconds to a thermal stimulus after 24 h of CFA-induced inflammation in proestrus, estrus, and diestrus female (open bars) compared with male (closed bars) rats. *C*, Percent change in paw diameter after 24 h of CFA-induced inflammation.



**Figure 5.** Illustration depicting localization of DersMAP (closed symbols) versus BlankSAP (open symbols) bilateral injection sites within the caudal ventrolateral PAG (bregma  $-7.04$  to  $-8.30$ ) of male (triangles) versus female (circles) rats.

Software (BD Biosciences). The vPAG was bilaterally sampled ( $35 \text{ mm}^2$  at  $10\times$  magnification) between bregma  $-7.04$  and  $-8.30$  (Paxinos and Watson, 1997) three times per section (6–8 sections per brain) and the average grayscale pixel value was recorded. Measures were corrected for nonspecific immunoreactivity background by subtraction of measures taken from areas of equal size lacking specific immunoreactivity adjacent to the vPAG in the same section. For autoradiography data, color images were captured and the brightness/contrast was adjusted using Fujifilm Multigauge software. The injection site area in the vPAG (bregma  $-7.04$  to  $-8.30$ ) was sampled three times per section (6–8 sections per brain), averaged and the photostimulated luminescence per  $\text{mm}^2$  (PSL/ $\text{mm}^2$ ) was recorded. Measures were corrected for nonspecific binding by subtraction of measures taken from a same section area adjacent to the vPAG of equal size.

#### Statistical analysis

For behavioral data analysis, data are expressed as either raw PWLs or percent maximal possible effect (%MPE), defined as  $[(\text{PWL} - \text{CFA baseline}) / (\text{maximal PWL} - \text{CFA baseline})] \times 100$  (Jensen and Yaksh, 1986). PWL data were analyzed for significant main effects of sex and estrous cycle using ANOVA. Percentile data were transformed into standard

scores. Unpaired  $t$  tests were used to determine significant differences in baseline data. Because no significant differences in %MPE were noted for the 30, 45, and 60 min time points, these values were averaged for derivation of  $\text{ED}_{50}$ , defined as the dose of morphine that produced 50% of the maximum possible increase in PWL, using Prism software and analyzed for significant reductions in values using ANOVA.  $p \leq 0.05$  was considered statistically significant. Fisher's *post hoc* tests were used to determine specific group differences when a main effect or interaction was observed.

#### Specific experiments

**Experiment 1. Do sex or estrous cycle influence MOR expression in the vPAG?** Male and female rats with an established 4 d estrous cycle were perfused transcardially with fixative on the morning of a specific stage of the estrous cycle (diestrus 1,  $n = 12$ ; diestrus 2,  $n = 12$ ; proestrus,  $n = 12$ ; estrus,  $n = 12$ ). Males ( $n = 12$ ) and females were killed at the same time. Tissue sections were processed immunohistochemically for MOR1 immunoreactivity (Arvidsson et al., 1995; Kalyuzhny et al., 1996; Kalyuzhny and Wessendorf, 1997; 1998; Wang and Wessendorf, 1999; Kalyuzhny et al., 2000; Wang and Wessendorf, 2002) and densitometry values were recorded across vPAG.

**Experiment 2. Do sex- and estrous-cycle-induced changes in PAG MOR expression influence the analgesic effects of morphine administered into the vPAG?** Intact male and cycling female rats were implanted with cannulas directed at the ventrolateral PAG. One week later, baseline PWLs and paw diameters were determined followed by induction of CFA-induced inflammation. Twenty-four hours later, PWL and paw diameters were re-determined to ensure the presence of hyperalgesia. Saline or morphine sulfate prepared fresh on the day of experiment (obtained from the National Institute on Drug Abuse, Rockville, MD) was injected into the PAG using a syringe pump attached to a 33-gauge injector (7 mm; Plastics One). Separate groups of animals received morphine (1.75, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 18.0  $\mu\text{g}$ ) or saline in a total volume of 0.5  $\mu\text{l}$  over

60 s. PWLs for the inflamed paw were determined at 15, 30, 45, 60, 90 and 120 min post injection to assess morphine analgesia. At the end of the experiment, Chicago Sky Blue dye (Sigma) was injected through the guide cannulas for injection site verification. Analysis was limited to those animals with injections into the caudal vPAG (bregma  $-7.04$  to  $-8.30$ ).

