8-8-2017

Sex Differences in Morphine Analgesia and the Role of Microglia in the Periaqueductal Gray of the Rat

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SEX DIFFERENCES IN MORPHINE ANALGESIA AND THE ROLE OF MICROGLIA IN THE PERIAQUEDUCTAL GRAY OF THE RAT

by

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Under the Direction of Anne Z. Murphy, PhD

ABSTRACT

Morphine has been and continues to be one of the most potent and widely used drugs for the treatment of pain. Clinical and animal models investigating sex differences in pain and analgesia demonstrate that morphine is a more potent analgesic in males than in females; indeed, we report the effective dose of morphine for female rats is twice that of male rats. In addition to binding to the neuronal mu opioid receptor, morphine binds to the innate immune receptor toll-like receptor 4 (TLR4) on microglia. Morphine action at TLR4 initiates a neuroinflammatory response and directly opposes morphine analgesia. Our recent studies demonstrate that administration of chronic morphine activates microglia within the ventrolateral periaqueductal gray (vlPAG), a critical brain

region for the antinociceptive effects of morphine, while blockade of vlPAG microglia increases morphine analgesia and suppresses the development of tolerance in male rats. Despite increasing evidence of the involvement of microglia in altering morphine efficacy, *no studies have examined sex differences in microglia within the PAG*. The present experiments seek to characterize the distribution and activity of vlPAG microglia in males and females using behavioral, immunohistochemical and molecular techniques, while demonstrating the sufficiency and necessity of vlPAG microglia to produce sex differences in morphine analgesia using site-specific pharmacological manipulation of TLR4. We also investigate a novel pharmacokinetic mechanism underlying the sexually dimorphic effects of morphine administration on microglial activity. Here, we address a fundamental gap in our current understanding of sex differences in morphine analgesia and establish a mechanistic understanding of how the activation of vlPAG microglia sex-specifically influences morphine analgesia.

INDEX WORDS: Opioids, Toll-Like Receptor 4, Glucuronide, Pain, Glia

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2017

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August 2017

DEDICATION

I dedicate this work to my parents Tom and Catie Doyle. Everything I do is for you, with all the love I can give.

ACKNOWLEDGEMENTS

I would like to thank my mentor, **Dr. Anne Murphy**, who gave me the support and freedom to explore and grow as a scientist. Anne allowed me to pursue my own research interests, and watching those research ideas grow has been one of the most incredible and rewarding experiences. I especially want to thank Anne for always being the person to ask “what about the females!?” Anne started a fire in my heart to pursue equality in research and I will take that passion with me wherever I go.

Thank you to my committee members, **Dr. Nancy Forger, Dr. Michael Morgan**, **and Dr. Sarah Pallas**, I am glad that I had the opportunity to know you all, and it has been an absolute pleasure to work with you. To **Nancy**, thank you so much for your trust and your compassion. Your approach to science is fascinating and unique, I think that my biggest regret in grad school is not collaborating with you on a microglia project! To **Mike**, thank you so much for your support. Your experience and knowledge in our field has been invaluable, and I sincerely appreciate your confidence in my work and my abilities. To **Sarah**, thank you so much for your feedback. Your thoughtfulness and diligence has improved my writing and my science, and your commitment to helping students (including me!) succeed is truly profound.

Thank you to the NI faculty and staff. To **Liz Weaver**, you have been an absolutely amazing friend and colleague. Your hard work and dedication inspires me--not just to be a better leader, but to be a better person. I am so fortunate to know you. To **Emily Hardy**, you are a wonderful friend (not just to me, but to all the NI students), and you brighten my days! I will dearly miss our talks! **Dr. Aras Petrulis**, I will never forget how much you’ve helped me. You are a wonderful mentor and I don’t think I

would even be getting a PhD without your encouragement when I was just starting out. To **Dr. Chuck Derby**, you have been incredibly supportive of me and I sincerely appreciate your guidance and understanding.

To my friends and colleagues, thank you! A special thanks to **Dr. Lori Eidson** for teaching me everything I know. You have been more than a lab-mate, you are a dear friend and I love you like a sister—you deserve all the happiness in the world. To **Lauren Hanus**, **William Bell**, and **Jonassie Zamor** mentoring you has been a blast! Thank you for all your help (Lauren especially for scoring those microglia!)! To my friends, **Mac McNeil, Emily Bruggeman, Richard and Michelle Campbell, Kate McCann, Amy Ross, and Mary Holder**: I adore you all, thank you so much for making research (and everything else) fun!

To **Johnny Garretson**, my rolling stone, I love you so much and I am so lucky to have you in my life. Your hard work and dedication has brought out a passion for science in myself that I didn't think was possible. You make me laugh without fail, you support me in all of my endeavors, and you love me more than I deserve. Being with you is the most fun I've ever had, and I can't wait to see what happens next.

I would like to give a special thank you to my dad, **Tom Doyle**. You have been there for me like no one else and I don't know where I would be without you. You pushed me to be the best I can be, and I would count myself lucky if I grow up to be half the person you are. Because of your example, I work hard to live a life you can be proud of. I love you!

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LIST OF ABBREVIATIONS

CFA, Complete Freunds adjuvant

ED50, 50% effective dose

IL [IL-1β, IL-6, IL-10], Interleukin [interleukin-1β, interleukin-6, interleukin-10]

LPS, Lipopolysaccharide

M3G, Morphine-3-glucuronide or morphine-3-β-D-glucuronide

M6G, Morphine-6-glucuronide or morphine-6-β-D-glucuronide

MD2, Myeloid differentiation factor 2

MOR, Mu (μ) opioid receptor

PAG, vlPAG, Periaqueductal gray, ventrolateral periaqueductal gray

RVM, Rostral ventral medulla

TLR, TLR4, Toll-like receptor, toll-like receptor 4

TNF, Tumor necrosis factor

UGT, Uridine 5'-diphospho-glucuronosyltransferase

1 INTRODUCTION

1.1 Sex differences in pain and treatment

Pain is a highly subjective and unpleasant experience arising from the central nervous system. The perception of pain evolved as a protective mechanism to avoid potentially fatal encounters, and to discourage or change harmful behavior. However, pain does not always occur as a result of an overt injury; in some cases, the cause of pain cannot be identified. In these instances, long-lasting pain may become a frustrating and debilitating disease in and of itself--and can be extremely difficult to manage, let alone treat.

Chronic pain--generally defined as pain lasting longer than three to six months--is one of the most commonly reported health problems in the US (Elzahaf et al., 2012; Kennedy et al., 2014). The National Health Interview Survey, conducted by the National Institutes of Health, reports that 55.7% of American adults had some level of pain within the previous three months, while 11.2 % had experienced chronic pain, that is, pain every day for the three months prior to the survey (Nahin, 2015). With so many individuals suffering from pain, it is no surprise that the economic burden of pain is massive. The National Academy of Sciences estimates the total annual cost of pain treatment to ranges from $560 to $635 billion annually in direct and indirect costs (Medicine, 2011).

Chronic pain is a social and economic burden that impacts a large number of Americans, however, women appear to be disproportionately affected. Indeed, women have a much higher incidence rate of chronic pain conditions such as fibromyalgia, migraine, temporomandibular disorder, and osteoarthritis (Unruh, 1996; Fillingim et al.,

2009; Mogil, 2012; Ruau et al., 2012; Buse et al., 2013; Kennedy et al., 2014). It is unclear if the female predominance of chronic pain conditions is due to higher susceptibility, or a greater likelihood for women to report pain, though many studies have attempted to tease apart these factors. Although women do use health care services more often than men for both painful and non-painful conditions (Bertakis et al., 2000; Prevention, 2014), a recent report demonstrates that among men and women with the same diagnosis, reported pain levels were significantly higher in women (Ruau et al., 2012).

Despite many years of study, it remains unclear if sex differences truly exist in pain perception. Human subjects show variable responses to experimentally induced pain, and sex differences in pain sensitivity are not always observed (Fillingim et al., 2009). This may be attributed to variability in testing conditions (*i.e.* the modality of pain being tested, reporting methods [self-report vs. threshold latency], or stage of menstrual cycle). However, when sex differences are observed, these studies overwhelmingly demonstrate that women are more sensitive to pain, and are more negatively affected by pain, than men [see (Fillingim et al., 2009; Mogil, 2012) for review].

1.1.1 *Opioids for the management of pain*

Opium derived from poppy plants has been used for thousands of years to alleviate pain and a variety of other ailments. Beginning in the early 1800's, with the extraction of morphine from opium, opioids have been—and continue to be—the most effective and widely used pain treatment in the world (Rosenblum et al., 2008; Trescot et al., 2008). Approximately 3-5% of the adult US population is currently prescribed long

term opioid therapy (Boudreau et al., 2009); however, opioids induce many negative peripheral effects that prevent them from being efficacious when used long term. For example, opioids can induce respiratory depression and gastric immotility (Pasternak and Pan, 2013). In addition, prolonged use of opioids reduces their analgesic efficacy over time, thus requiring steadily larger doses to maintain analgesia—a phenomenon known as tolerance (Trescot et al., 2006; Trescot et al., 2008). Analgesic tolerance is reported to significantly impair pain relief (Gulur et al., 2014), and subsequent dose escalation increases the risk of developing addiction (Trescot et al., 2006). Finally, both chronic and acute opioid administration may result in opioid induced hyperalgesia, a paradoxical effect of opioids resulting in enhanced sensitivity to pain (see (Angst and Clark, 2006) for review).

Of pain sufferers, women are more likely to be prescribed opioids—at higher doses and for longer periods of time—than men (Campbell et al., 2010; Frenk et al., 2015; Manubay et al., 2015). This may have potentially important implications for the development of tolerance and addiction to opioids. From 1999 to 2015, more than 183,000 people in the U.S. died from overdoses related to prescription opioids (Rudd et al., 2016), and in 2014, nearly two million Americans abused, or were dependent on, prescription opioids (2015). Although men are more likely to die from drug overdose than women, between 1999 and 2010, overdose deaths due to opioid pain relievers increased by 265% in men but increased by 415% in women ((CDC), 2013).

There is increasing evidence suggesting that neither chronic nor acute treatment with opioids is an effective strategy for pain relief in women. Women consistently experience a greater preponderance of the negative side effects associated with acute

opioid consumption, including nausea, dysphoria, headache, and vomiting than men (Myles et al., 1997; Cepeda et al., 2003; Fillingim et al., 2005; Comer et al., 2010). In addition, opioids may not offer the same degree of pain relief in women. Similar to sex differences in pain sensitivity, sex differences in clinical pain management are also varied and misunderstood [see (Fillingim et al., 2009) for review]. Although several studies report that there is no sex difference in the analgesic efficacy of opioids in humans (Sarton et al., 2000; Glasson et al., 2002; Fillingim et al., 2005; Bijur et al., 2008), when sex differences in opioid pain relief are detected, they demonstrate decreased analgesic efficacy in women (Cepeda and Carr, 2003; Miller and Ernst, 2004; Aubrun et al., 2005).

In contrast to clinical studies, preclinical research on pain relief using a variety of acute and persistent pain assays in rodents have *repeatedly and consistently* demonstrated that morphine is more effective in males than in females, with females requiring approximately twice as much morphine to achieve comparable pain-relief (Kepler et al., 1989; Boyer et al., 1998; Craft et al., 1999; Cicero et al., 2002; Krzanowska et al., 2002; Holtman et al., 2003; Ji et al., 2006; Loyd and Murphy, 2006; Wang et al., 2006; Loyd et al., 2008a; Posillico et al., 2015). Furthermore, several studies indicate that females also have greater morphine-induced hyperalgesia than their male counterparts (Holtman and Wala, 2005; Juni et al., 2008). Sex differences in opioid analgesia are not limited to morphine, indeed, greater pain relief is observed in male rats for almost every opioid tested (Barrett et al., 2002; Terner et al., 2003; Stoffel et al., 2005; Peckham and Traynor, 2006; Bai et al., 2015).

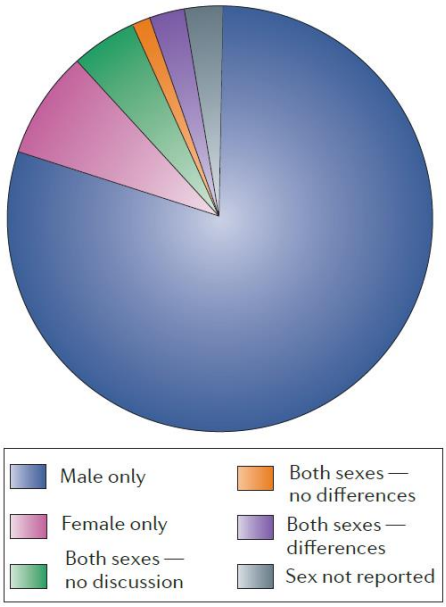

Figure 1.1.1 Inclusion of Sex in Preclinical Studies of Pain. (Mogil, 2012)

Together, these studies clearly illustrate the necessity of sex-specific research on pain and pain management; however, the overwhelming majority of preclinical studies of pain (approximately 79%; Figure 1.1.1) are conducted exclusively in males (Mogil, 2012). The study of pain and effective pain management in *both sexes* is not only important to ease the economic burden caused by chronic pain, but it is paramount to improve the lives of the immense number of men and women that suffer from chronic pain daily.

1.2 Mechanisms of pain and analgesia in the central nervous system

Painful stimuli originating from the periphery are transduced by nociceptive fibers of the spinothalamic tract that are able to detect thermal or mechanical pain. Activation of pain-sensing channels results in excitation of two main classes of nociceptive fibers: myelinated A-delta fibers and unmyelinated C-fibers; contributing to sharp momentary

pain and slow diffuse pain, respectively. The signal of pain is transmitted via nociceptive afferents from the periphery to lamina I and II of the dorsal horn of the spinal cord, then to laminae IV-VI. Here, the signal crosses the midline and ascends to the brainstem, thalamus, rostral ventral medulla (RVM), periaqueductal gray (PAG), and ultimately to the cortex where pain is perceived (Millan, 1999).

While ascending pathways alert the brain to painful stimuli (nociception), descending pathways carefully control and reduce pain (antinociception). The ventrolateral midbrain periaqueductal gray (vlPAG), and its descending projections to the rostral ventral medulla (RVM), are essential to exert control over antinociception (Basbaum et al., 1978; Behbehani and Fields, 1979; Fields and Heinricher, 1985). For example, electrical stimulation of the PAG is sufficient to induce robust analgesia, such that invasive surgery can be performed in the absence of anesthesia (Reynolds, 1969). This effect appears to be opioid-dependent, as intra-PAG injection of the mu opioid receptor (MOR) antagonist (-)-naloxone attenuates stimulation-induced analgesia (Akil et al., 1976).

Morphine and other opioids bind to neuronal MORs to modulate pain. (Jensen and Yaksh, 1986; Bernal et al., 2007; Loyd et al., 2008a). Activation of neuronal G-protein coupled MOR decreases the conductance of voltage-gated calcium channels and opens potassium channels, reducing the membrane potential to ultimately inhibit cell firing and decrease the probability of presynaptic neurotransmitter release (Millan, 2002). This effect occurs at several key sites (Figure 1.2.1):

1) In the spinal cord, MOR-induced inhibition of neurons reduces the release of pro-nociceptive transmitters (*e.g.* Substance P) from peripheral sensory neurons to

secondary ascending spinal neurons to decrease pain transmission, ultimately producing antinociception, or analgesia (Millan, 2002).

