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Murphy, A. Z., Suckow, S., Johns, M., & Traub, R. J. (2009). Sex differences in the activation of the spinoparabrachial circuit by visceral pain. *Physiology & Behavior,* 97(2), 205-212. doi:http://dx.doi.org/10.1016/j.physbeh.2009.02.037

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Elsevier Editorial System(tm) for Physiology & Behavior
Manuscript Draft

Manuscript Number: PHB-D-08-00625R1

Title: SEX DIFFERENCES IN THE ACTIVATION OF THE SPINOPARABRACHIAL CIRCUIT BY VISCERAL PAIN

Article Type: Special issue: Sex differences

Keywords: parabrachial nucleus, opiates, mu opioid receptor, morphine, colorectal distension, pain

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First Author: Anne Z Murphy, PhD

Order of Authors: Anne Z Murphy, PhD; Anne Z. Murphy, Ph.D.; Shelby K Suckow; Malcolm Johns, MS; Richard J Traub, PhD

Abstract: Women are more sensitive to most noxious visceral stimuli, both in terms of intensity and frequency. The spinoparabrachial (spino-PBn) pathway is an essential neural circuit for the central relay of viscerosensitive information, but studies characterizing the anatomical and physiological characteristics of this pathway have only been conducted in males. Sex differences in the anatomical and/or physiological organization of the spino-PBn may contribute to the sexually dimorphic incidence rate for visceral pain syndromes. Retrograde labeling and colorectal distention (CRD) induced Fos expression was used to delineate the spino-PBn circuit in male and cycling female Sprague-Dawley rats. The ability of morphine to suppress CRD was also examined. Neurons retrogradely labeled from the PBn were localized primarily within the superficial dorsal horn and sacral parasympathetic nucleus of the L5-S1 spinal cord. While no sex differences were noted in either the distribution of spino-PBn neurons or in CRD-induced Fos expression, significantly greater Fos expression was noted specifically in spino-PBn neurons in males compared to females. Morphine selectively attenuated Fos expression in spino-PBn neurons in

males, but not females. Subsequent anatomical studies showed significantly reduced mu opioid receptor protein levels and radioligand binding within the PBn of males in comparison to females. Together, these data indicate that there are profound sex differences in how visceral pain and opiates engage the spino-PBn pathway, which may account for the observed clinical differences in visceral pain sensitivity and morphine antinociception.

February 23, 2009

Eric G Krause, PhD
Editor, Special Issue, *Physiology & Behavior*

Dear Dr. Krause:

We submit the revised manuscript (PHB-D-08-00625), entitled:

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for publication in ***Physiology & Behavior, Special Issue on Sex Differences***.

We were very pleased with the positive and thorough review of our manuscript, and are submitting a revised version including all major and minor changes as recommended by the reviewers. A detailed list of all changes with a brief summary of the reviewers comment followed by the authors' response is given below.

Authors Response to Reviewers' comments:

Reviewer #1: General Comments:
Major Comments:

1. My only substantive suggestion is to pay at least 2-3 sentence lip service to the idea that other supraspinal regions involved in endogenous analgesia may be involved as well as PbN…
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2. "We have previously reported that in urethane anesthetized rats 30-40% of the lumbosacral dorsal horn neurons retrogradely labeled from the PBn were Fos+ following noxious colorectal distention [15], suggesting that visceral pain selectively activates the spinoparabrachial circuit." This, as stated, suggests visceral pain activates the spinoparabrachial circuit but I do not see how this implies any selectivity, which I take to mean that visceral pain but not superficial pain activates… Please clarify what is meant by selectivity and how such selectivity is supported by data.
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In summary, we would like to again thank the reviewers for taking the extensive time and effort to review our manuscript. The reviews and comments were very thorough and highly constructive, and we feel that the manuscript is much stronger with these revisions. We hope that you find our revised manuscript suitable for publication in *Physiology & Behavior*.

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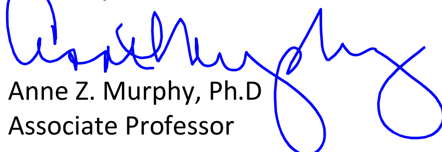

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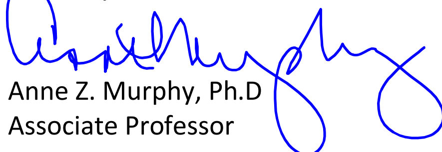

Anne Z. Murphy, Ph.D
Associate Professor

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SEX DIFFERENCES IN THE ACTIVATION OF THE SPINOPARABRACHIAL CIRCUIT BY VISCERAL PAIN

Anne Z. Murphy[1]*, Shelby K. Suckow[1], Malcolm Johns[1] and Richard J. Traub[2]

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[2] Department of Neural and Pain Sciences, University of Maryland Dental School, Baltimore, MD 21201

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Acknowledgements. The authors are grateful for the technical support of Ryan Castaniera.
Grant Support: NIH grants DA16272 and AR49555 to RJT & AZM.

Abstract. Women are more sensitive to most noxious visceral stimuli, both in terms of intensity and frequency. The spinoparabrachial (spino-PBn) pathway is an essential neural circuit for the central relay of viscerosensitive information, but studies characterizing the anatomical and physiological characteristics of this pathway have only been conducted in males. Sex differences in the anatomical and/or physiological organization of the spino-PBn may contribute to the sexually dimorphic incidence rate for visceral pain syndromes. Retrograde labeling and colorectal distention (CRD) induced Fos expression was used to delineate the spino-PBn circuit in male and cycling female Sprague-Dawley rats. The ability of morphine to suppress CRD was also examined. Neurons retrogradely labeled from the PBn were localized primarily within the superficial dorsal horn and sacral parasympathetic nucleus of the L5-S1 spinal cord. While no sex differences were noted in either the distribution of spino-PBn neurons or in CRD-induced Fos expression, significantly greater Fos expression was noted specifically in spino-PBn neurons in males compared to females. Morphine selectively attenuated Fos expression in spino-PBn neurons in males, but not females. Subsequent anatomical studies showed significantly reduced mu opioid receptor protein levels and radioligand binding within the PBn of males in comparison to females. Together, these data indicate that there are profound sex differences in how a noxious visceral stimulus and opiates engage the spino-PBn pathway, which may account for the observed clinical differences in visceral pain sensitivity and morphine antinociception.

Indexing Terms: colorectal distension, parabrachial nucleus, opiates, mu opioid receptor, morphine

Pain of visceral origin is a significant problem, both in terms of management and how it affects quality of life. It is the defining characteristic of functional bowel disorders, including irritable bowel syndrome (IBS), functional dyspepsia, ulcerative colitis, and acute and chronic pancreatic disease to name a few. Epidemiological studies have consistently shown that women are more likely to suffer from visceral pain than men [1-4]. Unfortunately, very little, if anything, is known regarding the etiology of these sex differences. Using colorectal distension as a model of visceral pain, we have recently shown that females have significantly lower visceral pain thresholds than males [5]. Our data further suggest that the gonadal steroid estradiol contributes to the observed sex differences in visceral sensitivity [5-7]. However, the anatomical and physiological basis for sexually dimorphic visceral pain thresholds is not known.