**Experiment 3. Do lesions of MOR-expressing neurons in the vPAG attenuate the analgesic effects of morphine?** After determination of baseline PWLs, animals were administered either DersMAP or BlankSAP bilaterally into the vPAG. Twenty-eight days later, PWLs were determined (baseline preinflamed PWL). CFA was then injected intraplantar to induce persistent inflammatory pain. Twenty-four hours later, PWLs were determined to ensure that DersMAP had no effect on baseline hyperalgesia. Animals then received cumulative doses of morphine every 20 min (1.8, 3.2, 5.6, 8.0, 10.0, 18.0 mg/kg), and PWLs were determined 5 min after administration. At the end of the experiment, animals were perfused and lesion sites verified immunohistochemically with antibodies directed against MOR1 and NeuN. An additional group of animals received either DersMAP or BlankSAP and were decapitated 28 d later for receptor autoradiography to examine the effectiveness of MOR lesioning.

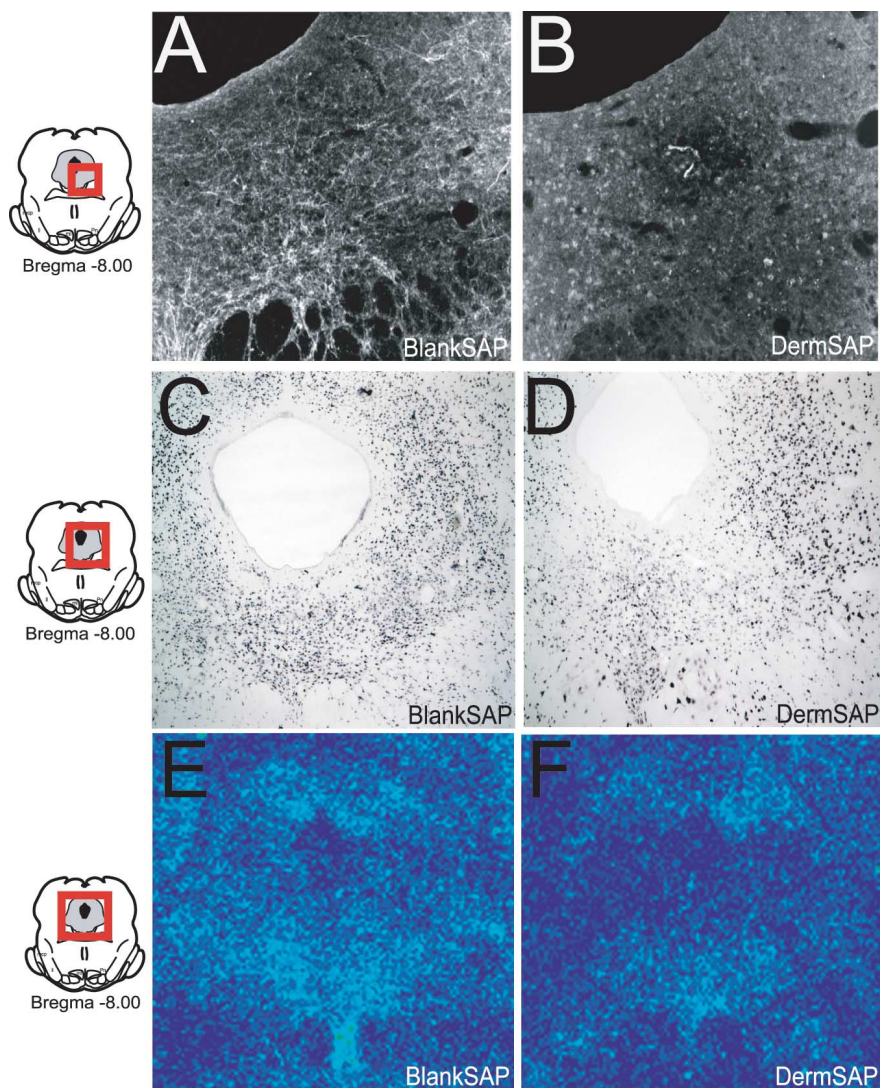
## Results

### Sex differences in $\mu$ -opioid receptor expression in the ventrolateral PAG

Expression of  $\mu$ -opioid receptor protein in the PAG of intact male and cycling female rats was examined immunohistochemically. Within the rostral PAG, MOR immunoreactivity was localized primarily within the dorsomedial and dorsolateral subdivisions of the PAG and was comparable between the sexes. Minimal labeling was observed in the lateral PAG. Moving caudally, MOR immunoreactivity shifted and was densely localized within the lateral and vlPAG, primarily at the level of the dorsal raphe (bregma  $-7.04$  to  $-8.30$ ). Very little to no labeling was observed in the dorsomedial and dorsolateral PAG. Overall, females had significantly less MOR immunoreactivity in the caudal vlPAG compared with males [ $t_{(58)} = 2.18$ ;  $p = 0.0333$ ] (Fig. 1*A, B*). As shown in Figure 1*C*, MOR expression fluctuated across the estrous cycle, with reduced MOR immunoreactivity noted in proestrus females when compared with both diestrus females ( $p = 0.0528$ ) and males ( $p = 0.0068$ ).

