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F. CERVERO

Sexually Dimorphic Activation of the Periaqueductal Gray – Rostral Ventromedial Medullary Circuit during the Development of Morphine Tolerance in the Rat

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Running Title: PAG-RVM Activation During Morphine Tolerance

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ABSTRACT

Previous studies have shown that tolerance develops to a greater degree in male compared to female rats. The midbrain periaqueductal gray (PAG), and its descending projections to the rostral ventromedial medulla (RVM), provides an essential neural circuit for the antinociceptive effects of opiates and has been implicated in the development of tolerance to morphine. We have previously reported that systemic morphine administration activates a greater proportion of PAG-RVM neurons in male versus female rats; our hypothesis is that if the PAG-RVM pathway is essential for the development of morphine tolerance, then (1) morphine activation of the PAG-RVM pathway should decline as tolerance develops, and (2) sex differences should be reflected as a greater decline in males. These hypotheses were tested using behavioral and neuroanatomical techniques to map the activation of the PAG-RVM pathway during the development of tolerance to repeated morphine administration (4.5 mg/kg; s.c.). We found that as male rats develop tolerance (D50 increased from 3.0 to 6.3 mg/kg), there was no significant decline in the overall activation of the PAG, however, there was a steady decline in the percentage of PAG-RVM output neurons activated by morphine. This reduction occurred in males only; there was no significant decline in the activity of PAG-RVM output neurons in females. These data demonstrate that the greater development of tolerance to morphine administration in male rats corresponds with a significant reduction in the activation of the PAG-RVM circuit. Our results provide additional data demonstrating a central role for the PAG in morphine tolerance.

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INTRODUCTION

It is estimated that as many as one in five adults currently experience chronic pain. Of that population, 41% are receiving treatment with opioid-based narcotics (Breivik *et al.*, 2006). While morphine and other opioid-based narcotics are the most effective treatment for pain, opiates do not provide adequate pain relief for many people. The sex of the patient and the development of tolerance to morphine's analgesic effects are two particularly important factors that limit the effectiveness of opiates. Men tend to experience greater analgesia following opiate administration compared to women (Cepeda & Carr, 2003; Miller & Ernst, 2004), a difference that has been reported for a wide range of other species using both somatic and visceral pain models (Craft, 2003; Ji *et al.*, 2006; Loyd & Murphy, 2006; Wang *et al.*, 2006; Loyd *et al.*, 2007).

Recent findings suggest that the midbrain periaqueductal gray (PAG), and its descending projections to the rostral ventromedial medulla (RVM), contribute to the sexually dimorphic actions of morphine. Microinjection of morphine directly into the PAG produces greater antinociception in male compared to female rats (Krzanowska & Bodnar, 1999; Wang *et al.*, submitted), and blocking PAG opioid receptors reduces morphine antinociception to a greater extent in female rats (Bernal *et al.*, 2007). Furthermore, both the anatomy and physiology of the PAG-RVM pathway is sexually dimorphic, with males having a significantly greater percentage of PAG-RVM neurons activated by either inflammatory pain (Loyd & Murphy, 2006) or systemic morphine (Loyd *et al.*, 2007).

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Tolerance is known to occur with repeated or continuous administration of morphine into the ventrolateral PAG of male rats (Jacquet & Lajtha, 1976; Siuciak & Advokat, 1987; Tortorici *et al.*, 1999; Tortorici & Morgan, 2002; Lane *et al.*, 2005; Morgan *et al.*, 2006). In addition, blocking opioid binding in the ventrolateral PAG attenuates tolerance to systemically administered morphine (Lane *et al.*, 2005). Tolerance appears to be mediated by a reduction in mu opioid receptor signaling efficacy in PAG neurons (Bagley *et al.*, 2005), an effect that is reversed when mu opioid receptor coupling is enhanced via upregulated cyclase activity (Hack *et al.*, 2003). These findings suggest that the PAG contributes to the decrease in antinociception that occurs with the development of tolerance.

Given that morphine activates a greater percentage of PAG-RVM output neurons in male compared to female rats (Loyd *et al.*, 2007), our working hypothesis is that if the PAG-RVM pathway is essential for the development of morphine tolerance, then (1) activation of the PAG-RVM pathway should decline as tolerance to repeated injections of systemic morphine develops, and given that males show greater antinociception and greater tolerance (Craft *et al.*, 1999; Barrett *et al.*, 2001), (2) sex differences should be reflected as a greater decline in the activation of the PAG-RVM pathway in male compared to female rats. These hypotheses were tested using behavioral assessment of nociception and neuroanatomical techniques to map the activation of the PAG-RVM pathway over the time course of the development of tolerance to repeated morphine administration.

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MATERIALS AND METHODS

Experimental Subjects

Thirty-five adult male and thirty-seven weight-matched (250-350g) cycling female Sprague-Dawley rats were used in these experiments (behavior subjects from Harlan, Indianapolis, IN and anatomy subjects from Zivic-Miller; Pittsburg, PA). Rats were housed in same-sex pairs on a 12:12 hour light: dark cycle. Access to food and water was ad libitum throughout the experiment except during surgery and testing. These studies were performed in compliance with the Institutional Animal Care and Use Committees at Georgia State University and at Washington State University. All efforts were made to minimize any possible suffering by the animal, and to reduce the number of animals used.

