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Advanced age attenuates the antihyperalgesic effect of morphine and decreases μ-opioid receptor expression and binding in the rat midbrain periaqueductal gray in male and female rats

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ABSTRACT

The present study investigated the impact of advanced age on morphine modulation of persistent inflammatory pain in male and female rats. The impact of age, sex, and pain on μ-opioid receptor (MOR) expression and binding in the ventrolateral periaqueductal gray (vlPAG) was also examined using immunohistochemistry and receptor autoradiography. Intraplantar administration of complete Freund's adjuvant induced comparable levels of edema and hyperalgesia in adult (2–3 mos) and aged (16–18 mos) male and female rats. Morphine potency was highest in adult males, with a greater than two-fold increase in morphine EC_{50} observed in adult versus aged males (3.83 mg/kg vs. 10.16 mg/kg). Adult and aged female rats also exhibited significantly higher EC_{50} values (7.76 mg/kg and 8.74 mg/kg, respectively) than adult males. The upward shift in EC_{50} from adult to aged males was paralleled by a reduction in vlPAG MOR expression and binding. The observed age-related reductions in morphine potency and vlPAG MOR expression and binding have significant implications in pain management in the aged population.

1. Introduction

The US population is rapidly aging, and in the coming decade, over 20% of US citizens will be 65 years of age or older (Centers for Disease Control and Prevention, 2013). It is estimated that over 50% of these individuals will experience persistent and/or severe pain, including pain associated with arthritis, cancer, diabetes mellitus, and cardiovascular disease (Centers for Disease Control and Prevention, 2016 Robeck et al., 2014). Persistent pain in the elderly typically involves multiple sites and is comorbid with a variety of other conditions, resulting in depression, sleep disruption, cognitive impairment, and decreased socialization. Together, these factors potentially decrease quality of life and increase the risk of early death (Patel et al., 2013; Makris et al., 2015; Reid et al., 2015; Domenichiello and Ramsden, 2019). Although opioids, including morphine and fentanyl, are the most potent and commonly prescribed analgesics for chronic pain management, pain in the elderly is typically undermanaged or ignored altogether (Pergolizzi et al., 2008). Several factors contribute to the undermanagement of pain in the elderly, including under-reporting of pain intensity by the patient, physician reluctance to prescribe opioids due to fears of adverse side effects including cognitive impairment and respiratory depression, and misconceptions on the part of the patient and physician regarding tolerance and addiction (Cavalieri, 2005; Miaskowski, 2011; Miaskowski et al., 2011; Naples et al., 2016; Prostran et al., 2016; Saunders et al., 2010; Schiltenwolf et al., 2014).

Although opioids for chronic pain management in the elderly are finally beginning to increase (Lapane et al., 2013; Pokela et al., 2010), there remains a dearth of information regarding the suitable dosing regimen (Campbell et al., 2010). Clinical studies examining opioid potency in the elderly are inconsistent, with reports of increased, decreased, or equivalent sensitivity compared with adults (Gagliese and Katz, 2003; Kaiko, 1980; Papaleontiou

Declarations of interest: None

Summary: Despite the recognition that undermanaged pain in the elderly is a growing clinical problem, remarkably little basic research has been conducted. The results of the present study establish that morphine's ability to attenuate persistent inflammatory pain is reduced in aged rats, likely due to a loss of density and functionality of vlPAG MOR. Together, these results suggest that PAG MOR may provide the anatomical basis for observed age differences in morphine analgesia and identify a potential novel target for the improvement of pain management in the aged population.

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et al., 2010; Prostran et al., 2016). Several factors likely contribute to these disparate results. First, although opioids are prescribed for the alleviation of severe and persistent pain, most of these studies were conducted in elderly patients experiencing acute pain. The presence of comorbid conditions such as diabetes and high blood pressure, and the use of concomitant medications, may also either augment or potentiate morphine's analgesic effects. These studies also typically fail to include sex as a biological variable, either not reporting it or focusing exclusively on males (Aubrun et al., 2005; Gagliese and Katz, 2003; Lautenbacher, 2012), leaving the impact of age on opioid modulation of pain in women largely unknown. Sex and age differences in the likelihood of self-reporting pain in a clinical setting, along with the subjective nature of pain, further contribute to the contradictory findings regarding effective dosing in the aged population (Dampier et al., 2013; Ferrell et al., 1991; Reddy et al., 2012).

Preclinical studies in rodents have also failed to provide insight regarding the impact of advanced age on opioid modulation of pain. First, very few studies have been conducted, and similar to their clinical counterparts, these studies report increased, decreased, and no change in opioid potency as a function of age (Chan and Lai, 1982; Gagliese and Melzack, 2000; Kramer and Bodnar, 1986). These studies also suffer from the same shortfalls identified for clinical studies, including acute rather than chronic pain assays (typically tail-flick or hot plate) and use of male subjects exclusively (Chan and Lai, 1982; Jourdan et al., 2002; Kavaliers et al., 1983; Smith and Gray, 2001). Therefore, a clear picture regarding the impact of advanced age on opioid potency has yet to emerge.

The endogenous descending analgesia circuit, consisting of the midbrain periaqueductal gray (PAG) and its descending projections to the rostral ventromedial medulla and dorsal horn of the spinal cord, is a critical neural circuit for exogenous pain modulation (Basbaum et al., 1976, 1978, ; Basbaum and Fields, 1979;Behbehani and Fields, 1979; Morgan et al., 1992, 2006). The ventrolateral PAG (vlPAG) contains a large population of mu-opioid receptor postitive (MOR+) neurons,- the preferred receptor for morphine (Martin, 1963; Wolozin and Pasternak, 1981), and direct administration of MOR agonists into the PAG produces potent analgesia (Bodnar et al., 1988; Jensen et al., 1986; Satoh et al., 1983). Conversely, direct administration of MOR antagonists into the PAG, or lesions of PAG MOR, significantly attenuate the analgesic effect of systemic morphine (Loyd et al., 2008; Ma and Han, 1991; Wilcox et al., 1979; Zhang et al., 1998). Despite high rates of chronic pain among the elderly, and reported age differences in morphine potency, surprisingly little is known regarding how advanced age influences the distribution and function of MOR in the PAG.