2) The PAG contains a high density of MOR-containing neurons (Gutstein et al., 1998; Commons et al., 1999; Wang and Wessendorf, 2002), and MOR binding at inhibitory GABAergic interneurons within the PAG decreases firing to descending pain-inhibiting neurons. This is thought to disinhibit the PAG-RVM circuit, producing a net activation of the PAG-RVM, and inhibition of pain transmission at the spinal cord (Al-Hasani and Bruchas, 2011; Stein, 2013; Lau and Vaughan, 2014). The importance of the PAG to opioid action and descending antinociception is further demonstrated as microinjection of opiate agonists directly into the PAG induces robust analgesia in male rats (Sohn et al., 2000; Loyd et al., 2008a). In addition, site-specific lesions of MOR-positive neurons within the PAG significantly reduce the analgesic action of systemic morphine in male rats (Loyd et al., 2008a).

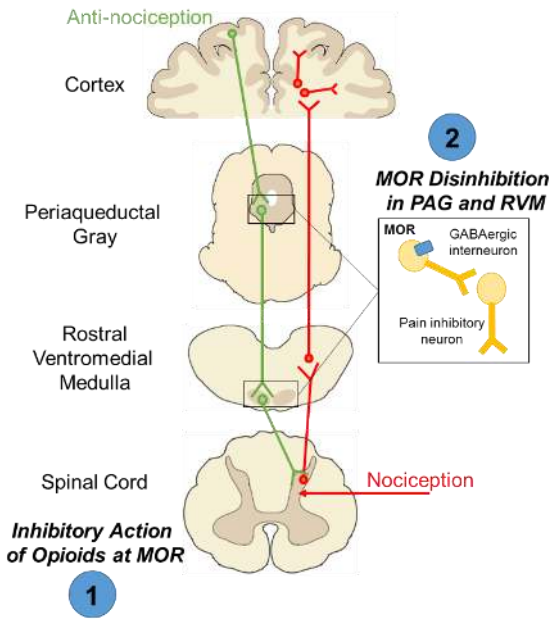

Figure 1.2.1 A Model of Descending Analgesia.
This figure demonstrates two key sites of opioid action in the CNS. Adapted from (Al-Hasani and Bruchas, 2011).

The PAG and its projections to the RVM and spinal cord possess innate sex differences in their anatomy and physiology that influence pain management and the effectiveness of opioid drugs. Specifically, our previous studies have found that females have approximately twice as many output neurons in the PAG-RVM pathway as their male counterparts; however, PAG-RVM output neurons are preferentially activated in males during persistent pain or following morphine injection (Loyd and Murphy, 2006; Loyd et al., 2007). In addition, males appear to have greater levels of MOR expression and binding within the PAG, likely contributing to the increased analgesic efficacy of morphine in males (Loyd et al., 2008a). However, selective ablation of vlPAG MOR-expressing neurons significantly attenuates the response to morphine exclusively in males, indicating that the density of PAG MOR expression is significantly correlated with the degree of opioid analgesia in male, but not female, rats (Loyd et al., 2008a). These studies indicate that although MOR tone in the PAG is sexually dimorphic, density of MOR alone cannot solely account for the decreased efficacy of morphine observed in females.

1.2.1 *Morphine action: pharmacokinetics, pharmacodynamics, and sex differences*

Following administration ~10% of morphine is directly absorbed or excreted unchanged (Christrup, 1997). The remaining ~90% of morphine is metabolized to form two active metabolites: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Metabolism occurs primarily in the liver (Coffman et al., 1997), but also by peripheral macrophages (Tochigi et al., 2005) and brain microglia (Togna et al., 2013),

indicating both peripheral and central metabolism of morphine. Isozymes in the uridine 5'-diphospho-glucuronosyltransferase (UGT) 1 and 2 subfamilies metabolize morphine by addition of a glucuronic acid to the morphine substrate via Phase II metabolism (Coughtrie et al., 1989; Christrup, 1997). Peripheral enzymes UGT 1A1, 1A3, 1A6, 1A8, 1A9, 1A10, and 2B7 are all capable of M3G glucuronidation, but M6G can only be formed by UGT2B7 in humans (Stone et al., 2003). In rats, the UGT 1A1, 1A6, 1A7, and 2B1 enzymes synthesize both M3G and M6G (Togna et al., 2013). Almost all UGT enzymes preferentially synthesize M3G over M6G (~45-55% and 15% of metabolized product, respectively; (De Gregori et al., 2012)).

Primary morphine metabolites have unique—and opposing—pharmacodynamic properties. M6G binds to MOR with high affinity and is a potent analgesic (Abbott and Palmour, 1988; Wittwer and Kern, 2006). Indeed, a recent meta-analysis suggests a substantial contribution of M6G to total analgesia following morphine administration (approx. 85-96%; (Klimas and Mikus, 2014)). On the other hand, M3G does not bind to MOR or produce analgesia (Loser et al., 1996). Rather, M3G produces allodynia (pain in response to a normally non-noxious stimuli) and hyperalgesia (exaggerated pain in response to a normally mild painful stimulus) (Lewis et al., 2010; Due et al., 2012). Further, administration of M3G is correlated with nociception (Smith and Smith, 1995) and behavioral excitation (*e.g.* “wet dog shakes”, excessive grooming) (Yaksh et al., 1986; Bartlett et al., 1994), and actively opposes the analgesic effects of morphine and M6G (Smith et al., 1990; Ekblom et al., 1993).

There is evidence to suggest sex differences are also present in morphine pharmacokinetics. For example, sexual dimorphism of UGT1 and 2 enzymes has been

observed in humans (Gallagher et al., 2010) and rats (Iwano et al., 2012); however, this is not surprising, given that these enzymes metabolize—and are directly influenced by—steroid hormones (Strasser et al., 1997). Although clinical studies show both the presence (Murthy et al., 2002) and absence (Sarton et al., 2000) of sex differences in glucuronide concentrations in humans, animal studies of morphine metabolites consistently show that M3G serum concentrations are ~2 times higher and the plasma ratio of M3G to morphine is ~5.5 times higher in female than in male rats (South et al., 2001; Baker and Ratka, 2002; South et al., 2009).

1.3 Immune modulation of pain

Pain transmission is dynamic and variable, and carefully modulated at every step from the periphery to the CNS. Pain can be "turned down" by descending antinociceptive processes such as endogenous opioid release and MOR binding, but it can also be "turned up" by pro-nociceptive processes stemming from the immune system. Although neurons have been the primary focus of pain transmission, non-neuronal glial cells of the CNS, primarily microglia and astrocytes, are in key positions to modulate neuronal signaling and influence nociception.

Astrocytes surround neurons at the synapse, and actively participate in neuronal signaling (Haydon et al., 2009). Microglia layer themselves across the brain and survey the neuronal space for pathogens or chemicals associated with cellular distress, known as "alarmins" (Davalos et al., 2005; Bianchi, 2007). In the event of cellular distress (*e.g.* tissue injury or the presence of pathogens/bacteria), neurons release "alarmins" such as fractalkine (CX3CL1), nitric oxide, substance P, calcitonin gene related peptide, ATP,

glutamate and prostaglandins (Watkins et al., 2007; Watkins et al., 2009). These "alarmins" bind to pattern recognition receptors, known as "toll-like" receptors (TLRs), located primarily on microglia, and to a lesser extent, on astrocytes (Hutchinson et al., 2008a; Watkins et al., 2009). Binding of pathogenic or damage-associated molecules to microglial toll-like receptor 4 (TLR4) results in the transition of microglia from a 'resting' to a 'reactive' (or 'activated') state (Watkins and Maier, 2003; Bianchi, 2007; Watkins et al., 2009; Buchanan et al., 2010).

'Activated' microglia are primary contributors to pain modulation. Microglia release pro-nociceptive molecules such as cytokines (interleukins [IL]-1, -6, and -10; tumor necrosis factor [TNF]), chemokines, cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and reactive oxygen species (Bonizzi and Karin, 2004; Doyle and O'Neill, 2006). These glial products cause down-regulation of inhibitory $GABA_A$ receptors and up-regulation of excitatory AMPA and NMDA receptors on neurons, and decrease glutamate transporters to increase extracellular concentrations of glutamate, together producing a net increase in neural excitability (Ogoshi et al., 2005; Stellwagen et al., 2005; Watkins et al., 2005; Yan et al., 2014; Eidson et al., 2016); also see (Tilleux and Hermans, 2007) for review. Glial release of pro-inflammatory molecules and subsequent neuronal excitability results in the sensitization of nociceptive neurons and enhanced pain (Watkins and Maier, 2003).

Activation of CNS microglia, and subsequent release of pro-inflammatory mediators, is now well-established to play a role in pain. For example, 'activated' microglia are observed in essentially every known animal model of clinical pain, including nerve damage, bone cancer, migraine, and many others [see (Watkins et al., 2007) for

review]. Furthermore, activation of glial cells with inflammatory CFA (complete Freund's adjuvant) or the TLR4 agonist lipopolysaccharide (LPS; a component of gram-negative bacteria), induces robust allodynia and hyperalgesia (Watkins et al., 1994; Raghavendra et al., 2004a; Sorge et al., 2011). On the other hand, blocking glial cell function or glial release of pro-inflammatory molecules prevents or reverses allodynia and hyperalgesia (Maier and Watkins, 1998; Plunkett et al., 2001; Raghavendra et al., 2003; Ledeboer et al., 2005; Hutchinson et al., 2008a). These data demonstrate that glial cells, via TLR4, communicate bidirectionally with neurons, and their activation is associated with increased nociception.

1.3.1 *Opioids bind to immune receptors on glia*

Microglia and astrocytes are relatively new targets in the search for improved pain therapeutics (Tanga et al., 2005; Milligan and Watkins, 2009; Nicotra et al., 2012). Morphine binds to the myeloid differentiation factor 2 (MD2) co-receptor of TLR4 (Hutchinson et al., 2010). Thus, similar to pathogenic molecules, morphine can initiate a pro-inflammatory cascade, ultimately leading to cytokine release and neuronal excitation that paradoxically reduces its own analgesic efficacy (Stellwagen et al., 2005; Hutchinson et al., 2007; Hutchinson et al., 2010; Franchi et al., 2012; Li, 2012; Eidson and Murphy, 2013a; Thomas et al., 2015). Unlike the MOR which can only bind the negative isomer of opioids, TLR4 binds opioids in a non-stereoselective fashion, such that both negative (-) and positive (+) isomers bind to TLR4 to affect glial signaling (Hutchinson et al., 2010) (Figure 1.3.1). It is important to note that although MORs have

been localized on microglia (Chao et al., 1997), acute morphine does not activate microglia via MOR (Gessi et al., 2016).

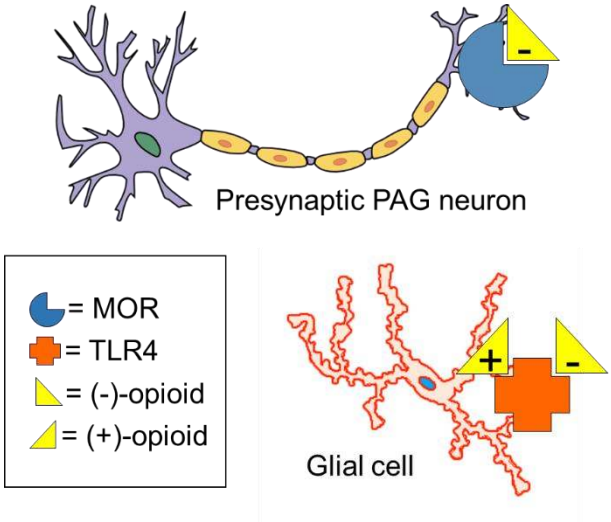

Figure 1.3.1. TLR4 Binds (-) and (+) Isomers of Opioid Ligands.
TLR4 binds opioids in a nonstereoselective fashion. Adapted from (Hutchinson et al., 2010) and (Eidson et al., 2016).

To date, many studies have shown a link between glial activation and decreased morphine efficacy. Activation of TLR4 by morphine has also been linked with many of the negative side effects associated with morphine use, such as morphine-induced hyperalgesia (Raghavendra et al., 2004b), withdrawal (Ledeboer et al., 2007), and tolerance (Song and Zhao, 2001; Raghavendra et al., 2002; Raghavendra et al., 2004b; Mika et al., 2009; Bai et al., 2014). Inhibition of microglia or TLR4 signaling, or blockade of proinflammatory cytokine production, result in a potentiation of morphine-induced analgesia (Raghavendra et al., 2004b; Watkins et al., 2005; Hutchinson et al., 2008b; Hutchinson et al., 2008a; Hutchinson et al., 2010; Li, 2012; Eidson and Murphy, 2013a; Bai et al., 2014).

We have recently reported that the PAG is a critical neural target for morphine-induced glial activation and pain modulation in male rats. Chronic systemic

administration of morphine activates TLR4 within the PAG to induce local cytokine release and oppose analgesia (Eidson and Murphy, 2013b; Eidson et al., 2016). Further, specific inhibition of TLR4 with (+)-naloxone directly into the PAG increases analgesia and blocks the development of tolerance to chronic morphine (Eidson and Murphy, 2013a; Eidson et al., 2016).

Morphine binding at TLR4 is now well accepted, but notably, many other clinically prescribed opioids, such as oxycodone and fentanyl, also bind to TLR4 (Hutchinson et al., 2010). In addition, the active morphine metabolite M3G binds to TLR4 with high affinity (Hutchinson et al., 2010), causing robust activation of microglia accompanied by cytokine release and the development of hyperalgesia (Lewis et al., 2010; Due et al., 2012). Together, this implies that many relevant opioids may have neuro-excitatory effects, and oppose analgesia via TLR4. Importantly, the analgesic metabolite of morphine, M6G, does not appear to bind to TLR4 (Hutchinson et al., 2010), and is not associated with pro-inflammatory glial activity (Carrigan and Lysle, 2001). Thus, M6G and potential drugs that do not activate immune cells may produce greater analgesia and represent a favorable alternative to the commonly used immune-activating opioids.

1.3.2 *Sex differences in innate immunity*

Sex differences in immunity have been well demonstrated, with females having a more robust immune system at baseline, and more reactive immune response to perturbation than males [for review, see (Marriott and Huet-Hudson, 2006), (Garcia-Segura and Melcangi, 2006), and (Schwarz and Bilbo, 2012)]. For example, in the peripheral immune system, female mice have higher baseline titers of immunoglobulins

(Klein, 2000), and splenocyte blastogenic responses to T and B cell mitogens than males (Schneider et al., 2006). Females also demonstrate increased resistance to bacterial and parasitic infection compared with males (Klein, 2004). Sex differences in the release of pro-inflammatory molecules (*i.e.* cytokines) following immune challenge has been well documented, however, the direction of the effect is not consistent--showing increased glial activity in both males (Drew and Chavis, 2000; Aulock et al., 2006) and females (Calippe et al., 2008; Calippe et al., 2010; Loram et al., 2012; Engler et al., 2016). In a recent clinical study, LPS immune challenge was shown to elicit a greater cytokine response in women that was accompanied by increased hyperalgesia to cold/heat pain suggesting that clinical reports of sex differences in pain sensitivity may be the result of a pro-nociceptive cytokine response that is potentiated in women (Karshikoff et al., 2015).

Although increased immune responsivity in females is thought to be neuroprotective, over-activation of the immune system can be pathological (Streit et al., 2004). Females demonstrate increased inflammation and hyperalgesia in response to immune challenge compared with males (Cook and Nickerson, 2005), which may make females more susceptible to long-term effects of inflammation than their male counterparts (LaPrairie and Murphy, 2007). Exaggerated inflammatory responses may also underlie the increased prevalence of auto-immune disorders (Whitacre, 2001; Cooper and Stroehla, 2003) and chronic inflammatory conditions observed in females.