Experiment 1: Behavioral Assessment of Morphine Tolerance

Thirteen male and fourteen female rats were handled daily for five days prior to initiation of drug administration. Morphine sulfate (5 mg/kg; provided by the National Institute on Drug Abuse, Bethesda, MD; Loyd et al., 2007) was prepared in a saline vehicle and administered systemically twice a day for three consecutive days to male (n=7) and female (n=7) rats. Control groups consisting of six male and seven female rats received daily injections of saline (1 ml/kg, s.c.). Tolerance was assessed on Day 4 by injecting cumulative doses of morphine every 20 minutes resulting in quarter log doses of 1.8, 3.2, 5.6, 10.0, and 18.0 mg/kg. Nociception was assessed using the hot-plate test 15 minutes after each injection. The hot plate test measures the latency to lick the hindpaw when the rat is placed on a 52.5^{o}C plate. If the animal did not respond within 50 seconds, it was removed from the plate and given a score of 50 seconds. The

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mean latency at each dose was calculated for male and female rats pretreated with morphine or saline.

Behavioral Data Analysis and Presentation

The half-maximal antinociceptive effect (D50; Tallarida, 2000) and 95% confidence intervals (CI) were calculated from dose-response curves generated using GraphPad software. The lower limit for calculating D_{50} values was the mean baseline score, and the upper limit was the mean hot plate latency following administration of the highest morphine dose. Changes in D50 between groups were assessed using ANOVA (GraphPad).

Experiment 2: Anatomical Assessment of Morphine Tolerance

Retrograde Tracer Injections

Twenty-two male and twenty-three female rats were deeply anesthetized with ketamine/xylazine (50 mg/kg / 10mg/kg; s.c.). When a surgical plane of anesthesia was reached each animal was placed in a stereotaxic frame and the skull was adjusted so bregma and lambda were at the same dorsal-ventral plane. Glass micropipettes (10-20 μM) filled with the retrograde tracer Fluorogold (FG; 2% soln. w/v in saline; Fluorochrome LLC; Denver, CO) were lowered into the RVM using the following coordinates (in mm): AP: -2.0 Lambda; ML: 0.0; DV: -8.5). FG was iontophoresed (50/50 duty cycle, 7.5 μA current) into the RVM for 25 minutes to facilitate neuronal uptake. The current was then turned off and the pipettes remained in place for an additional 5 minutes prior to removal to minimize backflow of the tracer along the pipette track. Following tracer injections, wounds were sutured closed, the antibiotic Neosporin was applied to the wound, and the animals were placed in clean cages to recover under

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a heat lamp. Upon complete recovery from the anesthetic, animals were returned to their original housing facilities.

Morphine Administration

Ten days following tracer injection, animals were administered morphine or saline for three consecutive days. Morphine sulfate was prepared fresh in a saline vehicle within one hour prior to administration. Animals were divided into two experimental groups: (1) eight male and eight female rats received one daily injection of morphine sulfate (4.5 mg/kg, s.c.; NIDA; Bethesda, MD) for three consecutive days and (2) five male and six female rats received two daily injections of morphine sulfate for three consecutive days. Injections were administered between the hours of $10{:}00_{A.M.}$ and $4{:}00_{P.M.}$ with multiple daily injections separated by six hours. Two control groups were used: (3) the morphine naïve group consisted of four male and four female rats injected with saline twice daily for three consecutive days and (4) the acute morphine group consisted of five male and five female rats injected with saline twice daily for 2.5 consecutive days followed by a final injection of morphine (see Table 1).

Perfusion fixation

One hour following the last injection of morphine or saline, all animals were given a lethal dose of Nembutal (160 mg/kg; i.p.). The animals were 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 following removal of blood, 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polyscience; Niles, IL) 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

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any residual acrolein. Immediately following perfusion, the brains were carefully removed, placed in a 30% sucrose solution and stored at 4^0C for at least one week prior to sectioning. Sucrose solutions were changed daily to optimize saturation of sucrose into the tissue. To section the brain, the dura and pia matter were carefully removed and the brains were cut into six series of 25 μm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution (Watson *et al.*, 1986) at –20°C until immunocytochemical processing.

Immunocytochemistry

A 1:6 series through the rostrocaudal axis of each brain was processed for FG and Fos immunoreactivity as previously described (Murphy & Hoffman, 2001). Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) to remove cryoprotectant solution immediately followed by a 20-minute incubation in 1% sodium borohydride to remove excess aldehydes. The tissue was then incubated in primary antibody solution rabbit anti-Fos (Oncogene; Cambridge, MA, lot no. 4194; 1:50,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. After rinsing out the primary antibody with KPBS, the tissue was incubated for one hour in biotinylated goat anti-rabbit IgG (Jackson Immunoresearch; West Grove, PA, 1:200), rinsed with KPBS, followed by a one hour incubation in avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), Fos was visualized as a black reaction product using nickel sulfate intensified 3,3'-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. After rinsing, sections were placed in primary antibody solution rabbit anti-FG (Chemicon; Billerica, MA, lot no. 25060005; 1:10,000) in

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KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. FG was visualized as a brown reaction product using 3,3'-diaminobenzidine containing 0.08% hydrogen peroxide in Trizma buffer (pH 7.2). After 15-30 minutes, three rinses in sodium acetate buffer terminated the reaction. Sections were then mounted out of saline onto gelatin-subbed slides, air-dried overnight, dehydrated in a series of graded alcohols, cleared in xylene, and cover-slipped using Permount.

