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Impact of Aging on Morphine Analgesia and Associated Changes in μ-Opioid Receptor Binding and Expression in the Ventrolateral Periaqueductal Gray

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IMPACT OF AGING ON MORPHINE ANALGESIA AND ASSOCIATED CHANGES IN μ-OPIOID RECEPTOR BINDING AND EXPRESSION IN THE VENTROLATERAL PERIAQUEDUCTAL GRAY

by

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Under the Direction of Anne Z. Murphy

ABSTRACT

Chronic pain in the aged is a widespread phenomenon, and morphine is the most commonly used narcotic analgesic for treatment. Despite that fact, there are relatively few published studies examining the impact of advanced age on morphine analgesia. We hypothesized that aged rats would be less sensitive to morphine than adults, and that aged animals would have reduced mu-opioid receptor (MOR) binding and expression in the ventrolateral periaqueductal gray, a brain region responsible for morphine analgesia. Using a model of persistent inflammatory pain, we found that morphine was significantly less effective in aged males compared to adult males, and that aged males and females experience a reduction in MOR binding and expression compared to adults. These results suggest that there are clear age differences in morphine efficacy, and that reductions in MOR binding and expression in the periaqueductal gray could underlie those differences.

INDEX WORDS: Pain, Aging, Morphine, Chronic pain

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DEDICATION

I would like to dedicate this to my mother. Without her constant love, support, and enthusiasm for science (and all of the “borology” and “monotony” lessons as a child) none of this would be possible.

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I would like to thank Aras Petrulis for opening this door for me. I would also like to thank Anne Murphy for her support and guidance through the years. I would like to thank Lori Eidson for being a wonderful friend and for all of her editorial assistance on this document. I would also like to thank all of the other Murphy lab members who helped make this possible (Nicole, Dayna, Malcolm, Brittany, Vincent, Whitney, and Amanda)

I would also like to thank my loving wife, Emma Adair. She has been a constant source of motivation and support, as well as a shoulder to cry on. You are my pants!

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1 Introduction

As many as 70 percent of individuals over the age of 60 experience persistent or chronic pain. While a significant portion of these individuals are treated with morphine, fewer than 1 percent of published pain studies include the aged as a study population (Zwakhalen, Hamers et al. 2006). To date, basic research on morphine analgesia has been carried out primarily on adult rats in good health, and none to our knowledge look at age-related changes in opioid receptors. Given that there are over 600 million people in the world over the age of 60 and that the 80+ age group is the fastest growing population segment in the developed world (WHO 2008), it is crucial that we gain an understanding of the effects of aging on the ability of morphine to modulate pain.

This project examined whether there were age related differences in morphine analgesia between adult and aged rats in response to morphine administration following inflammatory injury, and age related changes in the function of the μ opioid receptor (MOR) itself. Currently there are few published studies examining age related differences in morphine analgesia. Of the studies that exist, many examine only aged males (Goicoechea, Ormazabal et al. 1997; Ko, King et al. 1997; Jourdan, Pickering et al. 2002; Crosby, Knapp et al. 2006), and to our knowledge all published pain studies in adult and aged rats measure the effects of analgesics in uninjured animals. Clinically, analgesics are given to alleviate moderate to severe acute and chronic pain, and aged individuals are highly unlikely to self administer or be prescribed narcotics in non-pain states (Robinson 2008). To this end, subjects were tested using a model of persistent inflammatory pain.

In addition to examining analgesic response between aged and adult rats we looked at changes in brain regions responsible for morphine analgesia in the central nervous system. Morphine has been demonstrated to act centrally in the midbrain ventrolateral periaqueductal gray (vlPAG) (Yaksh, Yeung et

al. 1976), specifically on neurons containing MOR. This brain region is part of the descending pain modulation circuit, with MOR-containing cells in this region projecting to the rostroventromedial medulla (RVM) in the brainstem. Neurons project from this region to the dorsal horn of the spinal cord to modulate the sensation of painful stimuli (Basbaum, Clanton et al. 1978).

Previous work from our laboratory has shown sex differences in morphine analgesia in rats, as well as differences in MOR distribution and development of morphine tolerance (Loyd and Murphy 2006; Wang, Traub et al. 2006; Loyd, Morgan et al. 2007; Loyd, Wang et al. 2008; Loyd, Morgan et al. 2008).Our results have shown that morphine is less effective in adult (60-90 day old) female rats than male rats of the same age (Wang, Traub et al. 2006). Additional studies showed that sex differences in morphine analgesia were the result of decreased MOR in the PAG of female rats.

The present experiments were designed to determine the impact of advanced age on morphine analgesia. Additional experiments were conducted to determine whether observed changes in morphine analgesia were due to changes in PAG MOR binding and/or expression.

2 Materials and Methods

2.1 Animal Subjects

Sprague Dawley rats were obtained from Charles River Laboratories. Adult males and females were approximately 90 days of age. Aged subjects were retired breeders purchased at approximately 12 months of age and matured in-house to 18-24 months of age. This is commonly held in the literature as "aged" (Jourdan, Boghossian et al. 2000). All animals were housed in same sex pairs. A 12:12 light cycle was maintained throughout the experiment (lights on at 7:00 am EST), and food and water was

available *ad libitum*. Aged rats were fed a reduced calorie diet for weight control (in pilot experiments no differences were observed between aged rats fed standard chow vs. reduced calorie chow).