1.4 Dissertation aims

Previous research has investigated various factors potentially driving sex differences in morphine analgesia, with few conclusive results to a single underlying mechanism. Previous work from our lab demonstrates that males have more MOR in the vlPAG (Loyd et al., 2008a) and respond more robustly to morphine agonism than females (Zubieta et al., 2002; Loyd and Murphy, 2006, 2009); however, female analgesia appears to have a limited relation with MOR density (Loyd et al., 2008a). This suggests that despite physiological differences in MOR, additional or parallel underlying mechanisms should be considered.

Given robust sex differences in immune function, and known links between immune function and morphine efficacy, we suggest that immune modulation is a likely novel mechanism that may be decreasing analgesic efficacy of morphine in females. See summary Figure 1.4.1. **Thus, we propose our overarching hypothesis that sex differences in the analgesic effects of morphine are due to sexually dimorphic responses of vlPAG microglia.** Specifically, we hypothesize that females have increased activation of microglia compared with males, thus the analgesic efficacy of morphine is decreased to a greater degree in females. In addition, we hypothesize that sex differences in morphine pharmacokinetics exacerbate this effect via increased M3G binding to TLR4 (and subsequently increased glial reactivity) in females. *We will test these hypotheses in <u>two</u> specific aims:*

Specific Aim 1: Characterize vlPAG microglia activity in males and females and establish the role of TLR in mediating the sexually dimorphic effects of morphine.

Despite evidence of sex-specific responses to immune challenge (Calippe et al., 2008; Calippe et al., 2010), and a well-established relationship between immune activation and pain (Watkins et al., 2007), TLR4-mediated glial activity has not been examined in any brain region involved in pain processing in females, and the contribution of TLR4 to morphine efficacy in females remains to be tested**.** Our previous studies demonstrate that TLR4 signaling within the PAG modulates morphine analgesia in male rats (Eidson and Murphy, 2013b), however, no studies have examined the role of PAG TLR4 in females. *This aim will 1) examine sex differences in distribution and activation of vlPAG microglia using behavioral, anatomical, and molecular techniques at baseline or following immune activation by either the TLR4 agonist LPS, or morphine. Furthermore, 2) we will demonstrate a mechanistic role of PAG microglia in modulating morphine efficacy in males and females using site-specific injections of a TLR4 agonist and antagonist to examine the sufficiency and necessity of vlPAG TLR4 to produce sex differences in morphine analgesia.*

Specific Aim 2: Investigate a pharmacokinetic mechanism involved in the sexually dimorphic activation of microglia*.* Morphine is converted into two active glucuronides: M3G and M6G. M6G binds to MOR and is a potent analgesic, while M3G binds to TLR4 with high affinity to activate glia, thus opposing the analgesic effects of morphine and M6G. In rats, M3G serum concentrations are ~2 times higher and the ratio of M3G to morphine is ~5.5 times higher in females than males (Baker and Ratka, 2002). Despite known sex differences in morphine metabolism, a mechanistic investigation of morphine metabolites on microglial activation or morphine analgesia has

never been performed. *Therefore, this aim will 1) examine behavioral responses to M3G and M6G using vlPAG site-specific injections; and 2) examine the contribution of morphine metabolites to the activation of microglia.*

These studies are the first to describe a role of microglia in producing sex differences in morphine analgesia. Furthermore, we suggest novel methods to improve current opioid-based pain management via inhibition of glial TLR4, and illustrate the necessity for sex-specific research and individualized treatment strategies for the management of pain.

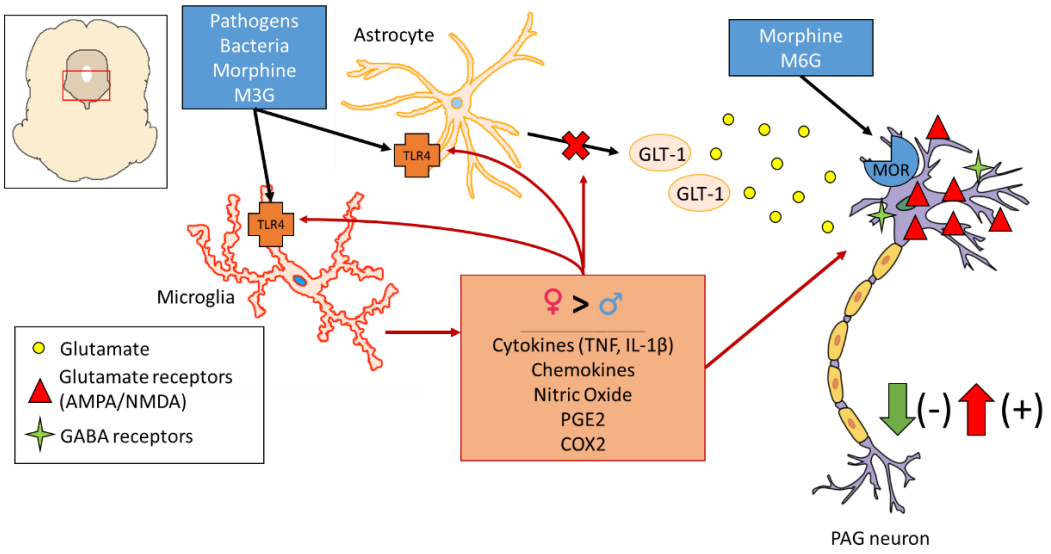

Figure 1.4.1. Summary Figure Describing a Potential Mechanism Underlying Reduced Morphine Efficacy in Females.

2 SEX DIFFERENCES IN MICROGLIA ACTIVITY WITHIN THE PERIAQUEDUCTAL GRAY OF THE RAT: A POTENTIAL MECHANISM DRIVING THE DIMORPHIC EFFECTS OF MORPHINE

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Published in *The Journal of Neuroscience* ©, 22 March 2017.

Abstract

Although morphine remains the primary drug prescribed for alleviation of severe or persistent pain, both preclinical and clinical studies have shown that females require 2-3 times more morphine than males to produce comparable levels of analgesia. In addition to binding to the neuronal μ opioid receptor (MOR), morphine binds to the innate immune receptor toll-like receptor 4 (TLR4) localized primarily on microglia. Morphine action at TLR4 initiates a neuroinflammatory response that directly opposes the analgesic effects of morphine. Here we test the hypothesis that the attenuated response to morphine observed in females is the result of increased microglia activation in the periaqueductal gray (PAG), a central locus mediating the antinociceptive effects of morphine. We report that while no overall sex differences in the density of microglia were noted within the PAG of male or female rats, microglia exhibited a more “activated” phenotype in females at baseline, with the degree of activation a significant predictor of

morphine ED50 values. Priming microglia with LPS induced greater microglia activation in the PAG of females compared with males that was accompanied by increased transcription levels of IL-1ß and a significant rightward shift in the morphine dose response curve. Blockade of morphine binding to PAG TLR4 with (+)-naloxone significantly potentiated morphine antinociception in females such that no sex differences in ED50 were observed. These results demonstrate that PAG microglia are sexually dimorphic in both basal and LPS-induced activation, and contribute to the sexually dimorphic effects of morphine in the rat.

2.1 Introduction

Opioid therapy remains a primary strategy for severe and chronic pain management with 3-4% of adults in the US receiving long-term opioid therapy (Dowell et al., 2016); however, preclinical studies using a variety of acute and persistent pain assays have repeatedly demonstrated that morphine is more effective in males than in females (Dawson-Basoa and Gintzler, 1993; Mogil et al., 2000; Wang et al., 2006; Craft et al., 2008; Loyd et al., 2008a). Decreased analgesic efficacy is often countered with dose escalation, leading to increased risk of negative side effects associated with opiate consumption, including respiratory depression, tolerance, and overdose (Trescot et al., 2006).

Several exogenous opioids, including morphine, bind to the myeloid differentiation factor 2 (MD-2) co-receptor of the innate immune receptor toll-like receptor 4 (TLR4) (Hutchinson et al., 2010). TLR4 is located primarily on microglia (Lehnardt et al., 2002; Lehnardt et al., 2003; Jou et al., 2006; Marinelli et al., 2015) and activation of the MD2-TLR4 complex promotes the expression of pro- and anti-inflammatory compounds including cytokines (tumor necrosis factor alpha [TNF], interleukins [IL-1β, IL-6, IL-10]), chemokines (CXCL3) and prostaglandin E2 (PGE2) (Bonizzi and Karin, 2004; Doyle and O'Neill, 2006; Hutchinson et al., 2008c; Hutchinson et al., 2010). Activation of TLR4 and the release of proinflammatory signaling molecules increases neuronal excitability via upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Ogoshi et al., 2005; Stellwagen et al., 2005), downregulation of glutamate transporter (Yan et al., 2014; Eidson et al., 2016), and decreased γ-Aminobutyric acid ($GABA_A$) receptor expression, that together, induce

hyperalgesia. Therefore, morphine paradoxically reduces its analgesic efficacy through TLR4-mediated inflammation (Song and Zhao, 2001; Hutchinson et al., 2007; Hutchinson et al., 2010; Franchi et al., 2012; Li, 2012; Wang et al., 2012; Eidson and Murphy, 2013b, a; Eidson et al., 2016).

The ventrolateral midbrain periaqueductal gray (vlPAG) is a critical neural locus mediating the antinociceptive effects of morphine (Morgan et al., 2005; Loyd et al., 2007). Direct PAG administration of morphine induces long-lasting analgesia, while identical administration of the opioid antagonist (-)-naloxone or lesions of PAG μ opioid receptor (MOR) completely abolish the antinociceptive effects of systemic morphine (Loyd et al., 2008a). The antinociceptive effects of intra-PAG morphine are highly sex-dependent, such that in males, the half-maximal antinociceptive dose (ED_{50}) of morphine ranges from 1.2-1.6 μg/μl, while in females, ED_{50} values range from 16 to >50 μg/μl (Krzanowska and Bodnar, 1999; Loyd et al., 2008a; Bobeck et al., 2009).

MD-2 is densely expressed within the PAG, and administration of morphine activates PAG microglia, opposing the analgesic effects of morphine and inducing tolerance in a TLR4-dependent manner (Eidson and Murphy, 2013b, a; Eidson et al., 2016). Similarly, blockade of PAG microglial activation in male rats via TLR4 inhibition potentiates morphine analgesia and suppresses the development of tolerance (Eidson and Murphy, 2013a; Eidson et al., 2016). Here, we test the hypothesis that the sexually dimorphic effects of morphine are due to sex differences in microglia activation in the PAG.

2.2 Materials and methods

2.2.1 *General methods*

Subjects. Age matched (60-90 day old) intact male and normally cycling female Sprague Dawley rats (Charles River) were used. Animals were pair-housed with the same sex on a 12:12 h light/dark cycle (lights on at 08:00). Access to food and water was available ad libitum throughout the experiments except during behavioral 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 animals used in these experiments and to minimize pain and suffering.

Vaginal cytology. Vaginal lavages were performed daily beginning 7 days prior to testing to confirm that all female rats were cycling normally and to record cycle stage at the time of testing. 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).

2.2.2 *Experiment 1: Influence of acute morphine administration on PAG microglia expression and morphology*

To determine if morphine activation of microglia was sexually dimorphic, male and female rats received a single ED_{50} injection of (-)-morphine sulfate (5 mg/kg; National Institute on Drug Abuse (NIDA), Bethesda, MD) or saline (1 mg/kg)

subcutaneously (s.c.) and were sacrificed by transcardial perfusion 15, 30, or 60 minutes post-injection. An additional group of animals served as handled controls, resulting in a total of 6-8 animals per sex per group, total number of animals 54 males, 48 females.

Immunohistochemistry. At the end of the experiment, animals were given a lethal dose of SomnaSol® (0.5-1.0 ml/kg; i.p.) and transcardially perfused with 200-250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator followed by 300 ml of 4% paraformaldehyde in 0.1M potassium phosphate buffered saline (KPBS). Brains were post-fixed in 4% paraformaldehyde and KPBS for 48 hours and then placed in a 30% sucrose solution and stored at 4°C. Brains were sectioned coronally at 25 µm with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution at -20°C. A 1:6 series through the rostrocaudal axis of each brain was processed for ionized calcium binding adaptor molecule 1 (Iba-1) immunoreactivity using standard immunhistochemical techniques as previously described (Loyd and Murphy, 2006). Iba-1 was selected as it is specific to microglia and is constitutively expressed across all stages of activation, allowing for both qualitative and quantitative assessment in both healthy/uninjured animals as well as endotoxin- and morphine-treated animals. Briefly, sections were rinsed extensively in KPBS to remove cryoprotectant and then incubated in a rabbit anti-Iba-1 (WAKO Chemicals, Richmond, VA; 1:10K) primary antibody solution in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 72 hours at 4°C. After rinsing with KPBS, the tissue was incubated for one hour in biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch; West Grove, PA, 1:600), then rinsed with KPBS and incubated for one hour in an avidin-

biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), microglia immunoreactivity was visualized as a black or purple reaction product using nickel sulfate intensified 3,3′-diaminobenzidine (DAB) solution (2mg/10ml) containing 0.08% hydrogen peroxide in sodium acetate buffer. Following incubation in DAB, tissue was rinsed in sodium acetate buffer followed by KPBS. Sections were then mounted out of KPBS onto gelatin-subbed slides, air-dried and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in xylene and glass cover-slipped using Permount mounting medium.

Densitometry and presentation. Levels of Iba-1 immunoreactivity in the vlPAG were compared across sex and treatment groups using semiquantitative densitometry as previously described (Loyd et al., 2008a; Laprairie and Murphy, 2009; Eidson and Murphy, 2013a). Twelve-bit grayscale images were captured using QImaging Retiga EXi CCD camera attached to a Nikon microscope and iVision Image analysis software (BioVision Technologies). The region of interest included sections through six representative levels of the rostrocaudal axis of the vlPAG (Bregma -6.72, -7.04, -7.64, -8.0, -8.30, -8.80), with 6-12 sections per brain as previously described (Loyd et al., 2007). As the distribution of glial cells was bilaterally symmetrical in the vlPAG, sections were sampled unilaterally.

Morphological Analysis of Microglia Subtype. Microglia morphology is highly representative of functional state (Karperien et al., 2013). Microglia were sampled as described above (2-3 representative sections of the caudal ventrolateral PAG (Bregma -7.64 to -8.3) per animal) and classified into one of three principle morphological subtypes: “non-ramified” cells, “intermediate” cells, and “ramified” cells. Non-ramified

cells are characterized as having a large circular or oval cell body that is either completely amoeboid in shape or with 1 or 2 thick primary processes and no secondary processes. Highly ramified "intermediate" cells were characterized as having many stout, thick processes arranged in bundles around a large cell body. "Intermediate" cells often have several thick primary processes, extending a relatively short distance from the soma; each primary process often has many short secondary processes, giving these cells a "bushy" appearance that are very darkly stained with Iba-1. In contrast to non-ramified and intermediate cells, highly ramified cells have small round cell bodies with many (generally 4-10) long, thin processes. The primary processes of highly ramified microglia have few secondary processes, extend a large distance from the soma, and are often lightly stained with Iba-1. Representative images of each cell type are provided in Figure 2.2.1. Non-ramified and intermediate microglia are considered to be "active" or "reactive", while highly ramified microglia with thin processes are considered to be quiescent (Frank et al., 2006; Bland et al., 2010; Colton and Wilcock, 2010; Kettenmann et al., 2011; Karperien et al., 2013; Lenz et al., 2013). Iba1-positive cells were counted only if the cell body and processes were completely visible within the image. Ambiguous cells with processes extending beyond the borders of the image, overlapping cells, and cells out of the plane of focus of the image were not counted.