Anatomical Data Analysis and Presentation

Data were analyzed across six representative levels through the rostrocaudal axis of the PAG (Bregma -6.72, -7.04, -7.74, -8.00, -8.30, -8.80). The number of PAG-RVM output neurons (FG+), the total number of activated PAG neurons (Fos+), and the number of activated PAG-RVM output neurons (Fos+FG) were quantified. Cell counts were conducted unilaterally as there are no differences in the number of FG+ cells for the left versus right side of PAG (Loyd & Murphy, 2006). The tissue was sectioned at 25 μm so that 125 μm separates each analyzed level of the PAG thus eliminating any possible bias from counting the same cell twice. Additionally, previous data have shown that there are no sex differences in total area (mm^2) of the PAG between weight-matched male and female Sprague-Dawley rats (Loyd & Murphy, 2006).

Data are expressed as the mean $\pm$ standard error of the mean from which percentages were calculated. A three-way analysis of variance (ANOVA) was used to test for significant main effects of sex (male, female), PAG level (Bregma –6.72 through -8.80) and treatment (two experimental groups of morphine administration and two control groups). A one-way ANOVA was used for post-hoc analysis to test for significant main effects of treatment and the Fishers PLSD was used to determine significant

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interactions between treatment groups when there was a significant main effect independent of sex. $P \leq 0.05$ was considered significant for all analyses.

Photomicrographs were generated using a Synsys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software, adjusted to figure format by adjusting brightness and contrast levels using Adobe Photoshop 7.0.

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RESULTS

Experiment 1: Behavioral Assessment of Morphine Tolerance

The objective of the present experiment was to compare the development of tolerance to morphine in male and female rats. Animals received daily morphine or saline injections for three days. On day 4, all animals received cumulative doses of morphine and tolerance was examined in each group using the hot-plate latency test. Administration of morphine on the day of testing produced a dose-dependent increase in hot-plate latency in all groups tested (Figure 1). The antinociceptive potency of morphine was greatest in male rats pretreated with saline on days 1-3 (D50 = 3.0 mg/kg). In contrast, the D50 for female rats pretreated with saline was 6.1 mg/kg, a 2.0 fold increase when compared to males. Consistent with the development of tolerance, male rats pretreated with morphine showed a significant rightward shift in the morphine dose-response curve compared to male rats pretreated with saline ([$F(1,74) = 20.04$, $p < .001$]; Figure 1A). Specifically, the D50 increased from 3.0 mg/kg (CI = 2.2 – 3.7) to 6.3 mg/kg (CI = 5.1 – 7.6) in rats pretreated with morphine, a 2.1 fold shift in the D50. By contrast, female rats showed significantly less tolerance compared to the male rats.

In females, pretreatment with morphine on days 1-3 resulted in a small rightward shift [F(1,115) – 5.63; $p < .05$] in the dose-response curve from 6.1 mg/kg (CI = 5.2 – 7.0) to 8.0 mg/kg (CI = 6.6 – 9.4; Figure 1B) indicating that females were much less sensitive to morphine tolerance.

Experiment 2a: Anatomical Assessment of Morphine Tolerance in the PAG

The next series of experiments were conducted to determine if there is a corresponding decrease in the activation of the PAG-RVM pathway during the development of tolerance to repeated systemic morphine administration in male and female rats. Animals were administered either one daily injection of morphine for three consecutive days (three doses total) or two daily injections of morphine for three consecutive days (six doses total). Control groups consisted of either two daily injections of saline for three consecutive days (saline only) or two daily injections of saline for two days and on the third day received one injection of saline followed by one injection of morphine (one dose total; See Table 1). All animals were perfused one hour following the last dose of morphine or saline and their brains were processed for Fos and FG immunoreactivity.

Across all groups, systemic morphine administration induced extensive Fos expression throughout the rostrocaudal axis of the PAG. Fos was evident in both intrinsic (non-FG+) PAG neurons and in PAG-RVM (FG+) output neurons. No significant main effect of sex [$F(1,208)= .764$, $p>0.05$] or PAG level [$F(5,208)= .0454$, $p>0.05$] was noted in the mean number of morphine-induced Fos in PAG neurons. However, there was a significant main effect of treatment [$F(3,208)= 8.733$, $p<0.05$] and a significant sex-by-treatment interaction [$F(3,208)= 10.617$, $p<0.05$]. Across all levels of the PAG,

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male rats treated with saline generally had significantly less Fos expression compared to all other treatment groups (Figure 2, $p<0.05$). In females, administration of one dose of morphine resulted in significantly less Fos expression than females treated with saline only (Figure 2, $p<0.05$). Total Fos expression was comparable between the three morphine treatment groups.