The parabrachial nucleus (PBN) has long been recognized as an important brainstem relay in visceral nociceptive sensory processing [8-10]. Nociceptive dorsal horn neurons, most notably from lamina I, project to the PBn [11-15] and relay noxious visceral input to the lateral parabrachial nuclei including the external, superior, dorsal and central lateral subnuclei [16, 17]. Neurons in the parabrachial nuclei that respond to noxious stimulation, in turn, project to the central nucleus of the amygdala [8, 18, 19], and this spino-parabrachial-amygdala pathway is thought to constitute an essential circuit for the affective-motivational component of visceral pain [18].

Lumbosacral spinal cord neurons retrogradely labeled from the PBn are located predominantly within lamina I and the lateral reticulated area of the neck of the dorsal horn. We have previously reported that in urethane anesthetized rats 30-40% of the lumbosacral dorsal horn neurons retrogradely labeled from the PBn were Fos+ following

noxious colorectal distention [15], suggesting that visceral pain activates the spinoparabrachial circuit. These studies were conducted in males and comparable studies have not been conducted in females even though the incidence rate for most visceral pain syndromes is significantly higher in females [1].

It is well established that systemic or central opioid administration significantly attenuates visceral pain. Indeed, opioid-based narcotics have long been recognized as the drug of choice for the attenuation of postoperative, obstetric and cancer pain that is visceral in origin [20-22]. Several studies have reported that intrathecally or systemically administered mu, delta or kappa agonists significantly attenuate both the visceromotor reflex and the pressor reflex evoked by CRD (rat: 6, 8, 18, 19, 23, 24; rabbit: 27, 28). Similar results have been reported using the acetic acid writhing test [25-27].

While the above studies indicate that morphine is an effective drug for the alleviation of visceral pain, it is becoming increasingly clear that there are profound sex differences in the antinociceptive potency of opioids. Indeed, we have recently reported that the ED_{50} for morphine is approximately two-times higher in female than male rats in either visceral [6, 24, 28] or persistent somatic [29, 37] pain assays. However, the locus for morphine suppression of visceral pain is unclear. Mu opioid receptors (MOR) are extremely dense within the rat dorsal horn and overlap with the distribution of spino-PBn neurons. However, as there are no sex differences in spinal morphine antinociception of CRD, suggesting that the spinal cord dorsal horn is not the locus underlying the sexually dimorphic actions of morphine [24]. Alternatively, MOR expression is extremely dense within the PBN, and overlaps with the distribution of spino-PBn afferents [30, 31]. Sex differences in morphine inhibition of visceroceptive input to the PBn would provide a

direct mechanism for the sexually dimorphic effects of morphine on visceral pain modulation.

The present study tested the hypothesis that sex differences in the anatomical organization of the spino-PBn circuit, and its activation by a noxious visceral stimulus, provide the biological bases for the observed sex differences in visceral pain. We also tested whether systemic administration of morphine differentially suppressed the activation of this pathway in male and female rats. Additional studies tested the hypothesis that sex differences in PBn MOR protein levels and binding contributed to the dimorphic effects of morphine observed in the present study.

MATERIALS AND METHODS

Experimental Subjects. Eighteen adult intact male Sprague-Dawley rats and eighteen adult cycling female Sprague-Dawley rats (Zivic-Miller) were used in these experiments. Animals were weight-matched (250-300g) and housed in same-sex pairs in separate rooms on a 12:12 hour light: dark cycle (lights on at $7{:}00_{A.M.}$). Rats had access to food and water *ad libitum*. These studies were performed in strict compliance with the Institutional Animal Care and Use Committee at Georgia State University. All efforts were made to minimize any possible suffering by the animal, and to reduce the number of animals used.

Vaginal Cytology. Vaginal lavages were performed daily beginning two weeks prior to 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 [58]. Diestrus 1 was differentiated from Diestrus 2 by the presence of leukocytes. As no differences were noted between Diestrus 1 and Diestrus 2 animals on any of our dependent measures, these groups are pooled (Diestrus). Rats that appeared between phases were noted as being in the more advanced stage.

Iontophoresis Injections. Male and female rats were anesthetized with ketamine/xylazine (50 mg/kg / 10mg/kg; s.c.) and placed in a stereotaxic frame upon achieving a deep surgical plane of anesthesia as previously described [15]. The skull was adjusted so that bregma and lambda were at the same dorsoventral coordinate. A glass micropipette (tip: 10-20 µM) was filled with the retrograde tracer Fluorogold (FG;

2% soln. w/v in saline; Fluorochrome Inc.) via capillary action and lowered into the parabrachial nucleus at the following coordinates (in mm): Lambda: -0.5; ML: 1.8; DV: 5.0 from brain. FG was iontophoresed (50/50 duty cycle, 7.5 μA current) for 15 min. Pipettes remained in place for five minutes after injection to prevent backflow of tracer along the pipette tract and to facilitate neuronal uptake. Attention to detail was taken to ensure that all injection protocols were comparable for males and females. Following surgery the animals recovered under a heat lamp and were returned to their original housing facilities upon recovery from the anesthetic.

Colorectal Distension & Morphine Administration. Animals were fasted 24 hrs prior to the experiment; water was provided ad libitum. On the day of the experiment, rats were briefly sedated with isofluorane and a 5-6 cm balloon attached to Tygon tubing was inserted through the anus into the descending colon and rectum. The distal end of the balloon was at least 1 cm proximal to the external anal sphincter. Rats were loosely restrained and allowed 30 min to recover from the isofluorane. Colorectal distention (CRD) was produced by inflation of the distention balloon with air. The pressure was monitored and kept constant by a pressure controller/timing device (Bioengineering, University of Iowa). Rats (male, n=16; females, n=16) were distended to 80 mmHg (noxious) for two hours (30 sec on; 90 sec off) and then perfused [32]. Control animals (n=2/sex) were treated identical to the experimental groups except that the balloon was not inflated.

Immediately prior to distention, animals were administered morphine sulfate subcutaneously (4.0 mg/kg, s.c.; 28, 34) or equivolume of sterile saline as a control.

Morphine sulfate, a gift from the NIDA Drug program, was prepared fresh in sterile saline on the day of the experiment.

Perfusion fixation. Two hours after morphine administration, animals were given a euthanizing dose of Nembutal (160 mg/kg; i.p.) and perfused transcardially with 150-200 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator, followed immediately by 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polyscience). Residual acrolein was removed from the animal with a final rinse with 150-200 ml of the sodium chloride/sodium nitrate solution. The brain and spinal cord were removed immediately following perfusion and the tissue was stored at 4°C in 30% sucrose solution. Tissue was cut into 25μm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution [33] at –20°C until immunocytochemical processing was initiated.