Here, we present a series of experiments delineating the impact of advanced age on opioid analgesia and the expression and binding of MOR in the vlPAG of the male and female rat. These studies provide the first data on age-mediated changes in the cellular and molecular processes of a central neural circuit governing pain and opioid analgesia in a persistent inflammatory pain model.

2. Materials and methods

2.1. Experimental subjects

Adult (2–3 mos) and aged (16–18 mos) male and regularly cycling female SAS Sprague Dawley rats were used in these experiments (Charles River Laboratories, Boston, MA). Rats were co-housed in same-sex pairs on a 12:12 hours light/dark cycle (lights on at 08:00 am). Access to food and water was ad libitum throughout the experiment, except during testing. All studies were approved by the Institutional Animal Care and Use Committee at Georgia State University and performed in compliance with Ethical Issues of the International Association for the Study of Pain and National Institutes of Health. All efforts were made to reduce the number of rats used in these experiments and to minimize pain and suffering.

2.2. Vaginal cytology

Beginning ten days before testing, vaginal lavages were performed daily on adult and aged female rats to confirm that all rats were cycling regularly and to keep daily records of the stage of estrus. Proestrus was identified as a predominance of nucleated epithelial cells, and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 was differentiated from diestrus 2 by the presence of leukocytes. Rats that appeared between phases were noted as being in the more advanced stage (Loyd et al., 2007).

2.3. Behavioral testing

Adult (2–3 mos) and aged (16–18 mos), male and female rats were used in the behavioral studies examining the impact of age and sex on morphine potency (n = 6–10/group; N = 32). As previously described, thermal nociception was assessed using the paw thermal stimulator (Univ. California San Diego) (Hargreaves et al., 1988; Wang et al., 2006). Briefly, the rat was placed in a clear plexiglass box resting on an elevated glass plate maintained at 30 °C. A timer was initiated as a radiant beam of light was positioned under the hind paw, and the time point at which the rat retracted its paw in response to the thermal stimulus was electronically recorded as the paw withdrawal latency (PWL) in seconds (s). A maximal PWL of 20s was used to prevent tissue damage due to the repeated application of a noxious thermal stimulus. Rats were acclimated to the testing apparatus 30–60 minutes before the start of the experiment on the day of testing. All behavioral testing took place between 08:00 am and 12:00 pm (lights on at 08:00 am). The maximum temperature of the thermal stimulus was recorded before and after each trial to maintain consistent recordings between groups and did not exceed a range of 60 °C–65 °C throughout the experiments. All testing was conducted blind with respect to group assignment.

2.4. Inflammatory hyperalgesia and edema

After baseline PWL determination, persistent inflammation was induced by injection of complete Freund's adjuvant (CFA; *Mycobacterium tuberculosis*; Sigma; 200 μL), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hind paw. Paw diameters were determined using calibrated calipers applied midpoint across the plantar surface of both hind paws before and after induction of inflammation.

2.5. Morphine administration

Twenty-four hours after CFA administration, 32 rats (n = 7–9 per group) were administered morphine using a cumulative dosing paradigm as previously described (Loyd et al., 2008). Briefly, rats received subcutaneous injections of morphine sulfate every 20 minutes (1.8, 1.4, 2.4, 2.4, 2.0, and 8.0), resulting in the following doses: 1.8, 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg (s.c; NIDA; Bethesda, MD, USA). PWLs were determined 15 minutes after each administration. Morphine sulfate was prepared in a saline vehicle within 24 hours of administration.

2.6. Perfusion fixation

Immunohistochemical localization of MOR was determined in a separate cohort of adult and aged male and female rats. To determine the impact of persistent inflammatory pain on MOR expression, a subset of rats in each treatment group received an intraplantar injection of CFA 24–72 hours before perfusion (total of 8 treatment groups; n = 5–8; N = 45). Rats were given a lethal dose of Euthanasol (i.p.) and transcardially perfused with 200–250 mL of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain, followed by 300–400 mL of 4% paraformaldehyde in 0.1 M phosphate as a fixative. Immediately after perfusion, brains were removed and stored in 4% paraformaldehyde solution. After 24 hours, brains were placed in a 30% sucrose solution and stored at 4 °C for at least one week before sectioning. Brains were cut coronally into 25-μm sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant antifreeze solution (Watson et al., 1986 1986) at −20 °C until immunohistochemical processing.

2.7. Immunohistochemistry

Coronal sections from the midbrain were removed from cryoprotectant and processed for MOR immunoreactivity, as previously described (Loyd et al., 2008). Briefly, sections were rinsed extensively in potassium phosphate–buffered saline (KPBS) to remove the cryoprotectant solution. After rinsing in KPBS, sections were incubated in a blocking solution of 5% Normal Donkey Serum in KPBS containing 0.5% Triton X-100 for 1 hour at room temperature. For immunofluorescence, the tissue was incubated in an antibody solution of rabbit anti-MOR (specifically targeting the extracellular N-terminus of the receptor; Alomone Labs, Jerusalem, Israel; 1:1000) in KPBS containing 1.0% Triton X-100 for 1 hour at room temperature followed by 48h at 4 °C. After rinsing in KPBS, sections were incubated in a Donkey anti-Rabbit 488 (1:200) solution for 2h at room temperature. Tissues were then rinsed using KPBS and mounted using SlowFade Diamond Antifade Mountant (Invitrogen). Images were processed on Zeiss LSM 700 Confocal Microscope at 40x.