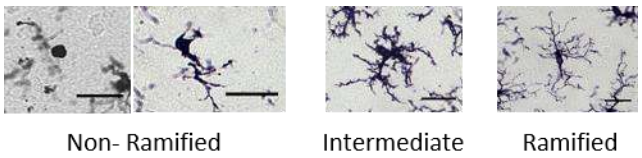

Figure 2.2.1. Microglial Subtypes.
Representative images of the three classifications of microglia based on morphology. "Non-ramified": round/amoeboid activated microglia; "Intermediate": reactive microglia with numerous thick processes; "Ramified" microglia with thin long processes. Scale bars represent 2µm in all images.

Data Analysis. Morphological cell counts were conducted using ImageJ Cell Counter tool by three independent investigators blind to treatment and sex of the animal. Total number of microglia were also determined to assess changes in microglia migration or proliferation. Morphological cell counts, and total microglia number are presented as the mean ± SEM. Statistical analyses were performed using SPSS software (Version 21); data were analyzed using an ANOVA to determine significant main effects of sex and treatment on microglia cell counts and morphology. Bonferroni's post hoc test was used to determine *a priori* specified specific group differences when a significant main effect was observed. $p \leq 0.05$ was considered statistically significant.

2.2.3 *Experiment 2: Correlation between microglia activation and morphine ED_{50}*

To determine if there was a relationship between microglia morphological subtype and morphine ED_{50} values, animals (n= 15 males, 8 females) were tested using our cumulative dosing paradigm (Morgan et al., 2006; Loyd et al., 2008a; Eidson and Murphy, 2013a; Eidson et al., 2016). Briefly, animals received an injection of morphine every 20 minutes, resulting in cumulative doses of 1.8, 3.2, 5.6, 8.0, 10.0, and 18 mg/kg. Control animals received repeated saline injections (1 ml/kg; s.c; n= 6 males, 7 females). Thermal nociception was assessed using the paw thermal stimulator 15 minutes after each injection (Hargreaves et al., 1988; Wang et al., 2006; Loyd et al., 2008b; Eidson and Murphy, 2013a). Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. A radiant beam of light is positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL) in

seconds. A maximal PWL of 20 s was used to prevent tissue damage due to repeated application of the noxious thermal stimulus. Animals were acclimated to the testing apparatus 30-60 minutes per day for three consecutive days prior to the start of the experiment and on the day of testing. All behavioral testing took place between 12:00 and 17:00 (lights on at 08:00). Temperature of the thermal stimulus was recorded before and after each trial to maintain consistent recordings between groups and did not exceed 64°C throughout the course of the experiments. All testing was conducted blind with respect to group assignment. To calculate ED_{50}, PWL data was normalized using GraphPad PRISM software, such that each individual animal's baseline PWL score = 0% and 20s = 100%. The normalized data was then plotted and ED_{50} was calculated as the point on the X-axis that corresponds with 50% maximal PWL (Morgan et al., 2006; Eidson and Murphy, 2013a; Eidson et al., 2016).

Immunohistochemistry. Within 60 minutes of the final morphine dose, animals were perfused and tissue was collected for immunohistochemical analyses. Microglia were categorized in the vlPAG as described above. To determine if morphine's impact on glia morphology was limited to the PAG or represented a more centralized effect, microglia were also characterized by morphological type for the following brain regions: rostral ventromedial medulla (RVM; Bregma -11.2:-12.2; implicated in pain modulation); superior colliculus (Bregma -6.9:-8.2; high MOR density but not implicated in pain); and substantia nigra (Bregma -5.0:-5.3; high levels of MOR and implicated in reward and addiction). The medial amygdala (Bregma -2.4:-3.0) was also analyzed as this region has a high density of microglia, but is not implicated in pain and is devoid of MOR.

Data analysis and presentation. Morphine ED_{50} values were compared between males and females using Mann Whitney U Test to confirm replication of previously observed sex differences (Loyd et al., 2008b; Loyd et al., 2008a). Analysis of covariation between ED_{50} values and microglial morphological subtype were assessed for each sex using SPSS statistical software; $p \leq 0.05$ was considered significant.

2.2.4 *Experiment 3: Influence of peripheral endotoxin LPS on microglia response in the vlPAG*

To determine if sex differences in microglia activation are driven by morphine or reflected a global sex difference in activation, animals received an injection of the TLR4 agonist lipopolysaccharide (endotoxin LPS; 1 mg/kg, i.p.; Sigma; *n*=7 males, 6 females) or equivolume saline (*n*= 7 males, 6 females). Comparable doses of peripheral LPS have been shown in male rats to increase the proinflammatory cytokine IL-1□ mRNA in the brain (Buttini and Boddeke, 1995; Nguyen et al., 1998; Quan et al., 1998; Quan et al., 1999) and alter microglia morphology (Buttini et al., 1996). LPS-induced febrile responses were monitored using Thermocron iButtons (Maxim Integrated, San Jose; model DS1921G), implanted into the abdominal cavity 7-10 days prior to testing. Body temperature was recorded in 30-minute intervals beginning 6 hours prior to LPS. Animals were sacrificed and transcardially perfused six hours following LPS administration, when central cytokine mRNA levels peak (Nakamura et al., 1999; Turrin et al., 2001; Qin et al., 2007; Czerniawski and Guzowski, 2014). Tissue was collected and processed for visualization of microglia (Iba-1) as described above. Iba-1 levels were analyzed for the PAG, RVM, SC, SN, and MeA. A subset of animals were

sacrificed by decapitation and their brains flash frozen for determination of cytokine levels using qPCR as previously described (Eidson et al., 2016).

Examination and presentation of immunomodulatory cytokines using qPCR. Flash frozen brains were sectioned at 300 µm on a cryostat (Leica) and mounted onto slides. One-millimeter bilateral micropunches were taken from 6 levels of the vlPAG (Bregma -6.72, -7.04, -7.64, -8.0, -8.30, -8.80) and RNA was extracted with TRIzol (Life Technologies; 15596026) using standard procedures, followed by the addition of Glycoblue (Life Technologies; AM5916) for visualization. Concentrations of RNA (ng/µl) were calculated using a NanoDrop ND-1000 Spectrophotometer (Version 3.8, Thermo Fisher; DE). Following RNA extraction, RNA was diluted to a standard concentration and converted to cDNA using an AMV First-Strand Synthesis Kit (Invitrogen). PCR was performed using FastStart Essential DNA Green MasterMix (Roche) and analyzed using a Roche LightCycler 96 and accompanying software (Version 1.1.0.1320, 2011 Roche Diagnostics; Switzerland). Data are presented as the normalized ratio of the target gene, with corrected amplification efficiency, relative to the GAPDH control gene. Primer sequences can be found in Table 1.

Data Analysis. The impact of sex and treatment on microglia morphology and cytokine mRNA levels were analyzed using an ANOVA, with Bonferroni post-hoc analysis where appropriate. Differences between males and females were compared using independent t-tests when k=2. Body temperature data were analyzed for significant main effects of sex and treatment across time using a mixed model ANOVA with Greenhouse-Geisser correction. Body temperature data are presented as the difference between saline-treated groups at 1-hour intervals post-LPS. Density,

morphology, and PCR data are presented as ΔCq means ±SEM; $p \leq 0.05$ was considered significant.

Table 1 Chapter 2 Primer Sequences for qPCR of Inflammatory Markers

GAPDH	Forward	GAG GTG ACC GCA TCT TCT TG
	Reverse	CCG ACC TTC ACC ATC TTG TC
IL-1β	Forward	CCC TGA AGG ATG TGA TCA TTG
	Reverse	GGC AAA GGG TTT CTC CAC TT
IL-6	Forward	AAG ACC CAA GCA CCT TCT TT
	Reverse	AGA CAG CAC GAG GCA TTT TT
IL-10	Forward	TGT ACC TTA TCT ACT CCC AGG TTC TCT
	Reverse	GTG TGG GTG AGG AGC ACG TA
TNF	Forward	TGT ACC TTA TCT ACT CCC AGG TTC TCT
	Reverse	GTG TGG GTG AGG AGC ACG TA
TLR4	Forward	TCC CTG CAT AGA GGT ACT TC
	Reverse	CAC ACC TGG ATA AAT CCA GC

2.2.5 *Experiment 4: Effect of TLR4 manipulation on morphine analgesia*

Intra-vlPAG cannulae implantation. To determine if PAG TLR4 activation or inhibition impacted morphine analgesia, animals were anesthetized to a deep surgical plane with 5% isoflurane (maintained at 2–5% isoflurane throughout surgery; Henry Schein Animal Health) and bilateral guide cannulae (22 gauge; Plastics One) aimed at the vlPAG (anterior—posterior: 1.7 mm; mediolateral: ±0.6 mm; dorsoventral: −5.0 mm from lambda) were implanted stereotaxically as previously described (Loyd et al., 2008a; Eidson and Murphy, 2013a). Animals were allowed to recover a minimum of 10 days post-cannula implantation before behavioral testing. Injection cannulae were

inserted into guide cannulae once a day for three days prior to testing to acclimate the animals to the injection procedure and maintain cannulae patency. Animals with blocked cannula were retained as no injection controls. No significant differences were observed between non-injected animals (n= 2 males, 5 females) and those animals receiving intra-PAG saline, so these groups are collapsed (data not shown).

Experiment 4.1: Effects of TLR4 agonism on morphine analgesia. On the day of testing, male and female rats received a single intra-PAG injection of LPS (5.0 µg/0.5 µl/side; Sigma) or saline (0.5 µl/side) into the PAG. This dose has been previously shown to increase glial activation and cytokine expression (Castano et al., 2002; Hernandez-Romero et al., 2008; Eidson and Murphy, 2013a). One hour later, morphine ED_{50} values were determined using the cumulative dosing paradigm described in Experiment 2. A total of 4 treatment groups were generated: Saline+Saline (*n*=5 males, 5 females), LPS+Saline (*n*=5 males, 5 females), Saline+Morphine (*n*=8 males, 11 females), and LPS+Morphine (*n*=7 males, 8 females).

Experiment 4.2: Effects of TLR4 antagonism on morphine analgesia. On the day of testing, animals received a single intra-PAG injection of (+)-naloxone (5.0 µg/0.5 µl/side; NIDA) or saline (0.5 µl/side); one hour later morphine ED_{50} values were determined as described above. This dose was chosen based on our previous experiments demonstrating inhibition of PAG microglia (Eidson and Murphy, 2013a). A total of 4 treatment groups were generated: Saline+Saline (*n*=5 males, 5 females), Naloxone+Saline (*n*=5 males, 5 females), Saline+Morphine (*n*=8 males, 11 females), and Naloxone+Morphine (*n*=7 males, 9 females).

Data analysis and presentation. At the end of the experiment, brains were removed, flash frozen, and sectioned at 25 μm with a Leica CM3050S cryostat. Sections from the injection site were mounted on to slides, Nissl stained, coverslipped, and cannulae placement verified using a Nikon microscope (10X magnification). Animals with bilateral cannulae located outside of the vlPAG (e.g., in the aqueduct or deep mesencephalic nucleus) were considered "cannulae misses" and analyzed for determination of site specificity.

Half-maximal antinociceptive effect (ED_{50}) and 95% confidence intervals (CI) were calculated from dose–response curves generated using Graph-Pad PRISM software as described above. Repeated measures ANOVA was used to assess for significant treatment effects, with Bonferroni's *post hoc* tests where appropriate. Cannula misses were compared to "hits" using a two-tailed Wilcoxon signed-rank test. Values of $p \leq 0.05$ were considered statistically significant.

2.3 Results

2.3.1 *Experiment 1: Sex, but not morphine treatment, affects microglia activation in the vlPAG*

Male and female rats were administered morphine or saline, and microglia were examined immunohistochemically within the caudal vlPAG at 15, 30, and 60 minutes post-injection. A separate group of rats served as handled controls. No significant effect of time post-injection was noted in microglia morphology for either the saline or morphine treated groups so these data are collapsed across time. Additionally, no significant differences were observed in total microglia cell counts in the PAG, regardless of sex ($F_{(1,68)}$=0.013, p= 0.91) or treatment ($F_{(1,68)}$=0.13, p= 0.720; Figure 2.3.1a).

Overall, females had significantly more non-ramified and intermediate type microglia in the vlPAG that was independent of treatment ($F_{(1,76)}$ = 13.10, p= 0.01; Figure 2.3.1b). We next determined if the observed sex difference in percentage of activated microglia was specific for a morphological subtype (non-ramified, intermediate or ramified; see Figure 2.3.1). The percentage of both non-ramified (t= 3.10, p< 0.001) and intermediate (t= 2.17, p= 0.03) microglia was significantly higher in females than males (Figure 2.2c). Females also had significantly fewer ramified microglia than males (t= 3.90, p< 0.001). Together, these data indicate that although there are no sex differences in the overall number of vlPAG microglia, the ratio of non-ramified and intermediate to ramified microglia are significantly greater in females than males, regardless of treatment.

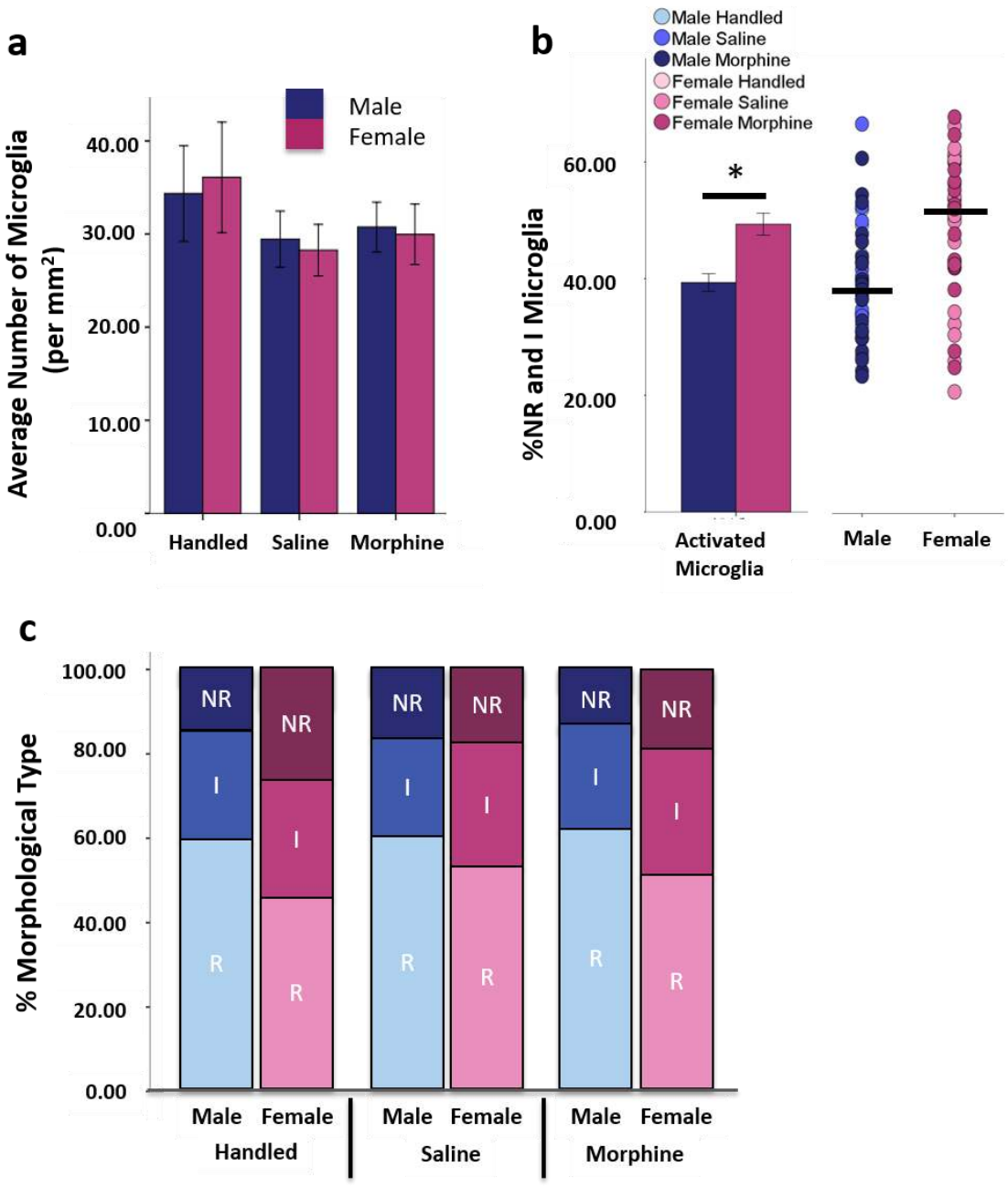

Figure 2.3.1. Sex, but Not Morphine Treatment or Time, Affects Microglia Activation in the vlPAG.
(a) No significant differences in microglia cell number were noted as a function of sex, time (collapsed) or treatment (handled [n= 5 males, 6 females], saline [*n*= 15 males, 17 females], morphine [*n*= 22 males, 17 females]). (b) Females had significantly more non-ramified/amoeboid [NR] and intermediate [I] microglia than males regardless of treatment ($t(80)$=3.90, p<0.001). As shown in the scatterplot at right, the percentage of non-ramified/amoeboid [NR] and intermediate [I] microglia were highly variable within each sex (line indicates median). (c) Females had significantly more non-ramified [NR] and intermediate [I], and fewer ramified [R] microglia in the vlPAG than males.

