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GLIAL CELL ACTIVITY WITHIN THE VENTROLATERAL PERIAQUEDUCTAL GRAY OF MALE AND FEMALE RATS

by

Jean-Marc A. Sauzier, Lori N. Eidson, Anne Z. Murphy

Under the Direction of Dr. Anne Z. Murphy

ABSTRACT

Morphine is one of the most commonly prescribed medications for the relief of prolonged pain. Both basic science and clinical studies indicate that females require 2-3 times more morphine than males to achieve the same analgesic effect. To date, the mechanisms underlying sex differences in opiate responsiveness are unknown. Recent studies suggest that glial cells are potent modulators of morphine-based analgesia, and in particular, decrease the analgesic effect of opiates. Therefore, we tested the hypothesis that the sexually dimorphic effects of morphine were due to sex differences in glial cell activity. Our studies focused on the midbrain periaqueductal gray (PAG) as this region of the brain is critical for the analgesic effects of morphine. Adult male and female Sprague Dawley rats (250g- 400g) were procured from Charles River Laboratories, and were allowed 7 days to acclimate to the new facility. On the day of the experiment, animals received a subcutaneous injection of morphine (5mg/kg) or were handled in a similar manner. Thirty or 60 minutes after injections or handling, animals were perfused with a 4% paraformaldehyde and 2.5% acrolein tissue fixative solution. Brains were removed and stored in 20% sucrose until ready for sectioning. Brains were sectioned at 25µm using a freezing microtome, and immunohistochemical localization of markers for astrocyte glial

cell activity was performed. Antibodies to glial fibrillary acidic protein (GFAP) were used to label activated astrocytes. If our hypothesis is correct, then females will have significantly greater density of the astrocyte cell activity marker GFAP as compared with males. Sex differences in PAG glial cell activity may provide the biological bases for the sexually dimorphic effect of morphine. This research may lead to better treatment for females experiencing prolonged chronic or neuropathic pain.

INDEX WORDS: Glia, Astrocytes, Chronic Pain

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by

Jean-Marc A. Sauzier

An Honors Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Biology Department

in the College of Arts and Sciences

Georgia State University

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Honors Thesis Director: Dr. Anne Z. Murphy
Honors College Dean: Dr. Larry Berman

Electronic Version Approved:

The Honors College
Georgia State University
April 2012

DEDICATION

To my family, immediate and extended, whose support is always constant. This would not have come to pass without all your input.

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INTRODUCTION

Morphine is one of the most commonly prescribed drugs for the relief of prolonged pain. Morphine does not produce the same degree of analgesia in males as compared to females (Kepler *et al*., 1989; Cicero *et al*., 1996). Previous work in our lab has shown that females require 2-3 times more morphine to produce the same analgesic effect as males (Fig 1; Wang *et al*., 2006.). To date, the mechanisms underlying sex differences in opiate responsiveness are unknown. The periaqueductal gray (PAG) is a brain region that has been shown to be important for the analgesic effects of morphine (Loyd and Murphy, 2008). The PAG sends extensive projections to the rostral ventromedial medulla (RVM) of the brainstem, which in turn sends descending projections to the dorsal horn of the spinal cord. The PAG-RVM-spinal cord pathway is an essential circuit for antinociception (A.I Basbaum *et al*., 1978; H.L Fields *et al*., 1991). These regions also contain a high density of mu opioids receptors (MOR) (Loyd *et al*., 2007). Opioids modulate pain by binding to the mu opioid receptor in the ventrolateral PAG (vlPAG). vlPAG projection neurons excite cells in the rostral ventromedial medulla (RVM) that project to the dorsal horn of the spinal cord (Fig 2: Loyd and Murphy, 2008) where they inhibit incoming pain signals.

Recent studies suggest that glial cells are potent modulators of morphine-based analgesia. In particular, several studies have now shown that glial cells decrease the analgesic effect of opiates (Hao *et al*., 2011; Wei *et al*., 2012). The two primary glia cells involved in this phenomenon are astrocytes and microglia. Microglia are shown to be the first responders to even minor pathological changes in the CNS (Kreutzberg G.W., 1996). Activation of these cells is a key factor in the defense against brain tumors, infectious diseases, inflammation, ischaemia, trauma, and neurodegeneration (Kreutzberg G.W., 1996). Astrocytes have also been implicated in the