Immunocytochemistry. Brain (1:6 series) and lumbosacral spinal cord (L5-S1; 1:4 series) were processed for FG and/or Fos immunoreactivity as previously described [28, 34]. Briefly, sections were removed from the cryoprotectant-antifreeze solution, rinsed extensively in potassium phosphate-buffered saline (KPBS), and then reacted for 20 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were then incubated in primary antibody solution directed against either Fos or FG in KPBS containing 0.1% Triton X for 1 hour at room temperature followed by 48 hours at 4°C. Cells containing Fos were identified using the polyclonal rabbit anti-Fos antibody (Oncogene, Cat. No. PC38; lot no. 4194) at a concentration of 1:50,000. This rabbit antiserum was prepared against the synthetic peptide (SGFNADYEASSSRC) corresponding to amino acids 4-17 of human c-Fos. In western blots, this antibody

recognizes the ~55 kDa c-Fos and ~62 kDa v-Fos proteins, and does not cross-react with the ~39 kDa Jun protein (manufacturer's technical information). FG containing cells were recognized using the polyclonal rabbit anti-Fluorogold antibody (Chemicon, Cat. No. AB153; lot no. 25060005) at a concentration of 1:10,000. This antibody was raised against the chemical compound hydroxystildamidine. No cytoplasmic FG staining was present in animals in which the tracer failed to be ejected from the electrode. In addition, the distribution of retrogradely labeled cells within the dorsal horn of the spinal cord is identical to published reports using alternative retrograde tracers.

After rinsing in KPBS, the tissue was incubated for 1 hour in biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, 1:600), rinsed in KPBS, and incubated for 1 hour 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. FG was always visualized as a brown reaction product using 3,3'-diaminobenzidine containing 0.08% hydrogen peroxide in Tris buffer (pH 7.2). The reaction product was terminated after 15-30 minutes by rinsing in sodium acetate buffer.

For labeling both antigens, sections were first processed for Fos immunoreactivity as described above producing a black reaction product. Following visualization of Fos, sections were rinsed in KPBS and then processed as above with the rabbit anti-FG producing a brown reaction product. Resulting double labeled neurons had black nuclei [35] surrounded by brown cytoplasm (FG) [15, 28]. Sections were 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.

For identification of mu opioid receptor containing neurons, 25 um tissue sections were incubated in primary antibody solution rabbit anti-MOR1 (Abcam, Cambridge, MA; 1,50,000) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. This antibody was prepared against the synthetic peptide **(**NHQLENLEAETAPLP) corresponding to amino acids 384-398 of rat MOR1 [36]. Secondary and avidin-biotin-peroxidase steps were identical to above. MOR1 was visualized as a black reaction product using nickel sulfate intensified 3,3'-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. Sections were rinsed, mounted and dehydrated in a series of graded alcohols, and then cover-slipped using Permount. Mu opioid receptor antibody specificity was confirmed by lack of MOR+ labeling in MOR knock out mouse tissue (kindly provided by Bridgette Kieffer, Ph.D., Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France).

Receptor Autoradiography. Intact male (n=4) and cycling female rats (n=4) were rapidly decapitated and brains prepared for autoradiography as previously described [37]. Briefly, 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 30 μm 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 50mM Tris buffer (pH 7.4) containing 100nM NaCl. Slides were then placed in a tracer buffer containing either tritiated DAMGO (1 nM; Amersham, Pittsburg, PA) or tritiated

Naloxone (0.5 nM; Amersham, Pittsburg, PA) for 60 minutes followed by a series of rinses in 50mM Tris buffer (pH 7.4) containing $MgCl_2$. Tissue was allowed to dry and placed on autoradiographic film for 5 weeks at which point films were developed with a FujiFilm BAS 5000 (Valhalla, NY).

Densitometry. For MOR immunohistochemistry data, 12-bit grayscale images were captured using a QImaging Retiga EXi CCD camera (Surrey, BC, Canada) and IPLab Image Analysis Software (BD Biosciences, Rockville, MD). The PBn was bilaterally sampled (35mm^2 at 10X magnification) between Bregma -9.16 to -9.68 three times per section and the average grayscale pixel value across the PBn (mean 6 sections per subject) was recorded. Measures were corrected for non-specific immunoreactivity background by subtraction of measures taken from areas of equal size lacking specific immunoreactivity adjacent to the PBn in the same section. For autoradiography data, color images were captured and the brightness/contrast was adjusted using Fujifilm Multigauge software (Vallhala, NY). Measures were corrected for non-specific binding by subtraction of measures taken from a same section area adjacent to the PBn of equal size.

Data Analysis and Presentation. The mean number of FG+, Fos+, or Fos+FG neurons was determined for three rostrocaudal levels of the spinal cord: L5, L6 and S1. These regions are easily distinguishable based on the following anatomical landmarks: L5 contains a prominent lateral column of motor neurons; L6 contains the retrodorsolateral (RDL) motoneuronal pool; S1 was defined as the absence of the RDL and the presence of the sacral parasympathetic nucleus (SPN) [38, 39]. The number of singly (FG or Fos) or dually (FG that contained Fos; FG/Fos) labeled cells was for

quantified for 5-6 sections per region per animal. The number of Fos+ cells within the PBn was also quantified on the side contra to the injection site using the above procedure. Results are expressed as the mean ± standard error of the mean. Only animals with comparable injection sites limited to the PBn were used for analysis (males, n=12; females, n=10). Analysis of variance was used to test for significant main effects of sex (male, female) and treatment (morphine, saline). $P<0.05$ was considered significant for all analyses.

For data presentation, a representative animal from each experimental group was selected and the distributions of FG and Fos within the L5-S1 spinal cord were plotted using a Nikon Drawing Tube attached to a Nikon Optiphot microscope. Plots were imported to the computer and finalized using Adobe Illustrator 10. Photomicrographs were generated using a Synsys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software and finalized using Adobe Photoshop 7.0. Alterations to the images were strictly limited to enhancement of brightness/contrast.