2.2 Inflammatory Injury

All groups were acclimated to testing equipment for two hours daily for a minimum of two weeks before testing began. Twenty-four hours prior to CFA administration mechanical and thermal baseline thresholds were measured (Paw thermal stimulator, UCSD; Dynamic plantar aesthesiometer, Ugo Basile). For thermal paw withdrawal latency (PWL), a focused beam of light was directed towards the animal's hindpaw, and the latency to remove their hindpaw was recorded as paw withdrawal latency (PWL). To assess mechanical thresholds, subjects were placed on a mesh platform and the dynamic plantar aesthesiometer (Ugo Basile) was applied to the ventral aspect of the hindpaw. The device automatically determined the force required to elicit a withdrawal. Following baseline measurement rats were given an intraplantar injection of Complete Freund's Adjuvant (CFA) (1 part CFA : 1part saline, Sigma), and allowed to return to their home cage. Twenty-four hours later and prior to testing, inflammation was verified by measuring the middle dorsoventral aspect of the injured hindpaw with a digital caliper and compared to a measurement made pre-injury.

2.3 Thermal and Mechanical Hyperalgesia

Following paw measurement, rats were randomly assigned into either morphine or saline groups and placed into the paw thermal stimulator. Following a 30 minute acclimation, post injury paw withdrawal latencies were measured, mechanical hyperalgesia was measured, and morphine or saline was

administered in an escalating dose (0, 1.8, 3.2, 5.6, 8, 10, and 18 mg/kg of morphine or 1ml/kg saline) (Loyd, Wang et al. 2008). Doses were administered every 15 minutes, and paw withdrawal latencies were measured immediately prior to next higher dose.

2.4 Sacrifice and Tissue Preparation

Animals used for immunohistochemistry were given an overdose of sodium pentobarbital (160 mg/kg, SleepAway, Fort Dodge (Wyeth), Madison, NJ) and perfused transcardially, first with 250-300ml of sodium nitrite (a vasodilator), followed by 300 ml of a solution of 2.5% acrolein (poly sciences), 4% paraformaldehyde (Sigma), and 0.175m potassium phosphate buffer. Brains were removed and placed in a 30% sucrose (Sigma) phosphate buffer solution at 4 degrees C until sunk, placed on a freezing stage microtome (Leics SM2000) and sectioned in a 1 to 6 series at 25 µm. Sections were stored in cryoprotectant until processing.

Subjects used for autoradiography were rapidly decapitated, brains were quickly extracted and snap frozen in isopentane chilled with dry ice. Following brain removal brains were sectioned in a 1 to 6 series with a cryostat (Leica CM3050S) at 20µm thickess and thaw mounted to slides. Slides containing brain sections were stored in boxes containing desiccant (dririte) at -80 degrees C until processing.

2.5 Immunohistochemistry

Briefly, free floating tissue sections from aged and adult males and females (n=6 per group) were rinsed of cryoprotectant with 0.175M KPBS (10x), incubated in 2% $NaBH_4$ in KPBS for 20 minutes to remove excess aldehydes, rinsed again, and incubated in primary antiserum (rabbit anti-MOR, 1:20,000 Chemicon) in the presence of 1%Triton x-100 at 4 degrees C for 48 hours. Next, tissue was rinsed of

primary and incubated in biotinylated goat anti rabbit antibody for 1 hr in 0.4% triton x-100+KPBS (biotinylated goat anti rabbit, Jackson Immunoresearch), rinsed again and incubated in in avidin biotin complex (ABC elite kit, Vector Labs, Burlingame, CA) for 1 hour. Bound antibody was visualized using nickel enhanced di-amino benzidine in a sodium acetate solution.

2.6 Autoradiography

Briefly, slides from animals in each group (n=6 per group) were air dried for at least 2 hours, then fixed for 2 minutes in a 0.1% paraformaldehyde/PBS solution. Subsequently, the slides were rinsed in a 50mM TRIS solution with NaCl (15 min.), 50mM TRIS (15 min.) and then placed in tracer buffer for 60 minutes (1nM 3H DAMGO (Perkin Elmer), Bovine Serum Albumin, and TRIS (sigma)). Following tracer incubation, tissue was rinsed (TRIS + MgCl 3x10 min. each, 30 sec dH2O) and slides were dried overnight. Following tracer incubation, slides were apposed to tritium sensitive imaging plates (Fuji BAS 2025 TR) and exposed for 30 days. At the end of the exposure period imaging plates were read using the Fuji BAS 5000 phosphoimager and resulting images were analyzed using Fuji Multi Gauge software.

2.7 Densitometry

To analyze tissue processed with immunohistochemistry grayscale images were obtained using a Qimaging Retiga Exi ccd camera controlled by iVision software. The correct region and level of the PAG was verified by comparison with Paxinos & Watson 1997. A square region of interest sample from the vlPAG was taken from each section under 10x magnification and the optical density of the labeled tissue was measured using iVision software. To control for non specific background labeling, an unlabeled

region was sampled from each tissue section analyzed, and the density value derived from this was subtracted from the optical density measurement value from the labeled area of interest (Loyd, Wang et al. 2008).

2.8 Statistical Analysis

All group differences were measured using analysis of variance (ANOVA) using Prism (Graph Pad). Post Hoc differences were calculated using Bonferroni's multiple comparison test in Prism when significant F scores were found. Dose response curves were plotted using the non-linear regression tool in Prism (GraphPad).

3 Results

3.1 Baseline and Post Injury Hyperalgesia

No significant differences between aged and adult rats of either sex were noted for baseline thermal and mechanical thresholds, indicating no effect of age on basal pain sensitivity. Indeed,

uninjured animals did not differ in thermal nociception [$F_{[3, 29]} = 2.170$, P = 0.113] (Figure 3.1.1). Additionally, we noted no group differences in baseline mechanical nociception [$F_{[3, 68]} = 1.260$, P = 0.2950] (Figure 3.1.2), change in thermal PWL following CFA [$F_{[3, 46]} = 0.5508$, P = 0.6502] (Figure 3.1.3), or change in mechanical hyperalgesia following CFA [$F_{[3, 39]} = 0.3450$, P = 0.7930] (Figure 3.1.4). CFA injections caused the same degree of inflammation in adult and aged rats [$F_{[3, 46]} = 1.313$, P = 0.2816] (Figure 3.1.5).