Experiment 2b: Anatomical Assessment of Morphine Tolerance in the PAG-RVM Circuit

Figure 3 shows an example of a typical iontophoretic injection of FG into the RVM of a male (top) and female (bottom) rat. All injections were located on the midline and dorsal to the pyramidal tract, at the level of the caudal pole of the facial nucleus (lambda –2.0mm). Injections outside of the RVM were not included for analysis. Injection of FG into the RVM produced dense retrograde labeling throughout the rostrocaudal axis of the PAG, with females having a significantly greater amount of labeling compared to males [$F(1,208)=59.610$, $p<0.05$] (data not shown; Loyd et al, 2006, Loyd et al., 2007).

Administration of one dose of morphine resulted in extensive Fos expression in PAG-RVM output neurons across all rostrocaudal levels of the PAG in male but not female rats (Figure 4). An analysis of the %Fos in FG+ cells revealed a significant main effect of sex [$F(1,208)= 41.194$, $p<0.05$], treatment [$F(3,208)= 43.607$, $p<0.05$] and a significant sex by treatment interaction [$F(3,208)= 19.440$, $p<0.05$]. There was no significant main effect of PAG level [$F(5,208)= .1883$, $p>0.05$] indicating that these results were consistent across the rostrocaudal axis of the PAG. The %Fos in FG neurons decreased as a function of morphine treatment in males only [$F(2,71)= 26.546$, $p<0.05$]. Overall, the average %Fos in FG neurons decreased from 44±6% in males

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receiving one injection of morphine to 8±2% in males following the sixth injection of morphine. In females, the %Fos in FG cells was low across all rostrocaudal levels of the PAG and no significant effect of treatment was observed [$F(2,83)= 1.919$, $p>0.05$]. Overall, the average %Fos in FG cells decreased non-significantly from 22±4% in females receiving one dose of morphine to 8±2% in females receiving six doses of morphine. Together, these results parallel our behavioral data indicating greater development of tolerance in male rats.

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DISCUSSION

In the present study, behavioral and neuroanatomical techniques were used to examine the role of the PAG-RVM circuit in the development of tolerance to systemic morphine in male and female rats. The results demonstrate that repeated administration of systemic morphine induces tolerance in male to a much greater extent than in female rats. In parallel, repeated morphine administration significantly reduces the activation of PAG-RVM neurons in males but not females. The effective dose for antinociception in saline treated animals was two times greater for female compared to male rats and is consistent with previous research (Cicero *et al.*, 1997; Cook & Nickerson, 2005; Ji *et al.*, 2006; Wang *et al.*, 2006). Sex differences in morphine potency are reflected in the greater activation of PAG-RVM output neurons in males following morphine administration reported here and previously (Loyd *et al.*, 2007). In addition, the greater tolerance to morphine in male compared to female rats is also consistent with previous research (Barrett *et al.*, 2001; Bernal *et al.*, 2007). Our data show that two injections of morphine per day for three consecutive days was sufficient to reduce morphine potency

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by 2.1 times in male rats. However, in females, the half maximal effective dose of morphine only increased from 6.1 to 8.0 mg/kg. Thus, not only do female rats show less antinociception in response to morphine, but they also show much less tolerance to morphine.

Our data indicate that the PAG and its projections to the RVM likely contribute to the observed behavioral differences between male and female rats. Although there was no significant main effect of sex in the number of Fos+ neurons in the PAG observed following morphine administration, the number of Fos+ neurons in the PAG that were retrogradely labeled from the RVM following a single dose of morphine was greater in male compared to female rats. Moreover, a progressive decrease in the number of Fos+FG neurons occurred following three or six repeated injections of morphine in male rats. This decrease in activation of PAG-RVM output neurons in male rats led to Fos+FG expression that was comparable to female rats. Moreover, the limited antinociceptive tolerance in females was consistent with a lack of change in the percentage of Fos+FG neurons following one, three, or six repeated injections of morphine. These data correspond very closely with the behavioral data showing that morphine antinociception is greater in male compared to female rats and tolerance causes a reduction in morphine potency so that tolerant male rats experience comparable analgesia to non-tolerant female rats. These results demonstrate that the development of tolerance to morphine is associated with a reduction in the activation of the PAG-RVM circuit in male rats and provide additional data demonstrating a central role for the PAG in morphine tolerance.

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It has been shown that tolerance occurs with repeated microinjections of morphine directly into the ventrolateral PAG (Morgan et al., 2006; Tortorici et al., 1999) and is associated with a reduction in mu opioid receptor signaling in the PAG (Bagley *et al.*, 2005). Importantly, blocking opioid binding in the PAG attenuates the development of tolerance (Lane *et al.*, 2005). While the PAG does not project directly to the spinal cord, it projects heavily to the RVM as a relay to the spinal cord (Beitz, 1982; Beitz *et al.*, 1983). The activity of RVM on-cells and off-cells that are normally inhibited and activated by morphine, respectively, become non-responsive to morphine following the induction of tolerance (Lane *et al.*, 2004). Given that tolerance develops to a greater degree when morphine is administered directly into the PAG compared to administration into the RVM (Morgan *et al.*, 2005) and there is a corresponding progressive decrease in the activity of PAG-RVM neurons, these data together indicate that a key mechanism for tolerance resides within the PAG. Although several neural structures contribute to tolerance to the antinociceptive effects of morphine, the PAG appears to play a prominent role in the development of tolerance to systemic morphine administration.