RESULTS

Anatomical and Functional Organization of the Spino-Parabrachial Circuit

The first series of experiments tested the hypothesis that sex differences in the anatomy and/or physiology of the spino-PBn pathway contributed to the previously observed sex differences in visceral sensitivity [24]. Figure 1 shows examples of a Fluorogold (FG) injections into the PBn. Spino-PBn neurons were localized within L5-S1 spinal cord, in lamina I and X, and the sacral parasympathetic nucleus (SPN). No sex differences were noted in either the distribution or number of FG+ cells, with an average of 10-15 retrogradely labeled cells per section (see Fig. 2A,B). Interestingly, while colorectal distention induced comparable Fos expression in the L5-S1 cord of males and females (mean cells/section for combined L5-S1: males, 79±7; females, 92±4; Fig. 2A, right panel and Fig. 2B), noxious CRD selectively activated spino-PBn neurons in males, but not females (Fig. 2C). Indeed, in males, approximately 70% of spino-PBn neurons expressed CRD-induced Fos; by contrast, less than 40% of spino-PBn neurons were Fos+ in females. Dual labeled cells were localized primarily within lamina I and SPN (Fig. 2B). Limited Fos expression was observed in control animals that did not undergo distension. Figure 3 shows an example of the high level of Fos expression in spino-PBn neurons in males versus females. These studies are the first to demonstrate sex differences in the activation of a visceral pain pathway; this sexually dimorphic activation of the spino-PBn circuit may provide the biological basis for our observed sex differences in visceral sensitivity.

Morphine differentially modulates visceral sensitivity in males and females.

In our previous studies, we found that systemic administration of morphine differentially suppressed the magnitude of the visceromotor response to CRD (ED_{50}: 3.8 for females vs. 1.5 mg/kg for males [24]). Given these significant sex differences in morphine attenuation of visceral pain, we next examined whether morphine preferentially suppressed CRD-induced Fos in spino-PBn neurons. As shown in Figure 4, morphine significantly suppressed CRD-induced Fos in males ($p<0.0001$) but not females ($p>0.05$). Similar to previous reports [42], morphine suppressed CRD-induced Fos in all laminar regions (Fig. 4B). Interestingly, the percentage of spino-PBn neurons expressing Fos was reduced from 70% to 40% in males, while no reduction in FG/Fos+ neurons was noted for females (Fig. 4C). Representative photomicrographs showing the sex difference in morphine suppression of CRD-induced Fos in spino-PBn neurons are shown in Figure 5.

Sex differences in mu opioid receptor expression in the PBn

In the present study, morphine was given systemically and therefore may be acting peripherally and/or centrally to suppress CRD-induced Fos and visceral pain. The lumbosacral dorsal horn and PBn are two likely sites for morphine action: both contain high levels of MOR and respond to noxious visceroceptive input. As we have previously shown no sex differences in spinal morphine suppression of visceral pain [24], our next series of experiments focused on the PBn, specifically examining whether MOR expression within the PBn was sexually dimorphic and may contribute to the observed sex differences in morphine suppression of visceral pain.

MOR immunoreactivity was extremely dense throughout the PBn, and was present in all PBn subnuclei. Figure 6 shows an example of MOR+ staining within the PBn of a

male (Fig. 6A) and diestrus female (Fig. 6B) rat. MOR expression fluctuated across the estrous cycle, with significantly reduced MOR immunoreactivity noted in proestrus females when compared males (p=0.007). This reduction in MOR expression in proestrus females was not limited to any particular subnuclei but was uniformly reduced throughout the PBn.

We next used autoradiography to determine if the sex differences in PBn MOR immunoreactivity were reflected as a decrease in ligand binding. Figure 7 shows representative autoradiograms of a male (Fig. 7A) and diestrus female (Fig. 7B). Quantitative analysis of tritiated DAMGO binding showed significantly reduced binding within the PBn of diestrus females compared to males (p=0.04).

Discussion

The present study tested the hypothesis that sex differences in the anatomy and/or physiology of the spino-PBn pathway contribute to sex differences in visceral pain. Two key observations are reported. First, no sex difference was observed in either the number or distribution of CRD-evoked Fos labeled neurons in the spinal cord, or in the number or distribution of spinal neurons retrogradely labeled from the PBn. However, noxious CRD preferentially activated the spino-PBn pathway in males, but not females. In contrast, the response of postsynaptic dorsal column neurons to noxious visceral stimulation is greater in females compared to males [55], suggesting that supraspinal relay of visceroceptive information is sexually dimorphic. These findings have significant implications for therapeutic strategies designed to suppress visceral pain.

The second major finding of these studies is that systemic morphine produced greater attenuation of CRD-induced Fos expression in the lumbosacral spinal cord in general, and spino-PBn neurons in particular, in male rats. This finding is consistent with previous studies reporting greater morphine antinociception in males compared to females [37, 44-46]. Indeed, we have recently reported that systemic, intracerebroventricular or intra-PAG morphine produces greater attenuation of visceral and persistent somatic inflammatory pain in male compared to female rats [24, 37]. Interestingly, the sex difference in the effect of morphine on the visceromotor response was not mediated at the level of the spinal cord as we have previously reported no sex difference following intrathecal morphine, although there was significant antinociception [24]. On the other hand, intracerebroventricular injection of morphine attenuated the

visceromotor response to a greater extent in male rats, suggesting supraspinal processing underlies the observed sex difference [37, 59, 60].

The present data are consistent with the hypothesis that the spino-PBn pathway contributes to the sex difference in CRD-evoked visceromotor response by more effectively activating a descending opioidergic inhibitory circuit in males. The PBn contains a dense population of both mu and delta opioid receptor immunoreactive fibers, as well as their respective endogenous ligands, endomorphin and enkephalin [10, 31, 49-51]. Activation of the spino-PBn pathway has been shown to modulate visceral pain, presumably via the release of endogenous opioids. Therefore, activation of the spino-PBn circuit by noxious colorectal distension would have the end result of decreasing visceral pain sensitivity. However, noxious CRD did not activate this circuit in females, and suggests that the failure of a noxious visceral stimulus to activate the spino-PBn pathway contributes to the overall increased visceral pain sensitivity observed in females.

In addition to the PBn, other brain regions may also contribute to the observed differences in viscerosensitivity and morphine analgesia noted in the present study, including the midbrain periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) which includes the nucleus raphe magnus. Indeed, the PAG, and its descending projections to the RVM and spinal cord constitute an essential pathway for the pain-suppressing effects of both exogenous and endogenous opiates. Similar to the PBn, sex differences in MOR expression have been reported within the PAG, with males having 1.5-fold higher levels than females [37]. In addition, persistent inflammatory pain has been shown to selectively activate the PAG-RVM pathway in males, but not

females [28, 59]. Together with the present data, this suggests that sex differences in the anatomical and physiological organization of pain pathways may be more common than previously realized, and highlights the need for the inclusion of female subjects in all experimental studies examining the biological bases of somatic, visceral or orofacial pain.

There are several additional mechanisms that may also contribute to the observed sex differences in morphine suppression of visceral pain. First, we show for the first time that overall, males have significantly higher levels of [3H]DAMGO binding within the PBn than females. Similarly, we show that MOR protein levels fluctuate across the estrous cycle, with overall lower levels of MOR immunoreactivity observed in females than males. As estradiol has been shown to induce rapid MOR internalization, thereby reducing available MOR for ligand binding [52,53], these results are not surprising. Estradiol has also been shown to uncouple MOR from GIRK channels [54], which would account for the decreased efficacy of morphine observed in the present study. Similar findings demonstrating reduced morphine potency during proestrus have also been reported following intra-PAG opiate administration [37]. Unfortunately, in our behavioral studies, we were not able to examine the impact of estrous on visceral sensitivity and activation of the spino-PBn pathway due to low numbers of animals in all three stages (estrus, diestrus and proestus).