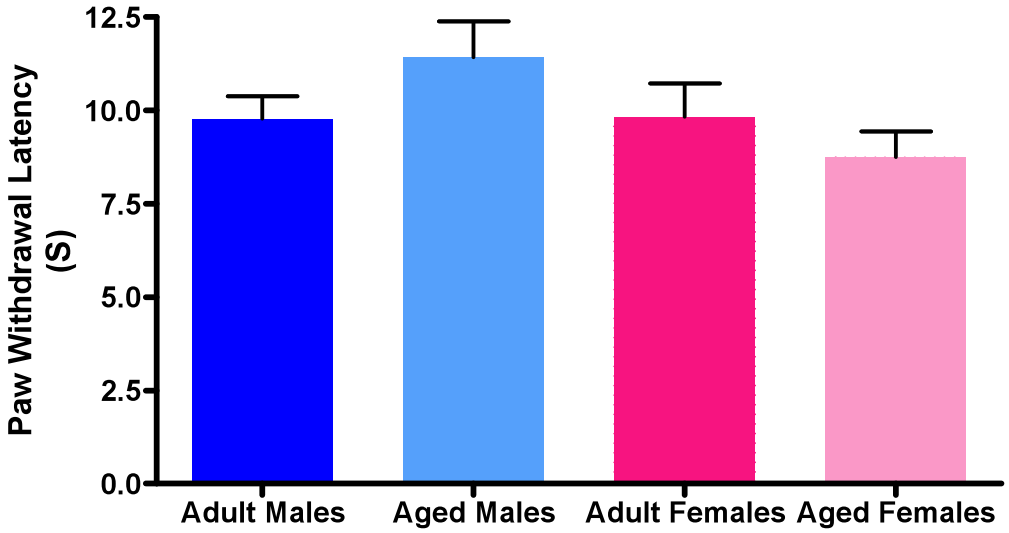

Figure 3.1.1 Baseline thermal hyperalgesia, reported as paw withdrawal latency (seconds)

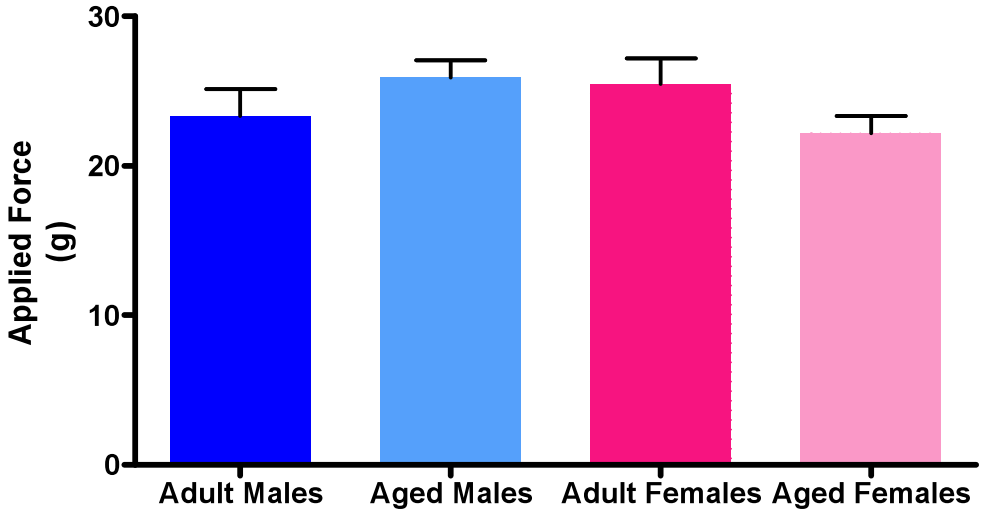

Figure 3.1.2 Baseline mechanical hyperalgesia, reported as grams of force required to elicit paw withdrawal

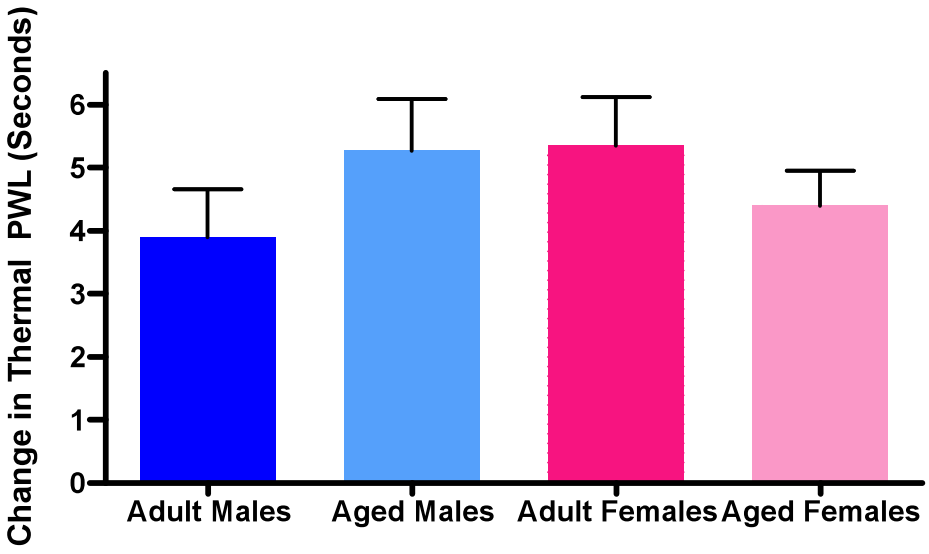

Figure 3.1.3 Thermal hyperalgesia (extent of inflammatory hyperalgesia), calculated by subtracting post inflammation PWLs from baseline values