To quantify neuronal number within the vlPAG, a second set of sections were stained using mouse anti-NeuN (Millipore Bioscience Research Reagents; 1:50,000). A third set of sections containing the inferior colliculus (IC; anatomical control) and PAG was stained using rabbit anti-MOR (Alomone Labs; 1:7500) and visualized using nickel sulfate intensified 3,3′-diaminobenzidine (Ni-DAB). After incubation in primary antibody solution as described previously, the tissue was rinsed with KPBS, incubated for 1h in biotinylated goat anti-mouse IgG or donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), rinsed again with KPBS, and then incubated for 1 hour in avidin-biotin-peroxidase complex (1:10; ABC Elite Kit, Vector Laboratories). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), NeuN and MOR immunoreactivity were visualized as a black reaction product using Ni-DAB containing 0.08% hydrogen peroxide in KPBS (pH 7.4). After incubating for 15–30 minutes, the reaction was terminated by rinsing in sodium acetate buffer. Tissue sections were mounted out of KPBS onto gelatin-subbed slides, air-dried overnight, dehydrated in a series of graded alcohols, cleared in xylene, and coverslipped using permount.

2.8. Anatomical data analysis and presentation

Fluorescent images were captured on Zeiss LSM 700 confocal microscope at 40x, and MOR intensity (signal intensity/signal volume) was calculated using Imaris software. MOR intensity values were determined for the left and right ventrolateral subdivision of each PAG image from 2 representative levels of the mid-caudal PAG (Bregma −7.74 and −8.00) and were averaged for each slice. Photomicrographs from Ni-DAB–stained sections were generated using a Sensys digital camera attached to a Nikon Eclipse E800 microscope. Images were captured with IP Spectrum software and densitometry of labeling assessed in ImageJ. Densitometry measurements were conducted bilaterally as separate images of the left and right side and averaged. As there was no significant effect of rostrocaudal level in the analyses, we then averaged the values from both levels to derive an average value per animal. Intensity and densitometry values are expressed as the mean ± standard error of the mean (SEM). Previous data have shown that there are no sex differences in total area (mm^2) of the PAG between male and female Sprague Dawley rats (Loyd and Murphy, 2006). All images were collected and analyzed by an experimenter blinded to the experimental condition.

2.9. Receptor autoradiography

To determine the impact of age, sex, and pain on vlPAG MOR binding, a separate cohort of adult and aged, male and female, and handled and CFA treated rats (total of 8 treatment groups; n = 6; N = 48) were used for receptor autoradiography. Rats were restrained using DecapiCones and decapitated. Brains were removed rapidly, flash frozen in 2-methyl butane on dry ice, and stored at −80 °C. Frozen tissue was sectioned in a 1:6 series of 20 μm coronal sections at −20 °C with a Leica CM3050S cryostat. Sections were immediately mounted onto Superfrost slides (20 °C) and stored at −80 °C until the time of the assay. Tissue was processed for autoradiography as previously described (LaPrairie and Murphy, 2009; Loyd et al., 2008). Briefly, sections were allowed to thaw to room temperature and fixed in 0.1% paraformaldehyde followed by rinses in 50 mM Tris buffer, pH 7.4. Slides were then placed in a tracer buffer containing tritiated DAMGO (100 nM; American Radiolabeled Chemicals) for 60 minutes followed by a series of rinses in 50 mM Tris buffer, pH 7.4, containing $MgCl_2$. After a final dip in cold dH_20, tissue was allowed to dry at room temp. Slides were then apposed to FujiFilm imaging plates along with [^{3}H]-microscale standards (PerkinElmer/NEN, MA, USA) for six weeks. Image plates were processed with a FujiFilm BAS 5000. Autoradiographic [^{3}H]-receptor binding was quantified from the images using Scion Image software. [^{3}H]-standards were used to convert uncalibrated optical density to disintegrations per minute (DPM). For analysis, DPM values were determined for the left and right ventrolateral subdivision of each PAG image from 2 representative levels of the mid-caudal PAG (Bregma −7.74 and −8.00) and averaged for each animal.

2.10. Statistical analysis and data presentation

All values are reported as mean ± SEM. For behavioral data analysis, data are expressed as either raw PWLs or percent maximal possible effect (%), defined as [(PWL–CFA baseline)/(maximal PWL–CFA baseline)] × 100. Significant main effects of sex, age, and treatment were assessed using ANOVA or repeated measures ANOVA; $p < 0.05$ was considered statistically significant. Tukey's post hoc tests were conducted to determine significant mean differences between groups that were *a priori* specified. Our behavioral data suggested that morphine was less potent in aged males and females, leading us to hypothesize that MOR expression and binding were reduced in the vlPAG of aged rats as well as females; thus, one-tailed post hoc tests were used for immunohistochemistry and autoradiography. All data were analyzed for identification of outliers using the ROUT method in GraphPad Prism, and data

points meeting the criteria were removed from analysis. Anatomical data are expressed either as percent area covered for Ni-DAB immunohistochemistry (IHC), intensity (signal intensity/signal volume) for fluorescent IHC, or DPM for autoradiography data. For data presentation, a representative animal from each experimental group was selected, and photomicrographs generated using a confocal microscope. Images were captured and processed with Imaris software (IHC) or processed with Scion Image (autoradiography). Alterations to the images were strictly limited to the enhancement of brightness and contrast.