proinflamatory response to injuy (Hao *et al*., 2011; Wei *et al*., 2012). Recent studies by our lab show that both microglia and astrocyte activity within the PAG increases in response to morphine (Fig 3 & 4: Eidson & Murphy; under review). As increased levels of glia activity are associated with decreased morphine analgesia, we tested the hypothesis that the sexually dimorphic effects of morphine were due to sex differences in glial cell activity within the vlPAG. If our hypothesis is correct, then administration of morphine to females should result in significantly higher levels of astrocyte activation as compared to males. For these studies we used immunohistochemistry to label glial fibrillary acidic protein (GFAP). GFAP is a commonly used marker of astrocyte activity, as it has been shown to correlate with a change in morphology and release of proinflammatory cytokines, indicative of an active phenotype (Raghavendra *et al*., 2004). Sex differences in vlPAG glial cell activity may provide the biological bases for the sexually dimorphic effect of morphine. Given the strong evidence indicating that glial cell activity prevents morphine from being an effective analgesic, this research may lead to better treatment for females experiencing prolonged, chronic, or neuropathic pain.

Materials and Methods

Subjects. Aged matched adult male and female (2 months; 150-350g) Sprague-Dawley rats (Charles River Laboratories; USA) were procured and were allowed 7 days to acclimate to the new facility. Same-sex rats were pair-housed in separate rooms with 12:12 hour light: dark cycle (lights on at $8{:}00_{\text{A.M.}}$). Vaginal lavages were taken, and vaginal cytology was analyzed daily (for 2-3 weeks) to ensure that females were cycling normally, and to determine the stage of the estrous cycle the rats were in on the day of sacrifice. Rats had access to food and water *ad*

libitum throughout the experiment. These studies were done in accordance with the Georgia State University Animal Care and Use Committee (IACUC).

Morphine Administration. Adult male and female Sprague Dawley rats were administered morphine sulfate (experimental group) in 0.9% sterile saline (5 mg/kg, sc; NIDA; Bethesda, MD) or were restrained in a similar manner (handled control). Following morphine administration, animals were placed back into their home cage until sacrifice.

Perfusion. The handled control and morphine groups were further assigned to two groups. The first group received a lethal dose of sodium pentobarbital (60mg/kg; ip) 15 mins after injection or handling. The second group was euthanized 60 mins following morphine administration. The animal was confirmed to be unconscious and was perfused transcardially with 200 ml of 0.9% sodium chloride containing 2% sodium nitrite solution to clear the blood from brain. Following the saline, brains were immediately fixed by perfusing 150 ml of 4% aqueous paraformaldehyde fixative solution containing 2.5% acrolein (Polysciences Inc.; Warrington, PA) into the heart. The fixative was rinsed using 200 ml of 0.9% sodium chloride/sodium nitrite solution. Immediately after perfusion, brains were removed and stored in a 30% sucrose solution at 4 C until the time of sectioning (at least 24 hours). Brains were coronally sectioned using a freezing microtome (Leica 2000R) at 25µm and stored in cryoprotectant solution (Watson *et al*., 1986) at -20 C until immunohistochemical staining.

Immunohistochemistry. Glial cell activity was localized to the vlPAG region. Tissue samples were rinsed in potassium phosphate buffer saline solution (KPBS) to remove cryoprotectant solution. Tissue was then incubated for 20 min in 1% sodium borohydride in KPBS. Astrocyte activity levels were determined by incubating the tissue samples in primary antibody, glial fibrillary acidic protein (GFAP), for one hour at room temperature followed by 48 hours at 4 C.

Rabbit α-GFAP (Abcam, 1:5000 for 3,3'-diaminobenzidine reaction, and 1:3000 for fluorescence) in KPBS containing 1% Triton-X solution. The primary antibody was washed out with KPBS, and the tissue was incubated for one hour in biotinylated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch, 1:600). Tissue was rinsed in KPBS and incubated in avindin-biotin peroxidase complex (ABC Elite Kit, Vector labs). Following rinsing in KPBS and sodium acetate solution (0.175M; pH 6.5), GFAP reactivity was visualized as a black chromagen reaction product using 3, 3'- diaminobenzidine solution containing nickel sulfate and 0.8% hydrogen peroxide in sodium acetate buffer. The reaction was terminated using three rinses of sodium acetate buffer. Sections were sorted to rostral-caudal levels, mounted onto gelatin-subbed slides, and allowed to air dry (at least 10 h). Tissue was dehydrated in a graded series of ethanol solutions, cleared in xylenes and cover-slipped using Permount.