As discussed above, the results of the present study parallel our recent findings demonstrating significantly higher levels of MOR immunoreactivity and binding within the PAG of male versus female rats [37]. Together, these studies suggest that, overall, females have lower levels of MOR within several key pain-related brain regions which

likely provide the biological bases for the sexually dimorphic action of morphine. These results further suggest that morphine may not be the drug of choice for the modulation of pain in females. Clearly, additional studies are warranted.

In summary, epidemiological studies have consistently shown that women are more likely to suffer from visceral pain than men [1]. Unfortunately, very little, if anything, is known regarding the etiology of these sex differences [21, 55-57]. The results of the present study demonstrate for the first time that noxious visceral stimulation selectively activates the spino-PBn pathway in males; as activation of this pathway would likely result in the release of endogenous opioid peptides in order to modulate the visceral pain, these studies suggest a potential anatomical circuit underlying sex differences in visceral pain.

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Figure Legends

Figure 1. Photomicrographs of Fluorogold (FG) injections into the PBn of male (A) and female (B) rats. LTDg, Laterodorsal tegmental nucleus; scp, superior cerebellar peduncle; Mo5, trigeminal motor nucleus.

Figure 2. (A) Mean number of retrogradely labeled neurons observed within the L5-S1 spinal cord following FG injection into the PBn of a male and female rat (left panel). The right panels show the mean number of Fos+ cells localized within the L5-S1 spinal cord following colorectal distention or control. (B) Representative plot of the distribution of FG+ cells (black) and Fos+ cells (grey) within the male and female L6 spinal cord following colorectal distension. Each dot represents one cell. (C) Percentage of spino-parabrachial neurons that contained Fos following noxious colorectal distention. Error bars represent S.E.M.

Figure 3. Photomicrograph of the spinal cord dorsal horn showing examples of neurons retrogradely labeled from the parabrachial nucleus (FG; brown), neurons expressing CRD-induced Fos (black) and dual labeled cells (FG+Fos; indicated by red arrows) in males (A) and diestrus females (B).

Figure 4. (A) Mean number of L5-S1 dorsal horn neurons expressing CRD-induced Fos following administration of saline or morphine in male and female rats. (B) Representative plots of the distribution of FG+ cells (black) and CRD-induced Fos (grey) within the male and female L6 spinal cord following administration of morphine. Each dot represents one cell. (C) Percentage of spino-parabrachial neurons that expressed CRD-induced Fos following administration of saline or morphine in males and females. Error bars represent S.E.M.

Figure 5. Representative photomicrographs of CRD-induced Fos (black dots) in the L6 spinal cord following administration of saline or morphine in male and female rats.

Figure 6. Mu opioid receptor expression within the parabrachial nucleus of the male (A) and female (B) rat. (C) Densitometric analysis of the mean optical density of PBn MOR immunocytochemistry showed a significant reduction of MOR in proestrus females in comparison to males. vsc, ventral spinocerebellar tract; scp, superior cerebellar peduncle; MO5, trigeminal motor nucleus; LPBv, lateral parabrachial nucleus, ventral; MPBe, Medial parabrachial nucleus, external; LPBc, lateral parabrachial nucleus, central LPBe, lateral parabrachial nucleus, external; ovx, ovariectomized; Di, diestrus; Pro, proestrus; Est, estrus. Error bars represent S.E.M. Optical density level for male = 526; for female = 364. * indicates a significant mean difference, $p<0.05$.

Figure 7. Photomicrograph depicting [3H]DAMGO receptor binding within the parabrachial nucleus of a male (A) and female (B) rat. (C) Optical density values of [3H]DAMGO binding within the PBn of males and females. Error bars represent S.E.M.

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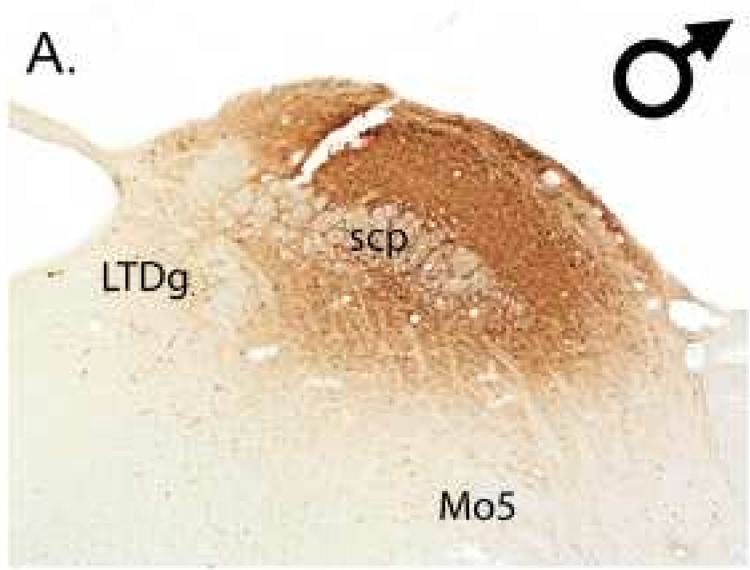

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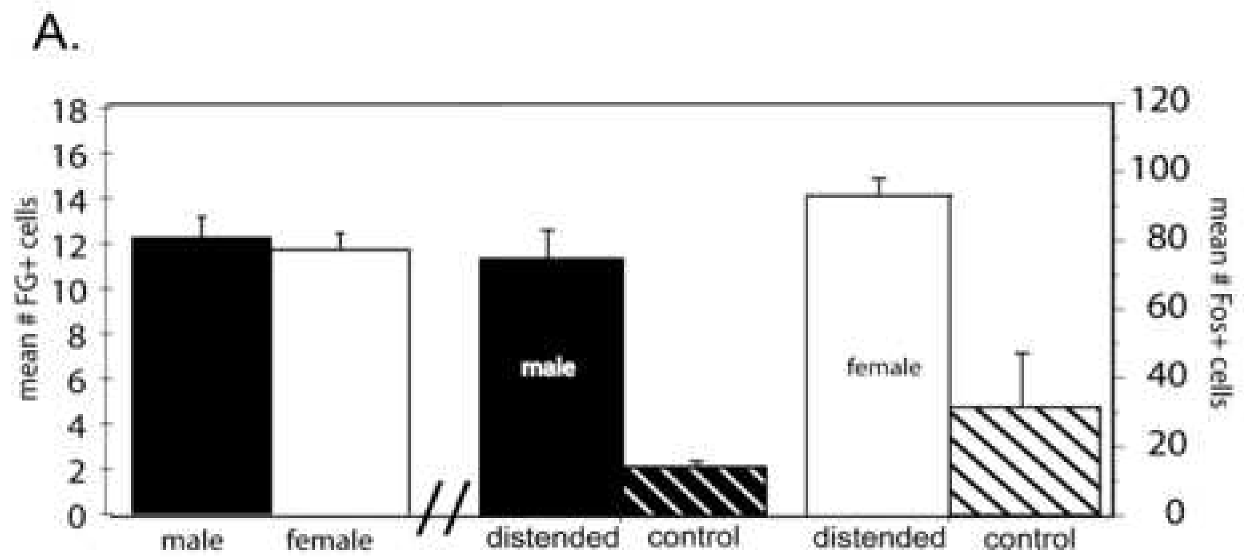