Previous data have shown that systemic morphine administration induces extensive Fos expression in the PAG that is comparable in male and female rats (Loyd *et al.*, 2007). Similarly, in the present study, total Fos expression in the PAG was not sexually dimorphic. However, unlike our previous study, morphine administration in males did not induce significantly greater Fos labeling compared to saline-treated rats. This difference between the effects of morphine in these two studies suggests that the multiple injections of saline over three days in the present study induces Fos expression that is independent of morphine. Given that Fos expression can be induced by a wide

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range of stimuli including fear and anxiety (Kim *et al.*, 1993), changes in respiration (Zhang *et al.*, 1990; Harper *et al.*, 1996), vocalization (Davis *et al.*, 1993; Zhang *et al.*, 1994), blood pressure (Carrive, 1991; Murphy *et al.*, 1995), and in fight or flight responses (Bandler *et al.*, 1985; Bandler & Carrive, 1988; Depaulis *et al.*, 1992), it is not surprising that repeated daily injections of saline induced Fos in PAG neurons. In contrast, PAG neurons retrogradely labeled with FG from the RVM were more likely to express Fos following morphine administration than saline treated rats. Moreover, this increase in Fos expression was greater in male compared to female rats. Both the increase in Fos+FG cells following morphine administration and the greater Fos labeling in male rats is consistent with our previous data (Loyd *et al.*, 2007). The greater Fos expression in PAG-RVM neurons correlates with the greater morphine antinociception produced in male compared to female rats.

Sex differences in mu opioid receptor (MOR) distribution and function in the PAG (Duncan & Murphy, 2005) may provide a mechanism for sex differences in morphine tolerance. Furthermore, cells that express both MOR and GABA are common in the PAG (Williams & Beitz, 1990; Kalyuzhny & Wessendorf, 1998) suggesting that morphine directly modulates the GABAergic neurons that tonically inhibit PAG-RVM neurons (Behbehani & Fields, 1979; Chieng & Christie, 1994; Vaughan & Christie, 1997; Commons *et al.*, 2000; Tortorici & Morgan, 2002). Administration of GABA agonists hyperpolarize PAG-RVM neurons (Osborne *et al.*, 1996) and microinjection of GABA antagonists into the PAG results in a net increase in membrane depolarization, firing frequency, frequency of EPSPs (Behbehani *et al.*, 1990) and antinociception (Morgan et al. 2005). In the present study, a potential mechanism of action may be that

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morphine is acting at mu opioid receptors present on GABA interneurons in the PAG. As tolerance develops, these GABAergic cells become unresponsive to morphine resulting in a return of tonic inhibition on PAG-RVM neurons and a corresponding decrease in the antinociceptive effects of morphine. This hypothesis is supported by the finding that repeated kainate-induced activation of PAG neurons is not sufficient to produce tolerance (Morgan *et al.*, 2003).

The PAG also contains a high density of estrogen (ERα) receptors (Murphy & Hoffman, 1999; Murphy & Hoffman, 2001; Marson & Murphy, 2006) and approximately 25% of PAG-RVM neurons contain ERα (Loyd and Murphy, unpublished observations). Estrogen has been shown to uncouple MOR from G protein-gated inwardly rectifying potassium channels, thus reducing morphine hyperpolarization of PAG-RVM neurons (Kelly *et al.*, 2003). In the present study, circulating estrogen acting at ERα receptors may be rapidly uncoupling MOR in the PAG of female rats, resulting in an overall reduction in the activity of these cells, thus preventing the development of tolerance. Cycling females were used in these studies and all rats continued to cycle normally during the three consecutive days of morphine or saline administration. The activation of PAG neurons in females tended to be more variable compared to males and stage of estrous may potentially affect morphine potency and tolerance. Interestingly, it has been shown that the excitability of neurons in the PAG is variable across the estrous cycle of the rat. During estrus and late diestrus, lower currents are required to stimulate PAG output neurons and GABA antagonists greatly increase PAG-RVM neuronal firing rates, suggesting a decrease in GABAergic tone (Brack & Lovick, 2007).

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In summary, the results of the present study indicate that the tolerance to repeated systemic morphine injections occurs in male rats, but not female rats. With repeated injections of morphine, the activation of the PAG-RVM pathway steadily declines such that males tolerant to the effects of morphine have a significantly reduced activation of the PAG-RVM pathway. Together, these data demonstrate that the development of tolerance to morphine is associated with a reduction in the activation of the PAG-RVM circuit in male rats only and provide additional data demonstrating a central role for the PAG in morphine tolerance.

DISCLOSURE/CONFLICT OF INTEREST

This work was fully supported by NIH grants to Anne Z. Murphy and Michael M. Morgan. The authors have no conflicts of interest to report.

