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The Impact Of Advanced Age On Morphine Anti-Hyperalgesia And The Role Of Mu Opioid Receptor Signaling In The Periaqueductal Gray Of Male And Female Rats

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by

Evan Fullerton

Under the Direction of Anne Murphy, PhD

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ABSTRACT

Opioids are among the most potent and widely used drugs for the management of chronic pain. Chronic pain is exceedingly prevalent in individuals over 65 years of age but is under-managed in this population, due in part to a lack of knowledge regarding the suitable dosing of opioids for chronic pain management. The present experiments first seek to characterize the impact of advanced age on opioid potency in male and female rats using intraplantar Complete Freund's adjuvant (CFA), a clinically relevant model of inflammatory pain. We report that advanced age and sex alter morphine modulation of persistent inflammatory pain, specifically, morphine potency was highest in adult male rats (2mos), with a 2-fold rightward shift in the dose-response curve for aged males (18mos), and females regardless of age. These findings suggest that opioids have decreased anti-hyperalgesic potency in aged rodents compared to adults, prompting us to determine the neural mechanisms underlying this attenuated response.

Morphine-induced analgesia is mediated primarily via binding to μ-opioid receptors (MOR) within the midbrain periaqueductal gray (PAG), a critical site for descending pain modulation. The present studies use cellular and pharmacological techniques to examine MOR binding and signaling within the PAG. The data in this thesis thoroughly characterize the impact of advanced and biological sex on PAG MOR expression, binding, and signaling within the PAG in the presence of chronic inflammatory pain, and thus have significant implications for pain management in the aged population. These studies contribute to our understanding of how advanced age and sex alter morphine anti-hyperalgesia and enhance our knowledge of age- and sex-induced changes in PAG MOR signaling. Our findings identify novel therapeutic targets to improve opioid signaling in the aged brain and develop effective chronic pain management strategies.

INDEX WORDS: Morphine analgesia, Chronic pain, Aging, Sex differences, Mu opioid receptor, Periaqueductal gray

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Evan Fullerton

Committee Chair: Anne Murphy

Committee: Kim Huhman

Aaron Roseberry

Shelley Hooks

Electronic Version Approved:

Office of Graduate Services

College of Arts and Sciences

Georgia State University

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DEDICATION

To my parents. Your confidence, wisdom, and support have shaped me into the person I am today. Thank you for all you have done for me.

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LIST OF ABBREVIATIONS

CFA	Complete Freund's adjuvant
EC_{50}	Median effective concentration
MOR	Mu (μ) opioid receptor
PAG	Periaqueductal gray
vlPAG	Ventrolateral periaqueductal gray
RVM	Rostral ventral medulla
DRG	Dorsal root ganglion
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
ATP	Adenosine Triphosphate
cAMP	Cyclic adenosine monophosphate
AC	Adenylyl cyclase
FSK	Forskolin
TRP	Transient receptor potential
GRK2	G-protein receptor kinase 2
PKC	Protein kinase C

1 INTRODUCTION

1.1 Chronic pain and pain management in the aged

Chronic pain, defined as severe pain persisting for longer than 3 months, is debilitating in nature, negatively impacting mental health and contributing to the increased risk of premature death (Patel et al., 2013; Treede et al., 2015; Domenichiello & Ramsden, 2019). It is among the most commonly reported health issue in the United States, with greater than 20% of adults experiencing chronic pain on a daily basis (Dahlhamer, 2018).

The United States population is rapidly aging. In 2019, 54 million Americans were 65 or older, comprising 16% of the population. By 2040, this demographic is projected to reach 80 million, at which time individuals over 65 years will represent nearly 25% of the United States population (*Promoting Health for Older Adults | CDC*, 2022). Those over 65 years of age experience chronic pain at a higher rate than their adult counterparts; nearly 40% of individuals over 65 report chronic pain (Larsson et al., 2017), and over 50% report experiencing at least one chronic pain condition within the past year (Tsang et al., 2008), including pain associated with arthritis, cancer, diabetes mellitus, and cardiovascular disease (Dahlhamer, 2018; Domenichiello & Ramsden, 2019). Elderly individuals residing in assisted living facilities report higher rates of chronic pain than community dwelling elderly, with upwards of 80% of nursing home residents reporting chronic pain (Higgins et al., 2004; Stompór et al., 2019). Thus, there is a growing need for adequate management of chronic pain in this vulnerable population (Miaskowski, 2011).

Chronic pain is typically managed by prescription medications called analgesics which reduce the severity of symptoms (Manchikanti et al., 2010; Sullivan et al., 2010). Opioids, including morphine and fentanyl, are among the most potent and commonly prescribed analgesics for chronic pain management (Mercadante et al., 2019). However, despite elderly

individuals suffering from chronic pain at a higher rate than young adults, chronic pain is undermanaged in the aged demographic, due in part to a dearth of knowledge regarding proper dosing and side effects of opioid use in the aged (B. A. Ferrell et al., 1990; Pergolizzi et al., 2008; Campbell et al., 2010; Barnett et al., 2017). Multiple clinical factors contribute to the undermanagement of pain in the elderly; most notable are under-reporting of pain intensity for fear of being institutionalized, physician reluctance to prescribe opioids to elderly individuals due to concerns over adverse side effects (including cognitive and physical impairment, respiratory depression), and misconceptions regarding tolerance and addiction (Cavalieri, 2005; Chau et al., 2008; Saunders et al., 2010; Miaskowski, 2011; Miaskowski et al., 2011; Reddy et al., 2012; Dampier et al., 2013; Schiltenwolf et al., 2014; Naples et al., 2016; Prostran et al., 2016). Studies that accurately assess the impact of advanced age on opioid analgesia are needed to identify potential mechanisms by which advanced age impacts opioid analgesia, thereby facilitating the development of therapeutics to effectively manage pain in this population while concurrently minimizing the negative side effects associated with opioid consumption.

1.2 Mechanisms of pain and opioid analgesia in the central nervous system

Painful stimuli originating in the periphery are initially detected by nociceptors, specialized sensory neurons with cell bodies in the dorsal root ganglion (DRG) and peripheral processes that innervate joints, muscle, and skin. Nociceptors, or primary afferents, transmit information from the periphery to the central nervous system to produce the sensory components of pain. Nociceptors are divided into 3 main categories: Aδ-, Aβ-, and C-fibers. Myelinated fast-conducting Aδ-fibers act as both thermal nociceptors that detect extreme temperatures and mechanical nociceptors that detect intense pressure on the skin. Myelinated Aβ-fibers act

primarily as mechanoreceptors but are also activated by noxious mechanical stimuli and noxious heat (Djouhri & Lawson, 2004). Unmyelinated slow-conducting C-fibers act as polymodal nociceptors that detect thermal, mechanical, and chemical stimuli. Noxious stimuli depolarize the endings of Aδ-, Aβ-, and C-fibers via transient receptor potential (TRP) ion channels, and the resultant action potentials are transmitted to the cell bodies or first-order pseudo-unipolar neurons in the DRG. The central branches of these DRG neurons terminate in the dorsal horn of the spinal cord: Aδ-fibers terminate preferentially within lamina I, II, and V; C-fibers terminate within lamina I, and II; and Aβ fibers terminate within lamina IV. (Millan, 1999; Lemke, 2004; Dubin & Patapoutian, 2010).

Nociceptive information predominantly ascends the spinal cord from the dorsal horn via the spinothalamic tract which relays nociceptive, thermosensitive, and somatosensory information. The axons of dorsal horn second order neurons cross the midline of the spinal cord at the anterior white commissure, project to the medulla and pons, and terminate supraspinally within thalamic nuclei. Third order thalamic nuclei relay nociceptive information to the somatosensory cortex and limbic system. Nociceptive information also ascends the spinal cord via the spinoreticular and spinomesencephalic pathways, projecting to the reticular formation and the midbrain periaquectuctal gray (Ab Aziz & Ahmad, 2006; Garland, 2012; Groh et al., 2018).

The nociceptive input from the periphery that projects to higher-order brain regions is subjected to tonic descending inhibition at the level of the dorsal horn of the spinal cord by the descending pain modulatory pathway (Melzack & Wall, 1965; Garland, 2012). The midbrain periaqueductal gray (PAG), the first region of the descending pain pathway, receives nociceptive input from multiple supraspinal regions. Projections from the PAG descend to the rostral ventromedial medulla (RVM), which sends bilateral projections via the dorsolateral funiculus to

terminate within the dorsal horn of the spinal cord (Basbaum et al., 1978; Behbehani & Fields, 1979; Fields & Heinricher, 1985).

The PAG, and in particular the ventrolateral periaqueductal gray (vlPAG), contains mu opioid receptors (MORs), proteins to which opioids bind to bring about the inhibition of ascending pain signals (Martin, 1963; Wolozin & Pasternak, 1981; Jensen & Yaksh, 1986; Morgan et al., 1992; Loyd et al., 2008). MORs are seven-transmembrane spanning proteins with an extracellular N-terminal domain and an intracellular C-terminal domain (Herman et al., 2022). MORs are G-protein coupled receptors (GPCRs), binding predominantly to inhibitory G-proteins $G_{i/o}$ (Goode & Raffa, 1997; Gintzler & Chakrabarti, 2004). Within the PAG, opioid binding to GABAergic interneurons promotes an intracellular G-protein-mediated signaling cascade whereby GABAergic neurons are hyperpolarized, inhibiting GABA release. Indeed, extracellular GABA in the PAG is reduced following administration of morphine (Stiller et al., 1996). In the absence of MOR binding, these GABAergic interneurons tonically inhibit the antinociceptive

PAG-RVM descending pathway. Thus, opioid-induced hyperpolarization of PAG GABAergic interneurons disinhibits the PAG-RVM descending pathway and enhances anti-nociceptive projections to the dorsal horn of the spinal cord (Stiller et al., 1996; G. W. Pasternak, 2001; Novak, 2007; Lau & Vaughan, 2014; Lueptow et al., 2018)(Figure 1.1).

1.2.1 Mechanisms of chronic inflammatory pain

Painful stimuli applied to the skin promote the local release of inflammatory mediators including bradykinins, prostaglandins, and adenosine triphosphate (ATP) (H. Wang et al., 2005; Amaya et al., 2013). Injury at the periphery activates not only nociceptive neurons but also innate immune cells, particularly microglia. Activated microglia, which are observed in virtually every known animal model of clinical pain, react to the presence of pathogens, including nitric

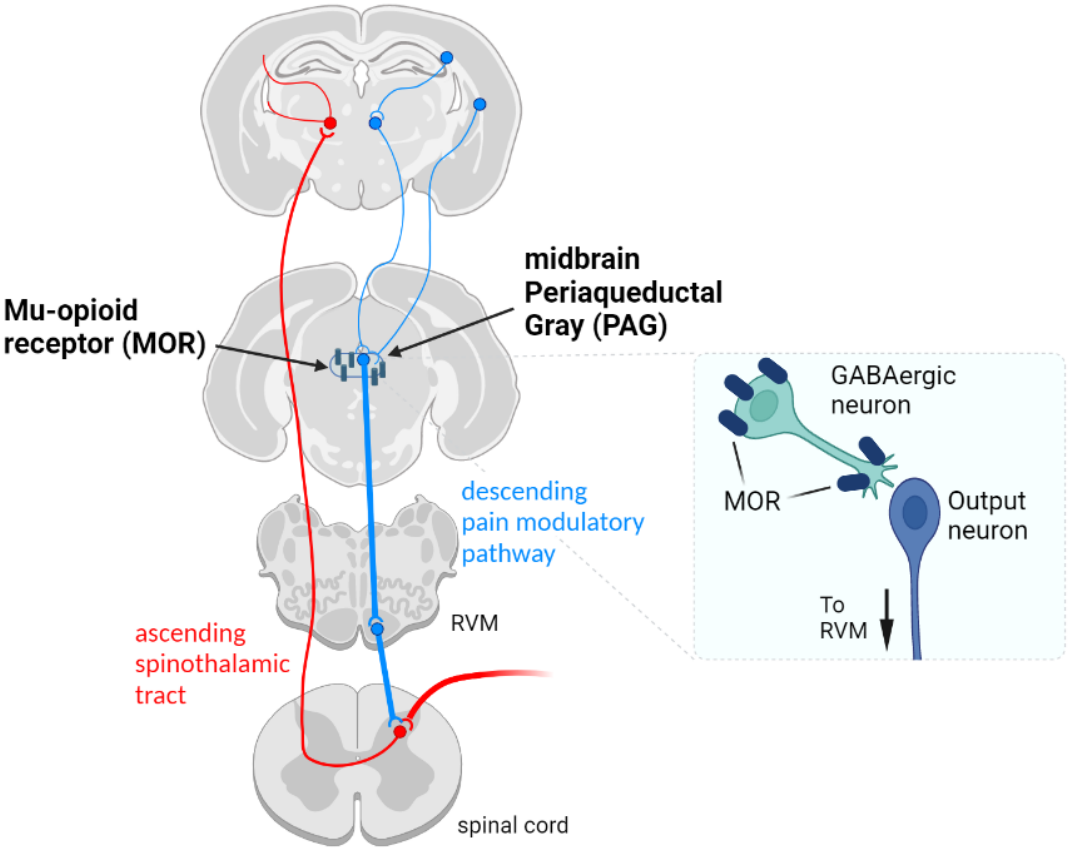


Figure 1.1 Descending pain modulatory pathway, critical role of PAG MOR

Afferent signals are inhibited by the downstream pain modulatory pathway. Opioids act at u-opioid receptors (MOR) within the periaqueductal gray (PAG) to inhibit pain at the dorsal horn of the spinal cord

oxide, substance P, and prostaglandins that are released at the time of injury (Watkins et al., 2009). These proinflammatory substances bind to receptors called toll-like receptors (TLRs) and in particular TLR4, located primarily on the surface of microglia. Binding to microglial toll-like receptor 4 (TLR4) results in the transition of microglia from a resting state to an activated state, a process called reactive gliosis (Watkins & Maier, 2003; Hutchinson et al., 2008; Watkins et al., 2009). In their activated state, microglia release pro-inflammatory molecules including cytokines (e.g. interleukins 1 and 6, (IL-1, IL-6), and tumor necrosis factor (TNF)), chemokines (e.g. IL-8, CCL2, and CCL5), cyclooxygenase-2 (COX-2), prostaglandins, and reactive oxygen species (ROS) (Bonizzi & Karin, 2004). Together, these molecules down-regulate inhibitory $GABA_A$ receptors, up-regulate excitatory neuronal AMPA and NMDA receptors, and decrease glutamate transporter activity, effectively increasing neural excitability and opposing opioid hyperpolarization (Watkins et al., 2005; Yan et al., 2014; Eidson et al., 2017).

One experimental method used to induce inflammatory pain in rodents is the intraplantar administration of the mycobacteria Complete Freund's Adjuvant (CFA). Intraplantar CFA is one of the most commonly used procedures to induce a persistent pain state and has been shown to reliably mimic the time course of postoperative pain as well as other types of persistent pain due to injury (Millan et al., 1988; Stein et al., 1988). The localized inflammation resulting from intraplantar CFA produces hyperalgesia that parallels the clinical experience of tissue injury induced by trauma or disease, for which morphine is typically prescribed. CFA-induced hyperalgesia is present within 2-6 hours of injection and is still evident 7-14 days following administration (Millan et al., 1988; Stein et al., 1988; Philippe et al., 1997; Goff et al., 1998; X. Wang et al., 2006).

The activation of glial cells directly contributes to the robust hyperalgesia produced by inflammatory CFA (Watkins et al., 1994; Raghavendra et al., 2004; Sorge et al., 2011). Indeed, inhibition of glial function or the blockade of pro-inflammatory cytokine release reverses hyperalgesia (Maier & Watkins, 1998; Plunkett et al., 2001; Raghavendra et al., 2004; Hutchinson et al., 2008), supporting a model whereby neurons and glia engage in bidirectional communication to promote hyperalgesia following inflammatory pain. Both the inhibition of microglial function and the blockade of pro-inflammatory molecule release prevent the development of hyperalgesia (Maier & Watkins, 1998; Ledeboer et al., 2005; Hutchinson et al., 2008).

1.3 Studies of opioid analgesia, effects of advanced age and biological sex

Several clinical studies have examined how the aging process affects opioid potency; unfortunately, conclusions drawn across studies remain inconsistent. For example, in post-operative cancer patients, morphine promoted greater pain relief in older (aged 50-89) versus younger patients (18-29) (Kaiko, 1980), and aged men self-administered lower concentrations of morphine to manage post-surgical pain compared to young men (Gagliese & Katz, 2003). Alternatively, a clinical assessment of opioid potency for cancer pain management in men and women found that morphine analgesia was attenuated in the aged. Here, analysis of self-reported pain and oral morphine concentration in 577 subjects ranging from age 17 to 91 revealed that men and women >74 years of age required higher daily doses of morphine to manage cancer pain (478.8 mg/day) compared to men and women <65 years of age (353.3 mg/day) (Loick et al., 2000).

This lack of consensus on how advanced age impacts opioid analgesia is due in large part to limitations of clinical trials and consequent issues in the interpretation of results. More

specifically, a primary limitation is that opioids are prescribed in the clinic to alleviate severe and persistent pain resulting from injury, surgery, or disability, but the majority of studies on aging and opioid efficacy were conducted on patients experiencing acute (rather than chronic) pain (Aubrun et al., 2005; Gagliese & Katz, 2003; Lautenbacher, 2012). Further, aged patients present high rates of comorbid health conditions, including cardiovascular disease, diabetes, and high blood pressure, all of which entail the use of concomitant medications that may attenuate or potentiate the analgesic effects of opioids (Naples et al., 2016; Prostran et al., 2016). In addition, aged populations underreport pain in a clinical setting, likely due to fears of institutionalization and concerns with addiction and overdose (Reddy et al., 2012). For example, many clinical studies use the McGill Pain Questionnaire, a verbally-based self-assessment consisting of questions regarding the affective qualities and intensities of pain (Perez-Lloret et al., 2016). Younger men are more likely to report pain using this type of assessment in comparison to a visual analog scale, in which there are no age differences in reporting (Gagliese & Katz, 2003).

To control for the issues associated with clinical studies, researchers have turned to rodent models to assess the impact of advanced age on opioid potency. Unfortunately, very few studies have been conducted that use aged animals, likely due to the increased costs in maintaining a long-term colony (Jackson et al., 2017). Of the studies that have been conducted, the results are often contradictory and thus, like the clinical studies, fail to provide a consensus on the impact of advanced age on opioid analgesia. For example, Chan and Lai reported that morphine (10mg/kg; sc) significantly increased the hot plate latency in adult male rats (54 days old), while this dose was without effect in middle aged (162 days) or elderly (327 days) rats (Chan & Lai, 1982). In female rats aged 4, 9, 14, 19, and 24 months, morphine was more effective in lowering withdrawal rates in the jump and tail flick tests in younger rats (Kramer &

Bodnar, 1986). Additionally, Crisp et al. reported that rats aged 26 months exhibited reduced opioid antinociception compared to rats aged 5-6 months, as evidenced by a rightward shift in the DAMGO dose-response curve measured using a tail-flick assay (Crisp et al., 1994). Similar results were reported by Islam et al., with male and female rats exhibiting higher morphine EC_{50} doses (lower potency) at 18 months versus 6 months of age (Islam et al., 1993). However, not all studies on rodents reveal age-dependent reductions in opioid potency. In a review of relevant literature on opioid analgesia, Gagliese and Melzack (2000) report that while the results of several studies suggest a decline in morphine analgesic potency with advanced age, several studies report no impact or even an improvement in opioid analgesia as a function of advanced age (Gagliese & Melzack, 2000).

Preclinical studies of morphine analgesia using rodents also present limitations that mirror some of the issues associated with clinical studies. Notably, researchers typically utilize acute (tail flick, hot plate) rather than persistent pain assays, and more often than not, elect to use exclusively male subjects in their studies (Chan & Lai, 1982; Kavaliers et al., 1983; Smith & Gray, 2001; Jourdan et al., 2002). As morphine is prescribed for the alleviation of persistent (and not acute) pain, it is important that experiments accurately model chronic pain conditions that necessitate opioid prescriptions in order to develop a definitive picture of the impact of advanced age on opioid potency.

The existence of sex differences in opioid analgesia in the clinic remains controversial. Although some clinical studies report no sex difference in the analgesic efficacy of opioids(Sarton et al., 2000; Fillingim et al., 2005; Bijur et al., 2008), other studies report that opioids exhibit decreased analgesic efficacy in women (Cepeda & Carr, 2003; Miller & Ernst, 2004). Importantly, these results are dependent on the setting (clinic versus laboratory), duration

of pain (acute versus chronic), and type of pain (visceral, inflammatory, or orofacial) for which opioids are administered. Preclinical studies using rodent models are more consistent, with most studies reporting that morphine is more efficacious in modulating persistent pain in males than females. Indeed, using a variety of acute and chronic pain assays, researchers have shown that morphine's median effective dose in female rodents is approximately twice the concentration needed for males to achieve comparable levels of pain relief (Kepler et al., 1989; Cicero et al., 2002; Ji et al., 2006; Loyd & Murphy, 2006; Loyd et al., 2008; Posillico et al., 2015). Sex differences in the antinociceptive effects of opioids have been reported for multiple MOR agonists, including levorphanol, dezocine, buprenorphine, butorphanol, and nalbuphine. (Barrett et al., 2002).