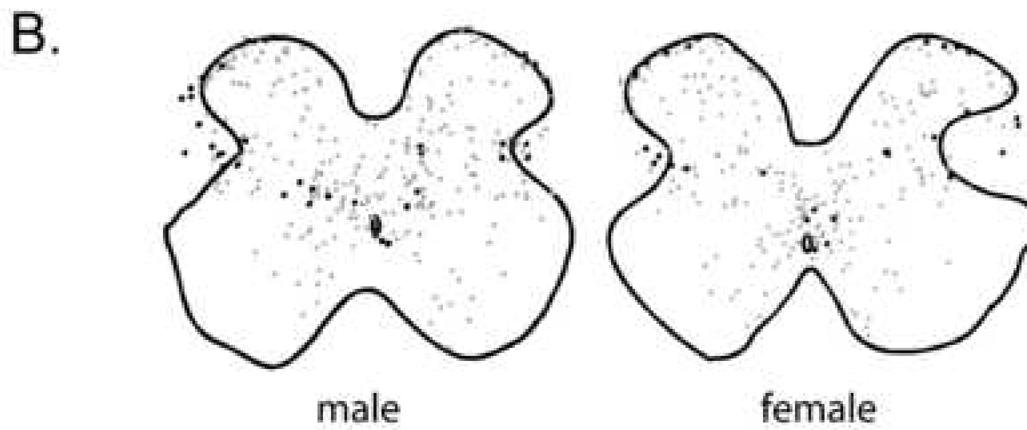

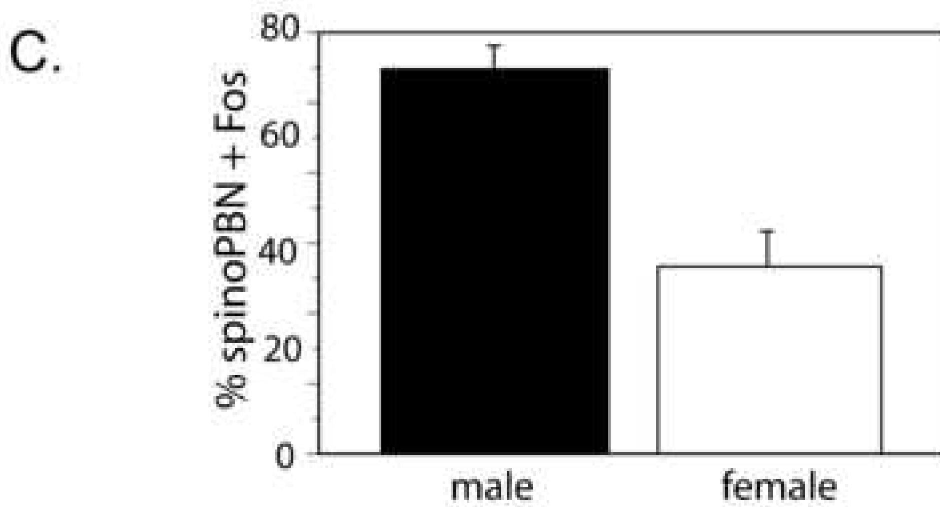

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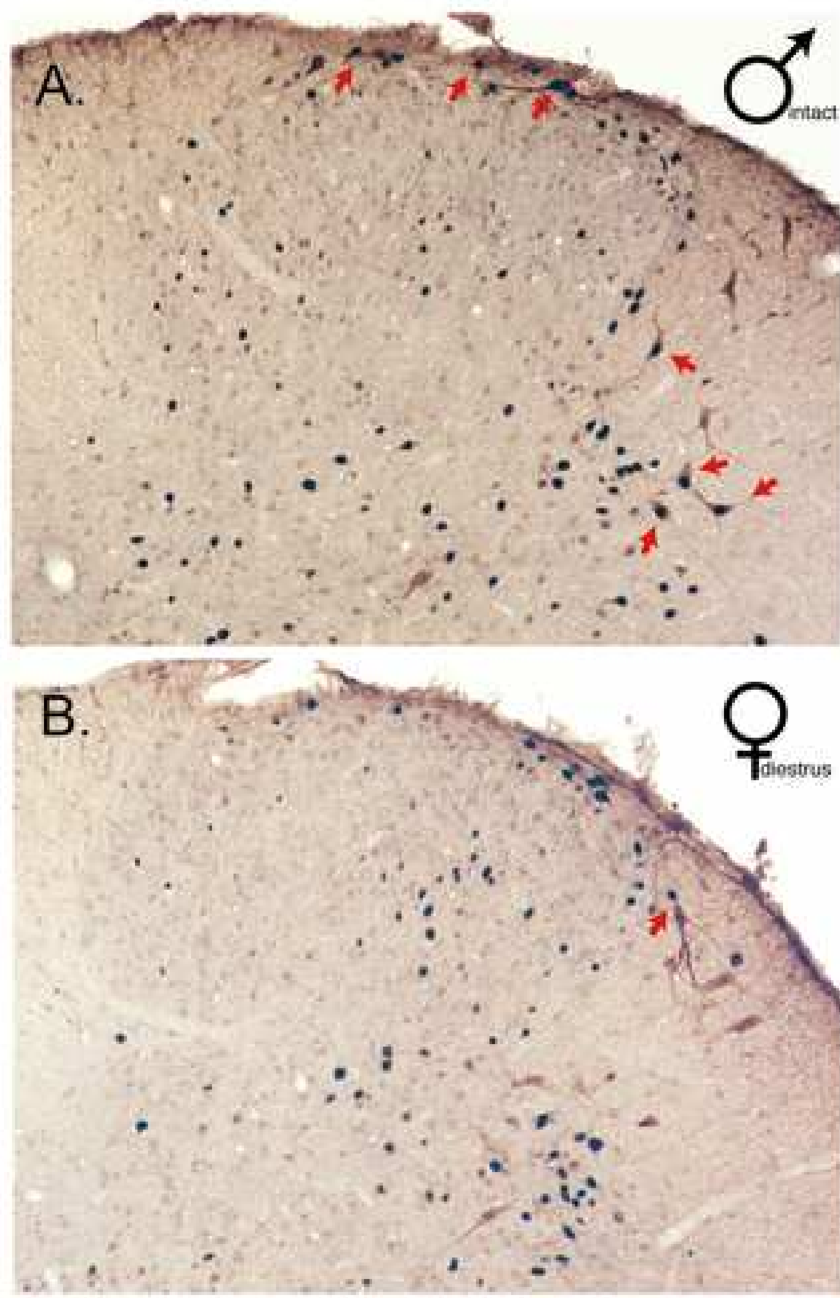