3. Results

3.1. No impact of advanced age or sex on basal thermal nociception or the magnitude of CFA-induced hyperalgesia

To assess the impact of age and sex on baseline thermal nociception, paw withdrawal latencies (PWLs) in response to a noxious thermal stimulus were determined (Hargreaves et al., 1988). No significant impact of age [$F_{(1,28)} = 3.384$, $p = 0.078$] or sex [$F_{(1,28)} = 1.362$, $p = 0.253$] was noted for baseline PWL (Fig. 1A). Similarly, no significant effect of age [$F_{(1,33)} = 2.323$, $p = 0.137$] or sex [$F_{(1,33)} = 2.533$, $p = 0.121$] on the magnitude of CFA-induced hyperalgesia was observed (Fig. 1B). In all 4 groups, CFA reduced PWLs from 8.26s to 3.86s, an average % change in PWLs of −53.4% (Fig. 1C). The inflammatory insult induced by CFA injection resulted in comparable inflammation in all experimental groups, with no significant impact of age [$F_{(1,40)} = 2.171$, $p = 0.149$] or sex [$F_{(1,40)} = 0.491$, $p = 0.487$]. In all 4 groups, CFA increased paw diameter by an average % change of 168.2% (Fig. 1D).

3.2. Age and sex differences in morphine antihyperalgesia

We next determined the impact of age and sex on morphine potency using a cumulative dosing paradigm to derive EC_{50} (Fig. 2A). Our analysis of opioid potency indicated a significant dose × age interaction, [$F_{(6, 174)} = 6.81$, $p < 0.0001$], a significant dose × sex interaction [$F_{(6, 174)} = 2.65$, $p = 0.0173$], and a significant age × sex interaction [$F_{(1, 29)} = 8.86$, $p = 0.0058$]. Further analysis of EC_{50} values from each animal indicated a significant age × sex interaction [$F_{(1, 28)} = 11.42$, $p = 0.0022$] (Fig. 2B). Aged male rats were less sensitive to morphine than their adult counterparts, with a two-fold increase in EC_{50} compared with adult males (EC_{50} = 10.16 mg/kg vs. EC_{50} = 3.83; $p = 0.0002$; Fig. 2C). Adult and aged females were also less sensitive to morphine than adult males (EC_{50} = 7.76 mg/kg and 8.74 mg/kg vs. EC_{50} = 3.83 mg/kg; p = 0.0076 and $p = 0.0103$, respectively). Females showed no age differences, with similar EC_{50} values observed for adult and aged rats (EC_{50} 7.76 mg/kg vs. EC_{50} 8.74).

3.3. Impact of advanced age, sex, and chronic pain on MOR expression in the midbrain vlPAG

We next sought to determine if age and sex differences in mu-opioid receptor levels in the vlPAG contributed to our observed behavioral differences. An example of MOR immunostaining in the vlPAG is shown in Fig. 3A. Our analysis indicated a significant impact of age on vlPAG MOR expression, with aged rats expressing a loss in vlPAG MOR density compared with adults [$F_{(1,37)} = 9.933$, p = 0.040] (Fig. 3B). No significant impact of sex [$F_{(1,37)} = 3.093$, p = 0.243] or treatment [$F_{(1,37)} = 0.855$, $p = 0.536$] was observed. Post hoc analysis showed that vlPAG MOR expression was significantly

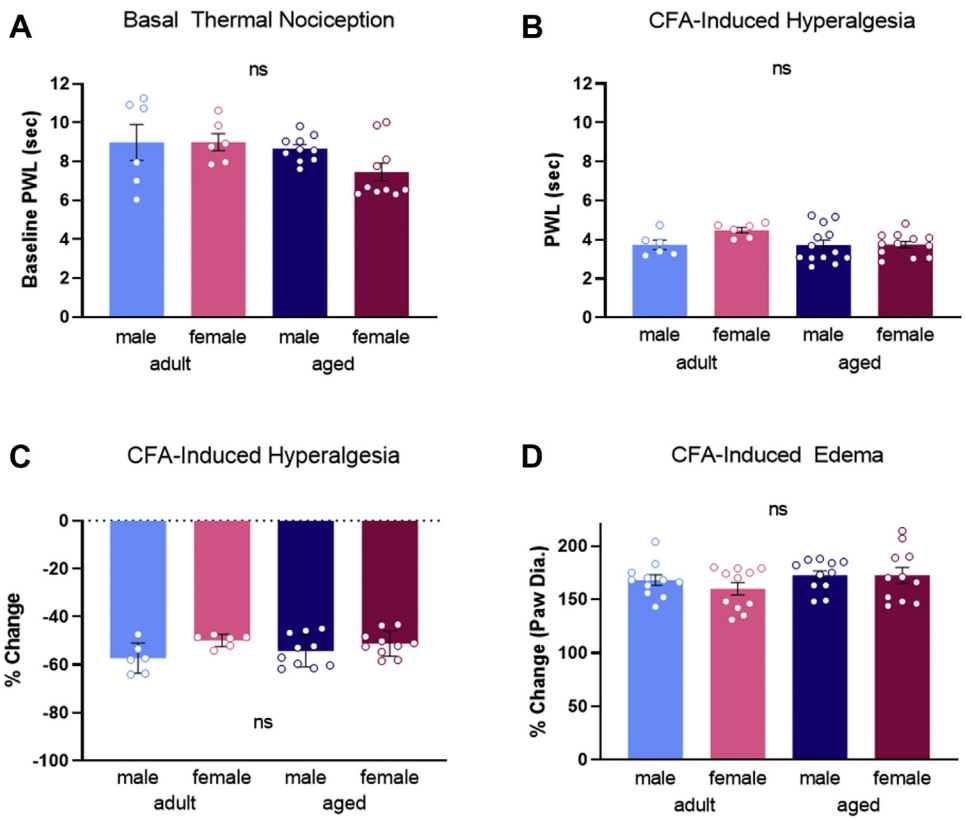

Fig. 1. No impact of age or sex was observed for basal thermal nociception (A) or post-CFA PWLs (B). Similarly, no age or sex difference in the magnitude of CFA-induced hyperalgesia (C) or CFA-induced edema (D). ns, not significant calculated by two-way ANOVA. Graphs indicate mean ± SEM.