The mechanisms underlying the attenuated response to morphine in females are not known, however several contributing factors have been identified, including sex differences in MOR expression, and sex differences in PAG-RVM projections (Loyd et al., 2007, 2008). Recent studies by our laboratory tested the hypotheses that sex differences in MOR-mediated analgesia are driven by sex differences in the activity of microglia. Morphine and other opioids bind not only to neuronal MOR but also to microglial TLR4; morphine binding to TLR4 within the PAG and spinal cord induces cytokine release, resulting in the attenuation of morphine analgesia (Eidson & Murphy, 2013; Eidson et al., 2017). Blockade of PAG TLR4 signaling, and the ensuing cytokine production, enhances morphine-induced analgesia (Eidson & Murphy, 2013; Eidson et al., 2017; Doyle et al., 2017). Although no sex differences in basal microglia expression (density) are present within the PAG, the percentage of microglia showing an 'activated' phenotype at baseline is significantly higher in females than males, suggesting that sex differences in microglia phenotype within the PAG contribute to the sexually dimorphic

effects of morphine (Doyle et al., 2017; Doyle & Murphy, 2017). Further, inhibition of PAG microglia with the selective TLR4 antagonist (+)-naloxone significantly potentiates morphine analgesia in females but not males, and thus abolishes the sex difference in opioid response (Doyle et al., 2017). These results suggest that PAG microglia are innately different in males and females in terms of their morphological state and implicate TLR4 in the attenuated response to morphine observed in females.

Experiments on possible sex differences in the inflammatory processes resulting from persistent inflammatory pain, the receptor-binding properties of exogenous opioids, as well as sex differences in the downstream activity of MOR, will provide a more detailed model of the mechanisms underlying sex differences in opioid analgesia. Moreover, continued study of the origins of these sex differences (be they hormonal, autosomal, or epigenetic) will provide novel information on the mechanisms underlying the phenotypical differences between males and females. The present studies represent the first known analyses of the impact of biological sex on PAG opioid signaling in the presence of chronic pain that use both adult and aged animals.

1.4 PAG neuronal MOR signaling

The PAG's role in descending pain modulation was first identified by Reynolds (1969) who reported that electrical stimulation of the rat PAG was sufficient to produce potent analgesia, such that abdominal surgery was performed without anesthesia or additional pharmacological intervention. Interestingly, experimental rats still oriented to non-painful stimuli, indicating that PAG stimulation selectively inhibits noxious stimulation (Reynolds, 1969). Analgesia produced by PAG stimulation is MOR-dependent, as intra-PAG injection of the MOR antagonist (-)-naloxone attenuates the analgesia produced by PAG stimulation (Akil et al., 1976). Further, direct administration of MOR agonists into the PAG promotes potent analgesia

(Satoh et al., 1983; Jensen & Yaksh, 1986; Bodnar et al., 1988). Analgesia produced by PAG MOR agonism, particularly morphine analgesia, is attenuated by PAG MOR antagonism via (-)-naloxone (Ma & Han, 1991; Y. Zhang et al., 1998) and by lesions of MOR+ PAG neurons in male rats (Loyd et al., 2008).

Opioid binding at vlPAG MOR+ GABAergic interneurons induces a downstream signaling cascade causing neuronal hyperpolarization which limits GABA output, thus disinhibiting neurons projecting to the RVM (H. Wang & Wessendorf, 2002; Al-Hasani & Bruchas, 2011; Samineni et al., 2017); however, the mechanism(s) by which PAG MOR activation hyperpolarizes GABAergic neurons depends on the neuronal location of MOR (presynaptic, postsynaptic) (Lueptow et al., 2018). Opioid agonist binding to *postsynaptic* MOR elicits direct neuronal hyperpolarization via activation of the Gα subunit of the coupled G-protein. This results in the opening of G-protein inwardly rectifying potassium (GIRK) channels, and the subsequent potassium efflux induces neuronal hyperpolarization (North & Williams, 1983; Pan et al., 2005). Concurrently with GIRK channel opening, the Gα and Gβγ subunits of the coupled inhibitory $G_{i/o}$ proteins inhibit the enzymatic activity of downstream effector adenylyl cyclase (AC). G-protein-mediated AC inhibition limits the conversion of adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP) (van Keulen & Rothlisberger, 2017).

Alternatively, opioid binding at presynaptic MOR inhibits the release of GABA directly at the synapse via activation of the Gβγ subunits of the coupled G-protein, which subsequently inhibit voltage-gated calcium channels and activate voltage-gated potassium channels to limit neurotransmitter release (Wilding et al., 1995; Vaughan et al., 1997; Connor & Christie, 1999; Williams et al., 2001) (Figure 1.2).

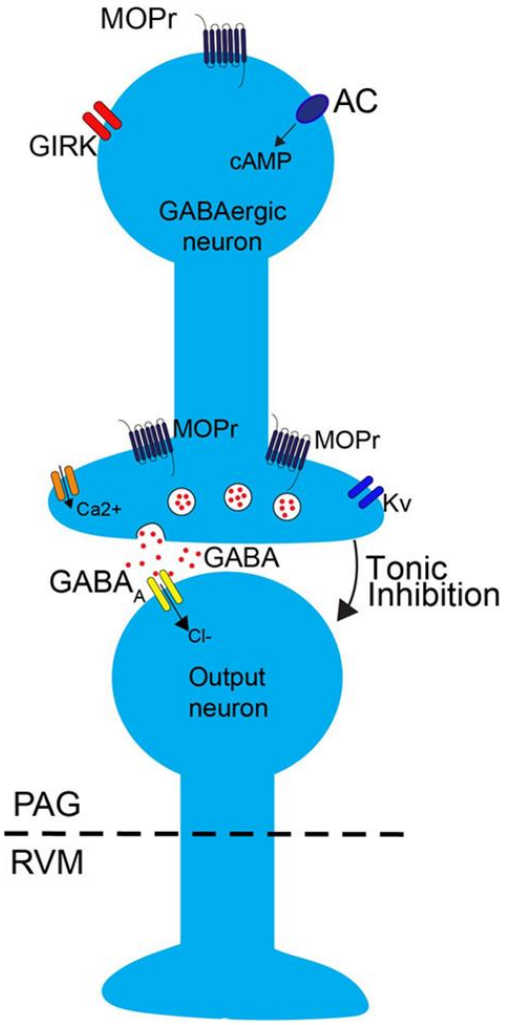


Figure 1.2 MOR Signaling within the PAG Pre- and post- synaptic MOR signaling in the PAG hyperpolarizes GABAergic neurons and disinhibits RVM output neurons. From Lueptow et al., 2018.

The present studies assess the impact of advanced age on PAG MOR agonist binding, G-protein activation, and cAMP inhibition; these processes are all critical for opioid signaling and subsequent pain inhibition. These studies also assess how advanced age impacts the regulation of PAG MOR signaling by measuring MOR phosphorylation and expression of Regulator of G-protein signaling proteins within the PAG.

1.4.1 MOR Agonist Binding

Opioid binding at MOR is critical for the promotion of analgesia (Roeckel et al., 2016; Ricarte et al., 2021). The magnitude of analgesia produced is dependent on the expression of functional MORs on the cell membrane surface as well as the affinity membrane-bound MORs exhibit for the agonist (Trescot, 2008). Receptor binding assays using a radioactive ligand are a

widely used tool for quantifying a ligand's efficacy to bind to its receptor (J. R. Traynor & Nahorski, 1995; Márki et al., 1999).This assay analyzes the interaction between a receptor and its ligand by providing a measure of total available receptor (B_{max}), along with a measure of a ligand's affinity for its receptor (K_d). The ratio of these values form the ligand-receptor binding potential, an index for determining the likelihood of an agonist to elicit a receptor-mediated response (Noël et al., 2001; Maguire et al., 2012).

Previous studies suggest that advanced age is concomitant with reduced receptor affinity; elderly humans exhibit reduced B-adrenergic receptor affinity (Feldman et al., 1984), and aging rats exhibit decreased affinity of cardiac muscarinic receptors (Baker et al., 1985). Indeed, in the spinal cord, male Fischer-344 rats aged 25 months exhibit significantly increased DAMGO K_d values compared to males aged 6 months, indicating reduced affinity of the receptor for the ligand in advanced age (Hoskins et al., 1998). This age-induced reduction in MOR affinity suggests a mechanism whereby MOR signaling is attenuated in advanced age, resulting in a reduction in the therapeutic efficacy of opioids in the elderly. The present studies assess how advanced age impacts MOR affinity and availability in the vlPAG in both male and female rats in the presence of chronic inflammatory pain.

1.4.2 G-protein activation

MORs primarily couple with G-proteins of the inhibitory type to carry out downstream neuronal hyperpolarization, primarily coupling the G-alpha-iota/o ($G\alpha_{i/o}$) class of adenylyl cyclase inhibitory Gα proteins. In the absence of a bound agonist (i.e., resting state), the 3 subunits of the G-protein form a heterotrimeric Gαβγ complex that couples to the MOR receptor. In this formation, the Gα subunit is bound to the inactive guanosine diphosphate (GDP). When an opioid agonist (such as morphine) binds to the extracellular N-terminus domain of MOR, the

G-protein complex uncouples from MOR. This binding promotes the dissociation of GDP from the Gα subunit and the subsequent binding of guanosine triphosphate (GTP). The binding of GTP to Gα catalyzes the subunit, resulting in the separation of Gα from the Gβγ heterodimer (Fig 1.3). The dissociated Gα-GTP and Gβγ complexes interact with unique downstream intracellular signaling pathways: $G\alpha_{i/o}$ inhibits AC, limiting the conversion of ATP into cAMP, and Gβγ inhibits calcium channels, limiting neurotransmitter release. G-protein intracellular signaling is terminated by the action of the Gα subunit's intrinsic GTPase, which hydrolyzes GTP to GDP (Ross & Wilkie, 2000; Hollinger & Hepler, 2002).

The present studies assess how advanced age affects opioid-induced G-protein activation in the vlPAG in the presence of chronic pain. MOR coupling to $G\alpha_{i/o}$ increases throughout postnatal development and stabilizes in adulthood (Talbot et al., 2005), and is then downregulated throughout the brain in advanced age, most notably in the prefrontal cortex (Young et al., 1991; Alemany et al., 2007; de Oliveira et al., 2019), with estimates of reduced expression as high as 65% in the frontal cortex, hippocampus, substantia nigra, and striatum (de Oliveira et al., 2019).

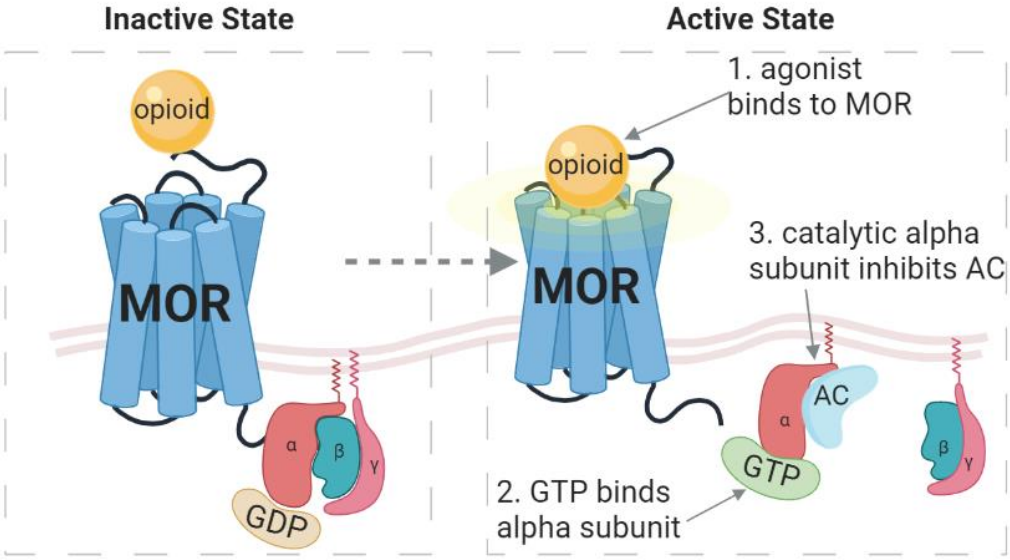


Figure 1.3 G-protein activation and signaling
MOR binding induces G-protein activation and dissociation by GTP and promotes second messenger signaling

1.4.3 cAMP inhibition

A primary target of MOR downstream signaling by $G\alpha_{i/o}$ is the effector adenylyl cyclase (AC). AC enzymatically converts adenosine triphosphate (ATP) to the excitatory second messenger cyclic adenosine monophosphate (cAMP). MOR initiation of $G\alpha_{i/o}$ signaling inhibits AC, thereby reducing cAMP expression (Santhappan et al., 2015)(Fig 1.3). Inhibition of cAMP limits the activation of cAMP-dependent protein kinase A (PKA), thus promoting inhibitory MOR signaling by limiting neuronal depolarization (Connor & Christie, 1999).

Several studies have assessed the impact of advanced age on cAMP production and signaling in the brain. Decreased cAMP expression has been reported in the cortex, thalamus, and midbrain of aged rats (Puri & Volicer, 1981; Titus et al., 2013; Kelly, 2018). Alternatively, age-dependent increases in cAMP activity, (assessed by measurement of cyclic AMP phosphodiesterase) have been reported in the rodent basal forebrain and hypothalamus (Stancheva & Alova, 1991; Asanuma et al., 1996). In addition to region-specific decreases in cAMP, widespread reductions in ATP, the precursor responsible for cAMP production, have

been reported in aged rodents (Błaszczyk, 2020). Further, AC binding shows age-related dysregulation, wherein the binding of forskolin, a nonspecific AC activator, is reduced within the cerebral cortex and hippocampus of 24-month-old rats (Araki et al., 1995).

To date, it is not known how advanced age (or sex) impacts cAMP expression in the vlPAG, nor whether vlPAG cAMP is altered as a function of chronic inflammatory pain. The present experiments assess whether the efficiency of opioid-induced cAMP inhibition within the PAG is impacted by advanced age, and thus determine whether advanced age alters opioid signaling via a cAMP-dependent mechanism.

1.4.4 MOR phosphorylation

The use of opioids for the management of pain is associated with the development of negative side effects including sedation, gastrointestinal immotility and respiratory depression (Koehl et al., 2018). These side effects are thought to be driven, in part, by the activity of a family of proteins called arrestins, particularly that of Beta-arrestin-2 (β-arrestin 2). β-arrestin 2 is an intracellular protein that acts to desensitize GPCRs. Opioid binding is thought to promote MOR phosphorylation at serine and threonine residues in the receptor carboxyl-terminal via G-protein receptor kinase 2 (GRK2) and protein kinase C (PKC), promoting the recruitment of β-arrestin 2, and subsequent clathrin-mediated receptor internalization (Lohse et al., 1992; DiCello et al., 2019). Thus, receptor phosphorylation limits agonist binding and attenuates opioid signaling (L. Zhang et al., 1996; Yu et al., 1997; Groer et al., 2011). In addition to desensitizing the receptor via internalization, β-arrestin binding to phosphorylated MOR is thought to promote the uncoupling of MOR from associated G-proteins, thus reducing the efficacy of MOR-mediated analgesia (Ferguson et al., 1996; Al-Hasani & Bruchas, 2011). Although the majority of studies assess agonist-induced receptor phosphorylation, basal phosphorylation (i.e., in the

absence of an agonist) also plays an important role in the maintenance of cell signaling (Rankin & Sibley, 2010). Basal, or constituent, receptor phosphorylation via GRK2 and PKC have also been shown to desensitize MOR signaling, suggesting that differences in opioid efficacy may result from differences in basal MOR phosphorylation in the PAG (Lemel et al., 2020). Previous studies have reported changes in receptor internalization (and thereby receptor phosphorylation) as a function of advanced age (L. Liu et al., 1998; Blanpied et al., 2003).

1.4.5 Regulator of G-protein Signaling (RGS) proteins

G-protein-dependent signaling pathways are tightly regulated to maintain receptor responsiveness to external stimuli. Regulators of G-Protein Signaling (RGS) proteins are a family of cellular proteins that primarily act to negatively modulate G-protein signaling by interacting with the GTP – GDP cycle. RGS proteins regulate G proteins by acting as GTPase-accelerating proteins (GAPs), whereby they enhance the intrinsic GTPase activity of Gα subunits, thus accelerating GTP hydrolysis. RGS proteins increase the intrinsic rate of GTP hydrolysis up to 100-fold, thereby aiding the hydrolyzation of the active GTP back into GDP and terminating downstream signaling (De Vries et al., 2000; Roman & Traynor, 2011; Gerber et al., 2016).

Several members of the RGS family have been reported to regulate G-protein-mediated opioid signaling (Ross & Wilkie, 2000; J. Traynor, 2012; Senese et al., 2020). Indeed, functional studies suggest that two RGS proteins in particular, RGS4 and RGS9-2, are key players in opioid receptor signaling (Xie & Palmer, 2005). RGS4 is expressed in many structures involved in pain transmission, including the dorsal horn of the spinal cord and the PAG (Avrampou et al., 2019). In-vitro studies in human embryonic kidney cells reported that overexpression of RGS4 attenuates morphine-induced adenylyl cyclase inhibition (Garnier et al., 2003). Further,

intrathecal inhibition of RGS4 reduced formalin nociception and enhanced DAMGO-mediated analgesia in male mice (Yoon et al., 2015). RGS9-2 is also expressed in the spinal cord and PAG, and its role in opioid analgesia has also been examined in rodent studies. Morphine analgesia in warm-water tail flick assays is potentiated in RGS9- global knockdown mice (Garzón et al., 2001). Additionally, morphine analgesia in both tail flick and hot plate assays is potentiated in RGS9- global knockout mice compared to controls (Zachariou et al., 2003).

Several studies have linked advanced age and RGS expression, suggesting that changes in the brain that develop with age-related diseases (Parkinson's Disorder and Alzheimer's disease) are dependent on RGS4 and RGS9-2 (Tekumalla et al., 2001; Muma et al., 2003). Using quantitative immunohistochemistry, Kim et al. (2005) reported higher RGS9-2 protein levels in the PAG of 1-year compared to 3-week-old male rats (Kim et al., 2005). Further, previous research suggests that chronic pain downregulates the signaling of G-proteins bound to MOR (Psifogeorgou et al., 2007; Avrampou et al., 2019). However, it is not known how advanced age and biological sex impact RGS levels in the PAG in the presence of chronic pain.

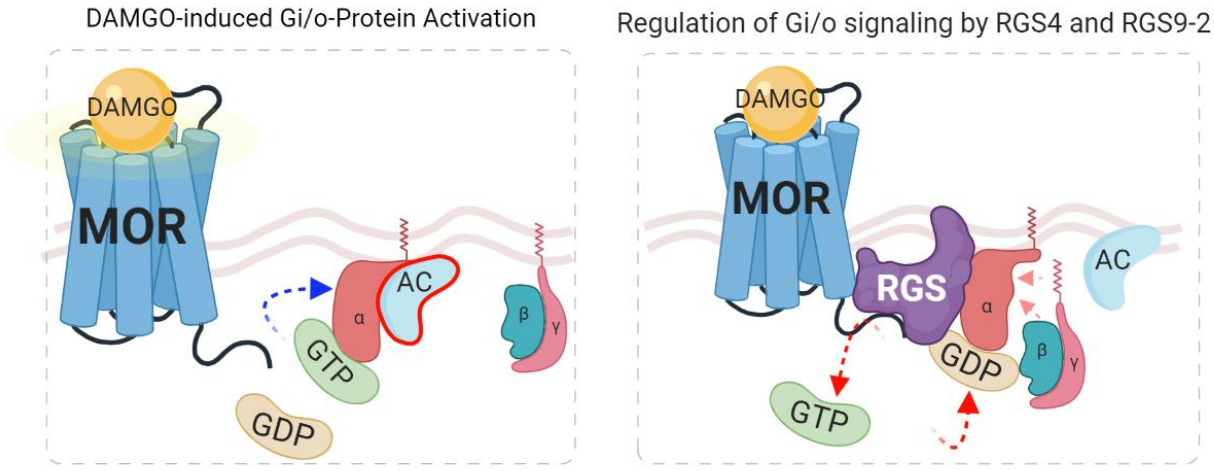


Figure 1.4 Regulator of G-protein signaling (RGS) proteins
RGS proteins promote the acceleration of GTPase, thus negatively modulating G-protein signaling

1.5 Aims

Preliminary studies in our laboratory assessed morphine anti-hyperalgesia 24 hours following intraplantar CFA. Adult and aged, male and female Sprague Dawley rats were administered subcutaneous morphine using a cumulative dosing paradigm. Withdrawal response to a noxious thermal stimulus were assessed at dosing intervals to determine median effective concentration of morphine (EC_{50}). These preliminary experiments show that aged males and females of both ages exhibit increased EC_{50} values compared to their adult male counterparts, indicating reduced morphine potency in aged males and females. In a second set of preliminary experiments, morphine and DAMGO were administered to adult and aged males via intra-PAG injection 24 hours post-CFA. Aged males exhibited reduced anti-hyperalgesic potency of both intra-PAG morphine and DAMGO, suggesting that age differences in opioid signaling in the PAG contribute to reduced morphine potency. Thus, the present studies were designed to test the hypothesis that advanced age and biological sex impact morphine analgesic potency by altering opioid signaling in the midbrain periaqueductal gray. Chronic pain is a debilitating disease that is pervasive in the aged population, and there exists a critical gap in research regarding adequate dosing of opioids for the management of chronic pain in the elderly.