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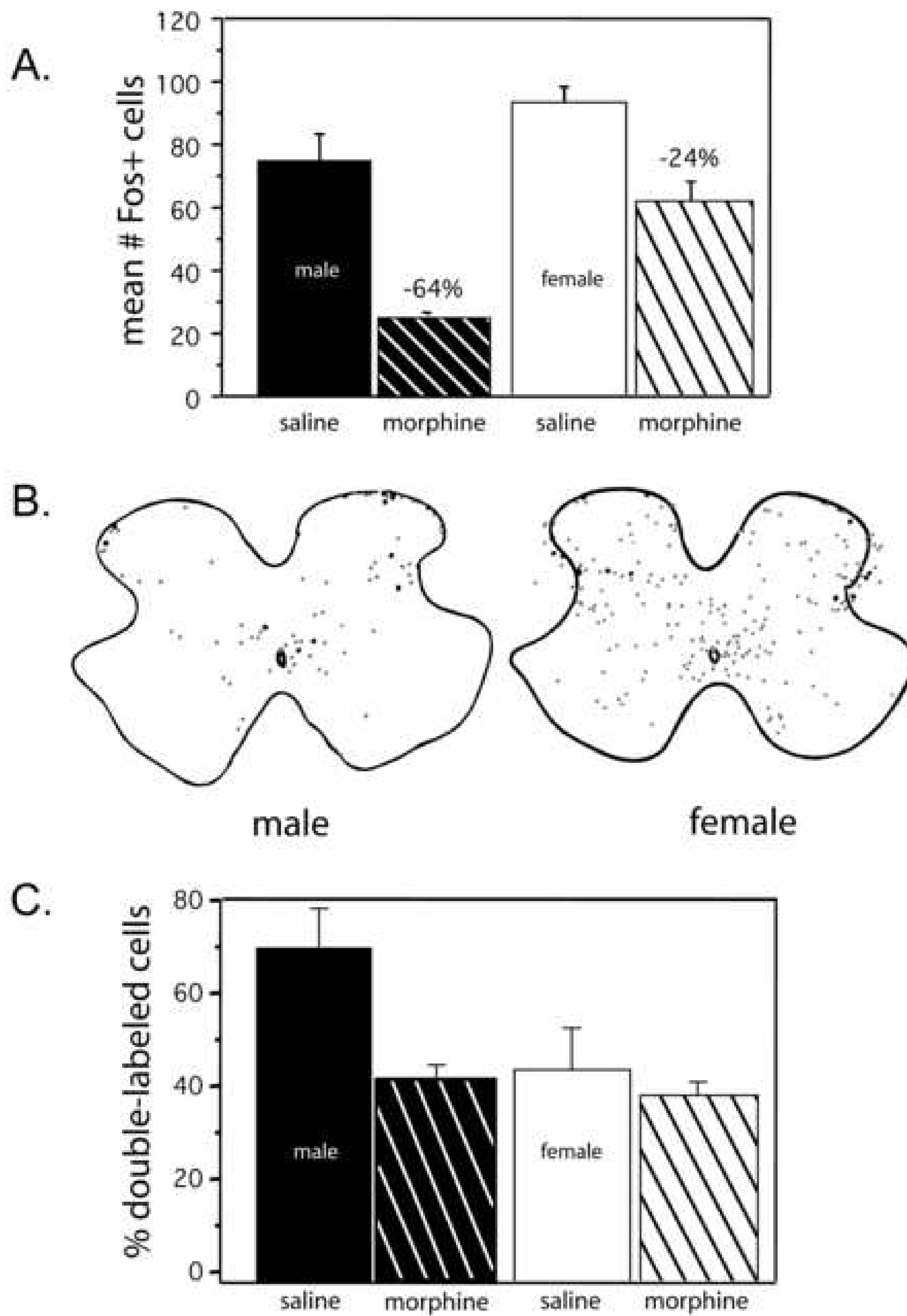

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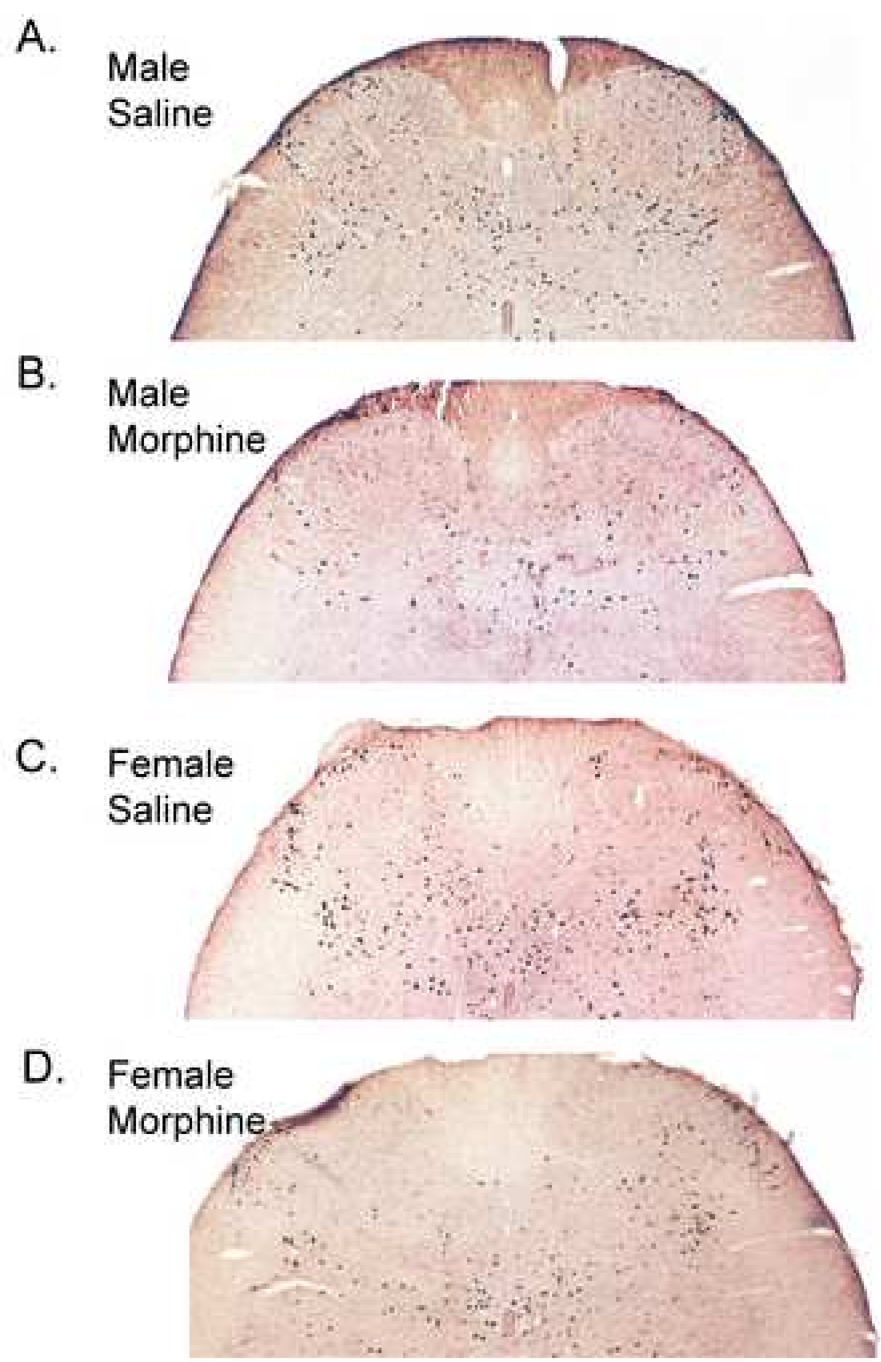