### Intra-vlPAG morphine produces greater analgesia in males

We have previously reported that morphine is significantly more potent in male versus female rats (Wang et al., 2006; Ji et al., 2007). Therefore, we sought to determine whether sex- and estrous cycle-induced differences in PAG MOR expression underlie behavioral differences in morphine potency. Intra-PAG cannulas were localized within the caudal vlPAG at the level of the dorsal raphe (bregma  $-7.04$  to  $-8.30$ ). Figure 2 shows a representative photomicrograph of the intra-vlPAG injection site in a male (*A*) and female (*B*) rat. Overall, morphine was significantly more potent in the reversal of hyperalgesia in males compared with females [ $F_{(1,21)} = 188.78$ ;  $p < 0.0001$ ], with a significant dose by sex interaction [ $F_{(1,21)} = 21.81$ ;  $p = 0.0001$ ]. In male rats, intra-vlPAG morphine produced significant analgesia with administration of  $10 \mu\text{g}$  resulting in 100%MPE at 30, 45, and 60 min after injection (Fig. 3*A*). No significant effect of morphine was observed in females at this dose. Because there were no significant differences in the %MPE observed for the 30, 45, and 60 min time points, these data are collapsed and presented for all doses (Fig. 3*B*). Intra-vlPAG morphine ( $1.75$ – $10 \mu\text{g}$ ) resulted in a dose-dependent analgesic response in males. The  $12.5 \mu\text{g}$  dose of morphine produced a 30%MPE in females, and increasing the dose of morphine from 15 to  $18 \mu\text{g}$  did not result in a significant increase in %MPE in females ( $\sim 55\%$  to  $40\%$ ). A small group of females were administered  $20 \mu\text{g}$ , however this dose was lethal in most animals so additional testing at this dose was terminated. Similarly, administration of the  $12.5$  and  $15 \mu\text{g}$  doses were lethal to all males so testing at this dose was terminated.