Our objectives are to test cellular and pharmacological activities integral to the generation of opioid analgesia in adult and aged, male and female rats in the presence of persistent inflammatory pain. We use a clinically relevant model of the persistent pain experienced by humans for which morphine treatment is indicated. This work characterizes age- and sex-induced changes in the mechanisms by which opioids induce analgesia, identifying potential targets for the enhancement of morphine analgesia in aged individuals.

Aim 1 – **Assess the impact of age and sex on sensitivity to peripheral inflammatory pain and the impact of advanced and sex on morphine anti-hyperalgesic potency**. Preclinical studies indicate that aged rats require higher doses of morphine for adequate analgesia compared to adults. We test the hypothesis that advanced age attenuates the modulation of chronic pain but not the nociceptive response to painful stimuli by assessing sensitivity to peripheral thermal pain and assessing morphine anti-hyperalgesia in the presence of chronic pain. Intraplantar injection of CFA is used to induce chronic inflammatory pain to test if age impacts the anti-hyperalgesic potency of morphine in male and female rats. Morphine effective concentrations (EC_{50s}) are determined for opioid potency using a cumulative dosing paradigm and paw thermal stimulation. These experiments performed on adult/aged, male/female rats provide behavioral evidence that opioid signaling is altered in aged rodents.

Aim 2 - Assess the impact of advanced age and sex on mu opioid receptor mRNA and protein expression and binding in the ventrolateral periaqueductal gray. Our lab has previously shown that administration of MOR antagonists into the PAG significantly attenuates the analgesic effects of systemic morphine, suggesting that functional MOR in the vlPAG is critical for opioid analgesia. Given the attenuation in morphine action observed in aged rats, the present studies test the hypothesis that the attenuated response to morphine observed in aged male rats is due to decreased MOR expression and binding in the vlPAG compared to young adults. We assess vlPAG MOR in adult/aged, male/female rats using several complementary techniques. MOR mRNA and protein levels are assessed using RNAscope and immunohistochemistry (IHC), respectively. These studies establish anatomical differences but do not address potential changes in the ability of the receptor to bind ligand. Thus, we also use autoradiography to assess vlPAG MOR binding. Together, these studies provide anatomical and

functional evidence of the impact of age, sex, and persistent pain on opioid signaling in the vlPAG.

Aim 3 - Identify age- and sex-induced differences in vlPAG MOR affinity, availability, G-protein activation, and cAMP inhibition. Agonist binding vlPAG MOR is critical for opioid analgesia. Binding induces the activation of coupled G-proteins that promote second messenger signaling cascades and ultimately inhibit afferent pain signals, notably via inhibition of cAMP production. The present studies examine the hypothesis that the attenuated response to morphine observed in aged rats is due to reductions in vlPAG MOR affinity and availability, dampened opioid-induced G-protein activation, and reduced cAMP inhibition. In the present studies, we assess MOR affinity and availability using radioligand-binding assays to generate K_d (receptor affinity) and B_{max} (receptor availability) values. We assess G-protein activation using the GTPγS assay to generate G-protein efficacy and potency values. And we assess opioid-induced cAMP inhibition using the cAMP assay. These data derived from adult/aged, male/female rats provide novel evidence on the impact of advanced age and sex on vlPAG MOR signaling.

Aim 4 - Determine if vlPAG MOR phosphorylation state or RGS expression are altered by advanced age and biological sex. MOR signaling is modulated by receptor phosphorylation, which limits agonist binding and recruits arrestin proteins, downregulating opioid signaling. Further, regulator of G-protein signaling (RGS) proteins attenuate G-protein activity by accelerating the hydrolysis of GTP. We test the hypothesis that the attenuated response to morphine observed in aged male rats is due to heightened MOR phosphorylation and greater expression of RGS proteins in the aged vlPAG. We assess MOR phosphorylation in

adult/aged, male/female rats using Western blot detection of immunoprecipitated proteins, and assess RGS mRNA using single-molecule fluorescent in situ hybridization.

The outcome of this work is a profile of the impact of advanced age and biological sex on opioid analgesic potency, namely on opioid signaling in the vlPAG. The present studies establish the impact of advanced age and biological sex on morphine anti-hyperalgesia, vlPAG MOR expression and binding, MOR signaling, and opioid signaling regulation.

2 ADVANCED AGE ATTENUATES THE ANTIHYPERALGESIC EFFECT OF MORPHINE AND DECREASES M-OPIOID RECEPTOR EXPRESSION AND BINDING IN THE RAT MIDBRAIN PERIAQUEDUCTAL GRAY IN MALE AND FEMALE RATS

Evan F. Fullerton, Myurajan Rubaharan, Mary C. Karom, Richard I. Hanberry, Anne Z. Murphy

[1]Neuroscience Institute, Georgia State University, Atlanta, GA, 30303, USA

2.1 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.

2.2 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 (Frieden, 2016.). 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 (Dahlhamer, 2018; Domenichiello & Ramsden, 2019). 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., 2014; Reid et al., 2015; Domenichiello & 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; Saunders et al., 2010; Miaskowski, 2011; Miaskowski et al., 2011; Schiltenwolf et al., 2014; Naples et al., 2016; Prostran et al., 2016)

Although opioids for chronic pain management in the elderly are finally beginning to increase (Pokela et al., 2010; Lapane et al., 2013), 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 (Kaiko, 1980; Gagliese & Katz, 2003; Papaleontiou 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 (Gagliese & Katz, 2003; Aubrun et al., 2005; 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 (B. R. Ferrell et al., 1991; Reddy et al., 2012; Dampier et al., 2013).

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 & Lai, 1982; Kramer & Bodnar, 1986; Gagliese & Melzack, 2000). 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 & Lai, 1982; Kavaliers et al., 1983; Smith & Gray, 2001; Jourdan et al., 2002). 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 & Fields, 1979; Behbehani & Fields, 1979; Morgan et al., 1992, 2006). The ventrolateral PAG (vlPAG) contains a large population of mu opioid receptor positive (MOR+) neurons,- the preferred receptor for morphine (Martin, 1963; Wolozin & Pasternak, 1981), and direct administration of MOR agonists into the PAG produces potent analgesia (Satoh et al., 1983; Jensen & Yaksh, 1986; Bodnar et al., 1988). Conversely, direct administration of MOR antagonists into the PAG, or lesions of PAG MOR, significantly attenuate the analgesic effect of systemic morphine (Wilcox et al., 1979; Ma & Han, 1991; Y. Zhang et al., 1998; Loyd et al., 2008). 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.

2.3 Materials and Methods

2.3.1 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.

2.3.2 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).

2.3.3 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; X. 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.

2.3.4 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.

2.3.5 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.

2.3.6 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) at −20 °C until immunohistochemical processing.

2.3.7 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.

2.3.8 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 (mm2) of the PAG between male and female Sprague Dawley rats (Loyd & Murphy, 2006). All images were collected and analyzed by an experimenter blinded to the experimental condition.

2.3.9 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 (Loyd et al., 2008; LaPrairie & Murphy, 2009). 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 MgCl2. After a final dip in cold dH20, tissue was allowed to dry at room temp. Slides were then apposed to FujiFilm imaging plates along with [3H]-microscale standards (PerkinElmer/NEN, MA, USA) for six weeks. Image plates were processed with a FujiFilm BAS 5000. Autoradiographic [3H]-receptor binding was quantified from the images using Scion Image software. [3H]-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.

2.3.10 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.

2.4 Results

2.4.1 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).

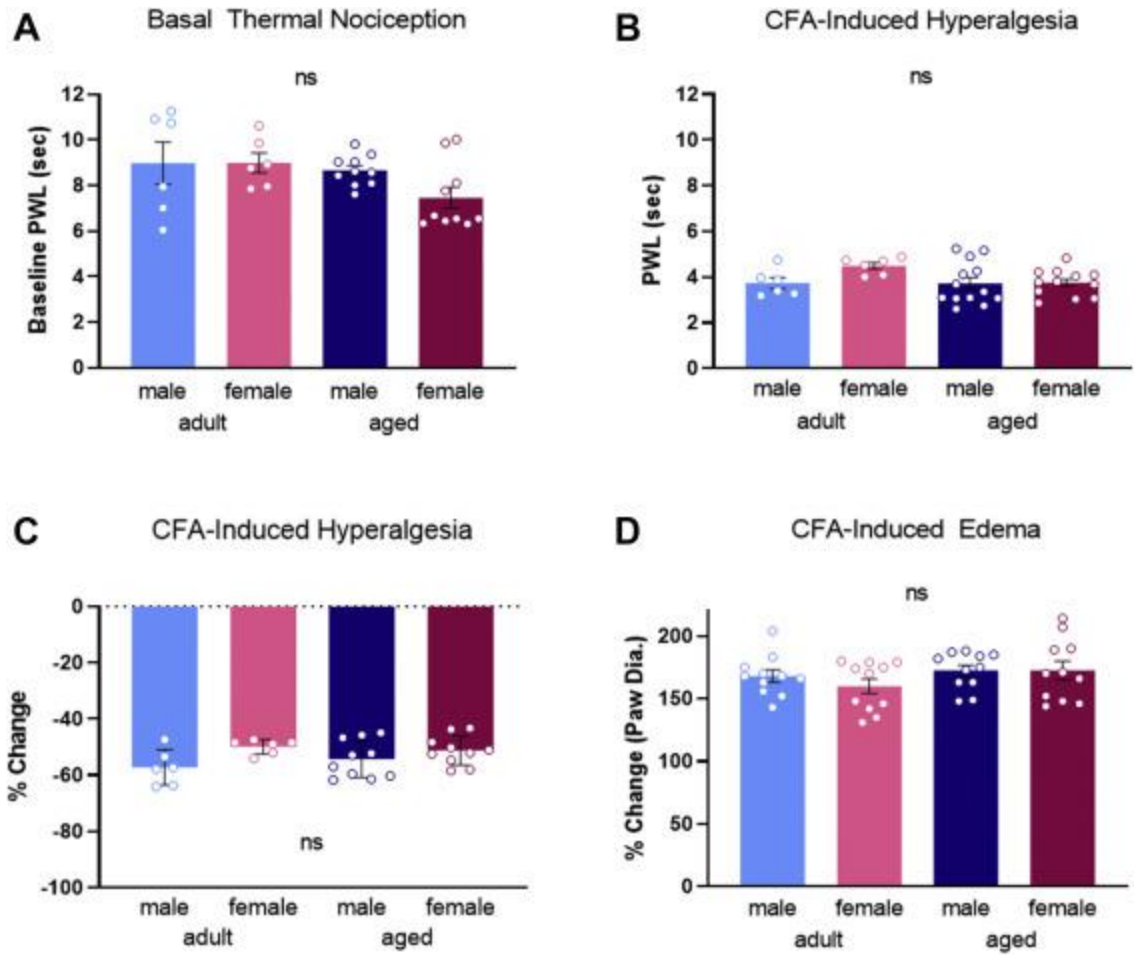


Figure 2.1 Basal thermal nociception and CFA-induced hyperalgesia

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.

2.4.2 Age and sex differences in morphine anti-hyperalgesia

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).

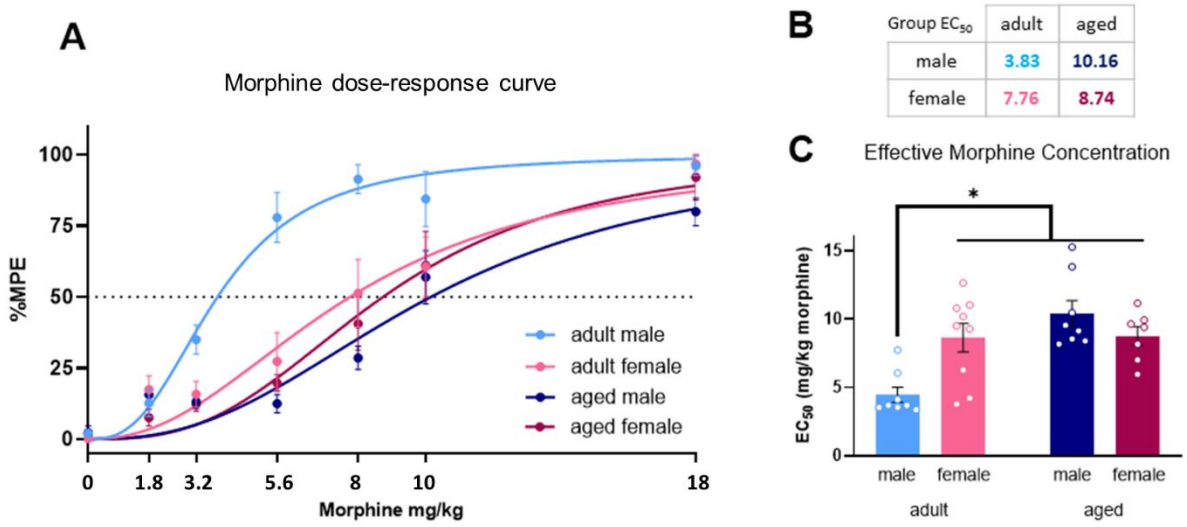


Figure 2.2 Morphine anti-hyperalgesia

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.

2.4.3 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 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 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).

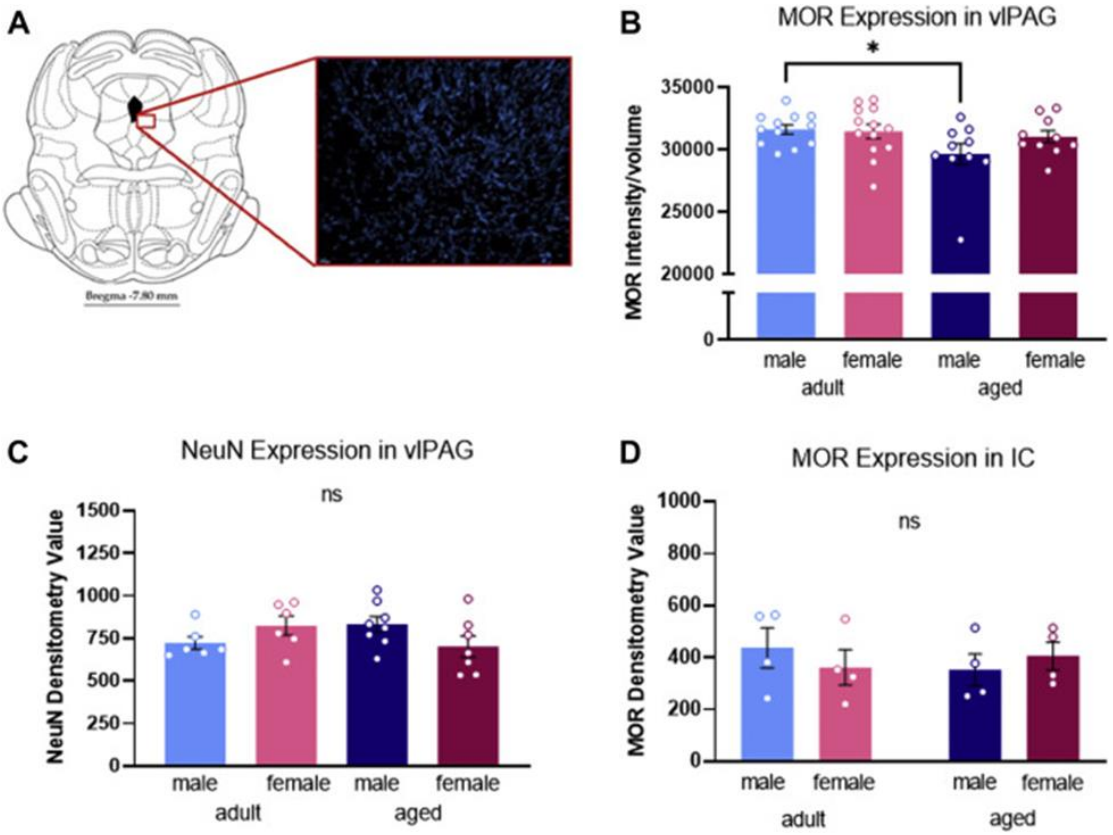


Figure 2.3 MOR expression

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.

2.4.4 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. [3H]-DAMGO was used to label MOR as previously described (LaPrairie & 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.

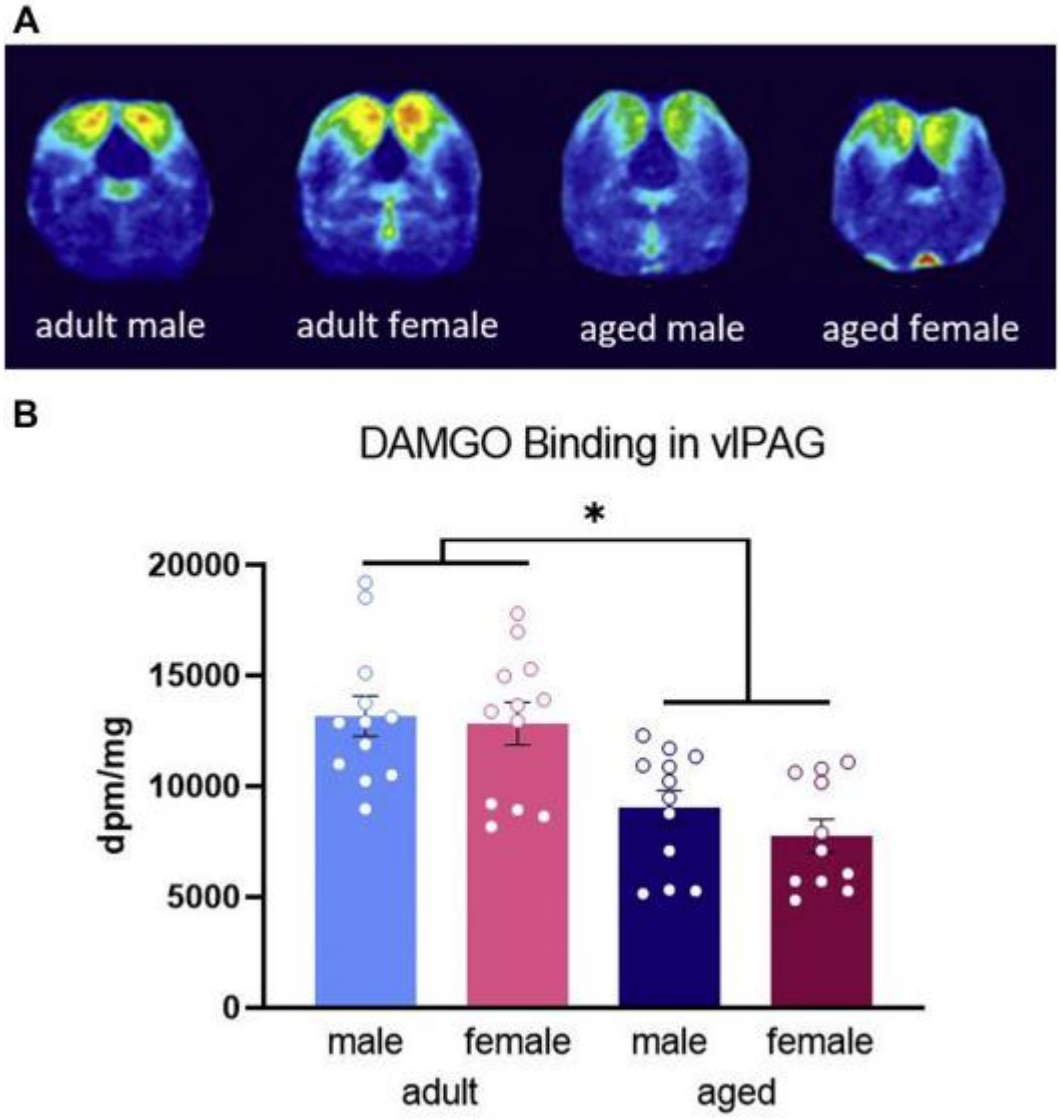


Figure 2.4 MOR saturation binding

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.

2.5 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.