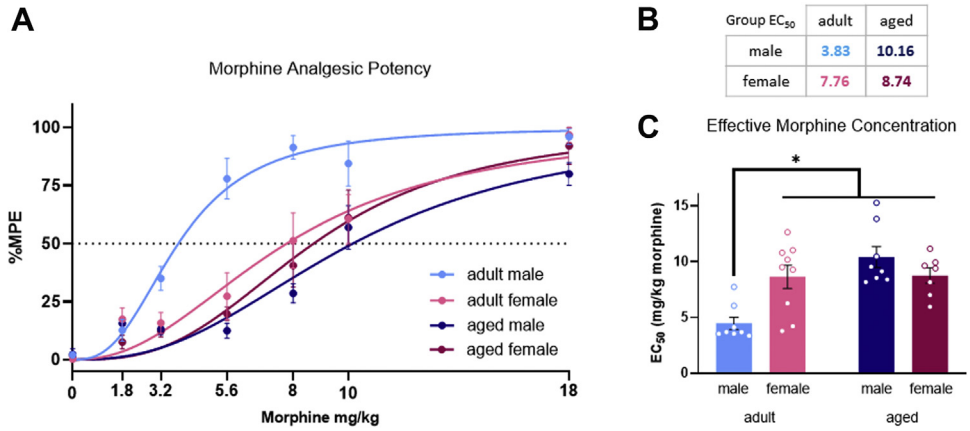

Fig. 2. Morphine dose response curve after cumulative administration (A). EC_{50} values were generated from nonlinear regression analysis (B). Adult males exhibit lower morphine EC_{50} values than females and aged males. (C) Data presented in milligrams of morphine per kilogram of body weight. *Significant differences between adult male and adult female, adult male and aged male, and adult male and aged female. $p < 0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM.

reduced in aged males compared with adult males ($p = 0.013$) (Fig. 3B). To ensure that our observation of reduced vlPAG MOR in aged males was not due to an age-induced decrease in overall cell number, adjacent sections through the PAG were immunohistochemically stained using the neuron-specific marker NeuN (Millipore; [75]) and the density of staining quantified using densitometry. No significant effect of age [$F_{(1,23)} = 0.133$, $p = 0.909$] or sex [$F_{(1,23)} = 0.072$, $p = 0.791$] was observed in NeuN immunostaining (Fig. 3C), indicating that the reduction in MOR density was not due to an overall age-related loss in neuronal number. We further examined if the reduction in MOR observed in aged males was limited to the PAG or present in other MOR-containing regions. Sections through the IC, a region rich in MOR, were stained and quantified. No significant effect of age [$F_{(1,12)} = 0.094$, $p = 0.764$] or