2.3.2 *Experiment 2: Microglial activation in the PAG correlates with morphine ED_{50}.*

PAG or spinal cord activation of microglia has been shown to oppose morphine analgesia (Watkins et al., 2005; Hutchinson et al., 2010; Eidson and Murphy, 2013b, a); therefore, we next examined the degree of association between morphine ED_{50} values and PAG microglia morphology for males and females. Animals were administered cumulative doses of morphine and sacrificed within sixty minutes of the final injection of morphine; microglia morphology was determined immunohistochemically. Consistent with our previous studies (Loyd et al., 2008b; Loyd et al., 2008a), ED_{50} values were significantly higher in females than males (female, 8.69 vs. male, 6.01 mg/kg; U = 30.5, p= 0.028), and no relationship was observed between ED_{50} and female estrus cycle (r= 0.19, p= 0.19). Morphine ED_{50} values were significantly correlated with the percentage of non-ramified and intermediate microglia, morphologies typical of "activated" microglia, in the vlPAG in both males (r=0.58, p=0.02) and females (r=0.68, p=0.047; Figure 2.3.2a). Further analysis by microglia morphological subtype showed no significant correlation for non-ramified microglia and morphine ED_{50} in males (r= 0.47, p= 0.051) or females (r= 0.32, p= 0.24). Intermediate microglia correlated significantly with ED_{50} in females (r= 0.679, p= 0.047) but not males (r= 0.316, p= 0.15), and ramified microglia negatively correlated with ED_{50} in both males (r= -0.58, p= 0.02) and females (r= -0.68, p= 0.047; Figure 2.3.2b). Together, these data show sex-specific positive correlations between morphine ED_{50} and percentage of activated microglia, and reciprocal negative correlations with morphine ED_{50} and ramified microglia, such that as the percent of

ramified microglia decreases, morphine ED_{50} increases (i.e. morphine is less efficacious).

We next determined if the relationship between microglia morphology and morphine ED_{50} values was specific to the PAG, or extended to regions also implicated in pain (RVM), and/or regions with high levels of MOR (SN, SC) or a high density of microglia (MeA). For females, no significant correlation between morphine ED_{50} and the percentage of non-ramified and intermediate microglia were observed in RVM (r= 0.21, p= 0.32), amygdala (r= 0.48, p= 0.12), superior colliculus (r= -0.05, p= 0.46), or substantia nigra (r= 0.09, p= 0.44). Similarly, in males, no significant correlations were observed in the RVM (r= -0.13, p= 0.35), amygdala (r= 0.23, p= 0.24), or substantia nigra (r= 0.32, p= 0.20). However, significant correlations between morphine ED_{50} and percentage of non-ramified and intermediate microglia were noted in superior colliculus of male rats (r= 0.53, p= 0.05; Figure 2.3.2c-g).

Similar to what was observed for the PAG, no significant differences were observed in the total number of microglia in any of the brain regions examined ($F_{(3,28)}$= 0.152, p= 0.93; data not shown), indicating that the difference in the number of activated cells between males and females is not due to increased proliferation or migration of activated microglia. Rather, these data indicate that morphine ED_{50} positively correlates with the overall percent of non-ramified and intermediate microglia, states typical of activation, in the PAG in males and females, with sex-specific differences in correlations noted for the superior colliculus.

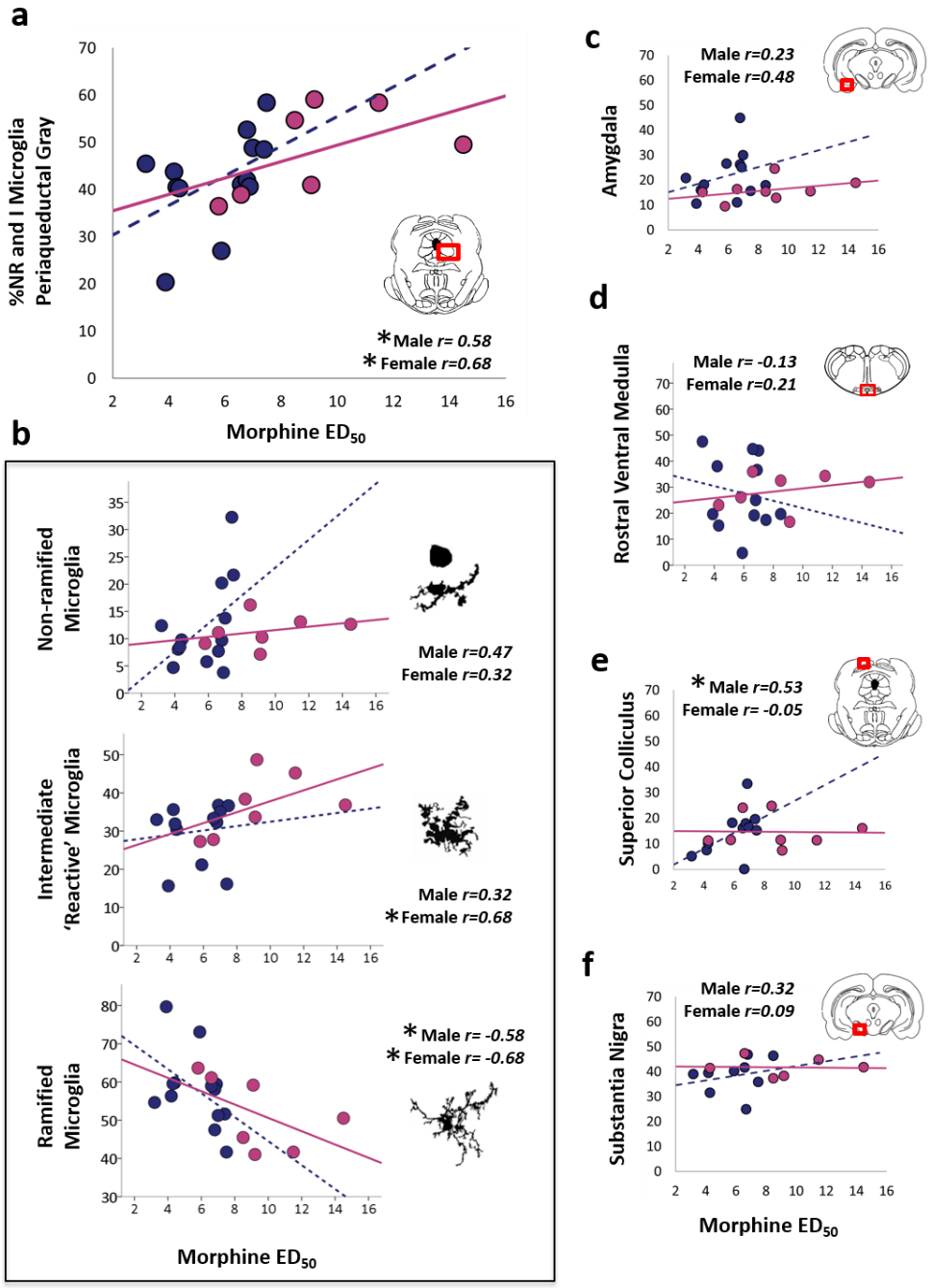

Figure 2.3.2. Percent Activated Microglia Correlate with Morphine Effective Dose.
Bivariate correlations of morphine ED_{50} for the percentage of (a) total non-ramified and intermediate microglia and (b) by morphological subtype within the PAG. Degree of relatedness for morphine ED_{50} and microglia morphology are also shown for the (c) amygdala, (d) rostral ventral medulla, (e) superior colliculus, and f) substantia nigra. *n*= 15 males, 8 females.

2.3.3 *Experiment 3a: Immune Challenge Activates vlPAG Microglia to a Greater Extent in Females than in Males*

The results from the above experiment show that although there were significantly more microglia with a non-ramified or intermediate morphology in females than males, these microglia were unaffected by morphine treatment. Therefore, we next tested if administration of LPS, a TLR4 agonist, activated vlPAG microglia in a sex-dependent manner. Peripheral administration of LPS induced a significant increase in the percent of non-ramified and intermediate microglia in the vlPAG of females (19%), but not males (12%; Figure 2.3.3a); significant main effect of sex ($F_{(1,22)}$=18.26, $p<$ 0.001) and treatment ($F_{(1,22)}$= 11.84, $p<$ 0.001). Similar to what was noted in Experiment 1, females had more non-ramified and intermediate microglia in the PAG at baseline (p=0.03).

Analysis of microglia by morphological subtype showed a significant main effect of both sex ($F_{(2, 44)}$= 13.14, p= 0.001) and treatment ($F_{(2,44)}$ = 6.44, p= 0.001) on microglia morphology and no interaction ($F_{(2,44)}$= 0.55, p= 0.58). Specifically, saline treated females had significantly more intermediate (t= 2.43, p= 0.05), and significantly fewer ramified (t= 2.83, p= 0.03) microglia than saline males. Similarly, LPS-treated females have significantly more intermediate (t= 2.95, p= 0.01), and significantly fewer ramified (t= 3.10, p= 0.01) microglia than LPS males (Figure 2.3.3b), suggesting a more "activated" morphological state in females. The observed sex difference in microglia morphology in the PAG was not accompanied by differences in sickness behaviors as no sex differences in core body temperature were observed in animals treated with LPS ($F_{(1,13)}$=2.22, p= 0.16; Figure 2.3.3d). These data suggest that in females, PAG microglia

may be more responsive to TLR4 activation (i.e. lower threshold) than their male counterpart.

No significant main effects of sex on total number of non-ramified and intermediate microglia were observed in any other brain region examined (Figure 2.3.3e). In contrast to what was noted with morphine, main effects of LPS-treatment were observed in several brain regions, including the superior colliculus ($F_{(1,20)}$=10.862, $p<$ 0.001) in males ($p=$ 0.01) but not females (p=0.08), and the RVM ($F_{(1,15)}$=5.76, $p=$ 0.03) in males ($p=$ 0.03) but not females ($p=$ 0.32). No significant main effects of LPS treatment were observed in the amygdala or substantia nigra, and no interaction effects were observed in any brain region tested. Finally, no significant differences were observed in the total number of microglia in any brain regions examined (data not shown).

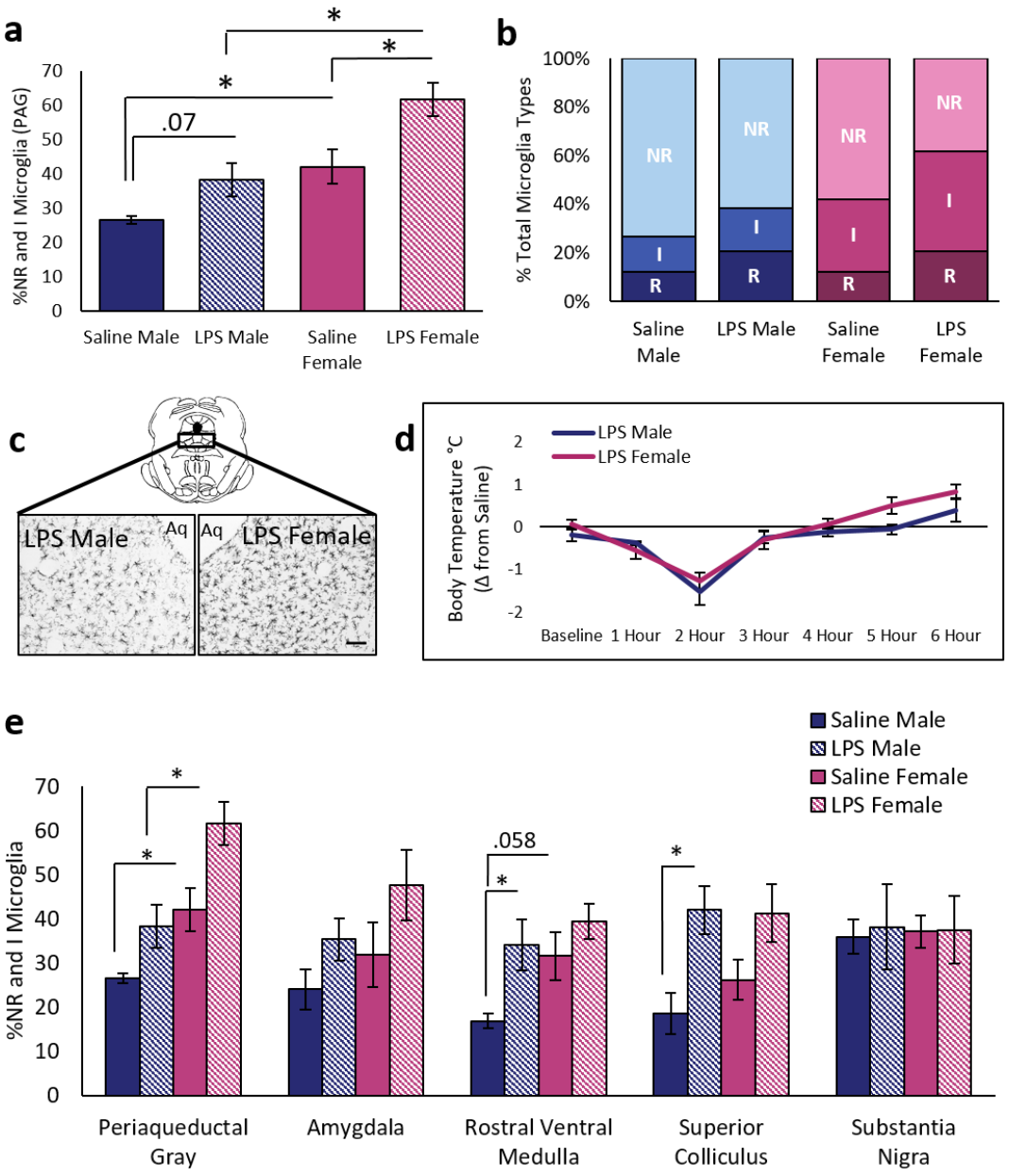

Figure 2.3.3. Treatment with TLR4 Agonist Lipopolysaccharide Increases Microglia Activation in Females. (a) LPS induces greater proportions of non-ramified/amoeboid [NR] and intermediate [I] microglia in the PAG of females than males. (b) LPS-treated females had a higher percent of intermediate (I) microglia in the vlPAG than saline-treated females and show decreased percent of ramified (R) microglia compared with all other groups. (c) Representative images of microglia in the vlPAG of an LPS-treated male and female. Note that females have a greater density of non-ramified and intermediate microglia than males. Scale bar = 5µm. (d) No sex differences in febrile response were noted following LPS administration. (e) Percentage of non-ramified and intermediate microglia for males and females following saline or LPS administration. LPS-induced increases in activated microglia were region- and sex-dependent. Endotoxin LPS, n=7 males, 6 females; saline, n= 7 males, 6 females.