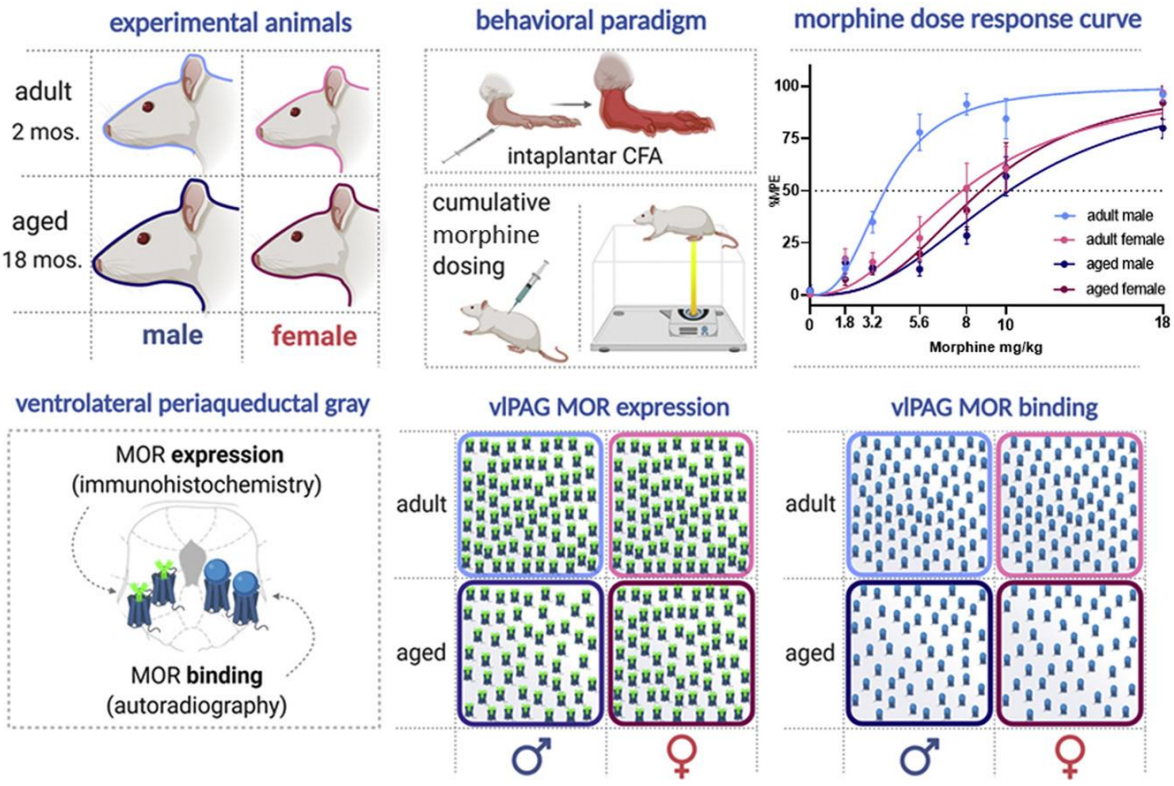


Figure 2.5 Study summary

Aged rats exhibit reduced morphine anti-huperalgesic potency and reduced vlPAG MOR expression and binding compared to adult males

2.5.1 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 & Milholland, 1998; X. Wang et al., 2006). Indeed, most rodent studies assessing the impact of sex on basal nociception report no differences(Mogil et al., 2000; X. Wang et al., 2006; Mogil et al., 2010; Racine et al., 2012; Mogil, 2020). 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 nociception, these reports are highly varied, with reports of increased (Hess et al., 1981; Kitagawa et al., 2005), decreased (Chan & Lai, 1982; Kramer & Bodnar, 1986; Garrison & Stucky, 2014; Muralidharan et al., 2020), and no change in mechanical or thermal pain sensitivity in aged rodents compared with adults (Crisp et al., 1994; Taguchi et al., 2010; Yezierski, 2012; Mecklenburg et al., 2017; Samir et al., 2017). Similarly, meta-analyses of both clinical and laboratory measurements in humans fail to report a consistent impact of age on nociception(Gibson & Helme, 2001; Weyer et al., 2016; Ostrom et al., 2017). 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 (Lautenbacher, 2012). 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 & Murphy, 2006; X. Wang et al., 2006; Loyd et al., 2008). 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 (Mecklenburg et al., 2017). 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 (Garrison & Stucky, 2014). 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).

2.5.2 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 (Kaiko, 1980; Gagliese & Katz, 2003; Keïta et al., 2008), 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 (B. R. Ferrell et al., 1991; Reddy et al., 2012; Dampier et al., 2013).

2.5.3 Sex and age differences in vlPAG MOR expression and binding

The PAG is a critical neural site for opioid modulation of pain (Reynolds, 1969; Behbehani & Fields, 1979; Morgan et al., 1991, 1992; Y. Zhang et al., 1998; Loyd et al., 2008). 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., 1981; 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 (Sorge et al., 2011; Rosen et al., 2017; Doyle & Murphy, 2017; Eidson & Murphy, 2019; Eidson et al., 2019). 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 GABAA receptor expression and upregulate AMPA receptor, resulting in an overall increase in neural excitability (Song & Zhao, 2001; Ogoshi et al., 2005; Stellwagen et al., 2005; Holdridge et al., 2007; X. Wang et al., 2012; Eidson et al., 2017). As morphine works primarily via hyperpolarization (Chieng & 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, 2010; VanGuilder et al., 2011; Norden & Godbout, 2013). 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.

3 AGE-INDUCED CHANGES IN MU OPIOID RECEPTOR SIGNALING IN THE MIDBRAIN PERIAQUEDUCTAL GRAY OF MALE AND FEMALE RATS

Evan F. Fullerton[1], Mary C. Karom[1], John M. Streicher[2], Larry J. Young[3], and Anne Z. Murphy[1]

[1]Neuroscience Institute, Georgia State University, Atlanta, GA, 30303, USA

[2]Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724, USA.

[3]Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA, 30322 USA

3.1 Abstract

Opioids have decreased analgesic potency (but not efficacy) in aged rodents compared to adults; however, the neural mechanisms underlying this attenuated response are not yet known. The present study investigated the impact of advanced age and biological sex on opioid signaling in the ventrolateral periaqueductal gray (vlPAG) in the presence of chronic inflammatory pain. Assays measuring mu opioid receptor (MOR) radioligand binding, GTPγS binding, receptor phosphorylation, cAMP inhibition, and regulator of G-protein signaling (RGS) protein expression were performed on vlPAG tissue from adult (2-3mos) and aged (16-18mos) male and female rats. Persistent inflammatory pain was induced by intraplantar injection of Complete Freund's Adjuvant (CFA). Adult males exhibited the highest MOR binding potential and highest G-protein activation (activation efficiency ratio) in comparison to aged males and females (adult

and aged). No impact of advanced age or sex on MOR phosphorylation state was observed. DAMGO-induced cAMP inhibition was highest in the vlPAG of adult males compared to aged males and females (adult and aged). vlPAG levels of RGS4 and RGS9-2, critical for terminating G-protein signaling, were assessed using RNAscope. Adult rats (both males and females) exhibited lower levels of vlPAG RGS4 and RGS9-2 mRNA expression compared to aged males and females. The observed age-related reductions in vlPAG MOR binding potential, G-protein activation efficiency, and cAMP inhibition, along with the observed age-related increases in RGS4 and RGS9-2 vlPAG expression, provide potential mechanisms whereby the potency of opioids is decreased in the aged population.

3.2 Significance Statement

Opioids have decreased analgesic potency (but not efficacy) in aged rodents compared to adults; however, the neural mechanisms underlying this attenuated response are not yet known. In the present study, we observed age-related reductions in vlPAG MOR binding potential, G-protein activation efficiency, and cAMP inhibition, along with the observed age-related increases in RGS4 and RGS9-2 vlPAG expression, providing potential mechanisms whereby the potency of opioids is decreased in the aged population. These coordinated decreases in opioid receptor signaling may explain the previously reported reduced potency of opioids to produce pain relief in females and aged rats.

3.3 Introduction

Clinical studies examining pain management in the elderly are challenging; co-morbid conditions such as diabetes and high blood pressure and participant use of concomitant medications affect patient outcomes, contributing to difficulties in the interpretation of results (Naples et al., 2016; Prostran et al., 2016). Additionally, there exist age- and sex-related individual differences in the likelihood of reporting pain in a clinical setting which may lead to misrepresentations of analgesic efficacy (Reddy et al., 2012; Dampier et al., 2013). Particularly, aged populations are known to underreport pain due to fears of institutionalization and concerns with addiction and overdose (B. A. Ferrell et al., 1990; Hofland, 1992).

Using a preclinical model of persistent inflammatory pain (intraplantar Complete Freund's Adjuvant; (CFA)), we have previously reported a significant impact of age and sex on morphine potency. Specifically, we showed that aged rats (18mos) exhibit decreased morphine potency compared to adults (2mos), with aged males requiring greater than 2x the concentration of morphine than their adult counterparts to produce equivalent analgesia (Fullerton et al., 2021). Similar results have been reported in prior preclinical studies, suggesting that aged rodents require higher doses of opioids to produce antinociception (Webster et al., 1976; Kavaliers et al., 1983; Kramer & Bodnar, 1986). The mechanisms underlying the attenuation of opioid potency in the aged population are currently unknown.

Morphine-induced analgesia is mediated primarily via binding to μ-opioid receptors (MOR), seven-transmembrane domain G-protein coupled receptors (GPCRs) located predominantly on neuronal cell membranes (Martin, 1963; Wolozin & Pasternak, 1981; Goodman & Pasternak, 1985; Serohijos et al., 2011). Following agonist binding, coupled G-proteins undergo a conformational change in which the guanosine diphosphate (GDP) bound to

the inactive alpha subunit is replaced by guanosine triphosphate (GTP), activating the subunit and promoting opioid signaling through interaction with downstream effectors (Senese et al., 2020). MORs are coupled to a family of G-proteins called Gi/o that signal via inhibition of adenylyl cyclase and subsequently decrease cyclic adenosine monophosphate (cAMP) (Koehl et al., 2018; Bouchet et al., 2021). This G-protein signaling is downregulated or terminated by **R**egulator of **G**-Protein **S**ignaling (RGS) proteins, which act as GTPase activating proteins (GAPS), hydrolyzing the active GTP back into GDP and terminating downstream signaling (Gerber et al., 2016). RGS proteins, particularly RGS4 and RGS9-2, have been previously implicated in opioid-mediated G-protein signaling, and have been shown to attenuate analgesia (Garnier et al., 2003; Psifogeorgou et al., 2007; Avrampou et al., 2019; Senese et al., 2020). A primary target of MOR downstream signaling is the effector adenylyl cyclase (AC). AC enzymatically converts adenosine triphosphate (ATP) to the excitatory second messenger cAMP. MOR binding inhibits AC, thereby reducing cAMP expression, facilitating neuronal hyperpolarization, and promoting analgesia (Santhappan et al., 2015). Like other G-protein coupled receptors, MOR signaling is subject to desensitization via the cellular mechanism of receptor phosphorylation, which limits agonist binding and recruits arrestin proteins and thus attenuates opioid signaling. (L. Zhang et al., 1996; Yu et al., 1997; Groer et al., 2011).

The present studies test the hypothesis that the observed age-induced reduction in morphine potency is mediated by changes in MOR signaling within the midbrain periaqueductal gray, a CNS region critical for the opioid modulation of pain. (Basbaum et al., 1976; Behbehani & Fields, 1979; Morgan et al., 1992, 2006). The ventrolateral PAG (vlPAG) contains a large population of MOR+ neurons, and direct administration of MOR agonists into the PAG produces potent analgesia (Satoh et al., 1983; Jensen & Yaksh, 1986; Bodnar et al., 1988; Loyd et al.,

2008) Intra-PAG administration of MOR antagonists or lesions of PAG MOR significantly attenuate the analgesic effect of systemic morphine, suggesting a critical role for PAG MOR in mediating morphine action (Ma & Han, 1991; Y. Zhang et al., 1998; Loyd et al., 2008).

We have previously shown that aged rats exhibit reduced vlPAG MOR protein expression and reduced MOR agonist binding in the vlPAG compared to adult rats (Fullerton et al., 2021), suggesting that diminished levels of functioning MOR in the vlPAG contribute to the attenuated opioid potency seen in the aged. The present studies build on these previous findings to assess age and sex differences in MOR availability, ligand affinity, phosphorylation, G-protein activation, cAMP inhibition, and expression of RGS proteins.

3.4 Materials and Methods

3.4.1 Experimental subjects

Adult (2-3mos) and aged (16-18mos) male and regularly cycling female 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 hour 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. All assays were performed on separate cohorts of rats (n=8/sex/age; N=32), with the exception of the radioligand binding and GTPγS assays, which were run simultaneously on PAG tissue from a single cohort.

3.4.2 Vaginal cytology

Beginning ten days prior to 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 estrous. 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).

3.4.3 CFA-induced chronic pain treatment

72 hours prior to experimentation, persistent inflammatory pain was induced by injection of complete Freund's adjuvant (CFA; Mycobacterium tuberculosis; Sigma; 200µl), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hind paw. Edema was present within 24 hours of injection, indicated by a >100% change in paw diameter, determined using calibrated calipers applied midpoint across the plantar surface compared to handled paw.

3.4.4 Membrane preparation for radioligand binding and GTPγS assays

vlPAG membrane protein lysates were prepared from adult and aged, male and female, handled and CFA treated rats to be used for radioligand binding and GTPγS assays. 72 hours post-CFA injection or handling, 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. Ventrolateral PAG tissue from caudal PAG (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -20°C. On the day of the assay, PAG sections were placed in ice-cold assay buffer (50 mM Tris-HCl, pH 7.4). Tissue was homogenized with a glass dounce and centrifuged at 20,000g at 4°C for 30 min. The supernatant was discarded and the pellet resuspended in assay buffer (Zollner et al., 2003; M. Shaqura, Li, Al-Madol, et al., 2016; M.

Shaqura, Li, Al-Khrasani, et al., 2016; X. Li et al., 2018). Membrane protein concentration was calculated using the Bradford Assay, and lysates of vlPAG membrane protein from adult and aged, male and female, naïve and CFA treated rats (n=4; N=32) were used immediately for radioligand binding and GTPγS assays.

3.4.5 Saturation radioligand binding assay

Saturation binding experiments were performed on vlPAG membranes using [3H]DAMGO (specific activity 50 Ci/mmol, American Radiolabeled Chemicals, Missouri). Briefly, 100 µg of membrane protein was incubated with various concentrations of [3H]DAMGO (0.5 to 5nM), in a total volume of 1ml of binding buffer (50mM Tris-HCl pH 7.4). Nonspecific binding was defined as radioactivity remaining bound in the presence of 10 µM unlabeled naloxone. At the end of the incubation period (1h at RT) bound and free ligands were separated by rapid filtration over Whatman brand Grade GF/C glass filters (Sigma-Aldrich) using a sampling vacuum manifold (MilliporeSigma). Filters were washed four times with 5ml of cold dH20. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in 3ml of scintillation fluid. All experiments were performed in triplicate. B_{max} and K_d values were determined by nonlinear regression analysis of concentration-effect curve in GraphPad Prism 9.1.

3.4.6 Agonist-stimulated [35S]GTPγS binding

DAMGO-stimulated [35S]GTPγS binding to PAG membrane protein was assessed by incubating membrane protein (100ug) in the presence or absence of [35S]GTPγS (0.1nM) (specific activity 1250 Ci/mmol, American Radiolabeled Chemicals, Missouri) and various concentrations of DAMGO (2 to 30,000 nM) in assay buffer (20mM Tris, 10mM MgCl2, 100mM NaCl, 0.2mM EGTA, pH 7.4) for 30min at 30°C. Stimulated [35S]GTPγS binding was

compared to unstimulated binding at each measurement point and presented as percent basal binding. At the end of the incubation period, bound and free ligands were separated by rapid filtration over Whatman brand Grade GF/B glass filters (Sigma-Aldrich) using a sampling vacuum manifold (MilliporeSigma). Filters were washed four times with 5ml of cold buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in 3ml of scintillation fluid. All experiments were performed in duplicate. Efficacy (E_{max}) is defined as the maximum percent stimulation by an agonist; potency (EC_{50}) is defined as the concentration of DAMGO required for half the maximal response. E_{max} and EC_{50} values were determined by nonlinear regression analysis of concentration-effect curves using Graph Pad Prism 9.1.

3.4.7 Phosphorylated MOR analysis

To assess the impact of advanced age and sex on MOR phosphorylation state, levels of MOR phosphorylation were analyzed by Western blot. The lysis buffers used and general methods are the same as reported in (Lei et al., 2017). Briefly, rat PAG samples were homogenized in ~500 μL of lysis buffer using a glass dounce and rotated overnight at 4°C. The samples were then spun down at 13,000g for 10 min at 4°C. The resulting lysates were quantified by DC Protein Assay Kit (Bio-Rad, #500-0111) according to the manufacturer's instructions. 50-75 μg of soluble protein per sample (same for all samples in a set) was run on 10% Bis-Tris Bolt PAGE gels (Fisher Scientific, #NW00100BOX) and wet-transferred to nitrocellulose membrane (Protran 0.2 μm NC, #45-004-001 from Fisher Scientific) at 30V at 4°C for 2-3 hours. Membranes were blocked using 5% nonfat dry milk in TBS, then blotted for primary antibody target in 5% BSA in TBST overnight rocking at 4°C. Primary antibodies used were: pMOR (1:1000, Bioss #bs-3724R), tMOR (1 μg/mL, R&D #MAB6866), and GAPDH

(1:1000, Invitrogen #MA5-15738). All 3 targets were always probed for on the same blot, using low pH stripping buffer between each set. Secondary antibodies were used at 1:5000 each in 5% nonfat dry milk in TBST, rocking for ~1 hr at room temperature. Secondary antibodies used were: Goat-α-Mouse-IRDye800CW (Fisher Scientific #NC9401841) and Goat-α-Mouse-IRDye680LT (Fisher Scientific #NC0046410). Target signal was acquired using an Azure Sapphire imager using the near-infrared channels (658 and 784 nm). Band density was quantified, and background subtracted using the onboard AzureSpot analysis software. Samples were run with at least two representatives of each experimental group on the same gel. Adult and aged, male and female, naïve and CFA treated rats (n=4; N=32) were used for these experiments. pMOR signal was normalized to the same sample tMOR or GAPDH as indicated, and all samples were normalized to the Adult Male average on each gel before combining data from different gels.

3.4.8 DAMGO-induced cAMP inhibition

DAMGO-induced cAMP inhibition was analyzed using a cAMP assay. vlPAG membrane protein lysates were prepared from adult and aged, male and female, handled and CFA treated rats. 72 hours post-CFA injection or handling, 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. Ventrolateral PAG tissue from caudal PAG (Bregma -7.5 through -8.5) was dissected from each brain using a straight edge razor at -20°C. Briefly, vlPAG tissues were homogenized in ice-cold lysis buffer containing 0.25-M sucrose, 50-mM Tris–HCl pH 7.5, 5-mM EGTA, 5-mM EDTA, 1-mM phenylmethylsulfonyl fluoride, 0.1-mM dithiothreitol, and 10 μg/ml leupeptin. The homogenized tissues were then centrifuged at 1000×g for 5 min (4 °C,) and the supernatant was centrifuged at 35000 ×g for 10 min (Viganò et al., 2003). vlPAG

membrane protein samples (100ug) from adult and aged, male and female, naïve and CFA treated rats (n=4; N=32) were incubated in 1uM forskolin (FSK) and in the presence or absence of 10uM DAMGO to stimulate adenylyl cyclase activity. cAMP levels in vlPAG tissue were determined using the LANCE Ultra cAMP Detection Kit (Perkin Elmer), a time-resolved fluorescence resonance energy transfer (TR-FRET) cAMP immunoassay. Data are plotted as % change in TR-FRET signal comparing FSK baseline measurements to measurements following DAMGO-stimulated cAMP inhibition.

3.4.9 Single-molecule fluorescence in situ hybridization

Single-molecule fluorescent in situ hybridization (RNAscope) assays were used to determine mRNA expression of OPRM1, RGS9-2, and RGS4 in the vlPAG. Rats were restrained using DecapiCones and decapitated. Brains were removed rapidly, flash frozen in 2-methyl butane on dry ice, and stored RNase free 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. vlPAG sections from adult and aged, male and female, naïve and morphine treated rats were used (n=4-7; N=45). For morphine dosing paradigm, see (Fullerton et al., 2021). Tissue was processed for single-molecule fluorescence in situ hybridization (smFISH) according to the RNAscope Multiplex Kit protocol (Advanced Cell Diagnostics) using probes for OPRM1, RGS4, and RGS9-2. To facilitate cellular mRNA quantification within the vlPAG, sections were counterstained with DAPI. mRNA puncta were visualized as fluorescent signals. Fluorescent images were captured on Zeiss LSM 700 Confocal Microscope at 40x, and mRNA expression (target puncta/DAPI) was calculated using Imaris software. To determine RGS4 and RGS9-2 expression levels in MOR+ neurons, quantification was restricted to puncta located within 10μm

of DAPI that co-expressed OPRM1 mRNA. mRNA expression values were determined for the left and right ventrolateral subdivisions of each PAG image from two representative levels of the mid-caudal PAG (Bregma −7.74 and −8.00). As there was no significant effect of rostrocaudal level in the analyses, data were collapsed and presented as vlPAG and were averaged for each rat. RGS4 and RGS9-2 mRNA values are expressed as the mean ± standard error of the mean (SEM). All images were collected and analyzed by an experimenter blinded to the experimental condition

3.4.10 Statistical analysis and data presentation

All values are reported as mean ± SEM. Data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Bartlett's tests. Significant main effects of sex, age, and treatment were assessed using 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. Data are expressed either as fmol/protein or nM DAMGO for radioligand binding assay or Emax or EC50 for GTPγS assay

3.5 Results

3.5.1 Advanced age and sex impact vlPAG MOR binding properties

To assess the impact of advanced age, biological sex, and pain on vlPAG MOR signaling, we first used radioligand saturation binding assays to determine MOR binding parameters. The saturation binding curves generated with [^{3}H]DAMGO are shown in Fig. 1A. CFA treatment did not significantly impact K_d [$F_{(1,22)} = 3.429$, $p=0.078$] or B_{max} [$F_{(1,24)} = 0.0023$, $p = 0.963$] so CFA and handled groups were combined.