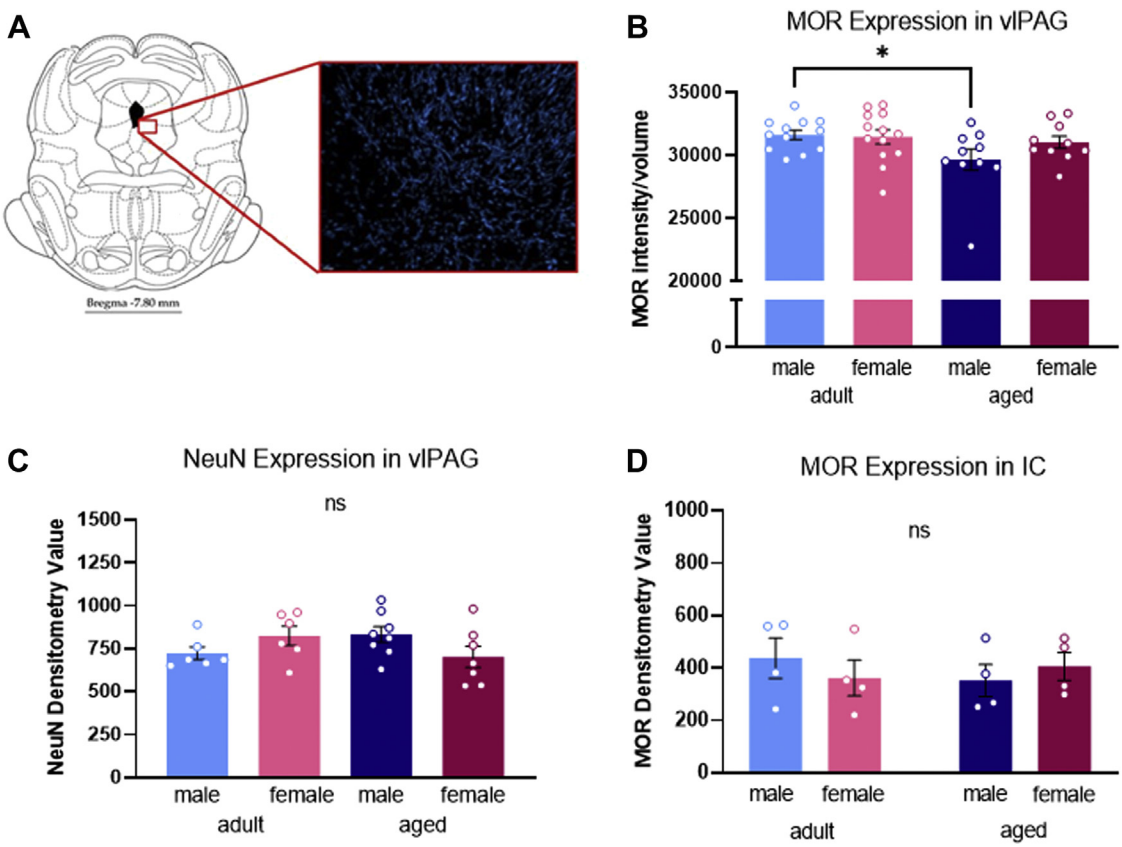

Fig. 3. Representative section from rat brain with red highlight showing the vlPAG region quantified for MOR immunoreactivity (A). MOR densitometry in the vlPAG was significantly lower in aged rats compared with adults (B). No significant impact of sex or treatment was noted. No differences MOR density was observed between handled and CFA treated groups, so these data are combined. No significant effect of age or sex was observed in NeuN densitometry in the vlPAG (C). No significant effect of age or sex was observed in MOR densitometry in the inferior colliculus (IC) (D). *Significant difference between adult and aged males; $p < 0.05$ calculated by Tukey's post hoc test. ns, not significant. Graphs indicate mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sex [$F_{(1,12)} = 0.029$, $p = 0.868$] on MOR expression was noted, suggesting the age-induced reduction in MOR expression was specific for the PAG (Fig. 3D).

3.4. Impact of advanced age, sex, and chronic pain on MOR binding in the midbrain vlPAG

We next used autoradiography to determine if the observed reduction in MOR expression in aged males reflected a reduction in MOR binding. [^{3}H]-DAMGO was used to label MOR as previously described (LaPrairie and Murphy, 2009) Representative autoradiograms are shown in Fig. 4A. Consistent with what we noted using IHC, there was a significant impact of age [$F_{(1,39)} = 30.08$, $p < 0.0001$], with aged rats exhibiting a loss of binding compared than their adult counterparts (Fig. 4B). Post hoc analysis indicated that aged males had reduced MOR binding compared with adult males [$p = 0.0067$]. MOR binding was also significantly reduced in aged females compared with adults [$p = 0.0009$]. There was no significant impact of sex [$F_{(1,39)} = 0.108$, $p = 0.306$] or treatment [$F_{(1,39)} = 0.021$, $p = 0.885$] (Fig. 4B), and no significant interactions were noted.

4. Discussion

The present studies were conducted to determine the impact of advanced age on opioid modulation of pain. Using a more clinically relevant model of persistent inflammatory pain, we report that aged rats require higher doses of morphine than their adult counterparts to achieve comparable levels of analgesia. The impact of age was sex specific, such that aged males required a significantly higher morphine dosage to reach analgesia than adult males; no impact of age was noted in morphine potency in females. Our results further demonstrate significant age differences in vlPAG MOR expression, with aged rats exhibiting reductions in vlPAG MOR protein and binding. As MOR expression and function in the vlPAG is critical for morphine attenuation of pain, the observed age-induced reduction in vlPAG MOR may drive the reduced morphine potency observed in aged rats.

4.1. No impact of advanced age or sex on baseline pain sensitivity and CFA-induced hyperalgesia

In the present studies, no effect of advanced age (or sex) was observed in baseline thermal sensitivity. This finding is consistent with our previous studies as well as those of others (Ali et al., 1995; Craft et al., 1998; Craft and Milholland, 1998; Wang et al., 2006). Indeed, most rodent studies assessing the impact of sex on basal nociception report no differences (Mogil, 2020; Mogil et al., 2000, 2010; Racine et al., 2012; Wang et al., 2006). In a recent meta-analysis of studies published as of August 2019, only 8% (41/526) reported a significant quantitative sex difference in pain sensitivity (Mogil, 2020). In regards to age-associated changes in basal