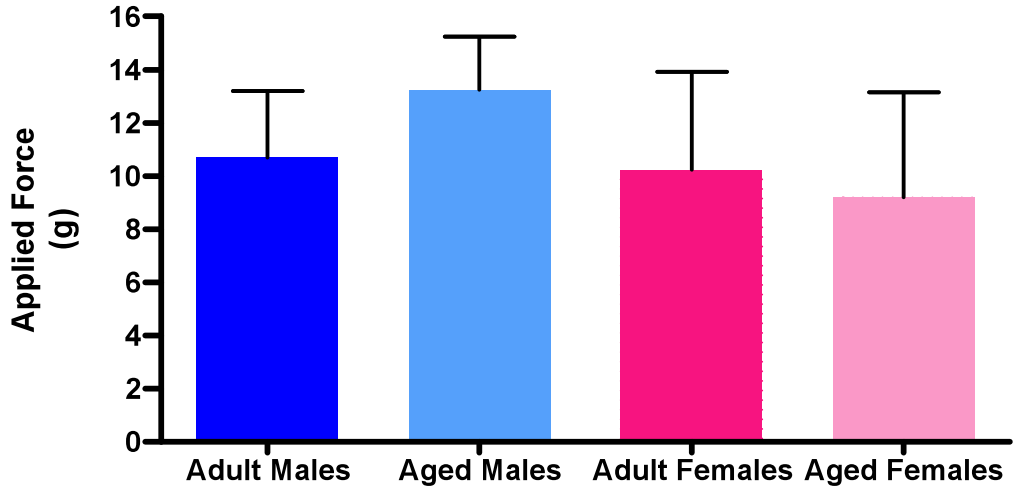

Figure 3.1.4 Mechanical hyperalgesia difference score (extent of mechanical hyperalgesia), calculated by subtracting post inflammation scores from baseline value

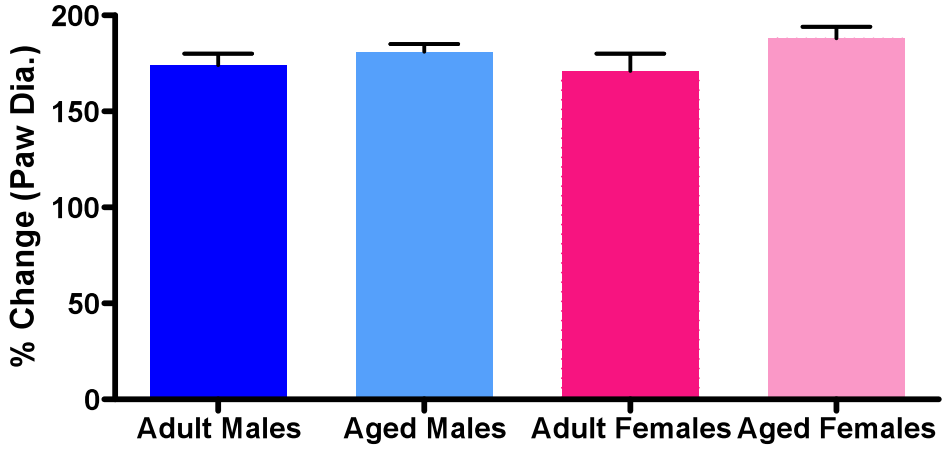

Figure 3.1.5 Extent of inflammation, reported as percent change in paw diameter following CFA administration

3.2 Morphine Antihyperalgesia

Adult males and females showed a clear sex difference in response to morphine administration, with females requiring a much higher dose (EC_{50}=10.51 mg/kg) than males (EC_{50}=3.82 mg/kg) to return to baseline PWL. Male aged rats were much less sensitive to morphine than their adult counterparts, with an EC_{50} of 9.153 mg/kg. Indeed, the EC_{50} of aged males was closer to that of adult and aged females (EC_{50}=10.51 mg/kg and 7.961 respectively). The decreased sensitivity of aged male rats to morphine administration suggests that morphine is a less effective analgesic in aged male rats than it is in adult males.