K_d values, indicative of receptor affinity, were determined using the concentration-effect curves generated from each sample. Analysis of K_d values indicated no significant impact of age [$F_{(1,26)} = 0.770$, p=0.388] or sex [$F_{(1,26)} = 1.241$, p=0.275], or a significant interaction [$F_{(1,26)} = 1.278$, p=0.269] (Fig. 1B).

Analyses of B_{max} values, indicative of receptor availability, showed no significant impact of age [$F_{(1,28)} = 2.661$, p=0.114] or sex [$F_{(1,28)} = 0.002$, p=0.967], but a significant interaction [$F_{(1,28)} = 4.995$, p=0.034]. Post hoc analysis showed a significant difference in vlPAG MOR availability between adult males and aged males, as evidenced by reduced B_{max} values in the aged males compared to their adult counterparts (p = 0.025)(Fig. 1C). B_{max} values for aged males were markedly reduced compared to adult males, while adult females and aged females exhibited smaller reductions in B_{max} compared to their adult male counterparts (%Δ -23.6 and -17.1, respectively. Fig. 1C).

Analyses of binding potential (BP) values, a measure that takes into consideration the density of available receptors and the affinity of the receptor for its agonist, showed a significant impact of sex, with males exhibiting greater binding potential than females [$F_{(1,28)} = 4.631$, p=0.040] and a significant interaction between age and sex [$F_{(1,28)} = 9.110$, p=0.005]. No significant impact of age [$F_{(1,28)} = 3.312$, p=0.079] was observed. Post hoc analyses showed a significant difference in vlPAG MOR binding potential between adult males and adult females (p = 0.006) and adult males and aged males (p = 0.010), indicating that adult males exhibit greater vlPAG MOR binding potential than their aged male counterparts and their adult female counterparts (Fig. 1D). Adult females, aged males, and aged females all exhibited marked

reductions in MOR binding potential compared to their adult male counterparts (%Δ -52, -48.7, and -40, respectively; Fig. 1D).

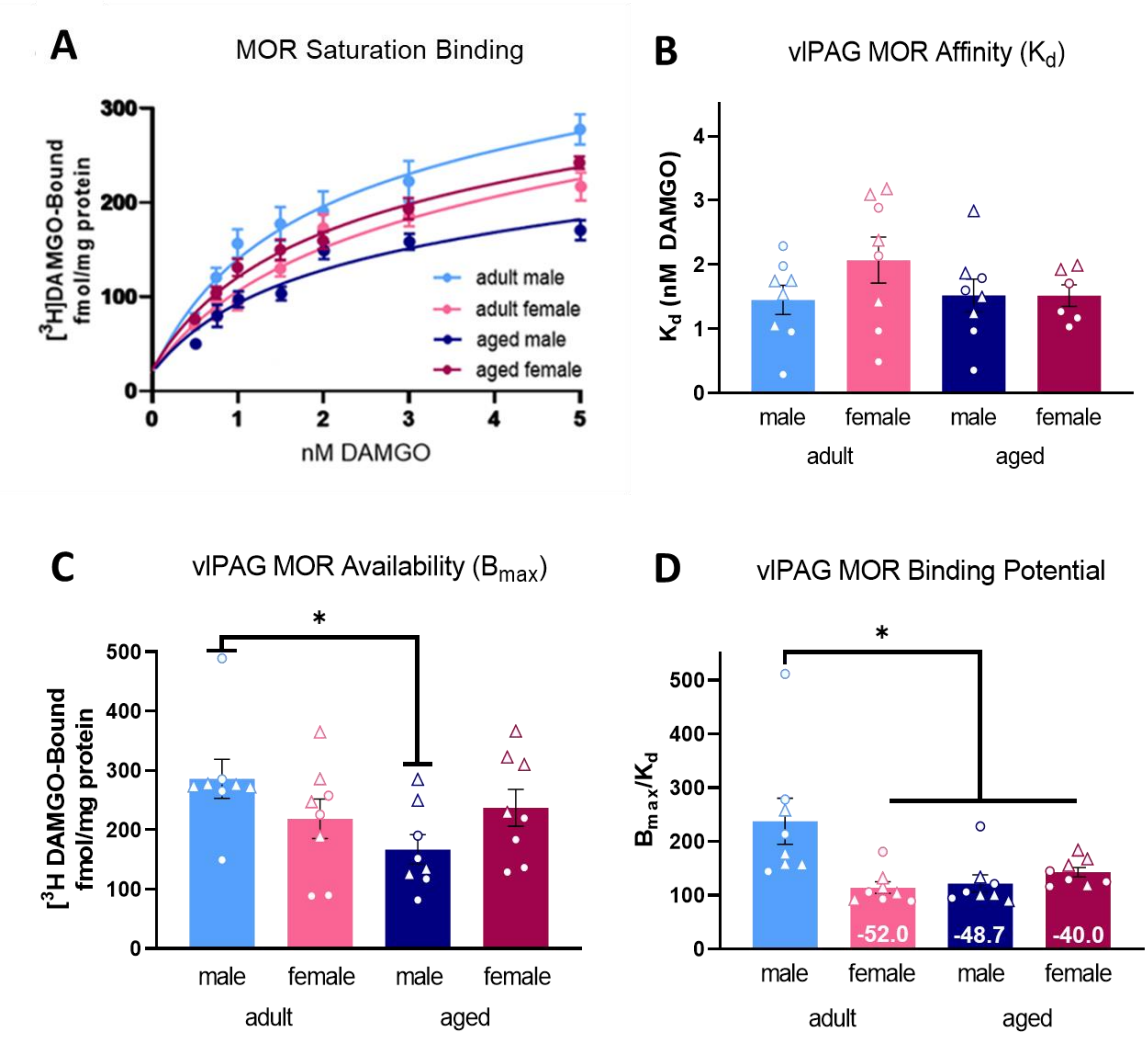


Figure 3.1 MOR binding properties

Saturation binding curve of bound [3H]DAMGO (A). No significant effect of age or sex was observed in MOR affinity (K_d) (B). MOR availability (B_{max}) was significantly lower in aged males compared to adult males (C). Adult females, aged males, and aged females exhibited reduced MOR binding potential (B_{max}/K_d) compared to adult males, indicating attenuated agonist interaction at the receptor level (D). CFA treated rats are represented as circles, handled rats are represented as triangles. ns, not significant. *Significant difference between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

3.5.2 Advanced age and sex impact opioid-induced G-protein activation in the vlPAG

We next conducted GTPγS binding assays to determine if advanced age or biological sex impacted MOR mediated G-protein activation. Our initial studies revealed a significant main effect of chronic pain [$F(1, 24) = 29.04$, $p < 0.0001$] (Fig. 2E). To improve the translatability of these results to the target population of aged patients suffering from chronic pain, all other data displayed from GTPγS experiments are exclusively from CFA-treated rats. Concentration curves generated from [35S]GTPγS assays are shown in Fig. 2A. Analyses of Emax values, a measure of G-protein availability combined with ligand efficacy, indicated no significant impact of age [$F(1,24) = 0.035$, $p = 0.8532$] or sex [$F(1,25) = 2.852$, $p = 0.1042$], or a significant interaction [$F(1,24) = 2.023$, $p = 0.1678$] (Fig. 2B).

Analyses of EC_{50} values, a measure of the concentration of DAMGO required for half-maximal G-protein binding, indicated a significant impact of sex, with males exhibiting lower effective concentration values than females, reflecting a higher potency of activation [$F_{(1,24)} = 5.177$, $p = 0.0321$]. There was no significant impact of age [$F_{(1,24)} = 2.339$, $p = 0.1393$], and no significant interaction [$F_{(1,24)} = 2.473$, $p = 0.1289$] (Fig. 2C). Although not statistically significant, aged males, adult females, and aged females all exhibited marked increases in G-protein EC_{50} compared to their adult male counterparts, as evidenced by their percent change values (%Δ 60.5, 114.8, and 98.6, respectively), reflecting a lower potency of activation (Fig. 2C). Together, these results suggest that opioid-induced G-protein signaling is impaired in the PAG of the aged rat and the female rat.

Next, we calculated the coefficient ratio of analysis of E_{max} / EC_{50}, which is indicative of overall G-protein activation efficiency. A significant impact of age was noted, with adults exhibiting higher vlPAG G-protein activation efficiency than aged rats [$F_{(1,24)} = 6.491$, $p =$

0.0177]. There was also a significant impact of sex, with males exhibiting higher vlPAG G-protein activation efficiency compared to females [$F_{(1,24)}$ =10.39, p = 0.0036]. A significant interaction was also observed [$F_{(1,24)}$ = 13.93, p = 0.0010]. Post hoc analyses showed a significant difference in vlPAG G-protein activation efficiency between adult males and aged males (p = 0.0009), with adult males exhibiting greater vlPAG G-protein activation efficiency than aged males. A significant difference in vlPAG G-protein activation efficiency between adult males and adult females (p= 0.0003) was also observed, with adult males exhibiting greater vlPAG G-protein activation efficiency than adult females (Fig. 2D). Aged males, adult females and aged females all exhibited marked reductions in G-protein activation efficiency compared to their adult male counterparts (%Δ -40.0, -52.1, and -41.9, respectively; Fig. 2D).

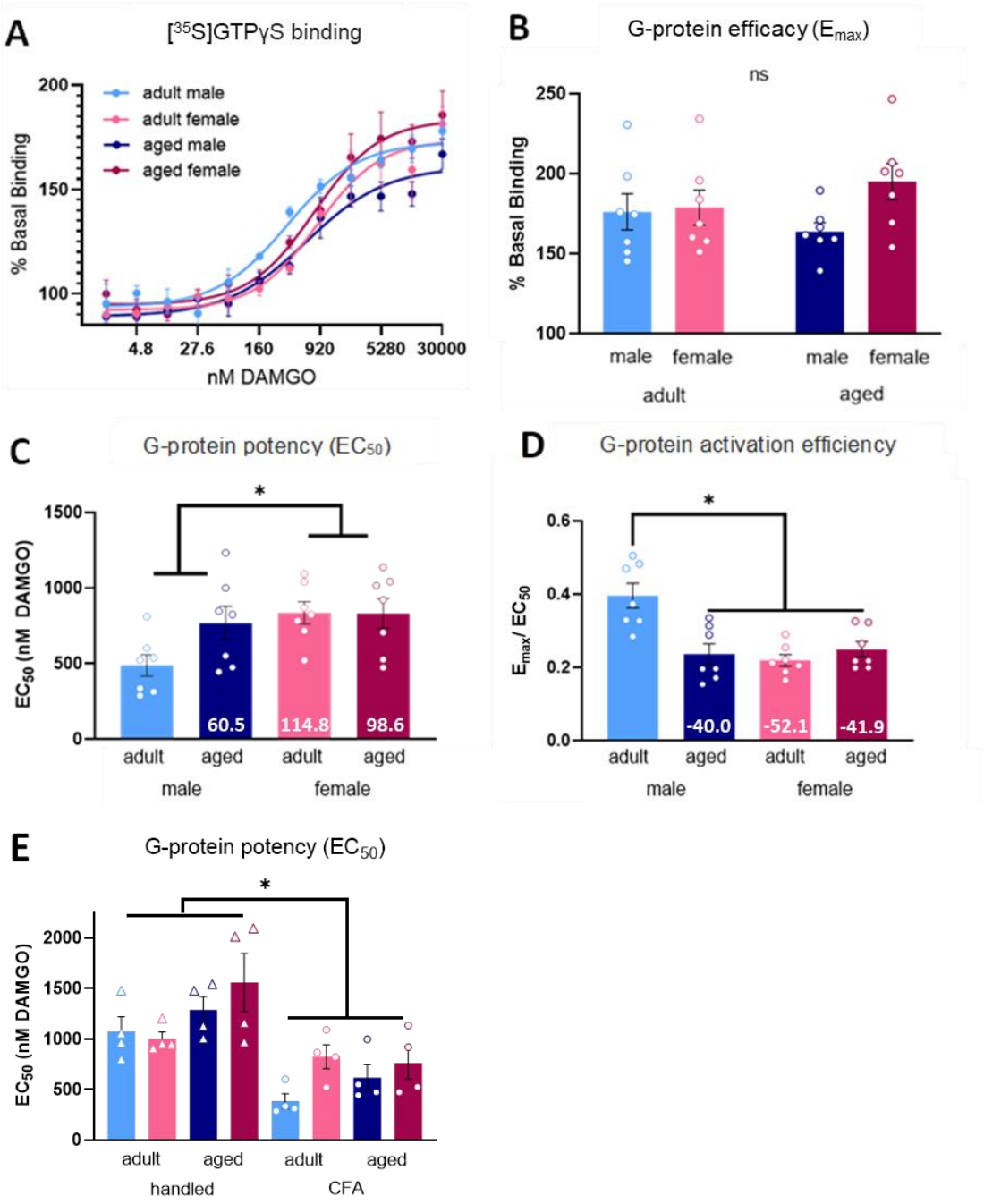


Figure 3.2 Figure 3.2 G-protein activation

[^{35}S]GTPγS binding curve of agonist-stimulated GTPγS binding (A). No significant effect of age or sex was observed in E_{max} (B). Aged males, adult females, and aged females exhibited increased EC_{50} values compared to adult males, indicating that adult males have the greatest potency (C). Aged males, adult females, and aged females exhibited reduced G-protein activation efficiency (E_{max}/EC_{50}) compared to adult males, indicating attenuated G-protein signaling (D). Initial studies using both handled and CFA treated rats indicated a significant main effect of chronic pain on EC_{50} values (E). CFA treated rats are represented as circles, handled rats are represented as triangles. ns, not significant. *Significant difference between adult and aged males, or adult males and adult females; $p<0.05$ calculated by Tukey's post hoc test. Graphs indicate mean ± SEM. Values indicate % change from adult male.

3.5.3 Advanced age and sex do not impact phosphorylated MOR in the vlPAG

The results above showed significant reductions in MOR binding potential and G-protein activation efficacy. Therefore, we next examined if there was an effect of age and sex on MOR phosphorylation, as increased levels of pMOR would likely contribute to these reductions. Western blots were used to determine the impact of advanced age on MOR phosphorylation at serine-375, a known site of phosphorylation-mediated desensitization (Schulz et al., 2004). There was no significant impact of treatment on our initial analyses [$F_{(1,16)} = 0.0033$, $p = 0.9547$], so CFA and handled groups were combined. Although increased pMOR/tMOR was observed in aged males compared to their adult counterparts (%Δ 34.0), no significant main effect of age [$F_{(1,41)} = 2.168$, $p = 0.1486$], sex [$F_{(1,41)} = 1.807$, $p = 0.1863$], or age x sex interaction [$F_{(1,41)} = 0.4615$, $p = 0.5008$] was observed (Fig. 3).

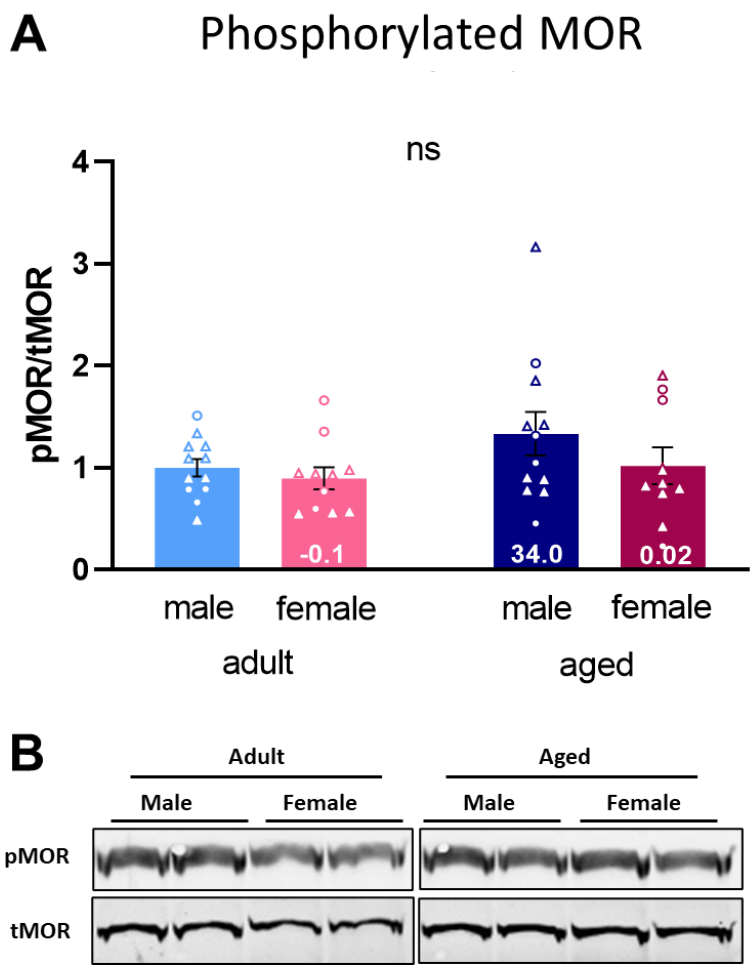


Figure 3.3 Phosphorylated MOR

No significant impact of advanced age or biological sex on the ratio of phosphorylated MOR/ total MOR (A). Representative phosphoMOR blots are shown below the graph (B). CFA treated rats are represented as circles are represented as triangles. ns, not significant. Graph indicates mean ± SEM. Values indicate % change from adul

3.5.4 Advanced age and sex impact opioid-induced cAMP inhibition in the vlPAG

We next determined if advanced age, biological sex, or persistent pain impacted opioid-induced cAMP inhibition in the vlPAG. Forskolin-induced cAMP release was used as a baseline measurement for each group, while forskolin + DAMGO was used to assess the degree to which cAMP was inhibited by DAMGO. Percent change from baseline was used to compare across treatment groups (Fig. 4A). There was no significant impact of persistent pain on percent change from baseline [$F(1,26) = 0.7619$, $p = 0.3907$], so CFA and handled groups were combined. Our

analyses revealed a significant main effect of age [F(1,30) = 9.314, p = 0.0047], a significant main effect of sex [F(1,30) = 10.31, p = 0.0031], and a significant interaction [F(1,30) = 14.24, p = 0.0007]. Post hoc analyses showed a significant difference in cAMP inhibition between adult males and adult females (p = 0.0002), adult males and aged males (p = 0.002), and adult males

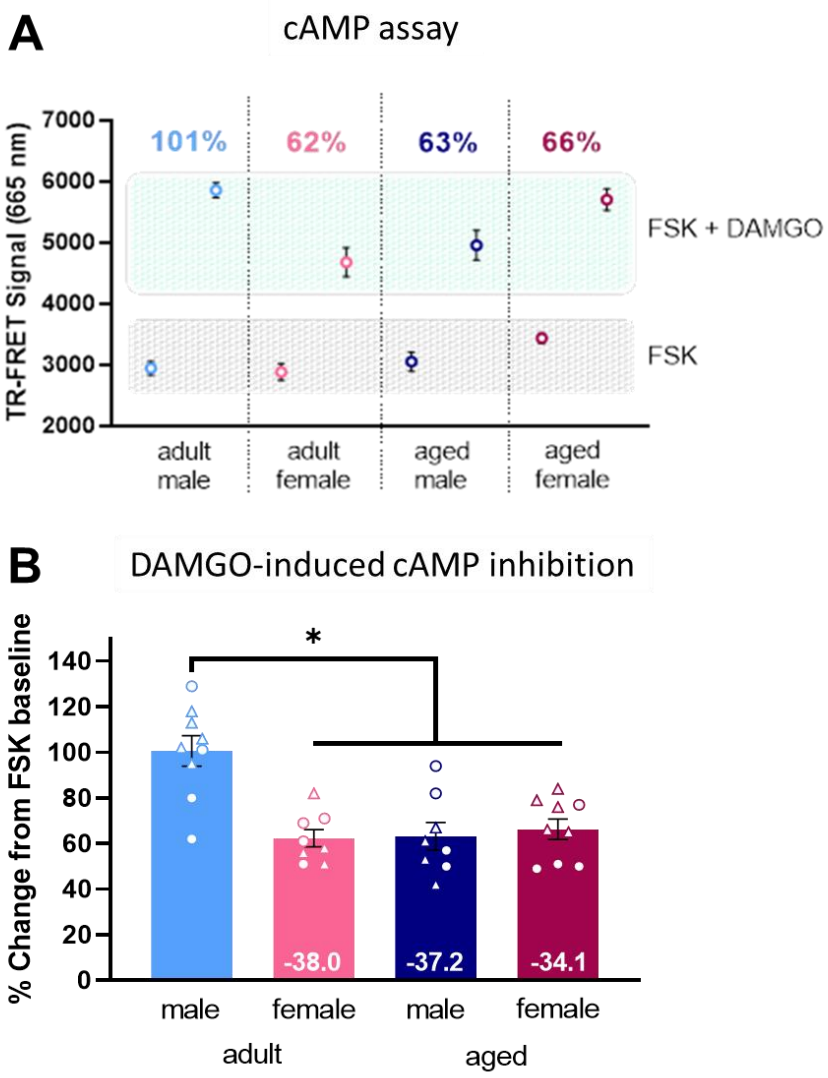


Figure 3.4 Opioid-induced cAMP inhibition

TR-FRET immunoassay showing % change from FSK stimulated baseline (1uM FSK) to DAMGO-induced DAMGO). cAMP levels are inversely proportional to TR-FRET signal. Adult males exhibited the highest level of ag cAMP (A). Individual values in 4B indicate each rat's % change from FSK baseline to DAMGO-induced cAMP inhi to compare across treatment groups. Aged males and females exhibited reduced agonist-dependent cAMP inhibition indicating attenuated downstream opioid signaling (B). CFA treated rats are represented as circles, handled rats are re *Significant difference between adult and aged males, or adult males and adult females; p<0.05 calculated by Tukey' indicate mean ± SEM. Values indicate % change from FSK baseline.

and aged females (p = 0.004), indicating that DAMGO elicits greater cAMP inhibition in adult males compared to aged males and females (both adult and aged) (Fig. 4B).