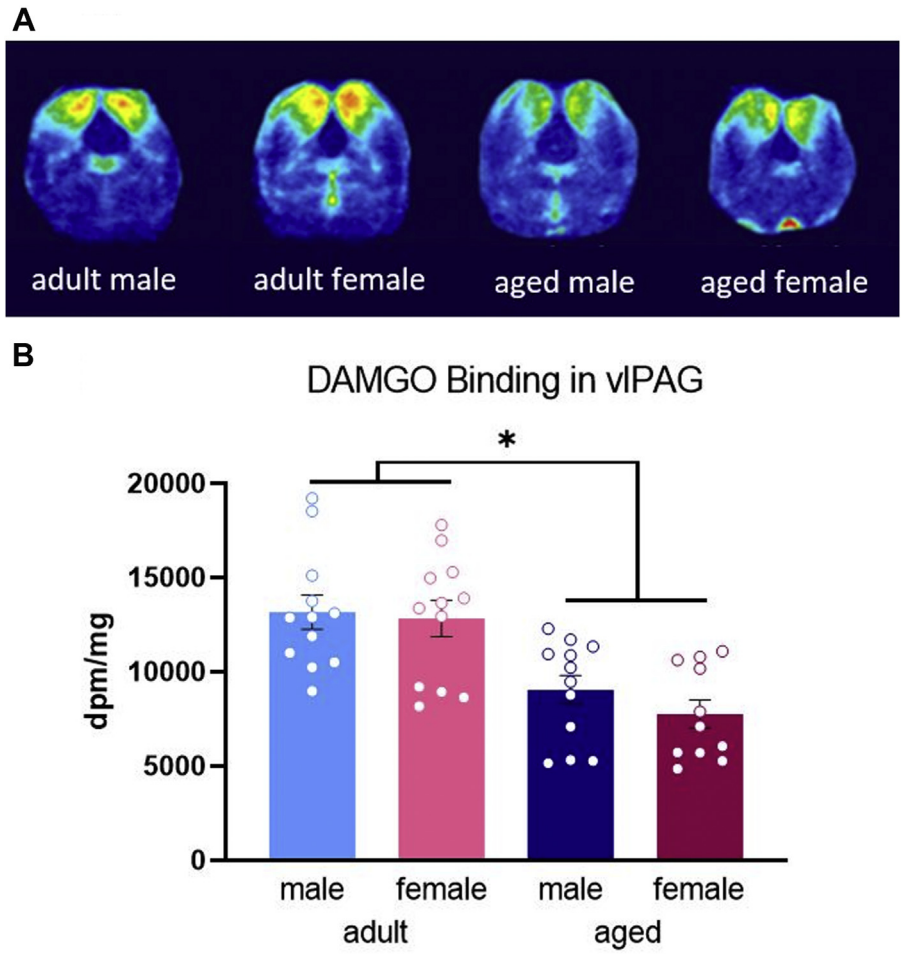

Fig. 4. Heat mapped representative images of DAMGO binding in the midbrain of experimental groups (A). Aged rats exhibited reduced PAG MOR binding compared with adults (B). No impact of sex was found. No differences in density were observed between handled and CFA-treated groups, so these data are combined. Data presented in disintegrations per minute per milligram. * Significant difference between adult males and females and aged rats; $p < 0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM.

nociception, these reports are highly varied, with reports of increased (Hess et al., 1981; Kitagawa et al., 2005), decreased (Chan and Lai, 1982; Garrison and Stucky, 2014; Kramer et al., 1985; Muralidharan et al., 2020), and no change in mechanical or thermal pain sensitivity in aged rodents compared with adults (Crisp et al., 1994; Mecklenburg et al., 2017; Samir et al., 2017; Taguchi et al., 2010; Yezierski, 2012). Similarly, meta-analyses of both clinical and laboratory measurements in humans fail to report a consistent impact of age on nociception (Gibson and Helme, 2001; Ostrom et al., 2017; Weyer et al., 2016). For example, in a meta-analysis summarizing the findings of 52 clinical research studies, Lautenbacher (2012) concluded that aged individuals exhibit increased pain thresholds (i.e., decreased sensitivity) compared with young adults. However, these data included both pain-free individuals and individuals who were experiencing chronic pain and did not include sex as a variable of analysis. A similar study by Ostrom et al. (2017) of over 3400 participants reported no impact of age on pain sensitivity across numerous modalities (pressure, mechanical, and thermal), although a limited age range was examined (18–44 years; Ostrom et al., 2017).

In the present study, intraplantar injection of the mycobacterium complete Freund's adjuvant was used to induce persistent inflammatory pain (Millan et al., 1988; Stein et al., 1988). We report no significant impact of either age or sex on the magnitude of thermal hyperalgesia elicited by intraplantar CFA or CFA-induced edema (Loyd et al., 2006, 2008; Wang et al., 2006). To date, very few preclinical studies have examined the impact of age on persistent pain. Mecklenburg et al. (2017) reported no impact of age (3, 6, 18, and 24 months) on either mechanical or thermal hyperalgesia in male mice using a plantar incisional model of pain. Similar results were reported by Garrison and Stucky (2014) in male mice aged 3 and 24 months after intraplantar CFA. This study also reported comparable levels of edema in adult and aged mice up to 8 weeks post-CFA. In contrast, Weyer et al. (2016) reported that aged male mice (18 weeks) showed decreased mechanical hyperalgesia in comparison with adults across all eight weeks examined. This attenuated hyperalgesic response was accompanied by an overall reduction in noxious stimulus-evoked action potentials in the dorsal horn. Similar to the present study, no impact of age on CFA-induced edema was reported (Weyer et al., 2016).

4.2. Impact of advanced age and sex on morphine analgesia

The present studies are the first to examine the impact of advanced age on opioid modulation of persistent inflammatory pain in male and female rats. We report morphine EC_{50} values dependent on age and sex; notably, aged male rats required significantly higher doses than their adult counterparts to produce comparable antihyperalgesia. Similar results were reported by Muralidharan et al. (2020), who noted a reduction in morphine's antiallodynic effect in male mice 54 weeks of age. Interestingly, the antiallodynic effects of the gabapentinoid pregabalin were also attenuated (Muralidharan et al., 2020), suggesting that the age-induced blunting of analgesics on pain is not limited to opioids, but rather, extend to other classes of drugs.

Clinically, several studies report that opioid requirements for postoperative pain are inversely related to age (Gagliese and Katz, 2003; Kaiko, 1980; Keita et al., 2009), although results to the contrary have also been reported (Papaleontiou et al., 2010; Prostran et al., 2016). The results from clinical studies on elderly individuals are more challenging to interpret due to the high degree of comorbidity with conditions such as heart disease, diabetes, and high blood pressure that may alter the perception of pain and necessitate the use of concomitant medications. Additional factors that have been shown to influence clinical studies on pain and analgesia are sex and age differences in the likelihood of self-reporting pain in a clinical setting and the subjective nature of pain (Dampier et al., 2013; Ferrell et al., 1991; Reddy et al., 2012).