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TITLES AND LEGENDS TO FIGURES

Table 1. Dosing Schedule for Experimental and Control Groups in Experiment 2

Figure 1. Comparison of tolerance to the antinociceptive effects of morphine in male (A) and female (B) rats. Rats were pretreated with morphine or saline twice a day for 3 days (see figure legend for pretreatment). Morphine dose-response curves for antinociception on the hot plate (HP) test were generated on Day 4 using a cumulative dosing paradigm. Both antinociceptive potency and tolerance to morphine were reduced in female (B) compared to male (A) rats.

Figure 2. A-F: Mean number of Fos-positive cells in male and female rats across the four experimental groups. Rats were injected with either morphine or saline once or twice daily for three days. Fos labeling was measured across six rostrocaudal levels of the PAG one hour following the last injection. Saline: morphine naïve; 1 Dose: saline pretreatment followed by one dose of morphine; 3 Doses: one dose of morphine per day; 6 Doses: two doses of morphine per day. Fos labeling did not vary across conditions or groups.

Figure 3. Photomicrograph of representative examples of FG injection sites at the RVM of a male (top) and female (bottom) rat. Injection sites were limited to localization within the bottom third of the medulla along the midline between the facial nuclei and dorsal to the pyramidal tract. Gi: gigantocellularis; py: pyramidal tract; 4V: fourth ventricle; 7: facial nucleus.

Figure 4. A-F: Percentage of Fos-positive neurons that were retrogradely labeled from the RVM (%Fos/FG) in male (solid bars) and female (open bars) rats for six rostrocaudal levels of the PAG. Saline: morphine naïve; 1 Dose: saline pretreatment followed by one dose of morphine; 3 Doses: one dose of morphine per day; 6 Doses: two doses of morphine per day. A decrease in labeling is evident with an increase in the number of morphine injections for male rats as would be expected with the development of tolerance. In contrast, activation of output neurons was relatively low in female rats regardless of the number of morphine injections.

TABLE 1. Daily Dosing Schedule

	Treatment Group	*Day 1*	*Day 2*	*Day 3*
Experimental	Morphine Pretreatment	1 Dose Morphine	1 Dose Morphine	1 Dose Morphine
	Morphine Pretreatment	2 Doses Morphine	2 Doses Morphine	2 Doses Morphine
Control	Morphine Naïve	2 Doses Saline	2 Doses Saline	2 Doses Saline
	Saline Pretreatment	2 Doses Saline	2 Doses Saline	1 Dose Saline/1 Dose Morphine

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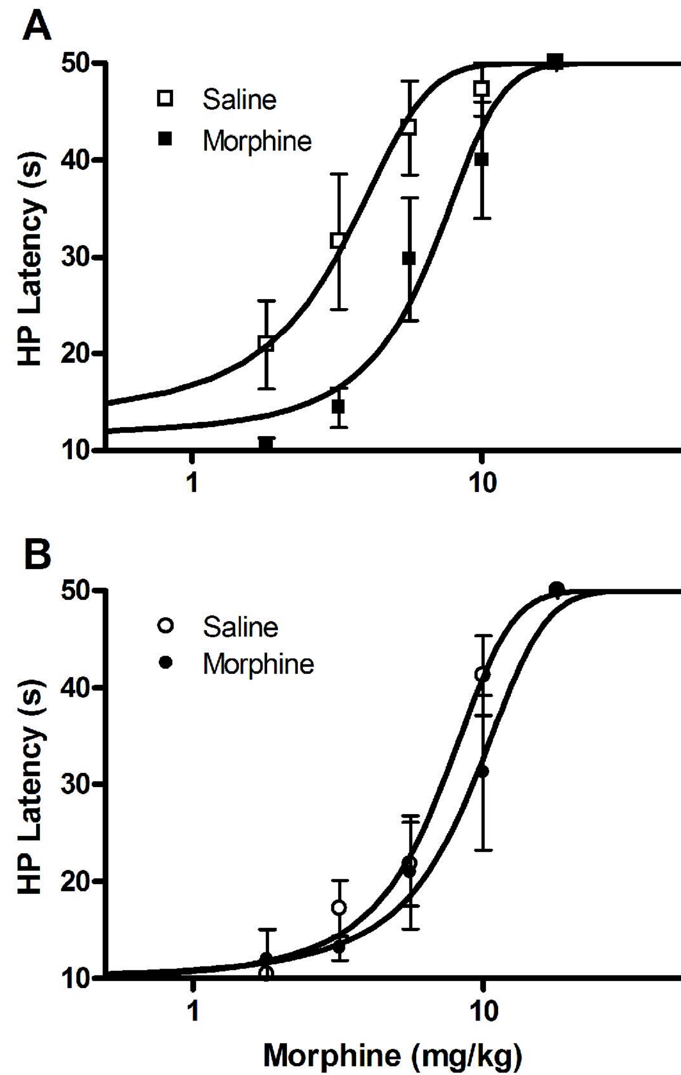