3.5.5 Advanced age and sex impact RGS4 and RGS9-2 expression in the vlPAG

Regulator of G-protein Signaling (RGS) proteins act as GAP accelerators to negatively modulate G-protein signaling. RGS protein family members RGS4 and RGS9-2 are expressed in the vlPAG and have both been shown to regulate opioid signaling by reversing G-protein activation. Therefore, we next used smFISH to determine if RGS4 and RGS9-2 expression in the vlPAG was altered by advanced age and biological sex. In these studies, following CFA administration, a cohort of rats was administered morphine to examine the relationship between morphine EC50 and RGS levels. No significant impact of morphine treatment on RGS4 [$F(1,22) = 0.8521$, $p = 0.3664$], RGS9-2 [$F(1,22) = 0.0258$, $p = 0.8739$], or OPRM1 [$F(1,22) = 0.3146$, $p = 0.5805$] was observed in our initial analyses, so naïve and morphine treated groups were combined. We first assessed total vlPAG expression of RGS4 and RGS9-2. These analyses revealed a significant main effect of age on both RGS4 [$F(1,37) = 18.15$, $p = 0.0001$] and RGS9-2 [$F(1,37) = 17.09$, $p = 0.0002$], with aged rats exhibiting increased levels of RGS4 and RGS9-2 mRNA compared to their adult counterparts (Fig. 5A & B). No significant main effect of sex on RGS4 [$F(1,37) = 0.0247$, $p = 0.8763$] or RGS9-2 [$F(1,37) = 0.4855$, $p = 0.4903$], or significant interactions between age and sex for RGS4 [$F(1,37) = 0.4994$, $p = 0.4842$] or RGS9-2 [$F(1,41) = 0.3246$, $p = 0.5723$] were observed (Fig. 5A & B).

Following our assessment of total RGS4 and RGS9-2, we next restricted our analyses to RGS4 and RGS9-2 mRNA expressed on MOR+ neurons. We first assessed overall OPRM1 mRNA expression in the vlPAG and found no significant main effect of age [$F(1,36) = 2.793$, p

= 0.1034], or sex [F(1,36) = 0.076, p = 0.7844], or significant interactions between age and sex [F(1,36) = 0.7626, p = 0.7892]. Similarly, no significant main effect of age [F(1,41) = 3.556, p = 0.0664], or sex [F(1,41) = 1.170, p = 0.2858], or significant interactions between age and sex [F(1,41) = 0.071, p = 0.7908] was observed for OPRM1 mRNA expressed specifically on MOR+ neurons. We next determined if RGS4 and RGS9-2 expression was increased preferentially in OPRM1+ neurons. Similar to what was noted above, a significant main effect of age on expression of RGS4 [F(1,41) = 26.47, p < 0.0001] and RGS9-2 [F(1,41) = 21.69, p < 0.0001] was observed, with aged rats exhibiting increased levels of RGS4 and RGS9-2 mRNA in MOR+ neurons compared to their adult counterparts (Fig. 5C & D). No main effect of sex for RGS4 [F(1,41) = 0.7881, p = 0.3799] or RGS9-2 [F(1,41) = 0.0006, p = 0.9799], or significant interactions between age and sex for RGS4 [F(1,41) = 0.6583, p = 0.4219] or RGS9-2 [F(1,41) = 0.0075, p = 0.9314] were observed (Fig. 5C & D).

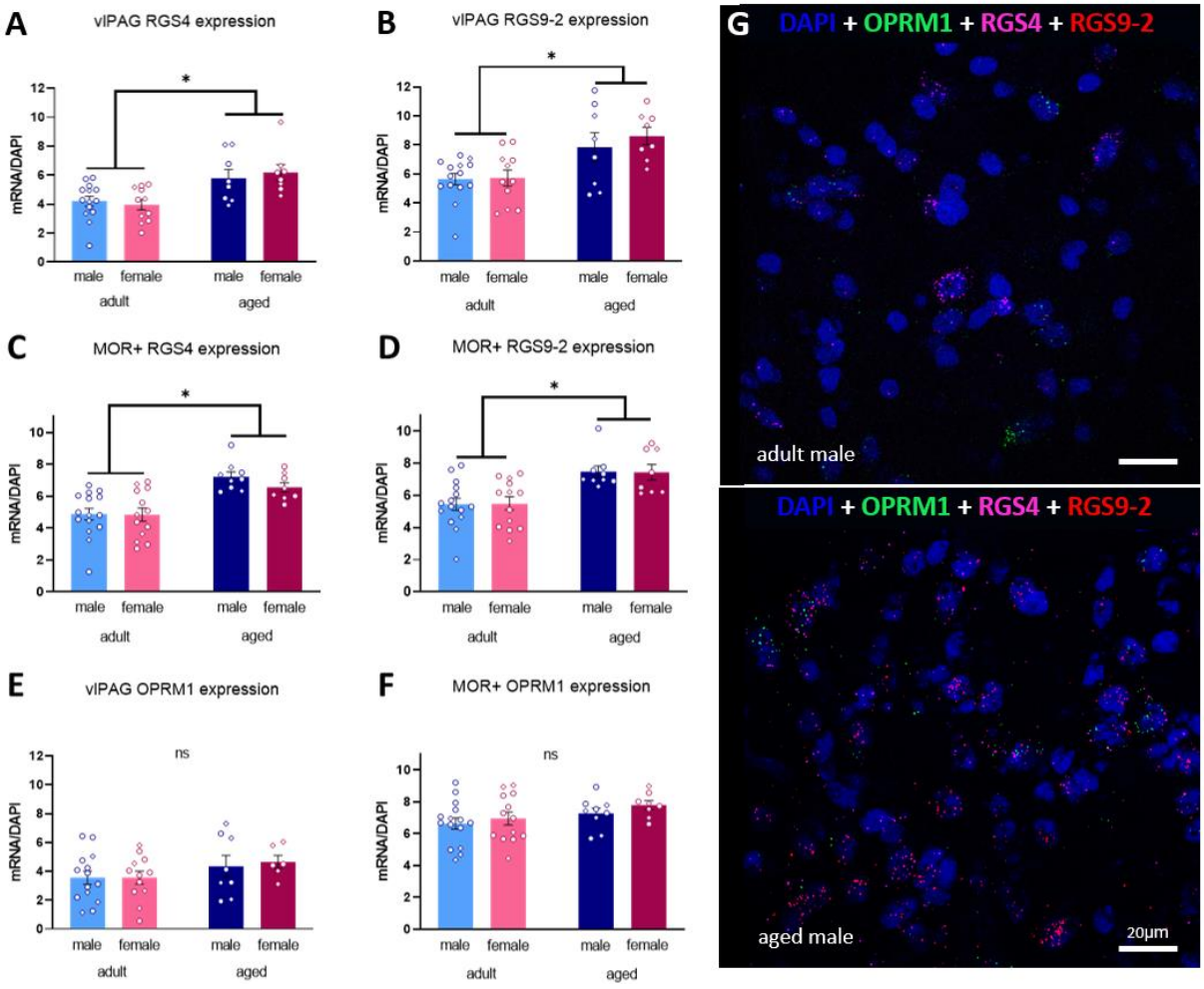


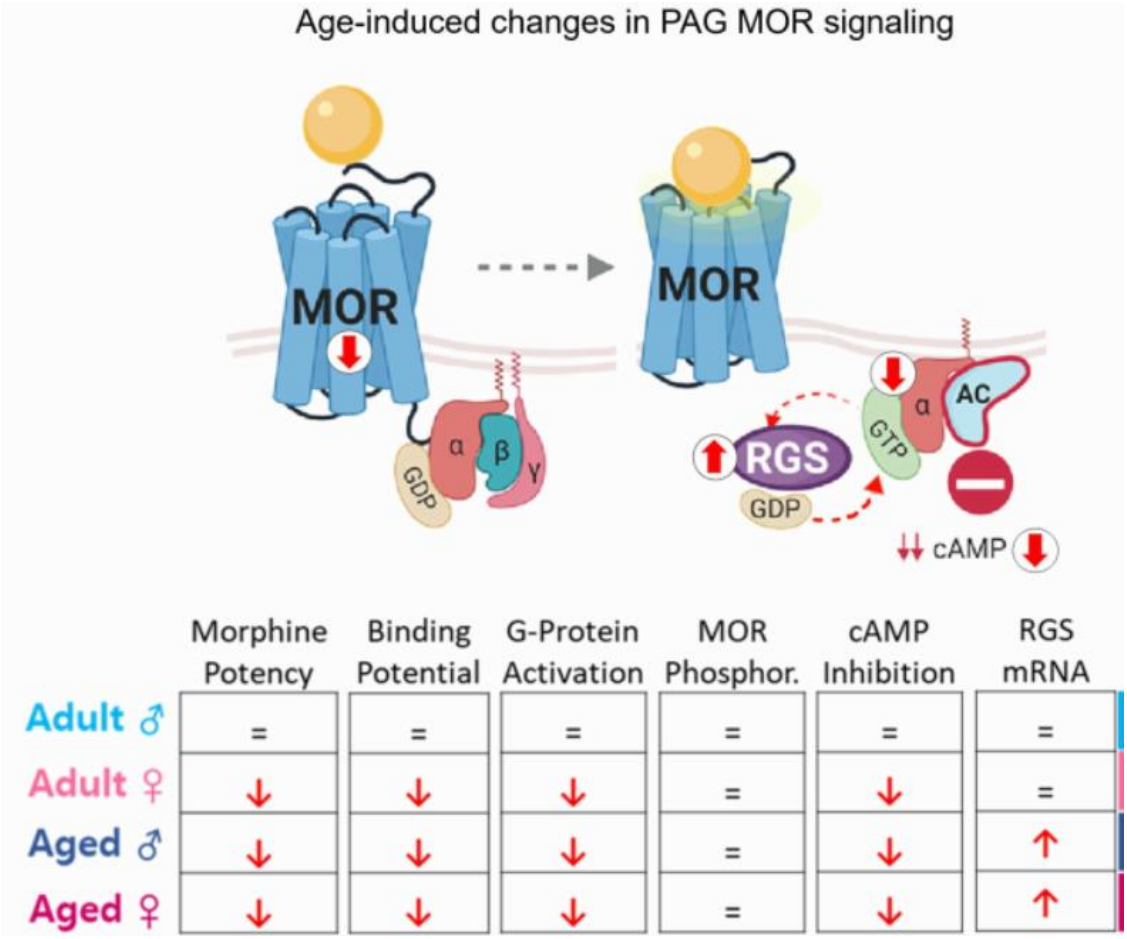
Figure 3.5 RGS4 and RGS9-2 expression

mRNA expression of RGS4 (A) and RGS9-2 (B) in the vlPAG was significantly greater in aged rats compared to adults. mRNA expression of RGS4 (C) and RGS9-2 (D) on MOR+ cells in the vlPAG was significantly greater in aged rats compared to adults. No significant impact of advanced age or biological sex on mRNA expression of OPRM1 (E) or mRNA expression of OPRM1 on MOR+ cells in the vlPAG (F) Photomicrograph of RGS expression compared an adult male and an aged male PAG section (G). CFA treated rats are represented as circles, CFA + morphine treated rats are represented as diamonds. *Significant difference between adults and aged rats; $p<0.05$ calculated by 2x2 ANOVA. Graphs indicate mean ± SEM.

3.6 Discussion

The present studies are the first to show that advanced age results in a significant attenuation in MOR signaling within the vlPAG of male and female rats. Specifically, aged males and females (regardless of age) showed decreased MOR binding potential, decreased G-protein activation, and decreased agonist-stimulated cAMP inhibition in comparison to adult

males (Fig. 6). These changes, along with the observed increase in RGS4 and RGS9-2 expression, provide a mechanism whereby morphine potency is significantly reduced in aged rats (Fullerton et al., 2021).



	Morphine Potency	Binding Potential	G-Protein Activation	MOR Phosphor.	cAMP Inhibition	RGS mRNA
Adult ♂	=	=	=	=	=	=
Adult ♀	↓	↓	↓	=	↓	=
Aged ♂	↓	↓	↓	=	↓	↑
Aged ♀	↓	↓	↓	=	↓	↑

Figure 3.6 Summary of MOR signaling impairments in the vlPAG of the aged and female rat. Aged and female rats exhibit decreased MOR binding potential, decreased G-protein activation, and decreased cAMP inhibition, while aged rats alone demonstrate increased RGS4 and RGS9-2 expression. No impact of age or sex was found in phosphorylation of MOR at serine-375

3.6.1 Impact of advanced age and sex on MOR binding potential

We previously reported that morphine potency is reduced in aged and female rats, likely due to a reduction in DAMGO binding in the vlPAG (Fullerton et al., 2021). Here we report significant reductions in vlPAG MOR binding potential in aged males and females of either age

in comparison to adult male rats, suggesting the observed decrease in opioid potency is driven, in part, by reduced expression/availability of MOR in the vlPAG. Notably, no impact of advanced age or sex on the MOR's affinity for its ligand (K_d values) was noted. However, aged males had reduced MOR availability as evidenced by lower B_{max} values compared to their adult male counterparts, a finding consistent with reports of reduced DAMGO binding in the vlPAG of aged males (Fullerton et al., 2021), and together suggest an age-induced downregulation of PAG MOR in males matching a consistently lower level of MOR expression in females of any age.

No impact of persistent inflammatory pain on vlPAG MOR binding potential was noted, consistent with previous studies. For example, patients with chronic fibromyalgia pain exhibit no changes in MOR binding in the PAG, despite significant reductions in the nucleus accumbens and amygdala (Harris et al., 2007). Similarly, no change in PAG MOR binding was noted following chronic pain induced by proximal nerve injury (Maarrawi et al., 2007). In rodents, persistent inflammatory pain increased MOR binding in the DRG at 24 and 96 hours post-CFA (Mousa et al., 2001; Zollner et al., 2003), with no change noted for the hypothalamus or spinal cord (M. A. Shaqura et al., 2004). Similarly, no change in PAG MOR availability or expression was observed following sciatic nerve injury, although reductions were noted for the insula, caudate putamen, and motor cortex (Thompson et al., 2018). These results, together, suggest that chronic pain alters MOR signaling in a CNS region-specific manner.

3.6.2 Impact of advanced age and sex on G-protein activation

Agonist binding at MOR activates Gαi/o -proteins and downstream signaling cascades, critical for opioid-mediated hyperpolarization (Laugwitz et al., 1993; Connor & Christie, 1999; Koehl et al., 2018; Mondal et al., 2020). Presently, adult males exhibited greater G-protein activation efficiency compared to aged males, suggesting that the decreases in opioid potency

observed in aged males are driven, in part, by attenuated G-protein-MOR coupling. A significant impact of biological sex on G-protein potency (EC_{50}) was also observed. The mechanism(s) by which age and sex attenuate potency is not known; these studies used membrane preparations that would be devoid of soluble signaling regulators. Similarly, the GTPγS molecule itself is not hydrolyzable and is thus not subject to regulation by GAPs (e.g., RGS proteins). This suggests that the observed decrease in G-protein activation potency may be due to decreased opioid receptor expression/availability observed in our binding studies.

Interestingly, the GTPyS assay was the only analysis where an impact of persistent inflammatory pain was observed and may contribute to previous findings that the analgesic effects of intrathecal DAMGO are potentiated following inflammation in male rats (Hurley & Hammond, 2000). While reductions in Gα subunit expression have been reported within the rostral ventromedial medulla and dorsal horn following intraplantar CFA in adult male rats (Wattiez et al., 2017), the mechanism whereby this would impact G-protein activation, and not the other pharmacodynamics of MOR, is unknown. Also unknown are the mechanisms by which advanced age and sex alter G-protein-MOR signaling. Advanced age results in a global downregulation of Gαi/o, most notably in the prefrontal cortex (Young et al., 1991; Alemany et al., 2007; de Oliveira et al., 2019), that is not associated with overall cell loss. Indeed, the Gαi/o subunit, in particular, appears susceptible to aging, with estimates of reduced expression as high as 65% in the frontal cortex, hippocampus, substantia nigra, and striatum (de Oliveira et al., 2019). Although age-induced changes in Gαi/o expression were not assessed specifically in PAG, a widespread reduction in Gαi/o would likely impact PAG, thereby limiting MOR-G-protein coupling and reducing both G-protein activation efficiency and opioid potency. Alternatively, an uncoupling between MOR and Gαi/o and/or a switch in Gα subunit from Gαi/o

to Gαs cannot be ruled out (Gintzler & Chakrabarti, 2000, 2004, 2006). Indeed, a shift from Gαi/o to Gαs would similarly increase adenylyl cyclase activity, resulting in reduced hyperpolarization and decreased morphine potency (Lamberts et al., 2011).

3.6.3 Impact of advanced age and sex on MOR Phosphorylation

The results of the radioligand binding and GTPγS assays suggested that aged and female rats exhibit decreased receptor expression and activation potency. As MOR can be desensitized by phosphorylation in its basal state, thereby limiting agonist activation (L. Zhang et al., 1996; Yu et al., 1997; Groer et al., 2011), we tested this possibility. Although MOR phosphorylation at serine-375 was higher in aged males compared to adults, this result was not significant, suggesting that our observed reductions in activation potency are driven by an alternative mechanism. Furthermore, this finding suggests that age and sex may not impact receptor desensitization and internalization, which are generally thought to be MOR phosphorylation-dependent.

MOR desensitization is canonically mediated by G protein-coupled receptor kinase (GRK)-dependent phosphorylation of the receptor (J. Zhang et al., 1998; Schulz et al., 2004; Dang et al., 2009). Although our results suggest no impact of advanced age or sex on GRK-mediated MOR phosphorylation, MOR signaling is also desensitized via phosphorylation through the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway (Dang et al., 2009). ERK1/2 phosphorylation stimulates the activity of Gα-interacting protein (GAIP), an RGS protein that acts as a GTPase activator to reduce opioid signaling at the level of G-protein activation (Ogier-Denis et al., 2000). Indeed, pharmacological inhibition of ERK1/2 phosphorylation in a rat model leads to improved morphine analgesia (Popiolek-Barczyk et al.,

2014; Melkes et al., 2020). Thus, the age- and sex-induced changes in morphine potency may be driven in part by age-induced hyper-phosphorylation of ERK1/2 and result in the downregulation of G-protein signaling. This represents an alternative explanation for our phosphorylation results, by which the receptor could still be functionally desensitized/downregulated without changing canonical phosphorylation.

3.6.4 Impact of advanced age and sex on cAMP inhibition

Agonist binding at MOR elicits a conformational change in the receptor to allow G-protein α and βγ subunits to interact with downstream effectors. Notably, the Gα subunit binds to adenylyl cyclase (AC) and inhibits the conversion of adenosine triphosphate (ATP) to cAMP, limiting the activation of cAMP-dependent protein kinase (PKA) and ultimately inducing higher levels of hyperpolarization (Christie, 2008; Seseña et al., 2014; Santhappan et al., 2015). Here, aged males and adult and aged females exhibited significantly lower levels of DAMGO-induced cAMP inhibition compared to adult males, suggesting an attenuated activity of the α subunit at the level of AC or a weakened relationship between AC and cAMP. This effect may result from decreased MOR expression and G-protein activation potency observed above.