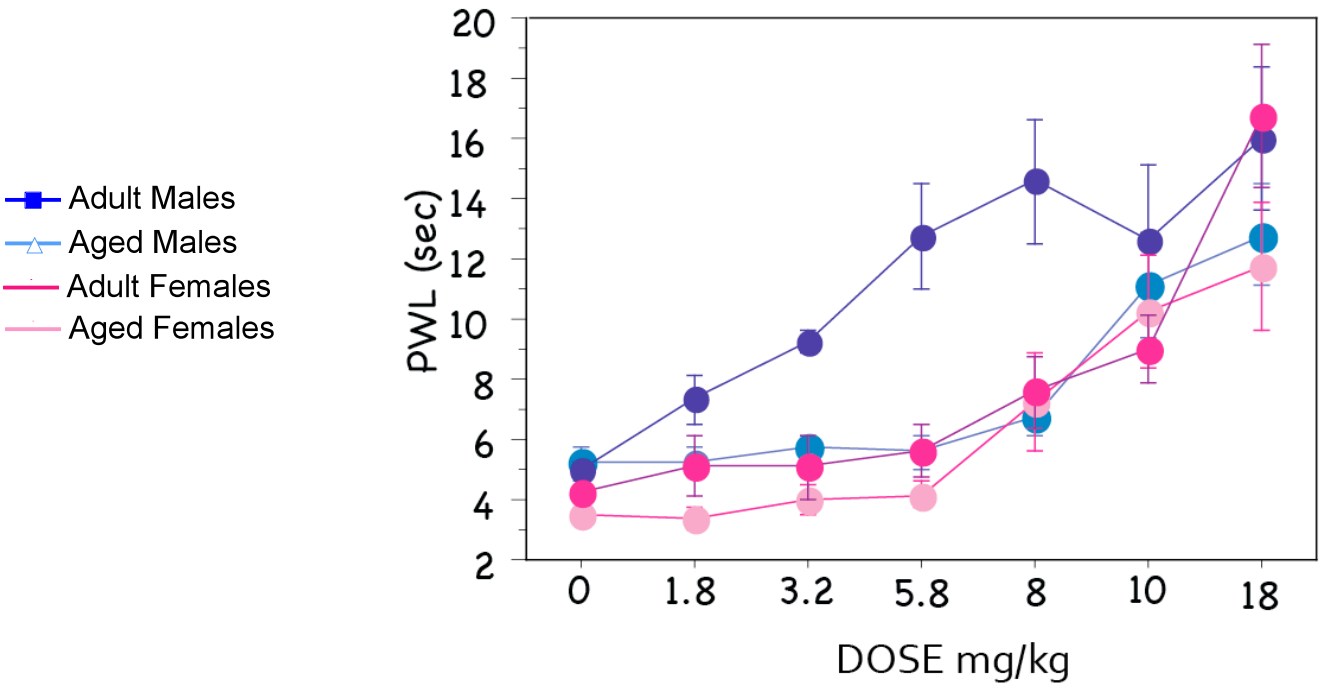

Figure 3.2.1 Thermal paw withdrawal latencies during cumulative morphine administration

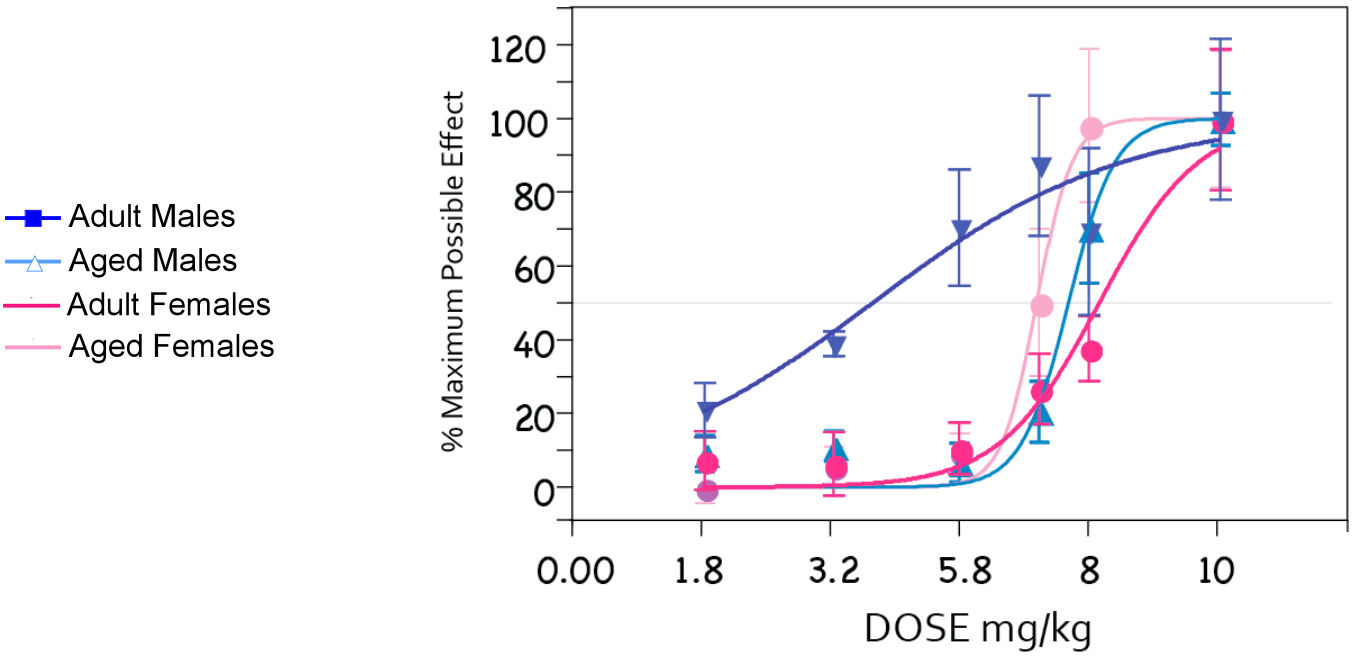

Figure 3.2.2 Response to cumulative morphine plotted as ED_{50}

3.3 Immunohistochemistry

Next we tested whether the reduction in morphine efficacy was caused by a decrease in MOR in the PAG. Immunohistochemical densitometry values indicated significant differences between aged and adult subjects in both MOR expression and binding in the PAG. Using ANOVA there were significant group differences in MOR distribution [$F_{[3, 23]} = 19.31$, $p<0.0001$]. Using Bonferroni's multiple comparison test aged males ($p <.001$) and females ($p <.001$) showed significantly less MOR labeling than their adult counterparts. Adult females exhibited reduced MOR labeling as well when compared to adult males ($p < .001$). Neuronal Nuclei immunostaining revealed no significant differences between groups [$F_{[3, 21]} = 1.858$, $p = 0.1677$]

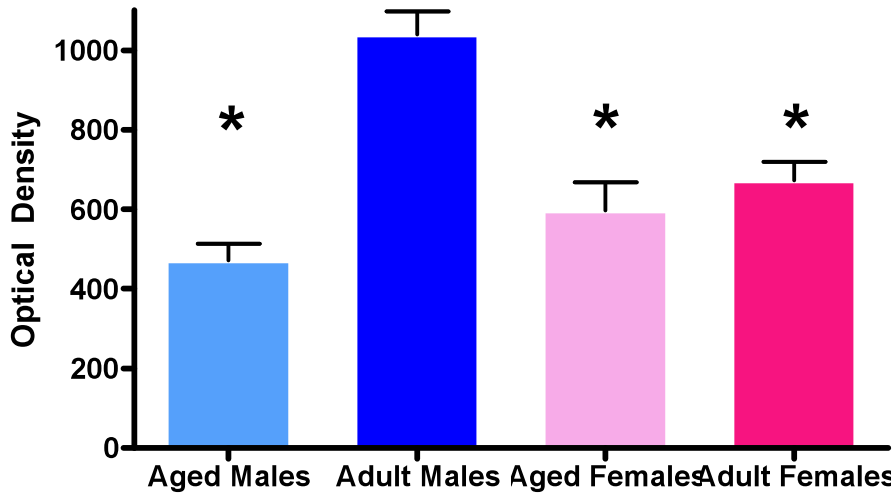

Figure 3.3.1 MOR densitometry in the vlPAG: Aged females, males, and adult females all exhibit significantly lower MOR labeling in vlPAG than adult males