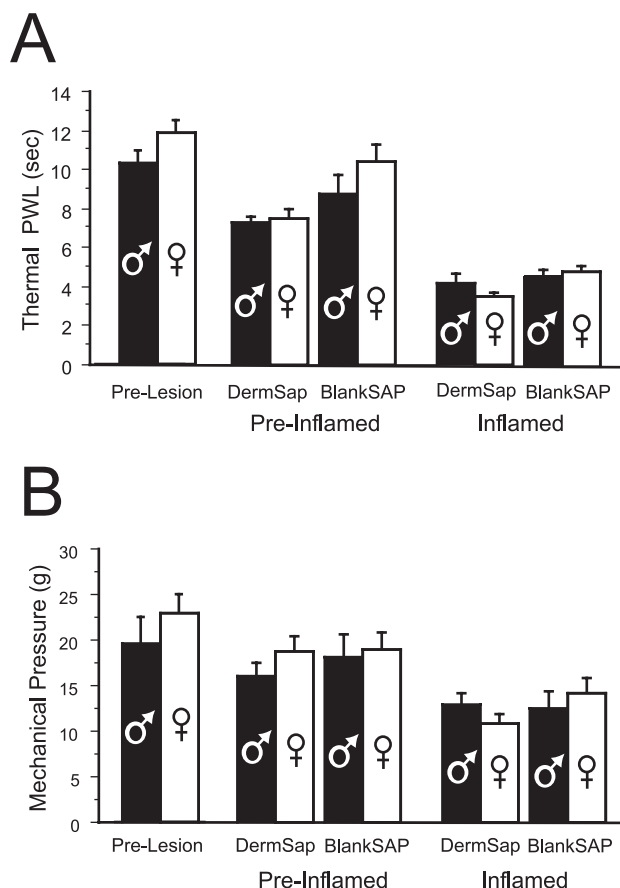


**Figure 6.** *A, B*, Photomicrograph depicting  $\mu$ -opioid receptor density in the vlPAG (bregma  $-8.00$ ) after BlankSAP (*A*) versus DermSAP (*B*) treatment. *C, D*, Photomicrograph depicting NeuN density in the vlPAG (bregma  $-8.00$ ) after BlankSAP (*C*) versus DermSAP (*D*) treatment. *E, F*, Photomicrograph depicting tritiated DAMGO binding in the vlPAG (bregma  $-8.00$ ) after BlankSAP (*E*) versus DermSAP (*F*) treatment.

Because there was no significant difference in the %MPE between the 12, 15, and  $18 \mu\text{g}/0.5 \mu\text{l}$  doses of morphine in females, these data were collapsed and examined for a main effect of the estrous cycle on morphine analgesia. There was a significant main effect of estrous stage [ $F_{(2,17)} = 15.35$ ;  $p = 0.0002$ ]; such that proestrus ( $p = 0.0001$ ) and estrus ( $p = 0.0005$ ) females had significantly lower levels of morphine analgesia (20–25%MPE), whereas diestrus females had the greatest levels of morphine analgesia (70%MPE) (Fig. 3*C*). Our observed sex differences in intra-vlPAG morphine analgesia are not due to sex or estrous cycle differences in baseline thermal sensitivity [ $F_{(3,60)} = 1.027$ ;  $p = 0.3870$ ] (Fig. 4*A*) or in CFA-induced hyperalgesia [ $F_{(3,60)} = 1.244$ ;  $p = 0.3017$ ] (Fig. 4*B*) and there was no difference in the degree of edema produced by intraplantar CFA [ $F_{(3,60)} = 1.842$ ;  $p = 0.1492$ ] (Fig. 4*C*).

### Lesions of MOR-expressing neurons in the vlPAG attenuate morphine antihyperalgesia in males only

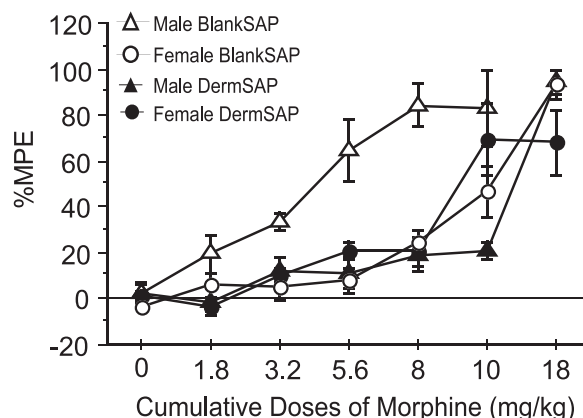
To test the role of vlPAG MOR in driving sex differences in morphine analgesia, we injected DermSAP into the vlPAG to site-specifically lesion MOR-expressing neurons. Lesions that were



**Figure 7.** Paw withdrawal latencies to a thermal (*A*) or mechanical (*B*) painful stimulus before surgical manipulation (Pre-Lesion), after DermSAP or BlankSAP treatment (Pre-Inflamed), and after 24 h of CFA-induced hyperalgesia (Inflamed) in male (closed) versus female (open) rats.