3.6.5 Impact of advanced age and sex on RGS protein levels

The inactivation of G-protein signaling is modulated by the activity of RGS proteins via enhancement of GTPase activity of the α subunit. By promoting the hydrolysis of the alpha-bound GTP during the active state, RGS proteins hasten the return of the α subunit to the GDP-bound inactive state (Roman & Traynor, 2011). RGS proteins play a critical role in negatively modulating opioid signaling, as morphine analgesia is increased in male mice lacking RGS9-2 (Garzón et al., 2001; Zachariou et al., 2003) and overexpression of RGS4 attenuates MOR signaling in reconstituted MORs in vitro (Ippolito et al., 2002). We observed increased

expression of RGS4 and RGS9-2 in the vlPAG of aged rats compared to adults, suggesting greater GTPase activity and reduced G-protein signaling. Similar results were observed when the analysis was limited to MOR+ cells, indicating opioid-induced G-protein signaling is subjected to greater negative regulation in the vlPAG of the aged rat. These results are consistent with Kim et al. (2005), who reported higher RGS9-2 protein levels in the PAG of 1-year old male rats compared to 3-week-old rats (Kim et al., 2005). In humans, advanced age is associated with increased RGS4 expression in the prefrontal cortex (Rivero et al., 2010), suggesting that our observed increase in RGS4 not be specific to the vlPAG.

No significant impact of advanced age on OPRM1 mRNA expression in the vlPAG was noted. This result is interesting given our finding of reduced MOR binding in aged rats and protein expression in aged rats (Fullerton et al., 2021), and suggests that the observed age-induced reduction of vlPAG MOR protein is a function of impaired translation. Ori et al., 2015 reported reduced expression of several markers necessary to initiate translation, namely EIF3A, EIF4G3, EIF4A1, and MDN1 in aged mice (Ori et al., 2015), and a recent study using both mice and fish found that aged brains exhibit decreased proteasome activity and decreased ribosome assembly (Sacramento et al., 2020). These results suggest an age-induced dysregulation of protein synthesis in the brain that is conserved across species.

3.6.6 Summary and Conclusions

The present studies are the first to show that sex and advanced age lead to attenuated vlPAG opioid signaling compared to adult male rats. Taken together with our previous findings, these results suggest that age- and sex-induced reductions in vlPAG MOR expression and binding, combined with attenuated downstream MOR signaling, contribute to the diminished opioid potency reported in aged and female rats (Fullerton et al., 2021). The results of our

analyses demonstrate that aged and female rats exhibit reductions in MOR expression/availability, G-protein activation, and cAMP inhibition, and increased G-protein regulation by RGS proteins, each of which provides potential therapeutic targets for improved pain management in the elderly.

4 GENERAL DISCUSSION

Chronic pain affects over 50 million persons daily, contributing to depression, increased disability, and early death (Yong et al., 2022). Older individuals suffer from higher rates of chronic pain than young adults, with over 40% of individuals over 65 suffering from chronic pain (Larsson et al., 2017). As the US population ages, these individuals represent a key demographic in chronic pain management. Further, women are more likely to experience chronic pain than men, indicating a critical need for individualized therapeutic strategies based on both age and sex for effective pain management. Opioids are among the most common and most effective medications for severe pain, with greater than 25% of Americans using opioids each year (Han et al., 2017). However, proper opioid use for the management of severe pain in the elderly remains a controversial topic due to a lack of knowledge regarding opioid pharmacodynamics in the aged.

As discussed in Chapter 1, a clinical assessment of opioid potency for pain relief reported that both men and women >74 years of age required higher daily doses of morphine to manage cancer pain compared to men and women <65 years of age (Loick et al., 2000). This suggests that opioid potency is attenuated in aged humans, however, the mechanisms of this reduced potency are not known. The present studies address a critical need for effective pain management in the elderly by defining the influence of advanced age on brain mechanisms subserving pain and analgesia.

Our findings indicate that aging is associated with decreased mu opioid receptor (MOR) availability, decreased opioid-induced G-protein signaling, and decreased opioid-induced cAMP inhibition within the ventrolateral periaqueductal gray (PAG). Taken together, these results suggest that advanced age causes a decrease in PAG MOR receptor density, resulting in a

decreased number of activated G-proteins and attenuated inhibition of downstream cAMP. Further, females exhibit decreased vlPAG MOR binding potential, opioid-induced G-protein signaling, and cAMP inhibition compared to males. No impact of advanced age or biological sex was observed on constitutive MOR phosphorylation. Finally, the present studies indicate that aged males and females exhibit increased vlPAG RGS4 and RGS9-2 expression compared to their young adult counterparts.

Our observed results suggest several potential mechanisms of action whereby sex and age impact analgesic potency. First, ligand affinity (K_d) and receptor phosphorylation state were not significantly different across age and sex suggesting that individual receptor functionality is comparable within the PAG. In contrast, we did observe a significant decrease in expression/availability (B_{max}) in aged males suggesting decreased available MOR, and not an attenuation in MOR sensitivity, contributes to the reduced potency of morphine. This age-induced reduction in PAG MOR signaling might explain the observed decrease in opioid-induced G-protein activation observed in the PAG of our aged rats. As soluble regulators (like RGS proteins) are absent in this assay and the ^{35}S-GTPγS is non-hydrolyzable, the decrease in G-protein coupling potency is likely due to decreased receptor availability. Downstream of the G-proteins, we also observed a decrease in cAMP inhibition. This result may be due to the observed increase in RGS4 and RGS9-2 expression which would decrease G protein activity, leading to less cAMP inhibition. However, RGS expression was not increased in adult females suggesting a contribution of additional mechanisms. Overall, our results identify two primary, and complementary, mechanisms whereby opioid signaling is reduced within the vlPAG: 1) decreased receptor expression/availability in adult females and aged males and females, and 2) increased RGS4 and RGS9-2 expression in aged males and females. Further investigation may

also uncover additional complementary sex- and age-related mechanisms that contribute to the lower analgesic potency of morphine in females and the aged.

4.1 Advanced age and sex alter morphine anti-hyperalgesia but not thermal nociception

The present studies use intraplantar administration of Complete Freund's Adjuvant (CFA) to induce persistent inflammatory pain. This method of peripheral pain induction has been used to model persistent pain in several mammalian species including humans and has been used extensively in mouse and rat studies, including multiple studies in our laboratory (Loyd & Murphy, 2006; X. Wang et al., 2006; Loyd et al., 2007). CFA injection induces moderate hyperalgesia and edema within hours of injection via local innate immune response (Watkins et al., 1994; Raghavendra et al., 2004). In rodents, CFA-induced hyperalgesia perpetuates for 7 days, at which point it beings to improve, ultimately resolving by 14 days post-administration (Millan et al., 1988; Stein et al., 1988; Philippe et al., 1997; Goff et al., 1998; X. Wang et al., 2006). Intraplantar injection of CFA emulates the time course of clinical pain produced by surgery and peripheral injury, conditions that are typically managed by opioids (Millan et al., 1988; Stein et al., 1988). Thus, intraplantar CFA provides a reliable and clinically relevant rodent model of persistent peripheral pain.

Studies described in Chapter 2 examined the impact of advanced age and/or biological sex on nociceptive thresholds. These studies included 4 treatment groups: adult males (2 months of age), adult females (2 months of age), aged males (16-20 months of age), and aged females (16-20 months of age). Basal withdrawal thresholds in response to a noxious thermal stimulus were determined using the Hargreaves assay (Hargreaves et al., 1988). Our results showed no significant effect of sex on baseline thermal sensitivity, a finding consistent with previous studies

from our laboratory in addition to those of others (Ali et al., 1995; Craft & Milholland, 1998; Craft et al., 1999; X. Wang et al., 2006). Indeed, meta-analyses find that the majority of rodent studies report no sex differences in basal nociception (Mogil et al., 2000, 2010; Racine et al., 2012; Mogil, 2020) and that only 8% (41/526) of such studies published as of August 2019 find a significant impact of biological sex on basal pain sensitivity (Mogil, 2020).

Our results further showed no significant effect of advanced age on baseline thermal sensitivity. Previous studies examining the impact of age on basal thermal pain sensitivity are contradictory, with reports of increased (Hess et al., 1981; Kitagawa et al., 2005), decreased (Chan & Lai, 1982; Kramer & Bodnar, 1986; Garrison & Stucky, 2014; Muralidharan et al., 2020), and no change in mechanical or thermal pain sensitivity in aged rodents compared to adults (Islam et al., 1993; Crisp et al., 1994; Jourdan et al., 2002; Taguchi et al., 2010; Yezierski, 2012; Mecklenburg et al., 2017; Samir et al., 2017). Multiple factors may contribute to these disparate results, notably the species used, the range of ages for aged and adult groups, the pain assay used (e.g., thermal, mechanical, visceral), and the chronicity of the pain (acute, persistent).

Clinical studies similarly fail to report a consistent impact of advanced age on basal pain sensitivity. A meta-analysis summarizing the findings of 52 clinical research studies reported that aged individuals exhibit decreased pain sensitivity compared to young adults (Lautenbacher, 2012). However, this analysis included both pain-free individuals and individuals experiencing chronic pain. This is a major confound as individuals experiencing chronic pain commonly use prescription medications and are more likely to suffer from additional disabilities.

In addition to basal pain, we also examined the impact of advanced age and sex on persistent inflammatory pain induced by intraplantar CFA. Pre-clinical studies in rodents from our lab and others demonstrate that females require approximately two times as much morphine as young

adult males to achieve comparable analgesia (Craft et al., 1999; Ji et al., 2006; X. Wang et al., 2006; Loyd et al., 2008). Studies conducted in a laboratory setting with aging rodents suggest that aged rats are also less sensitive to the analgesic effects of opioids, and thus require higher doses for adequate analgesia (Kavaliers et al., 1983; Jourdan et al., 2002). A primary flaw in the design of extant studies is the continued use of acute 'evoked response' pain assays rather than using a persistent pain assay. In addition, as with clinical reports, these aging studies fail to include sex as a variable of analysis. The inclusion of females and the use of animal models of pain that reflect the pathophysiological processes of clinically-diagnosed pain in humans are required to address the knowledge gap left by existing preclinical studies of pain (King & Porreca, 2014).

Consistent with our previous studies, we found no significant impact of sex on CFA-induced hyperalgesia or CFA-induced edema (Loyd & Murphy, 2006; X. Wang et al., 2006; Loyd et al., 2008). Further, no impact of advanced age on CFA-induced hyperalgesia or edema was noted, with adult and aged rats exhibiting equivalent nociceptive thresholds. Relatively few preclinical studies have assessed the impact of advanced age on hyperalgesia. Consistent with our results, (Garrison and Stucky, 2014) reported no differences in intraplantar CFA-induced hyperalgesia or edema in adult (3 mos) versus aged male mice (24 mos)(Garrison & Stucky, 2014). Similarly, no impact of age (3 to 24 months) on thermal or mechanical hyperalgesia was noted in a mouse model of plantar incisional pain (Mecklenburg et al., 2017). Alternatively, (Weyer et al., 2016) reported that following intraplantar CFA administration, aged male mice (18 mos) exhibited decreased mechanical hyperalgesia in comparison to adults. This hyperalgesia was accompanied by a reduction in noxious stimulus-evoked action potentials in dorsal horn neurons. Similar to the present study, no impact of age on CFA-induced edema was reported

(Weyer et al., 2016), suggesting that age-dependent changes in mechanical hypersensitivity are not dependent on CFA-induced edema.

Our studies examined the impact of advanced age on opioid modulation of persistent inflammatory pain in male and female rats. We report that aged male rats exhibit reduced morphine anti-hyperalgesic potency compared to young adult males. Indeed, aged males required significantly higher doses of morphine than their young adult counterparts to produce comparable modulation of persistent pain. These results are consistent with prior research on male mice; Muralidharan et al. (2020) reported that mice exhibit reduced morphine anti-allodynic potency at 54 weeks compared to 10 weeks. This group also reported attenuated anti-allodynia produced by the gabapentinoid pregabalin in aged male mice, suggesting that aged rodents are less sensitive to not only opioids but to other classes of analgesics as well (Muralidharan et al., 2020).

Importantly, this age-induced attenuation in morphine potency (Chapter 2) is not due to age/sex differences in basal thermal somatosensory thresholds or CFA-induced hyperalgesia or edema. Rather, our findings suggest that age has a specific impact on central opioidergic signaling.

4.2 Advanced age and sex modulate vlPAG MOR expression and ligand binding

The PAG, along with its projections to the rostral ventromedial medulla (RVM), is critical for opioid modulation of pain (Reynolds, 1969; Behbehani & Fields, 1979; Morgan et al., 1991, 1992; Y. Zhang et al., 1998; Loyd et al., 2008). Mu opioid receptor, the preferred receptor for morphine, is localized within the vlPAG, and several lines of evidence suggest that vlPAG MOR is critical for opioid modulation of pain. First, intra-PAG injection of the MOR antagonist (-)-naloxone attenuates the effects of stimulation-induced analgesia (Akil et al., 1976). Secondly, lesions of PAG MOR, induced by site-specific injections of the ribotoxin saporin conjugated with a potent MOR agonist dermorphin, completely attenuate systemic morphine-induced analgesia in male rats (Loyd et al., 2008). The present studies (Chapter 2) report that aged males and females exhibit reduced morphine potency, as indicated by increased morphine EC_{50} values. Further, our data (Chapters 2 & 3) indicate that aged males exhibit reduced PAG MOR expression compared to young adult males; similarly, aged males and females exhibit reduced MOR binding compared to adult males and females. Taken together, these findings suggest that age-induced changes in PAG MOR expression and binding contribute to reduced opioid potency in aged rodents.

Using immunohistochemistry (for protein localization) and autoradiography (for quantification of ligand binding) we report that aged rats exhibit reduced vlPAG MOR expression and binding compared to adults (Figure

Figure 4.1 MOR expression and binding MOR expression was assessed using immunohistochemistry, MOR binding was assessed using autoradiography

4.1). As a negative control, we also assessed the impact of advanced age on MOR expression within the inferior colliculus (IC), a brain region rich in MOR but not implicated in pain or opioid analgesia. We found no sex or age differences in IC MOR, suggesting that the age-associated reduction in MOR was limited to the PAG. However, as we did not conduct a widespread survey of CNS regions, we cannot definitively rule out if advanced age alters MOR expression in other brain regions.

The results of our autoradiography experiments showing reduced DAMGO binding in the vlPAG of aged rats (Chapter 2) were supported by parallel findings using saturated radioligand binding assays (Chapter 3). We report that aged males exhibit reduced MOR availability as evidenced by lower B_{max} values compared to young adult males. These results, together, provide corroborating evidence suggesting that the decrease in opioid potency observed in the aged rats is driven, in part, by reduced expression/availability of MOR in the vlPAG.

Interestingly, results from our radioligand binding assay show a significant reduction in B_{max} values for aged males compared to adult males; in contrast, aged females did not differ significantly from adult males. These results are inconsistent with those generated using autoradiography, which showed significantly reduced DAMGO binding in both aged males and aged females compared to young adults (Chapter 2). These conflicting results may be due to methodological differences between receptor autoradiography and radioligand binding. Receptor autoradiography was performed using a single concentration of DAMGO applied to 20-micron tissue sections, while the radioligand binding assays were performed on membrane protein lysates derived from dissected vlPAG tissue using 8 increasing concentrations of DAMGO. It is possible that by increasing the highest concentration of DAMGO and expanding our ligand binding curve we would reveal B_{max} values that more closely resemble our autoradiography

results (i.e., binding values of adult males and females continue to increase, while bound DAMGO in the aged females remains comparatively plateaued). However, it is also possible that the DAMGO binding values seen in the adult and aged female autoradiographs were due to non-specific binding. As the specific binding curves calculated with the radioligand binding assay were generated by removing non-specific binding, these values are more specific for MOR and thus better represent functional receptor.

The radioligand binding assay provides an important distinction between receptor availability (the total number of functional receptors to which agonist may bind; B_{max}) and receptor affinity (the strength of the agonist-receptor interaction; K_d). Our results indicate that advanced age and sex do not impact MOR affinity, as evidenced by no significant differences between K_d values. This suggests that unit receptor performance is not impacted in the aged vlPAG and that the changes imparted as a function of advanced age and sex are at the level of receptor downregulation rather than desensitization (Williams et al., 2013).

Previous studies examining the impact of advanced age on MOR binding report reduced MOR in the frontal cortex and striatum of aged male rats (Hess et al., 1981; Messing et al., 1981) and reduced MOR in the midbrain and thalamus of aged female rats (Messing et al., 1980). These studies support our findings (Chapters 2 & 3) and suggest age-induced downregulation of MOR in a sex- and site-specific manner. A recent clinical study using [^{11}C]carfentanil positron emission tomography reported that MOR binding in the thalamus, amygdala, and nucleus accumbens is reduced in aged males and females compared to young adults (Kantonen et al., 2020). Unfortunately, this study did not measure MOR binding in the PAG. In contrast, Kantonen et al. (2020) reported age-induced increases in MOR binding in the cingulate cortex,

orbitofrontal cortex, and temporal pole, indicating region-specific changes in MOR binding as a function of age.

Taken together, these findings suggest that MOR binding is downregulated in the aged brain in a region-specific manner. To determine if PAG MOR is downregulated in the aged as a function of age-induced changes in OPRM1 transcription, we used in situ hybridization. Quantification of OPRM1 transcripts and OPRM1+ neurons revealed no impact of advanced age on vlPAG OPRM1 mRNA expression suggesting that OPRM1 transcription within the vlPAG is not altered as a function of advanced age. Thus, although aged animals exhibit reduced MOR protein within the vlPAG compared to young adult rats, these deficits are not present in MOR mRNA and suggest that the reduction in vlPAG MOR protein is a function of impaired translation. Ori et al. (2015) compared RNA sequencing and ribosome profiling on 6 month old vs 24 months old rats and reported reduced expression of several markers necessary to initiate translation, namely EIF3A, EIF4G3, EIF4A1, and MDN1, in aged rats (Ori et al., 2015). Additionally, tissue from aged rats exhibited reduced levels of several markers that contribute to ribosomal regulation of translation, including BCCIP, Ngdn, RtCD1, and BOP1 (Ori et al., 2015). A study using *C. elegans* also reports that aging is associated with imbalanced protein regulation and increased mRNA degradation (Walther et al., 2015). However, if aged rats exhibit dysregulated translation of OPRM1 in the vlPAG is not known. The use of active mRNA translation sequencing or ribosome profiling to measure the rate of protein synthesis in specific brain regions will provide further insight into the impact of advanced age on MOR expression and availability.

No impact of persistent inflammatory pain on MOR expression or binding in the vlPAG was noted in the present studies. Our findings are in line with previous reports of no change in PAG

MOR availability or expression in adult male rats experiencing neuropathic pain (Thompson et al., 2018). This study did report reduced levels of MOR in the caudate-putamen and insula (Thompson et al., 2018). Intraplantar CFA has been shown to increase MOR binding in the dorsal root ganglia of adult male Wistar rats (Mousa et al., 2001; Zollner et al., 2003), but have no impact on MOR binding in the hypothalamus (M. A. Shaqura et al., 2004), suggesting that persistent inflammatory pain influences MOR binding in a region-specific manner.

Clinically, MOR binding is typically assessed using positron emission tomography (PET) measurements following administration of opioid agonists in the presence and absence of naloxone. Reduced MOR binding was reported in the straight gyrus and the frontal, temporal, and cingulate cortices of males and females experiencing rheumatoid arthritis pain (Jones et al., 1994); no change was found in the PAG. An additional study reported decreased MOR binding in the nucleus accumbens, amygdala, and cingulate cortex of females experiencing fibromyalgia pain, but the PAG was not included as a region of interest (Harris et al., 2007). No change in PAG MOR binding was noted in men experiencing chronic pain induced by proximal nerve injury (Maarrawi et al., 2007), however, neuropathic pain generated from a supraspinal injury was associated with reduced PAG MOR binding (Maarrawi et al., 2007). Together, these clinical findings suggest that the impact of pain on MOR binding is dependent on the modality of pain and the brain region examined. It is not known whether surgical pain or pain following a traumatic injury impacts PAG MOR expression or binding in humans.