4.3. Sex and age differences in vlPAG MOR expression and binding

The PAG is a critical neural site for opioid modulation of pain (Behbehani and Fields, 1979; Loyd et al., 2008; Morgan et al., 1991, 1992; Reynolds, 1969; Zhang et al., 1998). In the present study, using both immunohistochemistry and autoradiography, we report that aged rats exhibit reduced vlPAG MOR expression and binding compared with adults. This suggests that the age-induced decrease in morphine potency is driven, in part, by diminished vlPAG MOR. In the present study, morphine was administered systemically, and the analysis of MOR expression was limited to the PAG (and IC); therefore, a contribution from other pain-associated MOR+ regions (including the rostral ventromedial medulla and spinal cord dorsal horn) cannot be ruled out. Previous studies have reported an attenuation in MOR in the frontal cortex and striatum of aged male rats compared with adults (Hess et al., 1989; Messing et al., 1981). Reduced MOR binding in the midbrain and thalamus of aged female rats has also been shown (Messing et al., 1980). Together, these studies suggest that the reduction in MOR observed in aged rats is not specific to the PAG. Importantly, we did not find a significant reduction in MOR in the IC as a function of age or sex, suggesting that these age-associated changes may be limited to CNS sites implicated in pain and reward. No reduction in neuronal number within the vlPAG was observed, indicating that the observed reduction in MOR was not due to overall neuronal loss.

No impact of persistent inflammatory pain on MOR expression or binding in the vlPAG was noted. Similarly, Thompson et al. (2018) reported no change in MOR availability or expression in the PAG of male rats after induction of neuropathic pain, although decreased MOR was observed in the caudate putamen and insula (Thompson et al., 2018). In contrast, Zollner et al. (2003) reported increased MOR binding in the dorsal root ganglia after intraplantar CFA (Zollner et al., 2003), suggesting that the effects of persistent inflammatory pain on MOR are region-specific. Clinical studies utilizing positron emission tomography scans to assess for changes in MOR binding reported that individuals experiencing rheumatoid arthritis pain exhibit decreased MOR binding in the straight gyrus and the frontal, temporal, and cingulate cortices (Jones et al., 1994); in addition, individuals experiencing fibromyalgia pain exhibited reduced MOR binding in the nucleus accumbens, amygdala, and cingulate cortex (Harris et al., 2007). Neither of these studies reported an impact of pain on MOR binding in the PAG. A third study examining MOR binding in individuals experiencing poststroke neuropathic pain (centrally versus peripherally localized) reported reduced PAG MOR binding in individuals experiencing neuropathic pain localized centrally not peripherally, compared with controls (Maarrawi et al., 2007). Together, these results suggest that PAG MOR expression and binding may be altered as a function of the pain location or modality.

In the present study, we found no impact of sex on vlPAG MOR expression. These results are contradictory to our previous findings in which we reported significantly reduced levels of vlPAG MOR in adult females compared to males (Loyd et al., 2008). In that study, sex differences in MOR were primarily driven by diestrus females, with smaller, nonsignificant differences noted for the other stages of estrus in comparison with males. In the present study, although the estrus stage was determined, it was not included as a factor of analysis due to a lack of power. However, failure to include estrus as a variable of analysis is unlikely to account for the finding of no sex difference in MOR expression. Rather, methodological differences between the 2 studies likely contributed to these conflicting results,

including differences in antibody specificity and 3-dimensional confocal versus 2-dimensional light-field microscopy image acquisition and assessment of MOR density.

The finding of no significant effect of sex on vlPAG MOR expression or binding suggests that the observed sex differences in morphine potency are not driven by vlPAG MOR expression or binding as previously proposed (Loyd et al., 2008). Recent attention has focused on sex differences in neuroimmune signaling, and in particular, microglia, as a contributing factor to morphine's dimorphic effects (Doyle and Murphy, 2017; Eidson et al., 2019; Eidson and Murphy, 2019; Rosen et al., 2017; Sorge et al., 2011). In addition to binding to neuronal MOR, morphine has been shown to bind to the innate immune receptor toll-like receptor 4 (TLR4), localized primarily on microglia (Hutchinson et al., 2007, 2008, 2010). Morphine action at TLR4 has been shown to decrease both glutamate transporter (GLT1 and GLAST) and $GABA_A$ receptor expression and upregulate AMPA receptor, resulting in an overall increase in neural excitability (Eidson et al., 2017; Holdridge et al., 2007; Ogoshi et al., 2005; Song and Zhao, 2001; Stellwagen et al., 2005; Wang et al., 2012). As morphine works primarily via hyperpolarization (Chieng and Christie, 1996), this increased neural excitability within the vlPAG directly opposes morphine action (Ingram et al., 1998). Our laboratory has recently reported that blockade of vlPAG TLR4 signaling via (+)-naloxone results in a 2-fold leftward shift in the morphine dose response curve in females but not males (Doyle et al., 2017). Further, we report that pathogenic activation of TLR4 via systemic administration of the bacterium lipopolysaccharide results in a significantly higher vlPAG expression of the proinflammatory cytokine IL-1ß. Reduced vlPAG expression of the anti-inflammatory cytokine IL-10 was noted in females, but not males, after lipopolysaccharide. Increased levels of neuroinflammation have been reported in aged animals suggesting that changes in neuroimmune signaling, along with the observed reduction in vlPAG MOR binding, together contribute to the reduction in morphine efficacy (Gorelick et al., 2010; Norden and Godbout, 2013; VanGuilder et al., 2011). The impact of advanced age on morphine potency may also be driven by age differences in receptor affinity and opioid signaling in the vlPAG. Studies are currently underway to assess the impact of age and sex on vlPAG MOR binding potential and agonist stimulated G-protein activation.

Disclosure statement

The authors have nothing to disclose.

CRediT authorship contribution statement

Evan F. Fullerton: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Myurajan Rubaharan:** Investigation, Methodology. **Mary C. Karom:** Investigation, Methodology. **Richard I. Hanberry:** Investigation. **Anne Z. Murphy:** Conceptualization, Validation, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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