2.3.4 *Experiment 3b: Immune challenge induces greater cytokine expression in the vlPAG of females*

To determine if the observed sex differences in PAG microglia morphology was accompanied by sex-specific differences in proinflammatory cytokine transcription, qPCR was used to quantify PAG mRNA levels of pro-inflammatory TNF, IL-1β, IL-6, and anti-inflammatory IL-10 (Figure 2.3.4). LPS induced a significant increase in vlPAG TNF transcription in both males ($p < 0.001$) and females ($p < 0.001$) compared with saline controls ($F_{(1,42)}= 50.72$, $p< 0.001$). LPS treatment also induced in a significant increase in IL-1β transcription in females ($p<0.001$) and males ($p<0.001$); significant main effect of treatment ($F_{(1,38)} = 47.08$, $p= 0.00$). Notably, IL-1β levels were significantly higher in LPS treated females than males ($p=0.03$). LPS did not significantly change IL-6 in either sex ($F_{(1,34)}= 0.19$, $p= 0.67$). PAG transcription of the anti-inflammatory cytokine IL-10 was significantly higher in males than in females ($F_{(1,44)}= 14.99$, $p< 0.001$). LPS induced a significant decrease in IL-10 mRNA in females ($t=3.33$, $p< 0.001$) but not males (*$t=-0.19$, $p=0.86$*; Figure 2.3.4e). Together, these data demonstrate that PAG TLR4 activation induces an exaggerated pro-inflammatory response and a reduced anti-inflammatory response in females compared with males. Furthermore, these data support our immunohistochemical studies demonstrating that changes in microglial morphology are accompanied by measurable changes in pro-inflammatory cytokine mRNA.

We also examined LPS induced changes in PAG TLR4 transcription. LPS administration increased TLR4 transcription by 67% in females and 39% in males,

however these differences were not statistically significant: treatment, $F_{(1,40)}= 2.37$, $p= 0.13$; sex, $F_{(1,40)}= 2.37$, $p= 0.13$.

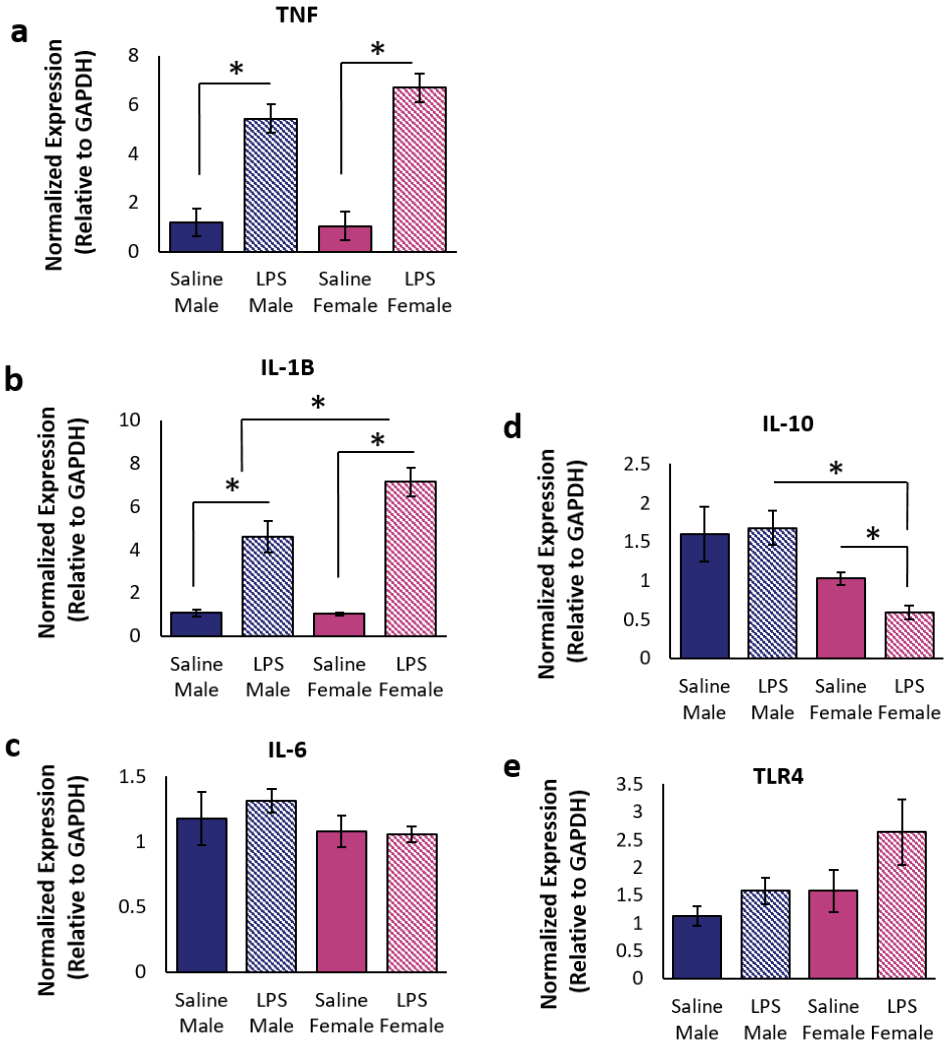

Figure 2.3.4. LPS-induced Changes in Cytokine and TLR4 Transcription in the vlPAG of Males and Females.

Peripheral administration of LPS increased the transcription of (a) TNF and (b) IL-1β in the vlPAG. No change in IL-6 was noted (c). Transcription of the anti-inflammatory cytokine IL-10 significantly decreased in females following LPS (d). No differences in TLR4 transcription (e) were noted for sex or treatment. Endotoxin LPS, n=5 males, 5 females; saline, n= 3 males, 3 females.

2.3.5 *Experiment 4. Manipulation of PAG TLR4 signaling has sex-dependent effects on morphine ED_{50} values*

Our previous studies established a direct link between microglia activation and morphine efficacy, such that site specific administration of the TLR4 agonist LPS resulted in a significant reduction in morphine efficacy (as indicated by a rightward shift in the morphine dose-response curve (Eidson and Murphy, 2013a). Given the results of the present experiments demonstrating that females show more non-ramified and intermediate morphologies than males, we next examined if this sex difference in microglia morphology would result in a sexually dimorphic shift in morphine ED_{50}. In particular, we tested if activation of vlPAG TLR4 via site specific administration of the endotoxin LPS would result in a larger rightward shift in the morphine dose-response curve in females compared with males. We further examined if blockade of TLR4 signaling with (+)-naloxone would result in a greater leftward shift of the morphine dose-response curve in females, indicative of potentiation of morphine antinociception in females.

Intra-PAG administration of LPS resulted in a significant decrease in morphine antinociception in both males and females, as indicated by a significant rightward shift in the morphine ED_{50} dose-response curve ($F_{(5,30)} = 18.01$, $p < 0.0001$; Figure 2.3.5a). LPS increased male ED_{50} values from 3.04 to 10.69 mg/kg, a 3.5 fold difference, $p < 0.001$; female ED_{50} values increased from 7.9 to 20.61 mg/kg, a 2.6 fold difference, $p < 0.05$. ED_{50} values were approximately two-fold higher in LPS+Morphine females than males (20.61 in females vs. 10.69 mg/kg in males; $p > 0.05$). Remarkably, following LPS priming, 6/8 females continued to respond at baseline levels following morphine

administration at 10 mg/kg; 2 of those 6 females responded near baseline following the 18 mg/kg dose. Indeed, females in the LPS+Morphine treated groups were not significantly different from saline control females that received no morphine (Saline+Saline females; $p>0.05$), indicating that LPS pre-treatment completely abolished the antinociceptive effects of morphine in a subset of females. No differences in response latencies were observed between LPS+Morphine males and Saline+Morphine females ($p> 0.05$), indicating that LPS activation of microglia reduced males to 'female-typical' levels of analgesia.

No sex differences were observed between Saline+Saline treated males and females ($p> 0.05$), and no significant differences were observed between LPS+Saline and Saline+Saline males ($p> 0.05$) or females ($p>0.05$), indicating LPS administration alone had no effect on basal PWL, consistent with what we have previously observed (Eidson and Murphy, 2013a). Together, these results indicate that microglia activation with LPS significantly decreases, or completely abolishes (in 33% of females), the antinociceptive effects of morphine.

We next determined if blocking microglia activation with the TLR4-specific antagonist (+)-naloxone potentiated morphine analgesia in a sex-dependent manner. In animals treated with morphine, intra-PAG administration of (+)-naloxone significantly increased antinociception in females, but not males, as indicated by a leftward shift in the morphine dose response curve ($F_{(3,18)} = 13.67$, $p<0.0001$; Figure 2.3.5b). Specifically, ED_{50} values for females treated with (+)-naloxone decreased from 7.9 mg/kg to 3.16 mg/kg ($p< 0.001$), a 2.5-fold reduction in ED_{50}. Importantly, ED_{50} values for (+)-naloxone+Morphine treated females are not significantly different from

Saline+Morphine treated males ($p > 0.05$), indicating that blocking microglia activation within the vlPAG completely abolishes sex differences in morphine analgesia. A moderate, but significant, increase in morphine ED_{50} was observed in males receiving (+)-naloxone+Morphine compared with Saline+Morphine males (ED_{50} increased from 3.04 mg/kg vs. 5.25 mg/kg; $p < 0.05$).

No sex differences between Saline+Saline treated males and females ($p > 0.05$) was observed. In addition, we observed no significant differences between (+)-naloxone+Saline and Saline+Saline controls in males ($p > 0.05$) or females ($p > 0.05$) indicating (+)-naloxone administration has no effect on basal nociceptive thresholds. The effects of (+)-naloxone on morphine analgesia are site-specific; male and female animals receiving (+)-naloxone outside the PAG showed significantly lower morphine antinociception relative to animals that received (+)-naloxone within the PAG ($F_{(3,18)} = 15.17$, $p < 0.001$; data not shown). Together, these data indicate inhibition of vlPAG microglia activity with the TLR4-specific antagonist (+)-naloxone is sufficient to reverse sex differences in morphine analgesia.

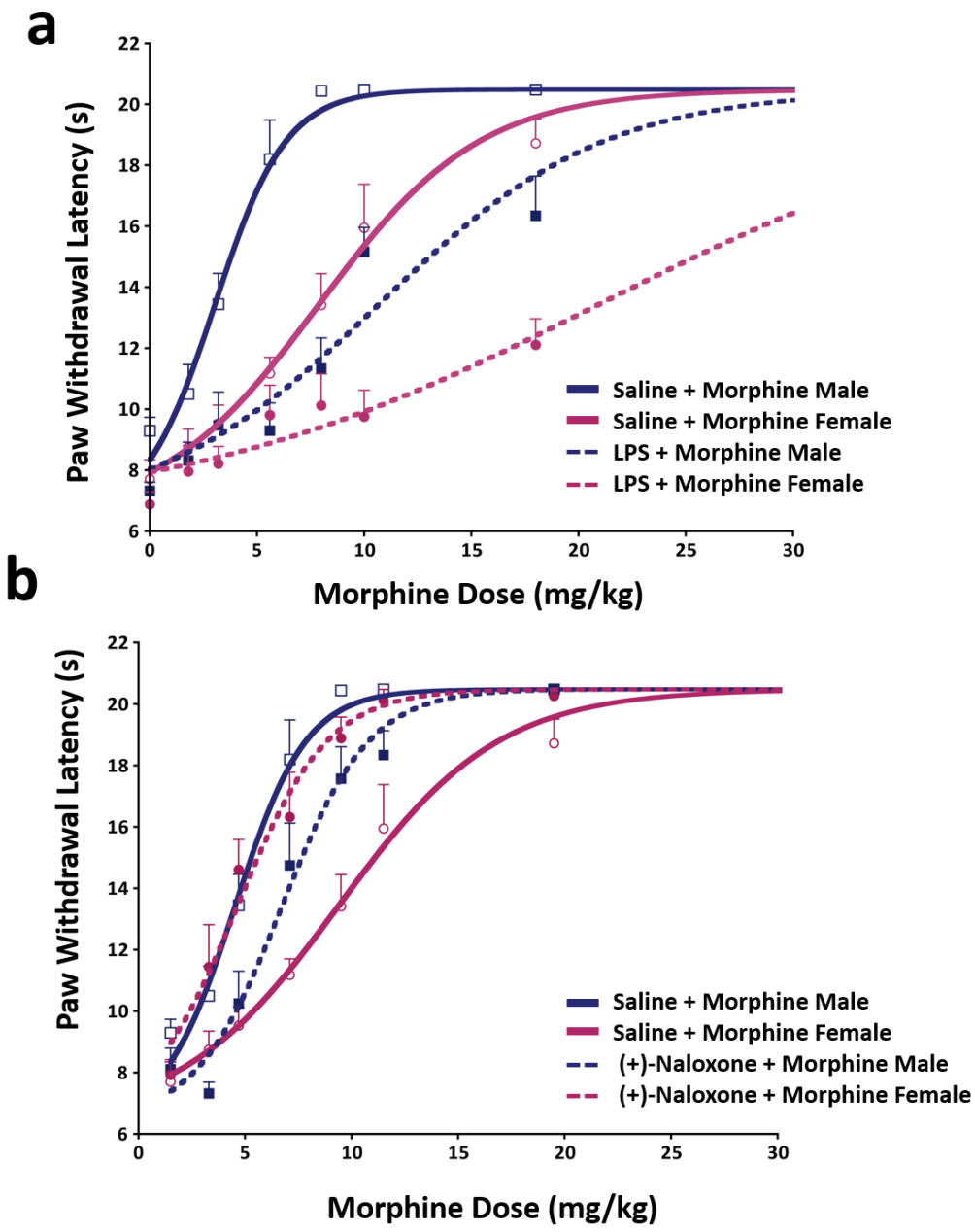

Figure 2.3.5. Pharmacological Blockade or Activation of vlPAG Microglia has a Sex-dependent Impact on Morphine Antinociception.

(a) LPS-induced activation of vlPAG microglia significantly attenuates the antinociceptive effects of morphine in both males and females, as indicated by a significant rightward shift in the morphine dose response curve. Saline+Saline (*n*=5 males, 5 females), LPS+Saline (*n*=5 males, 5 females), Saline+Morphine (*n*=8 males, 11 females), and LPS+Morphine (*n*=7 males, 8 females). (b) In contrast, inhibition of TLR4 via intra-vlPAG (+)-naloxone results in a significant leftward shift in morphine dose-response curves in females, such that (+)-naloxone + Morphine-treated females are not significantly different from Saline + Morphine-treated males. Naloxone+Saline (*n*=5 males, 5 females), Naloxone+Morphine (*n*=7 males, 9 females).