used for analysis were limited to localization within the vPAG between bregma  $-7.04$  and  $-8.30$  (Fig. 5). The effectiveness of the lesions were confirmed in three ways: (1) a significant reduction in MOR immunoreactivity (Fig. 6*A,B*), (2) reduced neuronal nuclei immunoreactivity without complete cell loss (Fig. 6*C,D*), and a significant reduction in tritiated DAMGO binding (Fig. 6*E,F*). DermSAP treatment significantly reduced MOR expression [ $F_{(1,19)} = 8.951$ ;  $p = 0.0075$ ] and MOR agonist binding [ $F_{(1,12)} = 115.468$ ;  $p < 0.0001$ ] in the vPAG compared with BlankSAP controls. There was no evidence of cell necrosis due to the injection procedure in any of the animals used for analysis, similar to previous reports (Porreca et al., 2001). Lesioning MOR-expressing neurons in the vPAG had no effect on baseline sensitivity to either a thermal [ $F_{(1,13)} = 0.193$ ;  $p = 0.6677$ ] (Fig. 7*A*) or mechanical [ $F_{(1,15)} = 2.833$ ;  $p = 0.1130$ ] (Fig. 7*B*) noxious stimulus in either sex. After 24 h of CFA-induced inflammation, there was no effect of lesions on thermal [ $F_{(1,16)} = 0.283$ ;  $p = 0.6018$ ] (Fig. 7*A*) or mechanical [ $F_{(1,16)} = 0.147$ ;  $p = 0.7064$ ] (Fig. 7*B*) hyperalgesia in either sex.

Twenty-four hours after the injection of CFA, the analgesic effects of morphine were assessed using a cumulative dosing paradigm. BlankSAP control male and female rats showed normal levels of morphine analgesia to cumulative doses of morphine (Fig. 8), with a mean average  $ED_{50}$  value of 4.07 mg/kg in males versus 10.39 mg/kg in females. There was a significant main effect of treatment [ $F_{(3,130)} = 4.750$ ;  $p < 0.0001$ ], such that DermSAP treated males displayed a significant rightward shift in  $ED_{50}$  from



**Figure 8.** Percent maximal possible effect of cumulative doses of systemic morphine (0, 1.8, 3.2, 5.6, 8, 10, 18 mg/kg) in inflamed male (triangles) and female (circles) rats after DermSAP (closed symbols) or BlankSAP (open symbols).

4.07 mg/kg in controls to 12.55 mg/kg; no significant change in  $ED_{50}$  was noted in females (10.39–9.21 mg/kg).