We report no sex differences in vlPAG MOR expression, a finding that contradicts previous results from our lab in which adult females exhibited reduced vlPAG MOR compared to males (Loyd et al., 2008). These contradictory results are likely due to methodological differences, and in particular, differences in antibody specificity and assessment of MOR density

from images acquired using a 3-dimensional confocal versus 2-dimensional light-field microscope. The lack of sex differences in PAG MOR expression found using immunohistochemistry was corroborated by our MOR saturation binding studies. These findings suggest that the reduced morphine potency seen in adult females compared to adult males is not driven by changes in vlPAG MOR availability as previously proposed (Loyd et al., 2008), but rather an alternative mechanism or mechanisms. Studies from our lab and others suggest that sex differences in neuroimmune signaling, particularly in microglia, contribute to the sexually dimorphic effects of morphine (Sorge et al., 2011; Eidson et al., 2017; Doyle & Murphy, 2017; Rosen et al., 2017; Eidson & Murphy, 2019). Morphine binds to neuronal MOR as well as to the innate immune receptor toll-like receptor 4 (TLR4), localized primarily on microglia (Hutchinson et al., 2007, 2008, 2010). Morphine promotes TLR4-mediated downregulation of GABA receptors and glutamate transporters (GLT1 and GLAST) and upregulation of AMPA receptors. These changes result in an overall increase in neural excitability, promoting depolarization and directly opposing morphine analgesia (Chieng & Christie, 1996; Ingram et al., 1998; Song & Zhao, 2001; Ogoshi et al., 2005; Stellwagen et al., 2005; Holdridge et al., 2007; X. Wang et al., 2012; Eidson et al., 2017). Activation of TLR4 via lipopolysaccharide results in increased pro-inflammatory IL-1ß and decreased anti-inflammatory IL-10 within the vlPAG of female but not male rats, and blockade of vlPAG TLR4 signaling via (+)-naloxone enhances morphine analgesia in females but not in males (Doyle et al., 2017), suggesting a sex-dependent mechanism for reduced opioid potency that is independent of PAG MOR. Neuroinflammation has been linked to many conditions concomitant with advanced age as well, suggesting that aged animals exhibit aberrant neuroimmune signaling (Gorelick, 2010; VanGuilder et al., 2011; Norden & Godbout, 2013). Thus, neuroinflammation via TLR4 activation may act in tandem

with changes in MOR binding and signaling to attenuate opioid analgesia in aged males and females.

To further elucidate the mechanisms by which advanced age and sex alter opioid analgesia, future studies should assess MOR expression and binding in additional regions along the descending modulatory tract. While the present studies represent a comprehensive assessment of MOR within the vlPAG, immunohistochemistry, autoradiography, and receptor binding assays performed on RVM and spinal cord tissue from rodents in a chronic pain state will further our knowledge of the impact of advanced age and sex on opioid analgesia.

4.3 Advanced age and sex modulate vlPAG MOR signaling

4.3.1 G-protein activation

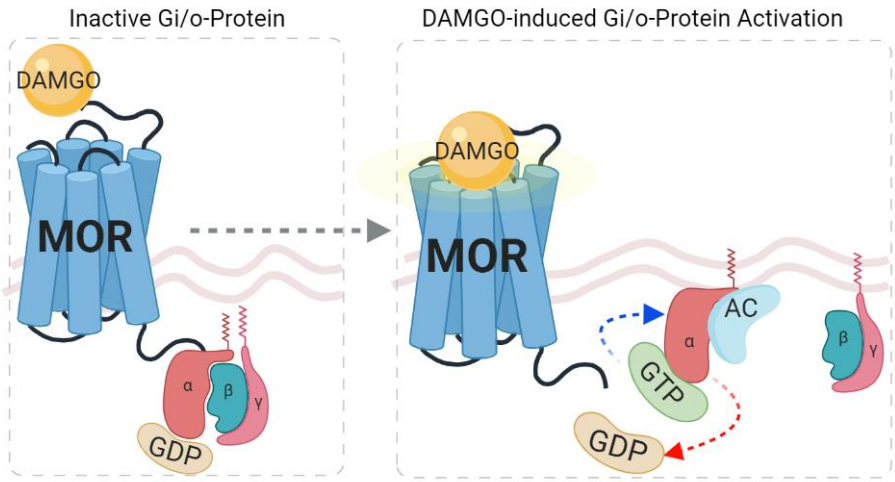


Figure 4.2 DAMGO-induced G-protein activation
DAMGO binding induces a conformational change in the MOR, promoting the dissociation of the Gαβγ complex, and subsequent activity at downstream effectors such as AC

G-protein-mediated signaling is critical for opioid-induced hyperpolarization (Laugwitz et al., 1993; Connor & Christie, 1999; Koehl et al., 2018). We report that adult males exhibit greater G-protein activation efficiency compared to aged males and adult and aged females (Chapter 3). A significant impact of biological sex on G-protein potency (EC_{50}) was also

observed; females of both ages exhibited increased EC_{50} values compared to males, indicating a reduction in the potency of opioid-dependent G-protein activation. This reduction in vlPAG G-protein activation likely contributes to our result (Chapter 2) and the findings of previous studies that females are less sensitive to the analgesic effects of opioids (Kest et al., 2000; Loyd et al., 2008; Y.-J. Wang et al., 2014). Whether these findings suggest an additional mechanism responsible for reduced opioid analgesia is not clear. The GTPγS assay uses membrane preparations; therefore, soluble signaling regulators (like kinases) are not present. In addition, the GTPS molecule itself is not hydrolyzable, and thus not regulated by GTPase-activating proteins (GAPs) such as RGS proteins (Strange, 2010). Therefore, the observed decrease in G-protein activation in the aged and adult females may be a result of the reduced opioid receptor expression/availability as noted in the autoradiography and radioligand binding studies (Chapters 2 and 3).

Our findings indicate reduced opioid-stimulated G-protein signaling within the PAG of aged and female rats. This reduced activation of downstream G-proteins may be due in part to age-induced downregulation of PAG Gi/o, or an uncoupling of MOR from Gi/o associated with aging. Several previous studies have reported an age-induced downregulation of Gi/o in the prefrontal cortex (Young et al., 1991; Alemany et al., 2007; de Oliveira et al., 2019) and the hippocampus, substantia nigra, and striatum in humans (de Oliveira et al., 2019). Whether Gαi/o expression is reduced in the PAG is not known, and thus further experimentation is needed to confirm the site-specificity of the observed age-induced downregulation. Age-induced uncoupling of MOR from Gi/o would reduce opioid-stimulated G-protein activation without changes in Gi/o tissue expression. Further, advanced age and/or biological sex may promote aberrant MOR-G-protein coupling in which MOR couples to the excitatory Gαs rather than the

inhibitory Gi/o (Gintzler & Chakrabarti, 2000, 2004, 2006). A shift from Gi/o to Gs would produce adenylate cyclase activation (rather than the requisite inhibition), resulting in cAMP-mediated depolarization and thus decreased opioid response (Lamberts et al., 2011). Experimentation assessing the impact of advanced age and sex on the expression and coupling of Gα isoforms would further elucidate the mechanisms whereby advanced age and sex attenuate opioid-induced G-protein activation.

The present studies used the high-affinity MOR agonist DAMGO for both the radioligand binding and GTPyS assay. Previous studies have reported that MOR pharmacodynamics are agonist-dependent; namely, MOR phosphorylation induced by morphine exposure is protein kinase C (PKC)-dependent, while DAMGO-induced MOR phosphorylation is PKC independent (Chu et al., 2010). Additionally, MOR is internalized by DAMGO and fentanyl binding, but not by morphine (Hashimoto & Kirihara, 2006). Therefore, future studies should consider incorporating at least two agonists in all assays to ensure any results are not ligand-dependent. Alternatively, advanced age may promote the expression of OPRM1 splice variants (Alvarez et al., 2002; Pan et al., 2005; Narayan et al., 2021). Alternative MOR isoforms are known to promote ligand-dependent regulation of opioid signaling (D. A. Pasternak et al., 2004; Oldfield et al., 2008). For example, variants of MOR-1B, a family of MOR isoforms expressed in the brain, are indistinguishable from MOR-1 at the extracellular surface and the agonist binding pocket but elicit alternative signaling properties that impact opioid-induced cell hyperpolarization, and dysregulation of these MOR splice variants has been implicated in advanced age (Latorre & Harries, 2017; H. Li et al., 2017; K. Wang et al., 2018). There are also reports of sex differences in the expression of MOR splice variants in the PAG, with male rodents expressing higher levels of MOR-1B5 and females expressing higher levels of MOR-

1B1 and MOR-1B2 (A. Liu et al., 2018). The unique conformation of MOR isoforms and subsequent alterations in receptor phosphorylation directly influence the G-proteins to which MORs couple, potentially resulting in biased agonism as a function of advanced age (Pan et al., 2005; Verzillo et al., 2014; Abrimian et al., 2021).

Our data (Chapter 3) indicate that CFA-treated rats exhibited reduced EC_{50} values, and thus increased G-protein activation efficiency, compared to handled rats, regardless of age or sex. These findings suggest that persistent inflammatory pain alters DAMGO-induced G-protein activation within the PAG. One study performed on male rats reported that Ga subunit expression is reduced in the RVM and dorsal horn of the spinal cord following intraplantar CFA (Wattiez et al., 2017), suggesting reduced DAMGO-induced G-protein activation in these regions. However, no impact of CFA on Ga subunit expression in the vlPAG was found. Thus, the mechanism whereby intraplantar CFA enhances G-protein activation is not known.

4.3.2 MOR phosphorylation

MOR desensitization is canonically mediated by G-protein-coupled receptor kinase (GRK)-dependent phosphorylation of the receptor, a mechanism by which β-arrestins bind to MOR, resulting in endocytosis (L. Zhang et al., 1996; Yu et al., 1997; J. Zhang et al., 1998; Schulz et al., 2004; Dang et al., 2009; Groer et al., 2011). The present studies tested the hypotheses that the observed reductions in G-protein activation efficiency were due to age- and sex-induced changes in basal or constitutive MOR phosphorylation by examining the phosphorylation state of serine-375 on PAG MOR in the absence of opioid agonist binding. We report no significant impact of age or sex on MOR basal phosphorylation (Chapter 3), suggesting that the reductions in G-protein activation potency observed in the vlPAG of aged and female rats are driven by an alternative mechanism.

In addition to GRK phosphorylation at Ser^{375} studies on rat MOR indicated that constitutive phosphorylation of the receptor is carried out by CAMKII phosphorylation at Thr^{370} and PKC phosphorylation at Ser^{363} (Chen et al., 2013; Lemel et al., 2020). MOR is also desensitized is via phosphorylation through the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway, in which ERK1/2 phosphorylation stimulates the activity of Gα-interacting protein (GAIP), an RGS protein that acts as a GTPase activator to reduce opioid signaling at the level of G-protein activation (Ogier-Denis et al., 2000; Dang et al., 2009; Melkes et al., 2020). This mechanism has been shown to contribute to opioid signaling, as pharmacological inhibition of ERK1/2 phosphorylation enhances morphine analgesia in a rat model of neuropathic pain (Popiolek-Barczyk et al., 2014). Thus, age- and/or sex-induced hyper-phosphorylation of ERK1/2 may result in reduced G-protein signaling and attenuated morphine potency observed in aged males and females.

In addition to lending insight into age-induced changes in G-protein signaling, investigation of MOR splice variant expression in the aged brain will provide insight into the mechanisms responsible for alternative receptor phosphorylation in the aged. The unique conformation of MOR isoforms promotes alternative receptor phosphorylation and influences biased agonism (Verzillo et al., 2014; Abrimian et al., 2021). Recent studies reported that multiple MOR splice variants, specifically MOR-1O, exhibit significant biases for β-arrestin2, suggesting that overexpression of certain MOR isoforms proteins promotes divergent opioid signaling processes (Xu et al., 2017; Narayan et al., 2021).

4.3.3 Opioid-induced cAMP inhibition

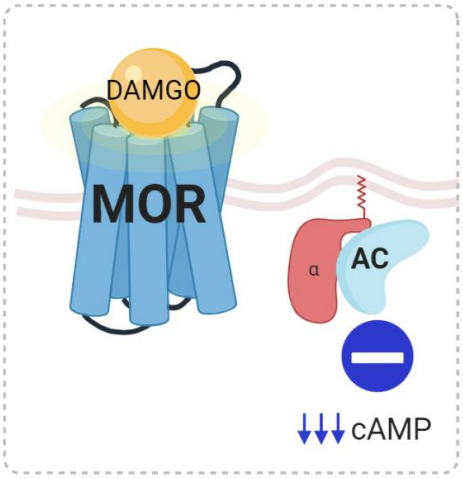


Figure 4.3 Opioid-induced cAMP inhibition
MOR binding induces Gai/o activation, promoting inhibition of AC and thus reduced cAMP

Opioid-induced G-protein mediated adenylyl cyclase (AC) inhibition plays a critical role in opioid analgesia. Exogenous opioids such as morphine hyperpolarize neurons in part by inhibiting the conversion of adenosine triphosphate (ATP) to cAMP (Christie, 2008; Santhappan et al., 2015). The present studies examine the hypothesis that the reduced opioid potency observed in our behavioral experiments is due to attenuated opioid-induced cAMP inhibition within the vlPAG of aged males and females regardless of age. In these studies, we used forskolin stimulation to boost cAMP levels in PAG tissue lysates and examined the impact of age and sex on the ability of DAMGO to inhibit AC-mediated cAMP production. Our results show that aged males and females (regardless of age) exhibit reduced DAMGO-induced cAMP inhibition compared to adult males, indicating attenuated downstream MOR signaling in the aged PAG. This attenuation may be due to the observed decreases in MOR availability and activation potency discussed above. Additionally, the reduced efficacy of DAMGO to inhibit cAMP may be due to advanced age- and sex-induced alterations to AC signaling mechanisms, such as aberrant phosphorylation of AC as a function of age (Yoshimasa et al., 1987). The observed reductions in DAMGO-induced cAMP inhibition may also be driven by dysregulated cAMP circulation via cAMP hydrolysis by phosphodiesterases (PDEs) (Goraya & Cooper, 2005). Indeed, expression of hydrolyzing PDEs PDE1C and PDE8A is increased in the striatum of aged rats (26 mos) compared to adults (5

mos). Further, PDE8A and PDE11A expression are increased in the hippocampus of aged rats, and PDE1C expression is increased in the cortex (Kelly et al., 2014).

Interestingly, previous studies have reported that basal cAMP levels are reduced in several brain regions of the aged male rats, including the cerebral cortex, midbrain-thalamus, and hypothalamus (Puri & Volicer, 1981; Titus et al., 2013; Kelly, 2018). However, these same studies reported no change in basal cAMP levels in the striatum, cerebellum, or hippocampus, suggesting the effects of aging on cAMP levels are region-specific. The impact of advanced age on basal cAMP levels within the rat vlPAG has not previously been addressed. Age-induced cAMP reduction within the vlPAG would likely result in a more effective DAMGO-induced cAMP inhibition, which is in direct contrast to our observed reduction in opioid potency. However, our present studies that measured forskolin-boosted cAMP suggest slight reductions in basal cAMP in the aged vlPAG, but only DAMGO-induced cAMP inhibition was statistically analyzed.

Previous studies assessing the impact of advanced age on cAMP were conducted exclusively in male rodents; thus, it is not known whether advanced age impacts cAMP signaling in both aged males and females. One recent study reported an impact of biological sex on kappa opioid receptor (KOR) signaling in the mouse spinal cord using a model of surgical pain, reporting that KOR signaling in males is PKA-dependent and KOR signaling in females is PKA-independent (Basu et al., 2021). It is not known, however, whether this sex difference is present in MOR signaling within the vlPAG.

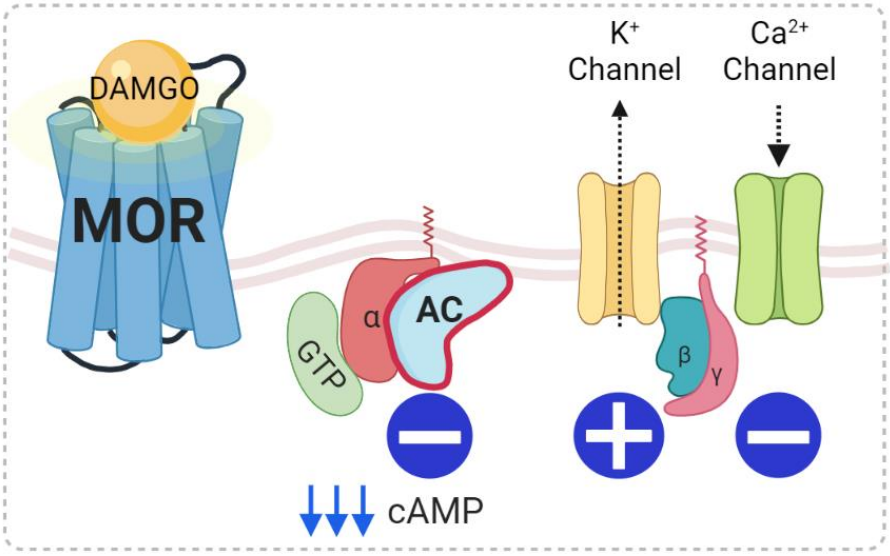


Figure 4.4 DAMGO-induced cAMP inhibition and βγ signaling
Opioid-induced Gai/o activation promotes AC-mediated cAMP inhibition, GIRK-mediated hyperpolarization, and calcium channel inhibition

Morphine binding to MOR results in the activation of Gai/o that primarily inhibits the activity of AC, thereby reducing cAMP production; in parallel, the βγ subunits activate G-protein inwardly rectifying potassium (GIRK) channels to induce hyperpolarization and decrease excitability (Law et al., 2000; Nobles et al., 2005; Yudin & Rohacs, 2018)(Figure 4.3). Advanced age is concomitant with increased levels of neuroinflammation and greater production of reactive oxygen species (ROS), both of which have been implicated in age-induced potassium channel dysfunction (Sparkman & Johnson, 2008; Cai & Sesti, 2009; Sesti, 2016; Lieberman et al., 2020). Consistent with this, aged Sprague Dawley rats (26-30 mos) exhibit reduced potassium currents in hippocampal neurons, with no effect of age on neuronal density or cell integrity (Alshuaib et al., 2001). The authors suggested that the observed reduction in potassium current is due to either age-induced alteration in potassium channel kinetics, or reduced density (number) of potassium channels. Taken together, these findings suggest that advanced age may induce aberrant GIRK channel-mediated hyperpolarization within the PAG, thereby limiting

opioid signaling in this region, and contributing to the reduced morphine potency observed in aged rats.

4.3.4 RGS protein expression

Opioid-induced G-protein signaling is modulated by the activity of Regulator of G-protein signaling (RGS) proteins (Cabrera-Vera et al., 2003; Xie & Palmer, 2005; Senese et al., 2020). Two members of the RGS protein family have been extensively studied for their role in regulating opioid signaling: RGS4 and RGS9-2. The overexpression of RGS4 attenuates MOR signaling in reconstituted MORs in vitro (Ippolito et al., 2002), and conversely, knockout of RGS9-2 enhances morphine analgesia (Garzón et al., 2001; Zachariou et al., 2003); thus, expression of RGS4 and RGS9-2 negatively correlates with opioid analgesia.

Our final experiments examined the hypothesis that age and sex-mediated changes in vlPAG RGS4 and RGS9-2 contribute to the reduced opioid potency observed in aged and female rats. In these studies, RNAscope was performed on vlPAG tissue sections from adult and aged, male and female rats. Our results showed increased vlPAG expression of both RGS4 and RGS9-2 in aged rats compared to adults. Increased expression of both RGS4 and RGS9-2 was specific for MOR+ neurons within the vlPAG, providing a mechanism by which advanced age could decrease analgesic potency and downstream cAMP signaling. However, as there was no difference in RGS expression as a function of sex in adults, these data do not explain why adult females demonstrate lower potency and signaling than adult males. These findings are consistent with Kim et al. (2005) who assessed the impact of advanced age using male Sprague Dawley rats and reported increased RGS9-2 expression within the PAG at 1 year vs 3 weeks (Kim et al., 2005).

To date, there is limited study on the impact of advanced age on RGS4 and RGS9-2 expression in the CNS. A clinical study of postmortem brain tissue of individuals aged 15-88 years used western blotting to examine age and sex differences in RGS4 along with RGS10, a protein whose dysregulation is implicated in neurodegenerative disorders such as Parkinson's disease and multiple sclerosis. Rivero et al. (2010) reported increased RGS4 expression in the pre-frontal cortex of aged brains, while no impact of advanced age on RGS10 was observed (Rivero et al., 2010). Unfortunately, the pre-frontal cortex was the only region examined so the impact of advanced age on RGS4 and RGS10 in other brain regions is unknown. Taken together with our findings, these data suggest that RGS4 expression is increased in multiple brain regions as a function of advanced age and identify RGS4 as a potential target for novel therapeutic strategies for improved opioid signaling in the elderly.

RGS proteins represent an interesting perspective for targeted therapy as their downregulation via specific pharmacological inhibitors could potentiate opioid analgesia, and as such, several RGS protein inhibitors have been developed. Recent studies have reported that intrathecal inhibition of RGS4 enhances the analgesic effects of opioids (Yoon et al., 2015). Interestingly, administration of intrathecal RGS4 inhibitors reduces hyperalgesia in the absence of opioids, suggesting RGS regulation of endogenous opioids (Bosier et al., 2015; Yoon et al., 2015). Taken together with our results (Chapter 3) these findings suggest that inhibition of RGS4 within the PAG may improve the pain-relieving properties of morphine following CFA injection. Based on our finding that RGS4 expression is upregulated in the aged PAG, we predict that pharmacological inhibition of vlPAG RGS4 in aged rats would amplify MOR signaling in this region and enhance morphine analgesia.

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