2.4 Discussion

Here we test the hypothesis that sex differences in vlPAG microglia contribute to the sexually dimorphic effects of morphine. We report that although no sex differences in overall number or density of microglia were noted for the vlPAG at baseline, the percentage of non-ramified and intermediate versus “ramified” microglia were significantly higher in females than males, suggesting that females have a more 'activated” microglial state within the vlPAG. Consistent with our previous studies, morphine ED_{50} values were significantly higher in females (Loyd et al., 2008b; Loyd et al., 2008a) , and a significant relationship was observed between morphine potency and percentage of reactive “intermediate” microglia in females, but not males. Although acute morphine treatment did not change microglia morphology in either sex, administration of the glial TLR4 agonist LPS increased the percentage of non-ramified and intermediate microglia in the vlPAG of females to a greater degree than males, an effect that was independent of febrile response. The LPS induced increases in microglia activation in females was accompanied by significantly increased proinflammatory IL-1β transcription and decreased anti-inflammatory IL-10 transcription. We further show that priming microglia with LPS significantly attenuates morphine analgesia in both sexes, and completely abolishes the antinociceptive response to morphine in a subset of females. Similarly, inhibition of vlPAG microglia with the TLR4 antagonist (+)-naloxone significantly potentiates morphine analgesia in females, but not males, abolishing the sex difference in opiate response. Together, these data indicate that vlPAG microglia are innately different in males and females in terms of their morphological state (both

basal and following immune activation with LPS), and implicate TLR4 in the attenuated response to morphine observed in females.

These studies are the first to implicate vlPAG microglial TLR4 in sex differences in morphine analgesia, and suggest that increased activation of vlPAG microglia contribute to the attenuated response to morphine observed in females. Importantly, these experiments establish that inhibition of vlPAG TLR4 in females reverses the observed sex differences in morphine analgesia. Together, these data suggest novel methods to improve current morphine-based pain management via inhibition of TLR4, and illustrate the necessity for sex-specific research and individualized treatment of pain in men and women.

2.4.1 *Sex differences in morphine analgesia and TLR4*

Preclinical studies utilizing orofacial, somatosensory or visceral pain assays typically report that morphine produces a significantly greater degree of analgesia in males versus females (Craft, 2003; Craft et al., 2004; Ji et al., 2006; Wang et al., 2006; Loyd et al., 2008a). Consistent with the present results, these studies also report morphine ED_{50} values that are 2-fold higher in females than in males. The present studies demonstrate that sex differences in PAG microglia, and in particular TLR4 signaling, contribute to the dimorphic response to morphine. TLR4 signaling has been previously shown to modulate morphine action in males (Tanga et al., 2005; Hutchinson et al., 2007; Hutchinson et al., 2008b; Hutchinson et al., 2008a), such that TLR4 activation with LPS reduces morphine efficacy (Johnston and Westbrook, 2005). The present studies confirm these previous findings in males, and further demonstrate that initiation of TLR4 signaling significantly, and almost completely, attenuates morphine

analgesia in females. In addition, blockade of vlPAG TLR4 increases the analgesic efficacy of morphine in females (2.5-fold reduction in morphine ED_{50}), reversing the observed sex difference in morphine response.

Several factors have been shown to contribute to the sexually dimorphic response to morphine. Interestingly, these factors are also heavily impacted by microglia. For example, blockade of N-methyl-d-aspartate (NMDA) signaling inhibits microglia activation (Thomas and Kuhn, 2005; Murugan et al., 2011) and enhances the antinociceptive effects of morphine to a greater degree in male than female rats (Holtman et al., 2003). Sex differences in MOR expression and signaling in the PAG (Bernal et al., 2007; Loyd et al., 2008a), as well as spinal MOR:KOR dimerization (Chakrabarti et al., 2010), have also been shown to contribute to the dimorphic effects of morphine. MOR expression is rapidly upregulated following peripheral inflammation and cytokine release in male rats (Ji et al., 1995; Mousa, 2003; Puehler et al., 2004), likely a homeostatic response that counteracts the excitatory effects of glial activation and thereby increases opioid efficacy. Interestingly, no change in MOR expression is noted in female rats following peripheral inflammation (Zhang et al., 2014), which may also contribute to the reduced antinociceptive effects of morphine observed in persistent pain assays (Cook and Nickerson, 2005; Loyd and Murphy, 2006; Wang et al., 2006; Murphy et al., 2009). We speculate that NMDAR and MOR activity represent downstream or parallel targets of microglia activation, and may explain the existence of multiple mechanisms underlying the sexually dimorphic response to morphine.

Sex-specific differences in TLR4 modulation of pain have been previously reported (Sorge et al., 2011; Sorge et al., 2015b). For example, in mice, inhibition or

transient depletion of spinal microglia reverses mechanical allodynia induced by spared nerve injury in males, but not females, suggesting that spinally-mediated chronic pain in females is maintained in a non-TLR dependent manner (e.g. an adaptive immune-dependent response) (Sorge et al., 2015a). In the present study, chronic pain was not induced; rather, LPS was administered to activate microglia in a TLR4-dependent manner. Sex-specific effects of TLR4 activity exemplify the need to consider both sexes when studying immune responses and the development of drugs impacting immune signaling. As TLR4 has also been shown to mediate the rewarding effects of morphine (Hutchinson et al., 2012) and the development of tolerance to morphine (Eidson and Murphy, 2013b; Eidson et al., 2016), sex differences in TLR4 signaling may have broad implications for the treatment of chronic pain in men and women.

2.4.2 *Sex differences in innate immune function*

In the present study, we show that female rats have significantly more “activated” microglia in the PAG than males at baseline, a difference that is potentiated following systemic LPS treatment. This finding suggests that females have a lower threshold for activation and/or launch a more robust proinflammatory response compared with males. Indeed, females of many species launch a more robust immune response than males (Gaillard and Spinedi, 1998; Klein, 2000; Schwarz and Bilbo, 2012). For example, in the peripheral immune system, female mice have higher titers of immunoglobulins (Klein, 2000) and splenocyte blastogenic responses to T and B cell mitogens than males (Schneider et al., 2006). Females often present with greater levels of pro-inflammatory markers following immune challenge (Tonelli et al., 2008; Bollinger et al., 2016; Morrison and Filosa, 2016). Interestingly, despite increases in PAG microglia activation

and IL-1β release in response to LPS immune challenge in females, we observed no sex differences in LPS-induced febrile response. However, IL-6, which correlates most closely with changes in body temperature (LeMay et al., 1990; Roth et al., 1993; Roth and De Souza, 2001), was not different in males and females, and may account for the lack of observed sex difference in fever response.

The present experiments used normally cycling female rats. Estrus cycle was monitored, but not controlled or manipulated, to demonstrate physiologically relevant changes in microglia activation as a function of estrus cycle. No significant effect of estrus cycle was observed in any of the present experiments. Immunomodulatory effects of sex hormones, specifically estradiol, have been shown to alter cytokine release and microglial activation in a concentration-, age-, and duration-dependent manner. For example, uterine TNF, IL-1β, and IL-6 levels increase during proestrus and estrus in rats (De et al., 1992), when estradiol is high. This coincides with our previous data demonstrating that morphine is least effective in reducing nociception during proestrus (Loyd et al., 2008a). Historically, estradiol is thought to be neuroprotective, but these effects may be biphasic (Whitacre et al., 1999) such that high levels of estradiol decrease inflammatory markers and attenuate the inflammatory response to LPS (Dimayuga et al., 2005), while low doses, comparable to normal circulating levels, increase concentrations of proinflammatory cytokines (Correale et al., 1998). Furthermore, chronic but not acute estradiol administration increases pro-inflammatory responses in peripheral immune cells of females compared with males (Calippe et al., 2008; Calippe et al., 2010), and potentiates LPS-evoked TLR4 immune responses *in vitro* (Loram et al., 2012). Consistent with clinical research demonstrating that women

have a much higher incidence rate of autoimmune disorders and chronic inflammatory conditions such as migraine (Buse et al., 2013), osteo- and rheumatoid arthritis (Whitacre, 2001) compared with men, these results suggest that women may have increased susceptibility to immune challenge and are at a greater risk for developing inflammatory pathologies than men.

2.5 Acknowledgements

This work was supported by the National Institutes of Health (Grant DA16272 to A.Z.M.) (-)-Morphine sulfate and (+)-naloxone were kindly provided by the National Institute on Drug Abuse drug supply program. We thank Lauren Hanus and Alyssa Bartlett for technical assistance.

3 OPIOID METABOLITES SEX-DEPENDENTLY IMPACT MORPHINE ANALGESIA IN THE RAT

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Abstract

Preclinical studies report that the effective dose for morphine is approximately 2-fold higher in females than males. Following systemic morphine administration, morphine is metabolized via Phase II glucuronidation in the liver and brain into two active metabolites: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), each possessing distinct pharmacological profiles. M6G binds to μ opioid receptors and acts as a potent analgesic. In contrast, M3G binds to TLR4, initiating a neuroinflammatory response that directly opposes the analgesic effects of morphine and M6G. M3G serum concentrations are 2-fold higher in females than males, however, sex-specific effects of morphine metabolites on analgesia and glial activation in vivo remain unknown. The present studies test the hypothesis that increased M3G, and subsequent TLR4-mediated activation of glia, is a primary mechanism driving the attenuated response to morphine in females. We demonstrate that intra-PAG M6G resulted in a greater analgesic response in females, with females requiring less than half the dose to achieve maximal analgesia relative to males. In contrast, intra-PAG M3G administration significantly attenuated the analgesic effects of systemic morphine

in males only, increasing the effective dose of morphine two-fold (5.0 vs 10.3 mg/kg). Together, these data implicate sex differences in morphine metabolism, specifically M3G, as a contributing factor in the attenuated response to morphine observed in females.

3.1 Introduction

Opioids such as morphine are widely used for the treatment of severe pain, with 3-5% of adults in the US receiving long-term opioid therapy (Dowell et al., 2016). Preclinical studies using both acute and chronic pain assays report that morphine is less effective in females than in males (Dawson-Basoa and Gintzler, 1993; Mogil et al., 2000; Craft et al., 2008; Loyd et al., 2008a). Indeed, greater antinociception is observed in male rats for almost every opioid tested (Barrett et al., 2002; Terner et al., 2003; Stoffel et al., 2005; Peckham and Traynor, 2006; Bai et al., 2015).

The midbrain periaqueductal gray (PAG) is a key neural locus for opioid action (Morgan et al., 2005; Loyd et al., 2007). Direct PAG administration of morphine induces long-lasting analgesia, while intra-PAG administration of the opioid antagonist (-)-naloxone or lesions of PAG μ opioid receptor (MOR) completely abolish the antinociceptive effects of systemic morphine (Loyd et al., 2008a). Sex differences are also evident following intra-PAG administration of morphine, with the half-maximal antinociceptive dose (ED_{50}) in males ranging from 1.2-1.6 μg/μl, while in females ED_{50} values range from 16 to >50 μg/μl (Krzanowska and Bodnar, 1999; Loyd et al., 2008a; Bobeck et al., 2009).

Recent data suggest that the innate immune receptor, toll-like receptor 4 (TLR4), contributes to the sexually dimorphic effects of morphine (Doyle et al., 2017). Many opioids, including morphine, bind to myeloid differentiation factor 2 (MD-2), a co-receptor of TLR4, located on glial cells (Hutchinson et al., 2010). Although the classical μ opioid receptor (MOR) binds only the (-)-stereoisomer of opioids, TLR4 binds opioids in a non-stereoselective manner (*i.e.,* both the (-) and (+) isomers of opioid ligands

modulate glial signaling) (Hutchinson et al., 2010). TLR4 activation initiates an inflammatory response that is characterized by the release of inflammatory compounds including cytokines (tumor necrosis factor alpha [TNF], interleukins [IL-1β, IL-6, IL-10]), chemokines (CXCL3) and prostaglandin E2 (PGE2) (Bonizzi and Karin, 2004; Doyle and O'Neill, 2006; Hutchinson et al., 2008c; Hutchinson et al., 2010). These inflammatory factors increase neuronal excitability, resulting in hyperalgesia (Ogoshi et al., 2005; Stellwagen et al., 2005; Yan et al., 2014; Eidson et al., 2016) and paradoxically reducing the analgesic efficacy of morphine (Hutchinson et al., 2007; Hutchinson et al., 2010; Franchi et al., 2012; Li, 2012; Eidson et al., 2016). Our previous research demonstrates that inhibition of TLR4 in the vlPAG with the TLR4-specific antagonist (+)-naloxone potentiates analgesia in females and abolishes the sex difference in morphine response (Doyle et al., 2017).

Recent studies suggest that morphine's primary metabolites contribute significantly to its immunomodulatory effects (Lewis et al., 2010; Grace et al., 2014). Following administration, ~90% of morphine is metabolized primarily in the liver (Coffman et al., 1997), as well as peripheral macrophages (Tochigi et al., 2005) and brain microglia (Togna et al., 2013), to form two active glucuronide metabolites: morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), each with distinct pharmacological properties (Coughtrie et al., 1989; Christrup, 1997). M6G binds to MOR with high affinity, and is a potent analgesic (Abbott and Palmour, 1988; Wittwer and Kern, 2006). M3G, on the other hand, does not bind to MOR and does not produce analgesia (Loser et al., 1996). Rather, M3G binds with high affinity to TLR4 (Lewis et al., 2010; Due et al., 2012) to upregulate pro-inflammatory IL-1 *in vitro*, induce allodynia

and hyperalgesia, and actively opposes the analgesic effects of morphine and M6G (Smith et al., 1990; Ekblom et al., 1993; Bartlett et al., 1994; Angst and Clark, 2006; Juni et al., 2006; Lewis et al., 2010). Importantly, unlike the vast majority of opioids, M6G does not bind to TLR4 and is not associated with a pro-inflammatory response (Carrigan and Lysle, 2001; Hutchinson et al., 2010).

Several studies in rats have observed sex differences in morphine metabolism, such that morphine administration results in approximately two-times greater concentrations of M3G in the plasma of females compared with males (Baker and Ratka, 2002; South et al., 2009). Though M3G concentrations are higher in females, there is no direct evidence that the increased M3G:M6G ratio contribute to the reduced efficacy of morphine observed in females. The present studies test the hypothesis that M3G specifically contributes to the observed sex differences in morphine antinociception by activating TLR4 and opposing morphine analgesia to a greater degree in females. Most importantly, the present studies investigate whether MOR activation with M6G, in the absence of morphine/M3G-induced glial activation, ultimately leads to equipotent analgesia in both males and females.

3.2 Materials and methods

3.2.1 *General methods*

Subjects. Age and weight matched (60-90 day old; 250-400g) intact male and normally cycling female Sprague Dawley rats (Charles River) were used. Animals were pair-housed with the same sex on a 12:12 h light/dark cycle (lights on at 08:00). Access to food and water was available *ad libitum* throughout the experiments except during behavioral 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 animals used in these experiments and to minimize pain and suffering.

Vaginal Cytology. Vaginal lavages were performed daily beginning 7 days prior to testing to confirm that all female rats were cycling normally and to record cycle stage at the time of testing (Loyd et al., 2007).

*Intra-vlPAG cannulae implantation and injections. A*nimals were anesthetized to a deep surgical plane with 5% isoflurane (maintained at 2–5% isoflurane throughout surgery; Henry Schein Animal Health) and bilateral guide cannulae (22 gauge; Plastics One) aimed at the vlPAG (anterior—posterior: 1.7 mm; mediolateral: ±0.6 mm; dorsoventral: −5.0 mm from lambda) were implanted stereotaxically and maintained as previously described (Loyd et al., 2008a; Eidson and Murphy, 2013a). Animals were allowed to recover 11-14 days post-cannula implantation before behavioral testing. Animals with blocked cannula were retained as no injection controls. No significant differences were observed between non-injected animals and those animals receiving

intra-PAG saline, so these groups are collapsed (data not shown). Animals with bilateral cannulae located outside of the vlPAG (e.g., in the aqueduct or deep mesencephalic nucleus) were considered “cannula misses” and were not included in any analyses.