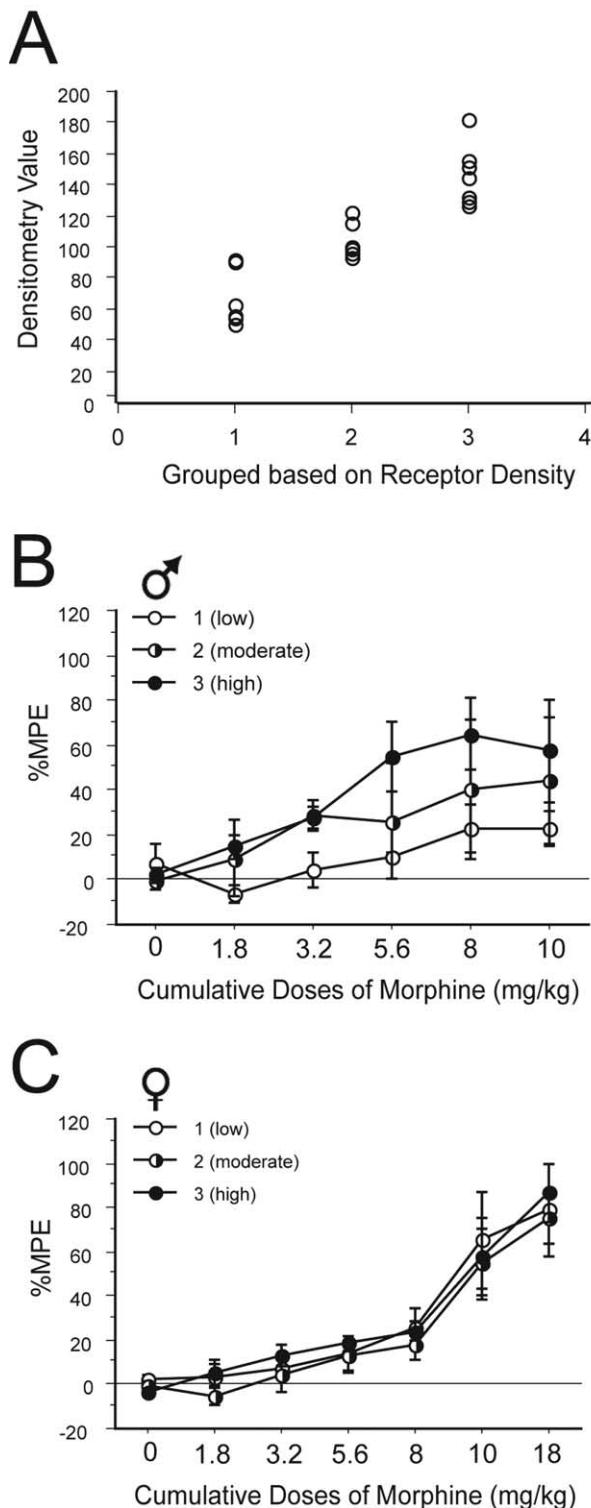
#### Positive correlation between morphine analgesia and vPAG MOR expression levels in males only

DermSAP treatment resulted in a differential loss of MOR protein across the caudal vPAG with levels ranging from abnormally low to normal or high levels. To further examine the effect of lesioning vPAG MOR on morphine analgesia, DermSAP and BlankSAP animals were pooled and grouped into the following three categories based on the extent of lesions: (1) abnormally low level of MOR immunoreactivity (densitometry value 49.80–91.82;  $n = 3$  males,  $n = 4$  females), (2) moderate reduction of MOR immunoreactivity (93.57–121.78;  $n = 3$  males,  $n = 4$  females) and (3) normal or high levels of MOR immunoreactivity (126.48–181.33;  $n = 4$  males,  $n = 3$  females). Normal density of MOR expression was over two-fold higher than that of abnormal levels of expression (Fig. 9*A*). In males, there was a positive correlation between the density of vPAG MOR-expressing neurons and the level of morphine analgesia (Fig. 9*B*). Reducing the expression of MOR in the vPAG caused a significant reduction in  $ED_{50}$  in males: the  $ED_{50}$  was reduced from 3.46 in males with a high expression (group 3; CI = 1.7–6.9) to 11.85 (group 1; CI = 7.04–13.9) in males with abnormally low levels of expression. There was no significant difference observed in females with a low density ( $ED_{50}$  9.46; CI = 7.8–11.6) versus a normal ‘high’ density ( $ED_{50}$  = 8.77; CI = 7.5–10.3) of MOR in the vPAG (Fig. 9*C*).

#### Discussion

Many factors limit the potency of opiates, including tolerance (Christie et al., 1987; Morgan et al., 2003; Bagley et al., 2005; Lane et al., 2005; Lloyd et al., 2008), negative side effects (Cepeda and Carr, 2003; Cepeda et al., 2003; Filligim et al., 2005; Panchal et al., 2007) and more recently recognized, “gender” or “sex” (Wang et al., 2006). It is now well known that morphine is more potent in males compared with females; however, the mechanism(s) driving this phenomenon is unknown. The present experiments were designed to test the hypothesis that the expression of MOR in the vPAG was sexually dimorphic and essential for eliciting sex differences in morphine potency. Here we report that (1) males have significantly higher levels of MOR protein in the vPAG compared with females; (2) intra-vPAG administration of morphine produces significantly greater analgesia in males; (3) lesions of





**Figure 9.** *A*, Densitometry value of MOR in the vPAG of each individual male and female rat (open circle) after DermSAP or BlankSAP treatment within each group based on  $\mu$ -opioid receptor density. *B*, *C*, Percent maximal possible effect of cumulative doses of systemic morphine (0, 1.8, 3.2, 5.6, 8, 10, 18 mg/kg) in inflamed male (*B*) and female (*C*) rats expressing low (1), moderate (2), and high (3)  $\mu$ -opioid receptor density.