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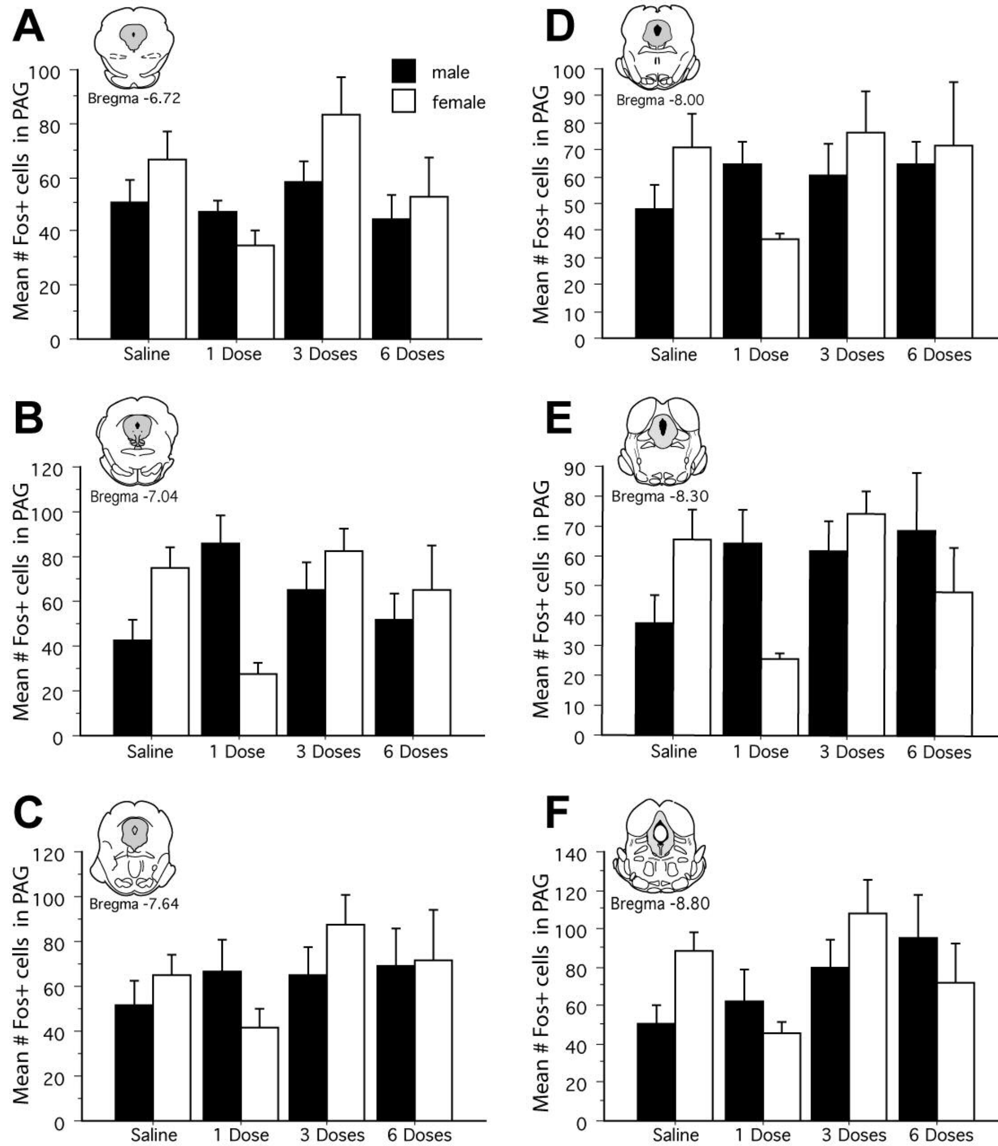

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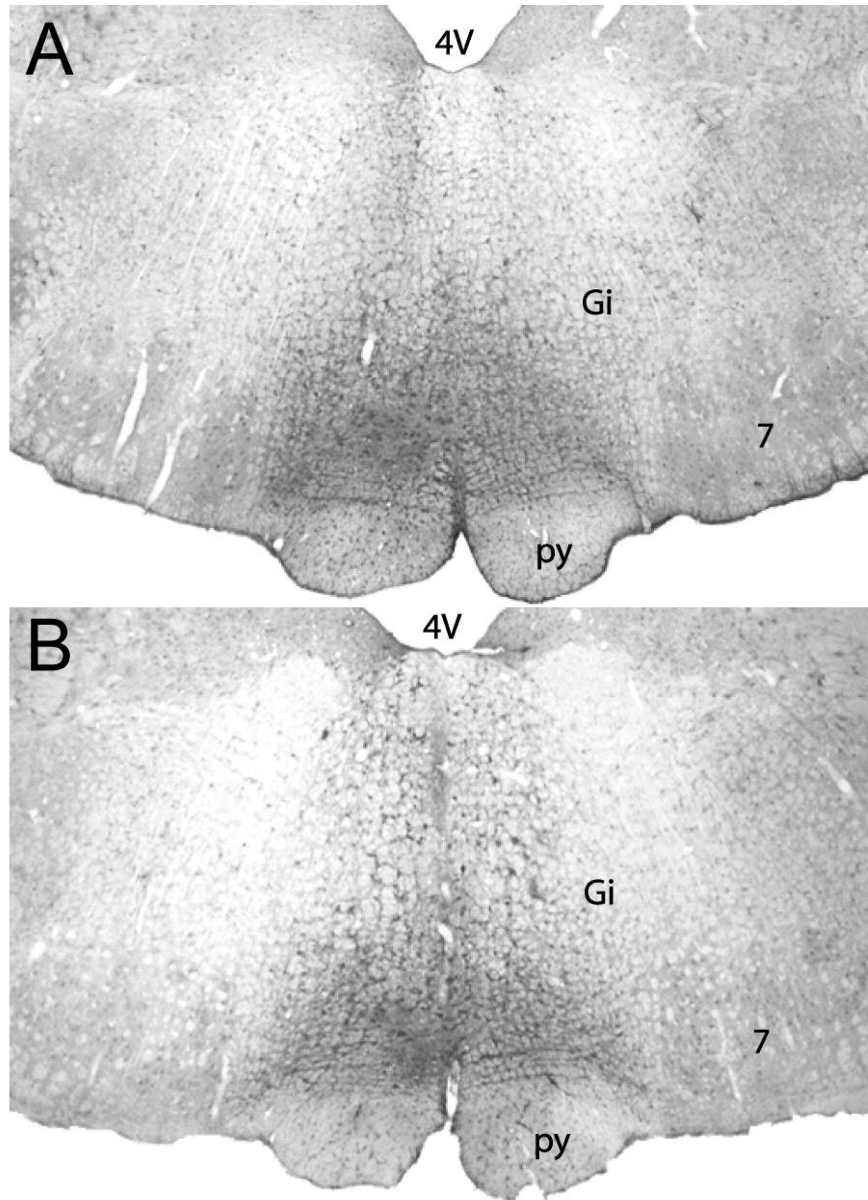