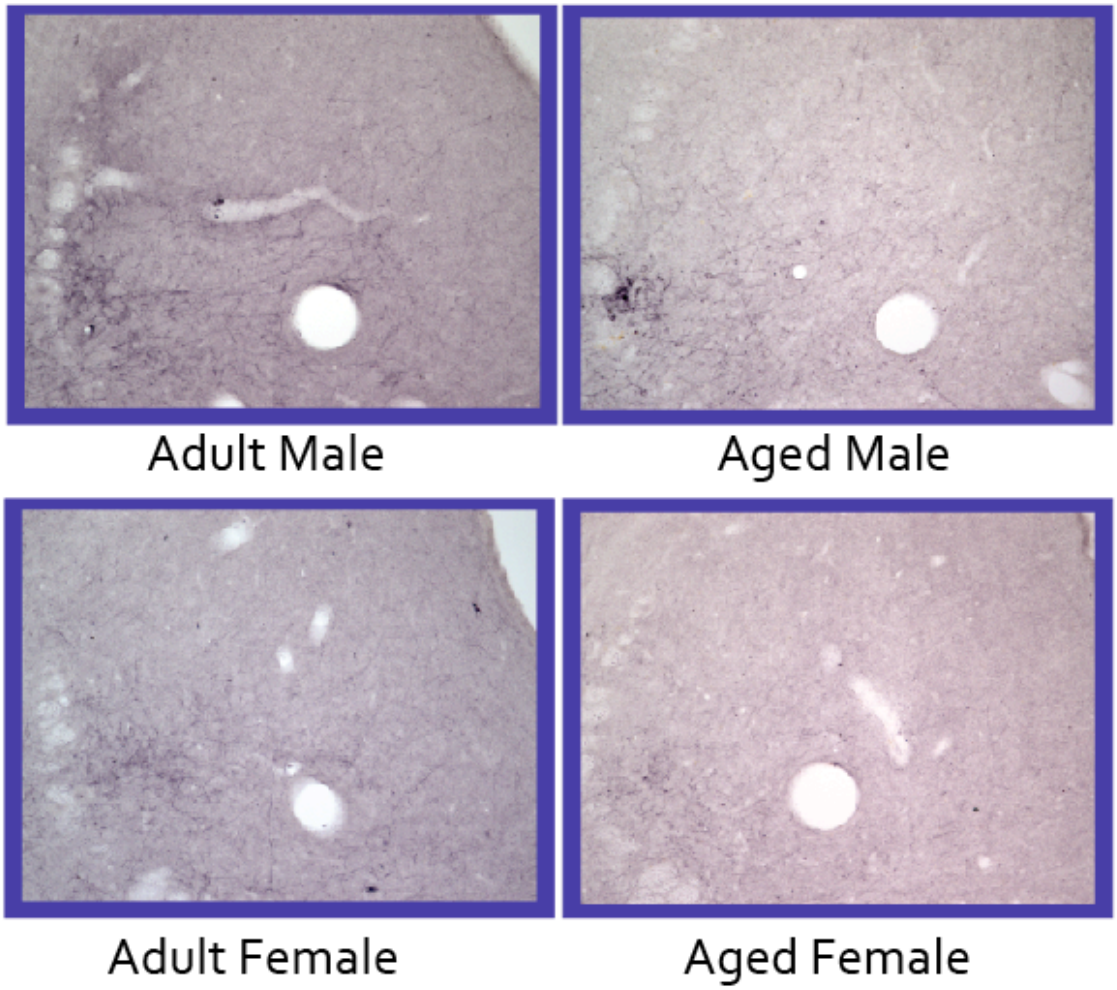

Figure 3.3.2 Representative sections from MOR immunohistochemistry in vlPAG

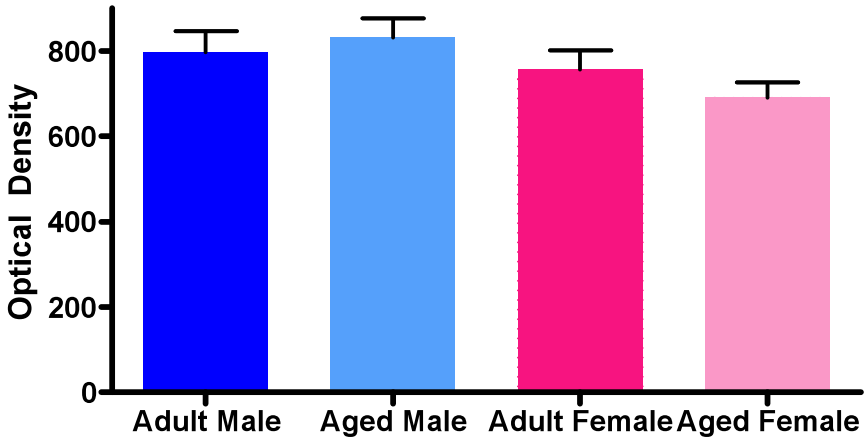

Figure 3.3.3 Neuronal nuclei densitometry: there are no significant differences in neuronal density in vlPAG between adult and aged animals of either sex