MOR-expressing vPAG neurons attenuate systemic morphine analgesia in males only; and (4) the degree of morphine analgesia is positively correlated with the density of MOR immunoreactivity in the vPAG.

### Sex differences in $\mu$ -opioid receptor expression in the ventrolateral PAG

In male rats, the PAG contains a high density of MOR (Mansour et al., 1986; Mansour et al., 1987; Arvidsson et al., 1995; Kalyuzhny et al., 1996; Gutstein et al., 1998; Commons et al., 1999; Commons et al., 2000; Wang and Wessendorf, 2002). Although Western blots would have provided a more quantitative approach, the present study used immunohistochemistry so that both qualitative and semiquantitative sex differences in MOR expression could be examined simultaneously within functionally distinct regions of the PAG. Here we report that the highest density of MOR immunoreactivity was observed in the caudal vPAG, similar to previous reports (Kalyuzhny et al., 1996; Commons et al., 2000). Overall, female rats had significantly less MOR immunoreactivity in the vPAG, with the greatest difference observed in proestrus females with approximately one-third less labeling compared with males. Interestingly, females in diestrus, the stage in which estrogen and progesterone are the lowest, had comparable levels of MOR immunoreactivity compared with males. Overall, these results are consistent with a recent Western blot study that reported twofold lower MOR protein expression in the female rat midbrain compared with males (Kren et al., 2008). These findings indicate that steroid hormones may play a role in MOR expression in the region of the PAG that is essential for analgesia and further suggests that the actions of morphine are estrous stage dependent.

### Intra-vPAG morphine produces greater analgesia in males compared with estrus and proestrus females

The antinociceptive effects of morphine are mediated primarily by the MOR, which is expressed in several supraspinal sites including the habenula, striatum, hippocampus, locus ceruleus, RVM and PAG (Arvidsson et al., 1995). Given that (1) up to 50% of PAG-RVM projection neurons contain MOR (Commons et al., 2000; Wang and Wessendorf, 2002) and (2) female rats have reduced levels of MOR in the vPAG, we hypothesized that differences in MOR within the PAG were sufficient to account for the sex differences in morphine analgesia. Microinjection of morphine directly into the vPAG produced a significantly greater degree of analgesia in males compared with females at all doses tested. These results are consistent with our previous studies using systemic morphine in which ED<sub>50</sub> values were twofold higher in female compared with male rats (Ji et al., 2006; Wang et al., 2006).

In the present study, morphine analgesia was reduced during both proestrus and estrus in comparison to diestrus and is consistent with previous studies (Kepler et al., 1989; Islam et al., 1993; Krzanowska and Bodnar, 1999; Krzanowska et al., 2002). In particular, increased levels of morphine analgesia were observed during diestrus when estrogen and progesterone are lowest. In fact, morphine analgesia during diestrus was not significantly different from males. These results parallel our findings of reduced MOR protein levels during proestrus compared with diestrus, and provide further support that the amount of available MOR is positively related to the degree of analgesia produced by morphine although other variables likely contribute. For example, while there was no significant difference in MOR expression between estrus females and males, estrus females displayed significantly less analgesia compared with males after intra-PAG morphine. This suggests that additional factors such as the activation state of the receptor also impact the ability of morphine to induce analgesia. In the present study, MOR was detected immunohistochemically, which would identify all receptors, re-

ardless of their ability to respond to exogenous opiates. Future studies comparing the functional state of the receptor between males and females are clearly warranted.

Between 20–50% of PAG-RVM neurons retrogradely labeled from the RVM contain receptors for the steroid hormones estrogen and androgen (Lloyd and Murphy, 2008), whereas 27–50% express MOR (Commons et al., 2000; Wang and Wessendorf, 2002); therefore it is likely that a proportion of PAG-RVM projection neurons express both receptor types. There are several mechanisms whereby changes in gonadal steroid levels could influence MOR expression and ultimately, morphine analgesia. Increased levels of estradiol result in MOR internalization (Eckersell et al., 1998; Micevych et al., 2003) and administration of estradiol results in the rapid uncoupling of MOR from G-protein-gated inwardly rectifying potassium channels (Kelly et al., 2003). Obviously, further research on the mechanism(s) whereby estradiol alters MOR expression and function is warranted.