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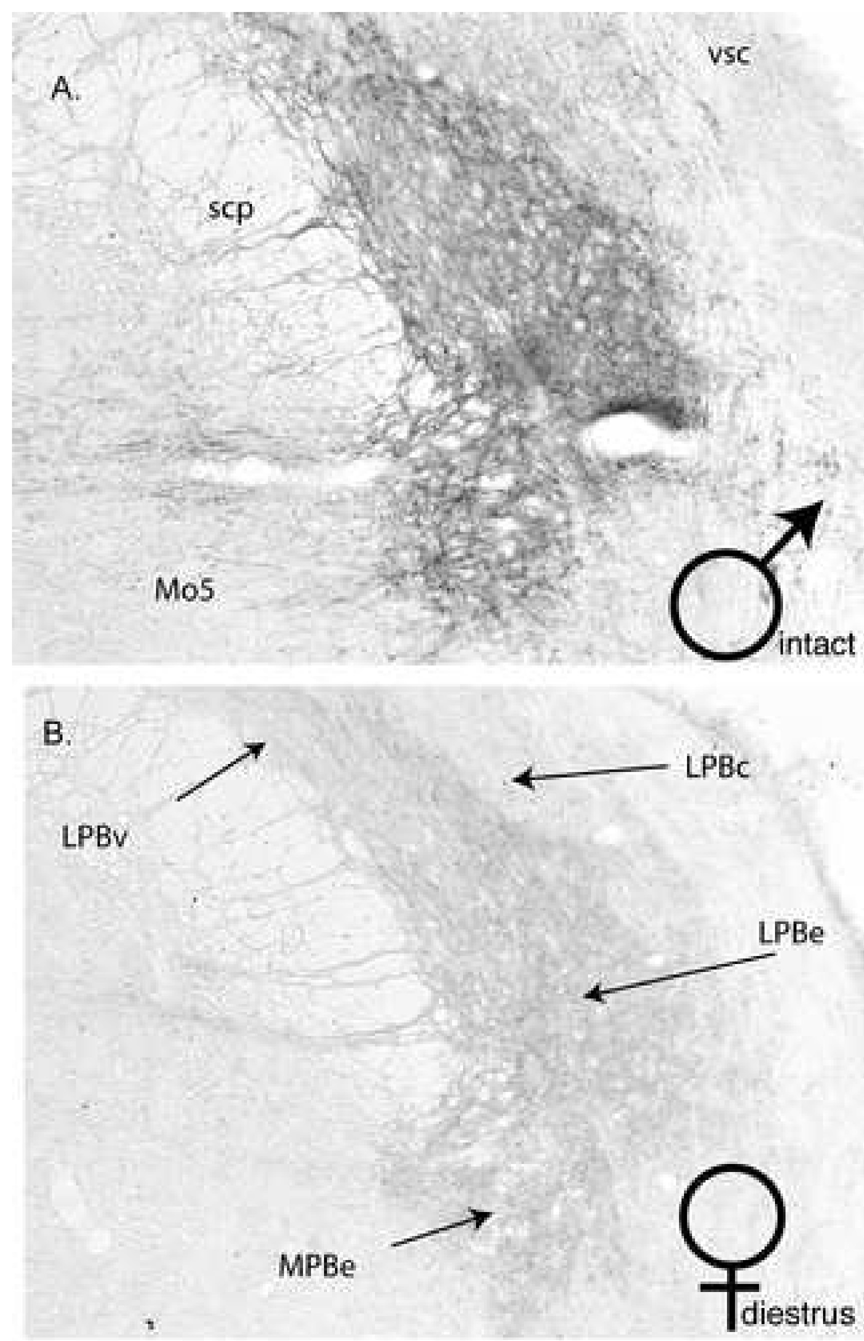

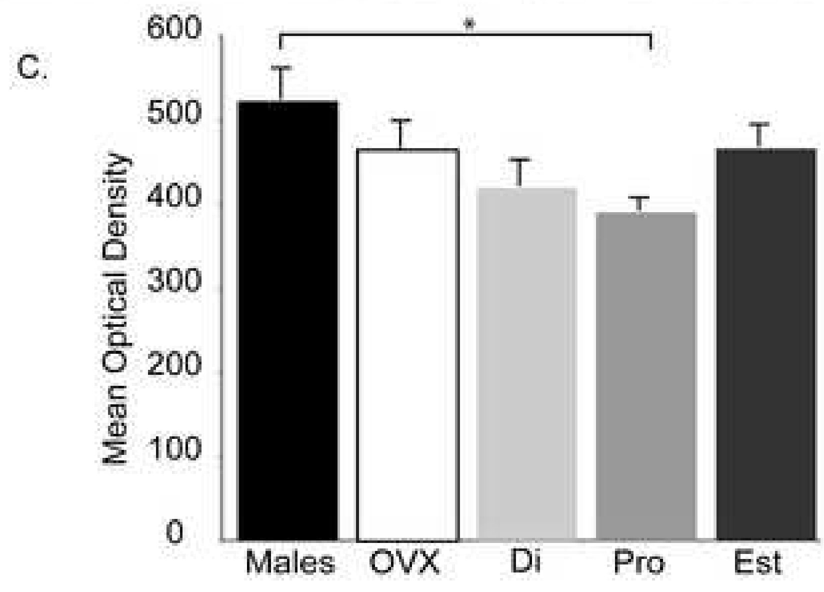

Figure 7
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