3.4 μ-opioid Receptor Autoradiography

In addition to examining differences in MOR expression, we also wanted to know whether there were differences in MOR binding in the PAG. DAMGO autoradiography revealed significant differences in MOR binding in the vlPAG [$F_{[3, 21]} = 11.69$, $p = 0.0001$]. Post hoc, Bonferroni's multiple comparisons test revealed a significant age difference in males ($p < 0.01$), and a significant difference between adult males and aged females ($p < 0.05$). This suggests a selective reduction in MOR binding in the vlPAG in aged animals as compared to adult males (Figure 3.3.4)

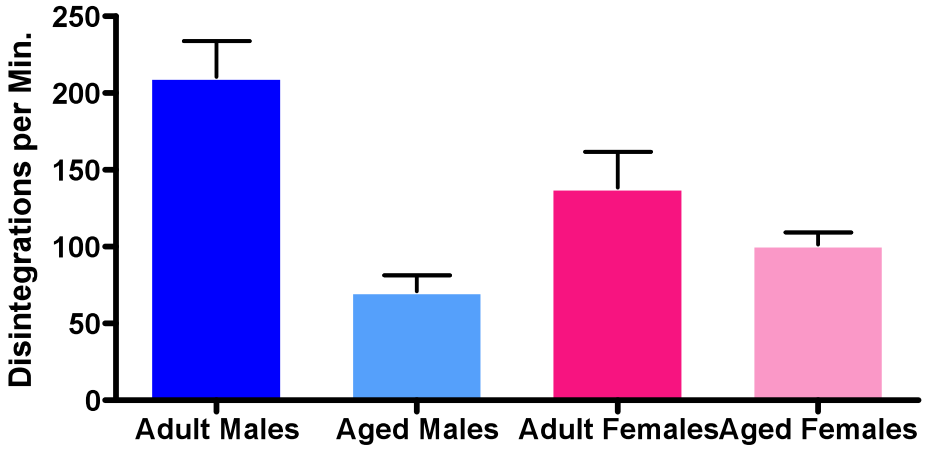

Figure 3.3.4 DAMGO binding in vlPAG: Aged males and females have significantly lower binding compared to adult males

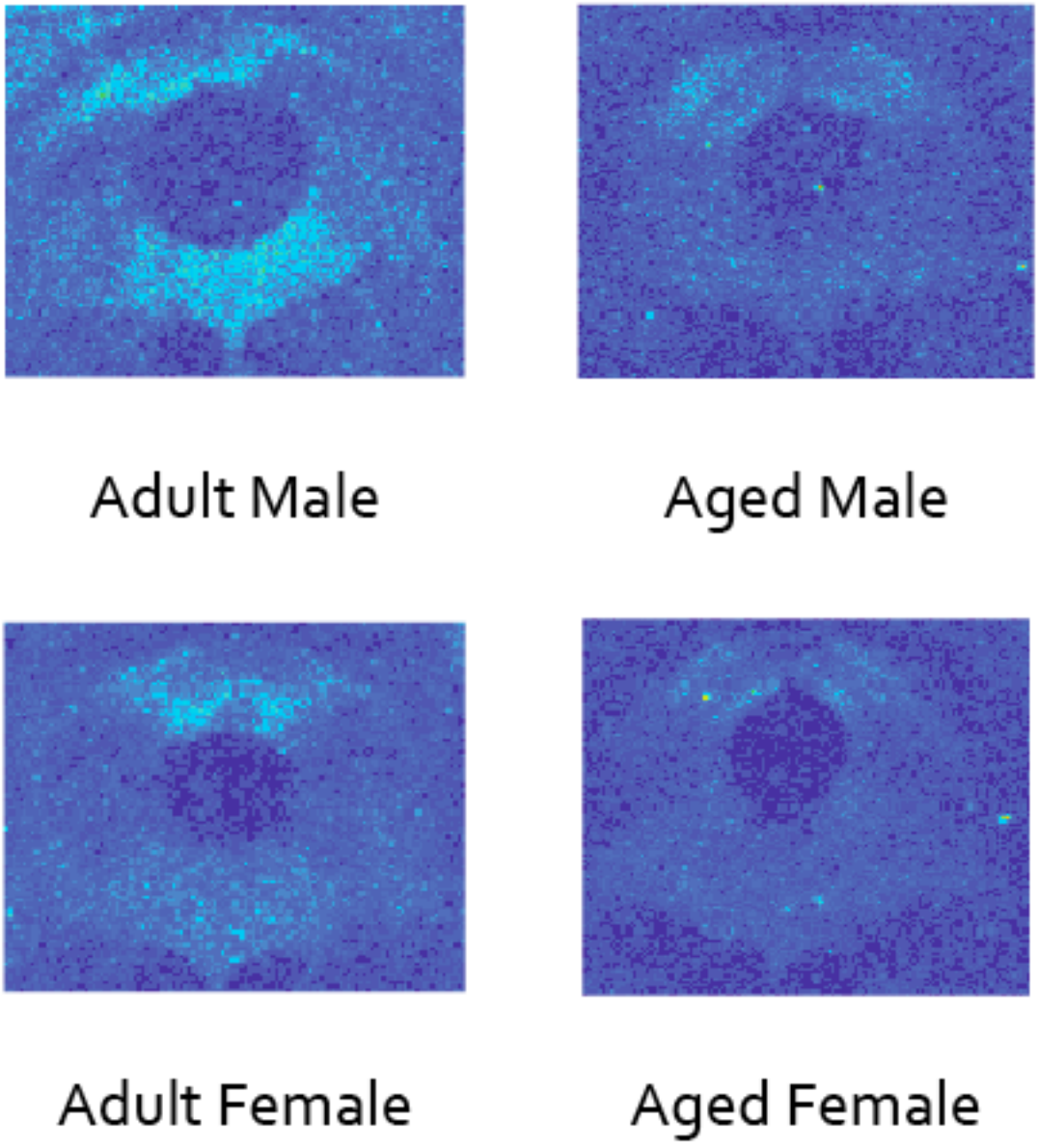

Figure 3.3.5 Representative DAMGO autoradiograms in vlPAG: aged males and females show reduced binding compared to adults