#### **$\mu$ -Opioid-expressing neurons in the ventrolateral PAG are necessary for sex differences in morphine analgesia**

Using site-specific lesioning techniques to test the necessity of MOR-expressing neurons in eliciting sex differences in morphine analgesia, we found that reducing the density of MOR in the caudal vlPAG significantly attenuated morphine analgesia to systemic morphine in male but not female rats. The ED<sub>50</sub> in males shifted from 4.07 mg/kg in controls to 12.55 mg/kg, whereas no significant shift was noted in the ED<sub>50</sub> in females. These data provide evidence that MOR-expressing neurons in the vlPAG are necessary for eliciting sexually dimorphic morphine potency. Removal of MOR in the vlPAG of females had no impact on morphine-induced analgesia; however, at high doses of systemic administration, morphine still produces analgesia. This suggests that in females, the PAG is not the primary anatomical substrate for the analgesic effects of morphine. Both the RVM (Porreca et al., 2001; Burgess et al., 2002) and the dorsal horn of the spinal cord (Kline and Wiley, 2008) contribute to morphine antihyperalgesia and perhaps these sites are more critical in females. In support, we have recently reported no differences in MOR expression within the lumbosacral spinal cord and similarly, no differences in ED<sub>50</sub> values for morphine when administered intrathecally (Ji et al., 2006).

In addition, we found that the density of vlPAG MOR immunoreactivity was positively correlated with morphine analgesia in male, but not female rats. Males with normal levels of MOR immunoreactivity in the vlPAG had significantly lower ED<sub>50</sub> values compared with males with two-fold less MOR immunoreactivity. These results further indicate that MOR-expressing neurons in the PAG are essential for morphine analgesia in male but not female rats.

Our observed sex difference in the actions of morphine is not to due sex differences in nociceptive threshold or inflammatory hyperalgesia. Interestingly, removal of vlPAG MOR with DermSAP had no effect on baseline PWL or inflammatory hyperalgesia to either a noxious thermal or mechanical stimulus. Thus, although vlPAG MOR obviously contributes to the effects of exogenous morphine, its reduction does not appear to alter endogenous pain modulation during inflammatory hyperalgesia suggesting that other pain inhibiting regions, including the RVM and spinal cord are involved. In support, previous studies have reported that DermSAP lesions of MOR-expressing neurons in the RVM (Porreca et al., 2001; Burgess et al., 2002) and dorsal horn neurons (Kline and Wiley, 2008) attenuate hyperalgesia in

male rats. Together, these data indicate that the RVM and the dorsal horn of the spinal cord, but not the PAG, are essential for driving descending facilitation (Terayama et al., 2000; Ren and Dubner, 2002; Dubner and Ren, 2004; Wei et al., 2008).

Overall, our results indicate that morphine is a remarkably ineffective opiate for the alleviation of persistent pain in female rats. Sex differences in morphine potency are well known in animal research, and have also been widely reported in humans (Kest et al., 2000; Sarton et al., 2000; Zacny, 2001; Cepeda et al., 2002; Cepeda and Carr, 2003; Miller and Ernst, 2004). Interestingly, sex is not the only factor that has been shown to affect the potency of various pharmacological agents. Recent studies have reported an influence of age (Smith and Gray, 2001; Aubrun and Marmion, 2007; Gagliese et al., 2008; Hanberry and Murphy, 2008) and ethnicity (Kaiko et al., 1983), and further argue for the inclusion of a wide range of study subjects in pain management research. In addition, despite the rapidly mounting evidence of limitations of opiates in treating persistent pain in females, opioid-based drugs remain the primary pharmacological tool for pain management. Clearly additional research with the inclusion of female subjects needs to be devoted to determining a more potent treatment for persistent pain in women.

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