Densitometry. Previous studies have shown that the PAG is not a homogenous structure (Van Bockstaele *et al*. 1991, Bandler *et al*., 1994). In this study the densitometry of GFAP staining was determined for four rostro-caudal levels of PAG (Bregma -6.24, -6.72, -7.04, -8.00). GFAP immunoreactivity in the vlPAG was compared across treatment groups using previously described semi-quantitative densitometry (loyd *et al*., 2008, Laprairie *et al*., 2009). Images, 12-bit grayscale, including the region of interest (ROI) were captured using a QImaging Retiga EXi CCD camera (Surrey, BC, Canada) and iVision Image analysis software (Biovision Technologies, Exton, PA). Grayscale values were inverted for each image so that higher values represent increased staining levels. Drawing tools were used in iVision to outline the ROI for data sampling, and using the "measure" function determine an average grayscale pixel value for the outlined area. Values were corrected for nonspecific binding by subtracting an adjacent measure of gray matter in the ROI to represent background. Densitometry values are presented

as mean ± S.E.M. Analysis of variance (ANOVA) was used to test for significant main effects of sex (male, female), PAG level (Bregma –6.24 through –8.00); treatment (Handled, sc morphine 15 min, sc morphine 60 min) where relevant. $P \leq 0.05$ was considered significant for all analyses.

RESULTS

Male and female vlPAG GFAP immunoreactivity increased in a similar pattern in response to morphine, with no significant differences noted at different Bregma levels within vlPAG region (Fig 4). There was a significant main effect of treatment (handled v. morphine; ANOVA: $F_{(2, 24)} = 5.767$; $p = 0.009$). Post-hoc analysis revealed that 60 minutes of morphine increased vlPAG astrocyte activity as compared with handled controls (t-test; $p = 0.0131$), and sc morphine 15 minute (t-test; $p = 0.0136$) in both males and females. There was no significant difference between the handled and sc morphine 15 groups (t-test; $p=0.6204$). There was not a significant main effect of sex (ANOVA: $F_{(1, 24)} = 0.148$; $p=0.7034$), and no significant interaction (ANOVA: $F_{(2, 24)} = 0.079$; $p=0.9245$).

DISCUSSION

Recent studies suggest that glial cells are potent modulators of morphine-based analgesia, and in particular, decrease the analgesic effect of opiates (Raghavendra *et al*., 2002, Wei *et al*., 2008, Watkins *et al*., 2001). Morphine acts through the PAG-RVM pathway to produce analgesia. The results of our study indicate that morphine does not differentially activate vlPAG astrocytes in male and female rats. Indeed, both males and females showed a similar activation pattern in response to morphine. Administration of morphine resulted in a 2 fold increase in activation at 60 mins as compared to handled. Consistent with previous work done in our lab, morphine causes increased vlPAG glial cell activity in males (Eidson & Murphy; under review). Our

hypothesis that female rats have increased glial cell activation in the vlPAG as compared with males was not supported. In future studies, microglial cell number and function will be determined within the PAG. Microglia have also been shown to inhibit morphine analgesia (Guo *et al*, 2012).

Morphine causes the activation of glia and leads to the release and upregulation of proinflammatory cytokines (Johnston *et al*., 2004) that oppose morphine analgesia (Raghavendra et al., 2002, Watkins et al., 2001, Hutchinson *et al*., 2008). Cytokines, or more specifically tumor necrosis factor alpha (TNFα), have been shown to suppress morphine analgesia (Hao *et al*., 2011). Studies of the role of cytokines in response to morphine analgesia in both the male and female models could also prove to be beneficial in better understanding mechanisms of vlPAG glial cell activity.

FIGURES

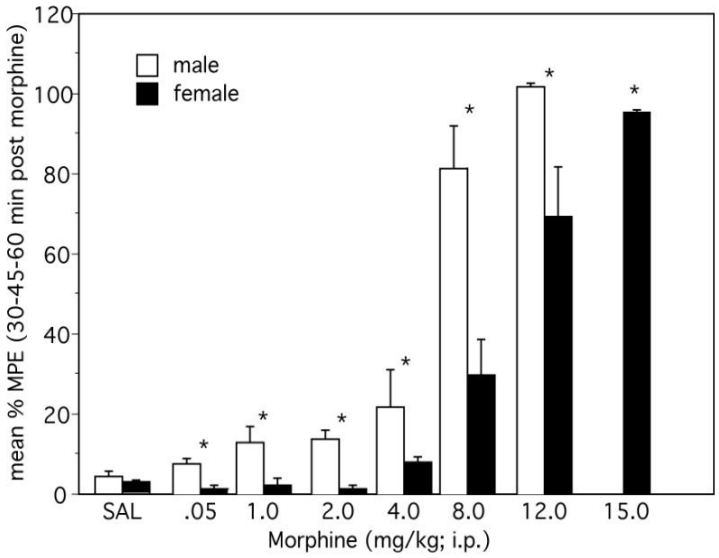

Fig. 1. The antinociceptive response to different morphine dosage, expressed as %MPE, in male and female CFA-treated rats (N = 54). PWL were measured for each dosage. A light cut off of 20 sec was implemented to reduce possible harm to animals. $P < 0.05$ for male-female comparison was used.