4 Discussion

4.1 Overview

The purpose of these experiments was to examine age related differences in morphine analgesia using a model of persistent inflammatory pain. Previous lab members have reported significant sex differences in morphine analgesia using this model (Wang, Traub et al. 2006), and identified the vlPAG as a critical region underlying the observed sex differences (Loyd and Murphy 2006; Loyd, Wang et al. 2008). The current experiments were designed to test the hypothesis that aged rats will be less sensitive to morphine than their adult counterparts, and that there will be observable age differences in MOR expression and binding in the vlPAG. The results of these experiments suggest that (A) Aged animals do not have a different threshold for pain than their adult counterparts (figs. 3.1.1, 3.1.2); (B) Aged animals did not differ in the hyperalgesia produced by inflammatory injury (figs. 3.1.3, 3.1.4); (C) Morphine was less effective in aged males compared to adult males (fig. 3.2.1); (D) Aged males and females have significantly lower MOR expression and binding in the vlPAG, a brain area shown to underly sex differences in morphine analgesia (figs. 3.3.3, 3.3.4) (Loyd, Wang et al. 2008).

The results of these experiments indicate that there are clear age differences in morphine analgesia, as well as differences in MOR expression and binding in brain regions shown to underlie morphine analgesia. Further study is required to determine whether the observed changes in the brain are the cause of the observed behavioral differences, but the experiments presented here provide a foundation for future studies in the area.

4.2 Thermal and Mechanical Hyperalgesia

Thermal and mechanical pain testing revealed no differences between aged and adult animals at baseline and following inflammatory injury with CFA. This suggests that aged animals' endogenous pain modulation mechanisms are unaltered by age. This also indicates that the changes that we observed behaviorally were due to actual differences in pain processing, rather than mechanical differences (i.e. different skin thickness, fat pad thickness, etc.)

4.3 Morphine Antihyperalgesia

While others have reported sex and age differences in morphine analgesia in rats (Jourdan, Boghossian et al. 2000; Jourdan, Pickering et al. 2002), none of the published studies were conducted using a model of persistent inflammatory injury. CFA inflamed rats make a good model for analyzing morphine efficacy because it more closely resembles the real-world clinical conditions under which morphine is administered (Robinson 2008). The results that we obtained working within this model support the hypothesis that there are age differences in morphine antihyperalgesia. While there were no significant changes in morphine antihyperalgesia in aged females as compared to adults, aged males were much less sensitive to morphine than their adult counterparts. Indeed, at 24 months of age, male rats responded to a cumulative dose of morphine in a similar fashion to adult and aged females (Figure 3.2.1). Indeed, compared to adult males, aged males experienced a two-fold rightward shift in ED_{50}, or the dose morphine required to return injured animals' PWL to pre injury levels (Figure 3.2.2).

4.4 Changes in MOR in vlPAG

Age differences in morphine analgesia are not dependent on pharmacokinetic differences (Wang, Mitchell et al. 2005), so we sought to identify age and sex differences in MOR-containing neurons in brain regions previously shown to mediate sex differences in morphine analgesia in adults, specifically the caudal portion of the vlPAG (Basbaum, Clanton et al. 1978; Loyd and Murphy 2006; Loyd, Morgan et al. 2007; Loyd, Wang et al. 2008). We sought to test the hypothesis that age subjects will exhibit reduced MOR labeling in the vlPAG first with immunohistochemistry, where we showed that there was a reduction in MOR protein levels, and second with Autoradiography, where we showed a corresponding reduction in binding.

The results of the immunohistochemistry indicated reduced levels of MOR protein in aged subjects as compared to adults. This result, however, should not be taken to mean that there is any change in receptor binding, as immunohistochemistry uses an IgG antibody directed towards a unique amino acid sequence in a receptor. As such, bound antibody indicates the location and density of the receptor, but not its function. To test the hypothesis that aged rats will have reduced receptor function compared to adults we used tritium labeled DAMGO autoradiography. Autoradiography is a better functional assay of MOR activity because the labeled molecule is a MOR agonist, and binding of an agonist is a direct biochemical measure of receptor function.

Previous lab members have demonstrated that vlPAG MOR containing neurons are essential for systemic morphine analgesia (Loyd, Wang et al. 2008). In aged subjects, the reduction in MOR binding that we observed in the vlPAG suggests that this could be the neural substrate for the behavioral changes that we observed, particularly in males. In females there was a lack of behavioral differences between aged and adult subjects, but aged males responded much differently than adult males. Loyd, Wang, et. al. 2008 demonstrate that site specific ablation of vlPAG MOR containing neurons is sufficient to reduce morphine antihyperalgesia in males, but female adults receiving a MOR specific lesion to the vlPAG do

not show a noticeable behavioral change. This suggests that other brain regions could be more extensively involved in female morphine antihyperalgesia, and that mechanism is less affected by old age.

To control for the possibility that the changes that we observed could be due to a global loss in neurons rather than a selective loss of MOR from aged and adult animals were stained for neuronal nuclei (NeuN). We found no differences in the neuronal staining of aged animals as compared to adults, and no sex differences. This suggests that the histological changes that we observed are consistent with an age related decline in MOR function and expression levels rather than a generalized loss of neurons.

4.5 Concluding Remarks

The relative lack of studies examining age-related changes in morphine analgesia and associated changes in descending pain circuits is alarming, and leaves little scientific background to inform clinicians in the treatment of the aged. The rapidly aging global population deserves to be treated with the same level of care with which adults and children are treated, and a better understanding of the aging process and pain in the aged in basic models will help guide clinicians in their research.

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