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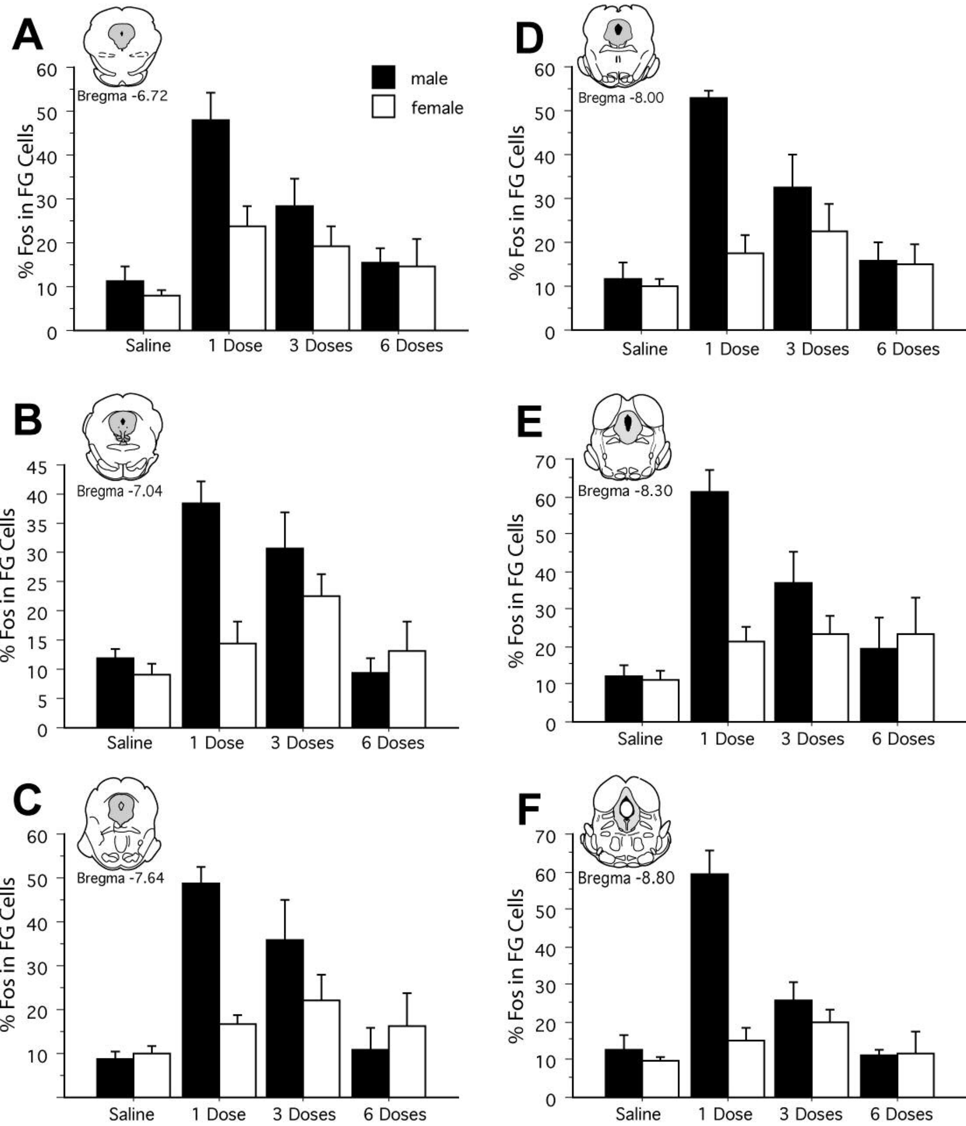

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