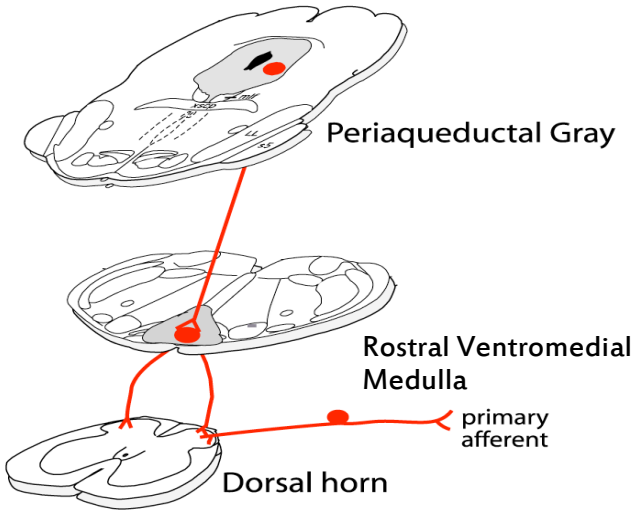

Fig. 2. A schematic of the neuronal projections from the midbrain periaqueductal gray to the brainstem RVM and spinal cord dorsal horn illustrating the descending pain pathway.

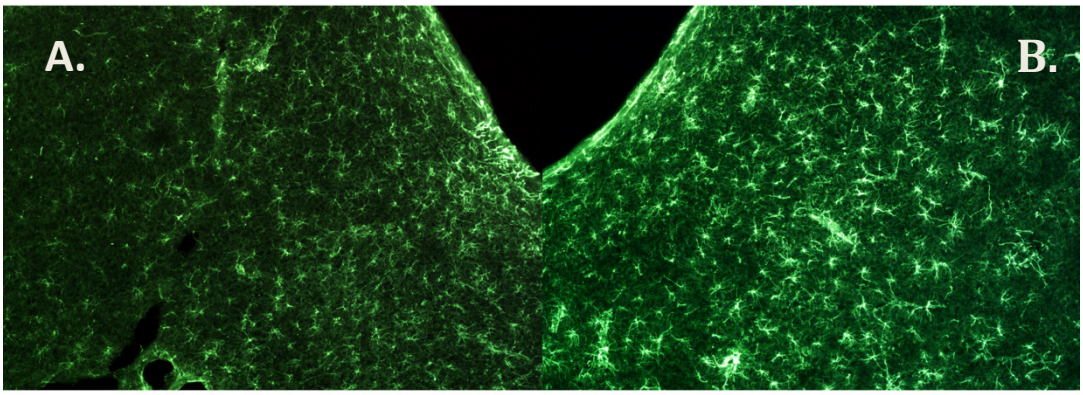

Fig. 3. Glial fibrillary acidic protein (GFAP) florescent-immunoreactive vlPAG of male rats. Animals were either administered **A.** s.c saline (5ml/kg) or **B.** s.c morphine (5mg/kg).

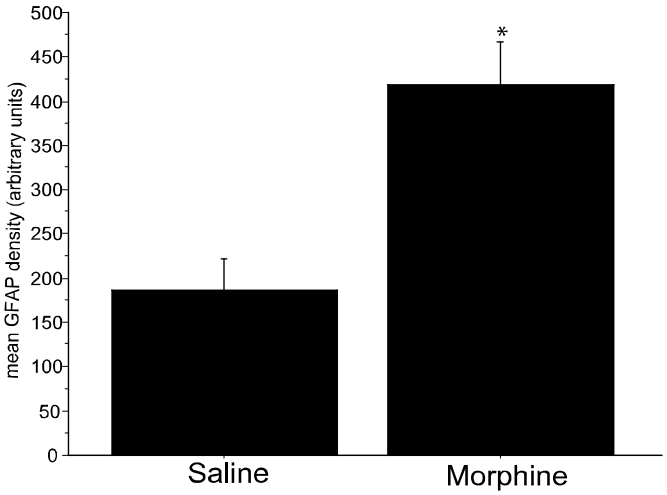

Fig. 4. Glial fibrillary acidic protein (GFAP) -immunoreactive cells in the midbrain periaqueductal gray (PAG) of male rats. Values are expressed as the mean ± SEM percentage of all levels. Animals were either administered s.c saline (5ml/kg) or s.c morphine (5mg/kg).

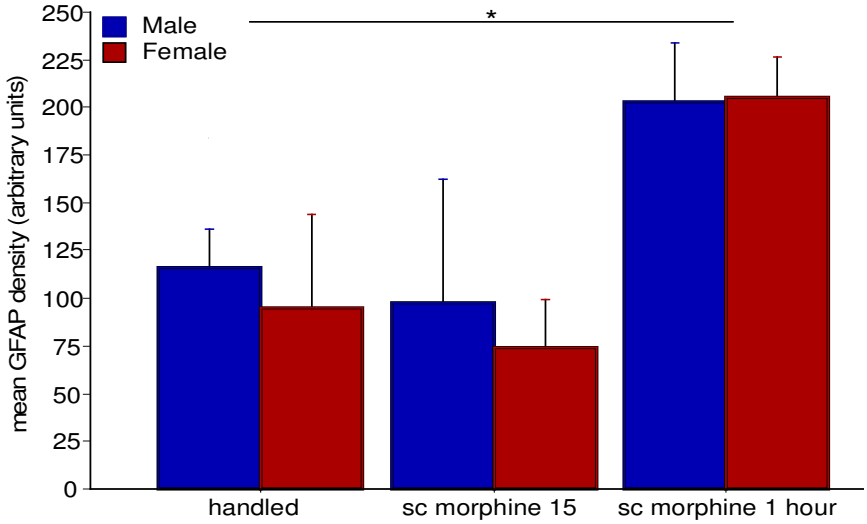

Fig. 5. Glial fibrillary acidic protein (GFAP) -immunoreactive cells in the midbrain periaqueductal gray (PAG) of male and female rats. Values are expressed as the mean ± SEM percentage of all levels. Animals were either administered s.c morphine (5mg/kg) or were handled. Both treatment groups were perfused either 15 min or 30 min after treatment.

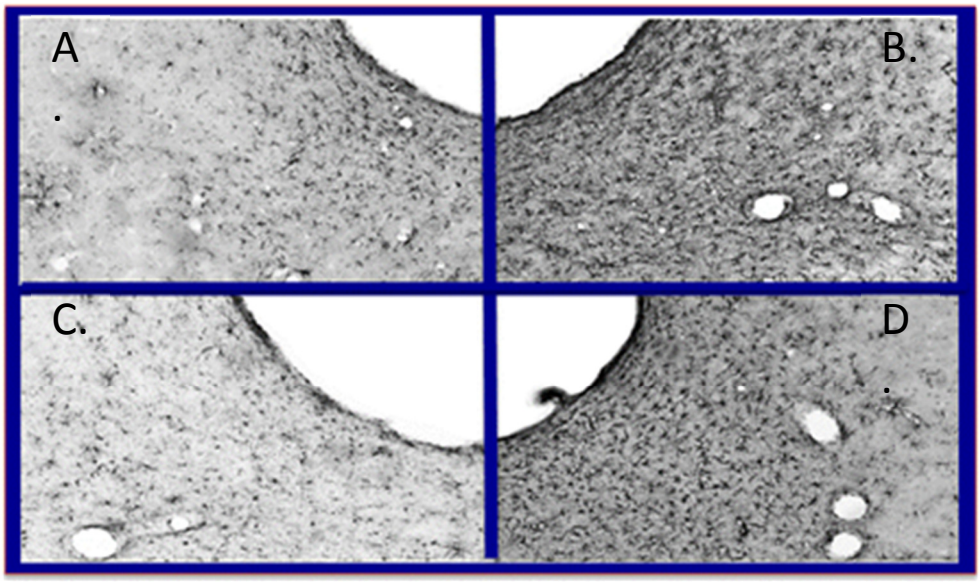

Fig. 6. Glial fibrillary acidic protein (GFAP) immunoreactive cells in the midbrain periaqueductal gray (PAG). Representative PAG images from Sprague-Dawley rats. **A.** Handled male **B.** s.c morphine (5mg/kg) male **C.** handled female female **D.** s.c morphine (5mg/kg) female. Animals were perfused 15 or 30 min after treatment.

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