Behavioral Testing and data presentation. Thermal nociception was assessed using the paw thermal stimulator (Hargreaves et al., 1988; Wang et al., 2006; Loyd et al., 2008b; Eidson and Murphy, 2013a; Doyle et al., 2017). Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. A radiant beam of light is positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL) in seconds. A maximal PWL of 20 s was used to prevent tissue damage due to repeated application of the noxious thermal stimulus. Animals were acclimated to the testing apparatus 30-60 minutes per day for three consecutive days prior to the start of the experiment and on the day of testing. All behavioral testing took place between 10:00 and 15:00 (lights on at 08:00). 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 60-64°C throughout the course of the experiments. All testing was conducted blind with respect to group assignment. Data were normalized to the percent maximum possible effect (%MPE) using the following formula:

$$\%MPE = \frac{Paw\ Withdrawal\ Latency}{20\ s}\ (100)$$

Tissue analysis and data presentation of qPCR. At the end of each experiment, brains were removed, flash frozen, and sectioned at 300 µm with a Leica CM3050S cryostat and mounted on to sterile slides. One-millimeter bilateral micropunches were

taken from 6 levels of the vlPAG (Bregma -6.72, -7.04, -7.64, -8.0, -8.30, -8.80) and RNA was extracted with TRIzol (Life Technologies; 15596026) using standard procedures, followed by the addition of Glycoblue (Life Technologies; AM5916) for visualization. Concentrations of RNA (ng/µl) were calculated using a NanoDrop ND-1000 Spectrophotometer (Version 3.8, Thermo Fisher; DE). Following RNA extraction, RNA was diluted to a standard concentration and converted to cDNA using an AMV First-Strand Synthesis Kit (Invitrogen). PCR was performed using FastStart Essential DNA Green MasterMix (Roche) and analyzed using a Roche LightCycler 96 and accompanying software (Version 1.1.0.1320, 2011 Roche Diagnostics; Switzerland). Primer sequences can be found in Table 1.

qPCR data are presented as the normalized ratio of the target gene relative to the GAPDH control gene using ΔCq to retain saline control groups. Data shown represent normalized values obtained using $2^{-(\Delta Cq)}$. The impact of sex and treatment on activated microglia and cytokine mRNA levels were analyzed by two-way ANOVAs, with Tukey's post-hoc analysis, using SigmaPlot. Bivariate correlations were performed for estrus cycle and cytokine expression using Spearman's test. PCR data are presented as $2^{-(\Delta Cq)}$ normalized means ±SEM; $p \leq 0.05$ was considered significant.

Table 2. Chapter 3 primer sequences for qPCR of inflammatory cytokines

GAPDH	Forward	GAG GTG ACC GCA TCT TCT TG
	Reverse	CCG ACC TTC ACC ATC TTG TC
IL-1β	Forward	CCC TGA AGG ATG TGA TCA TTG
	Reverse	GGC AAA GGG TTT CTC CAC TT
IL-6	Forward	AAG ACC CAA GCA CCT TCT TT
	Reverse	AGA CAG CAC GAG GCA TTT TT
IL-10	Forward	TGT ACC TTA TCT ACT CCC AGG TTC TCT
	Reverse	GTG TGG GTG AGG AGC ACG TA
TNF	Forward	TGT ACC TTA TCT ACT CCC AGG TTC TCT
	Reverse	GTG TGG GTG AGG AGC ACG TA

3.2.2 *Experiment 1: Does the administration of M6G eliminate sex differences in analgesia?*

Cannula implantation and behavioral testing. To determine if M6G produces equipotent analgesia in males and females, thermal nociception was assessed using the paw thermal stimulator immediately following a single injection of M6G into the vlPAG. Following baseline PWL measures, animals received a single injection of M6G (0.2ug or 0.7ug) into the PAG (Mathes and Kanarek, 2006) and immediately placed back into the testing chamber. PWL was measured every 10 minutes for 120 minutes following injection. A separate group of animals received a single injection of morphine as a positive control (7 µg/0.25 µl/side; (Loyd et al., 2008a)), or saline as a negative control (0.25 ul/side), resulting in 4 total groups: Saline (*n*=9 males, 7 females),

Morphine (*n*= 8 males, 16 females), M6G Low-Dose (*n*=9 males, 10 females), M6G High Dose (*n*=10 males).

To confirm action of M6G at μ opioid receptor and not TLR4, animals received a single injection of M6G at the effective dose (0.2 ug in females, 0.7ug in males), followed by a single subcutaneous injection of (-)-naloxone (3.7 mg/kg; (Wu et al., 1997)) or (+)-naloxone (8.0 mg/kg) immediately following the PWL measurement at the 20-min time-point. This resulted in 4 additional groups: Saline + (-)-naloxone (*n*= 6 males, 4 females), M6G + (-)-naloxone (*n*= 7 males, 5 females), Saline + (+)-naloxone (*n*= 5 males, 5 females), and M6G + (+)-naloxone (*n*= 7 males, 6 females). Animals treated with Saline and (-)-naloxone or (+)-naloxone are not statistically different from Saline-only animals, therefore, these groups are pooled for analysis and presentation (data not shown).

Data analysis and presentation. Paw withdrawal latency was normalized to %MPE and analyzed using SigmaPlot. Maximum analgesic effects of M6G and morphine were observed between 10-60 minutes post-injection, therefore, data are analyzed across the first 60 minutes. Data were analyzed by group across time using a two-way repeated measures ANOVA with Greenhouse-Geisser correction when appropriate using SigmaPlot. Area under the curve (AUC) was also calculated using %MPE data and analyzed using two-way ANOVAs. Tukey's post hoc tests were used for all ANOVAs; values of $p \leq 0.05$ were considered statistically significant.

Examination and presentation of immunomodulatory cytokines using qPCR. To determine if M6G activates microglia via TLR4, brain tissue was collected from animals in Experiment 1.1 for analysis of inflammatory cytokines using qPCR.

3.2.3 *Experiment 2: Does M3G alter morphine analgesia via TLR4?*

Intra-vlPAG cannulae implantation and injections. To determine if PAG TLR4 activation with M3G impacts morphine analgesia, animals were implanted with bilateral cannula aimed at the vlPAG. On the day of testing, animals received a single intra-PAG injection of M3G (0.075 µg/0.25 µl/side; NIDA) or saline (0.25 µl/side). Comparable doses have been shown to increase glial activation and cytokine expression when administered intrathecally (Lewis et al., 2010).

Approximately 45 minutes following the initial injection of M3G, behavior testing was used to calculate morphine ED_{50} values using our cumulative dosing paradigm. Briefly, animals received an injection of morphine every 20 minutes, resulting in cumulative doses of 1.8, 3.2, 5.6, 8.0, 10.0, and 18 mg/kg. Control animals received repeated saline injections (1ml/kg; s.c). A total of four primary treatment groups were generated: Saline+Saline (*n*=5 males, 5 females), M3G+Saline (*n*=8 males, 7 females), Saline+Morphine (*n*=5 males, 7 females), and M3G+Morphine (*n*=9 males, 7 females).

To determine if M3G activation occurs via TLR4, a separate group of animals receiving intra-PAG M3G also received a peripheral injection of the TLR4 antagonist (+)-naloxone (s.c.; 8mg/kg) immediately prior to behavioral testing. This dose was chosen based on previous studies demonstrating inhibition of morphine-induced TLR4 activation of microglia within the PAG (Eidson and Murphy, 2013a). Two additional groups were generated to test the effects of M3G at TLR4: Saline + (+)-naloxone (*n*=4 males, 4 females), and M3G + (+)-naloxone (*n*=8 males, 8 females). Animals treated

with Saline + (+)-naloxone were not statistically different from animals treated with Saline-only, therefore these groups are pooled (data not shown).

Data analysis and presentation. For morphine-treated animals, half-maximal antinociceptive effect (ED_{50}) and 95% confidence intervals (CI) were calculated from dose–response curves using Graph-Pad PRISM software. To generate curves, data was normalized such that each individual animal's baseline PWL score = 0% and 20s = 100% (Morgan et al., 2006; Eidson and Murphy, 2013a; Eidson et al., 2016). Repeated measures ANOVA was used to assess for significant treatment effects, with Bonferroni's *post hoc* tests where appropriate. GraphPad PRISM does not generate exact p-values, therefore these values are presented as $p<$ or >0.05; values of $p \leq 0.05$ were considered statistically significant.

Examination and presentation of immunomodulatory cytokines using qPCR. To determine of M3G sex-specifically alters glial activation via TLR4, brain tissue was collected from animals in Experiment 2.1 for analysis of inflammatory cytokines using qPCR.

3.3 Results

3.3.1 *Experiment 1a: M6G is more effective in females*

Exogenous M6G produces robust analgesia, and is reported to have greater potency than morphine presumably by acting exclusively via MOR, therefore bypassing the opposing effects mediated by TLR4 (Abbott and Palmour, 1988; Christrup, 1997; Kilpatrick and Smith, 2005)]. To test the hypothesis that M6G activation of MOR (and not TLR4), results in equipotent analgesia, animals received a single injection of M6G (0.2ug), morphine (7.0ug) or saline directly into the vlPAG.

Across the initial 60 minutes following intra-PAG injections, a significant interaction was observed between group and time ($F_{(36,306)}$= 5.80, p<0.001); Figure 3.3.1a. As expected, morphine significantly increased analgesia in both males (p=0.002) and females (p<0.001) compared with saline controls. Morphine produced greater analgesia in males than in females 30-60 minutes post-injection, with males having significantly greater analgesia at 40-minutes post-injection (p=0.04; Figure 3.3.1b).

Administration of M6G to females resulted in robust analgesia. Indeed, this response was greater than females receiving morphine alone, however, these groups were not statistically different (Average MPE: 69% vs 87%; p=0.07). Interestingly, M6G at low doses was significantly more effective in females than in males (p= 0.007). Although males receiving low-dose M6G had greater analgesia than saline-treated males, these groups were not statistically different (Average MPE: 59% vs 34%; p=0.08). To determine if the discrepancy in M6G analgesia between males and females was due to differences in drug potency, a higher dose of M6G (0.7ug) was also administered. Males were maximally analgesic at high doses of M6G, and did not

significantly differ from females receiving low-dose M6G (p=0.99) or from males receiving morphine (p=0.46). High-dose M6G was fatal in females, and this dose was discontinued following pilot studies.

3.3.2 *Mechanisms of M6G analgesia*

Administration of (-)-naloxone significantly and completely antagonized the analgesic effects of M6G in both males (p<0.001) and females (p<0.001); $F_{(15,147)}$=2.12, p=0.02, Figure 3.3.2a. Indeed, within 20 minutes of administration, PWLs were not significantly different from saline in males (p=0.54) or females (p=0.49). By contrast, administration of the TLR4 antagonist (+)-naloxone had no effect on M6G-induced analgesia in either males (p=0.90) or females (p=0.99); $F_{(15,153)}$=2.17, p=0.018; Figure 3.3.2b. Administration of either (-)-naloxone or (+)-naloxone had no effect on PWL latency alone in males (p>0.99 and p>0.99) or females (p>0.99 and p>0.99, respectively). Together, these results suggest that the analgesic effects of M6G are mediated via an action at MOR, and not TLR4.

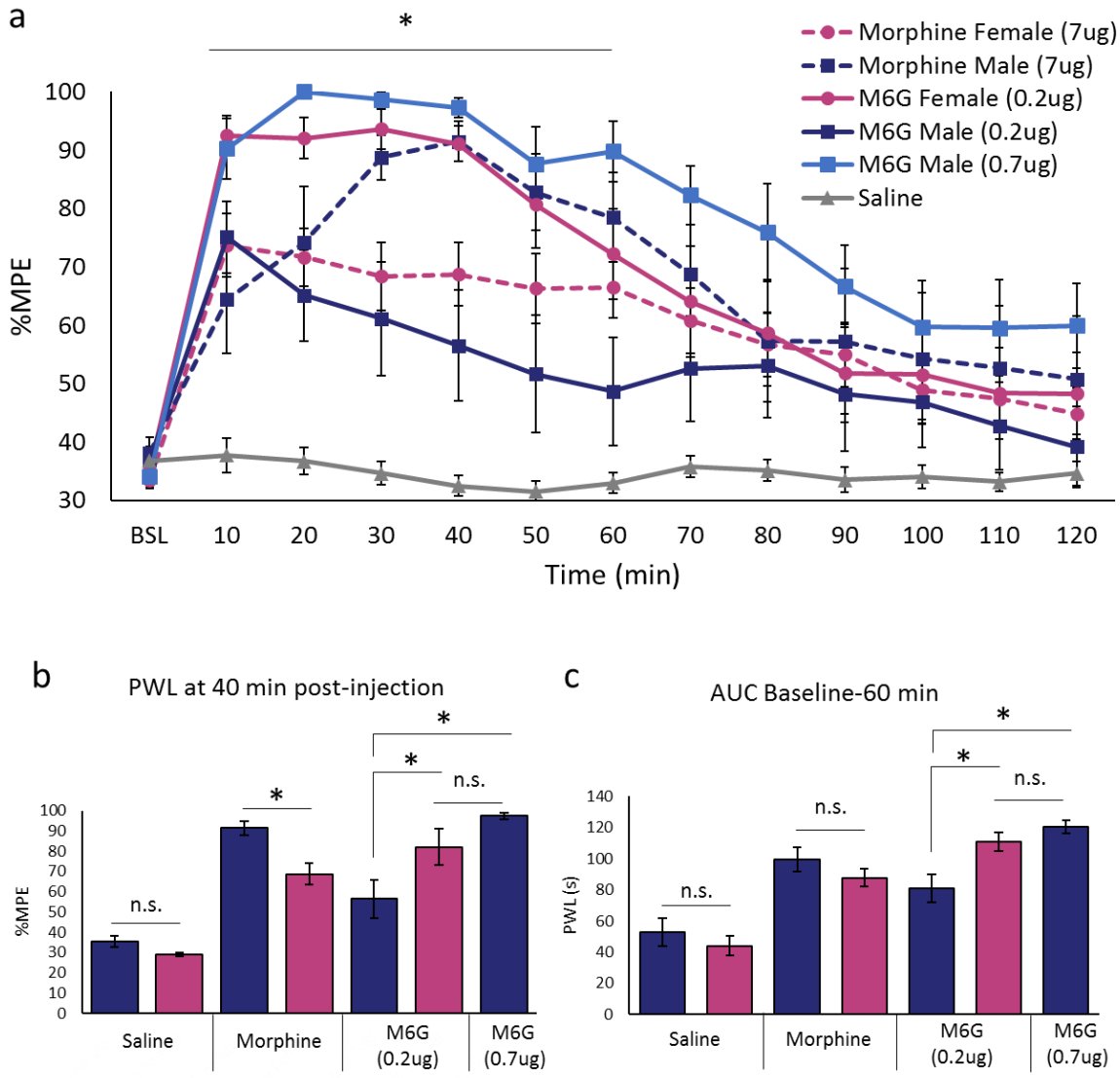

Figure 3.3.1. M6G Induces Robust Analgesia in Both Sexes, but is More Potent in Females.
a) M6G induces robust analgesia in both sexes. Low dose M6G (0.2ug) was effective in females but not males. b) At 40 minutes post-injection, at the time of peak morphine analgesia, significant sex differences were observed between morphine treated males and females. Effective doses of M6G (0.2ug in females, 0.7ug in males) did not show significant sex differences at 40 minutes, or c) across the first 60 minutes of the test. Saline (*n*=9 males, 7 females), Morphine (*n*= 8 males, 16 females), M6G Low-Dose (*n*=9 males, 10 females), M6G High Dose (*n*=10 males).

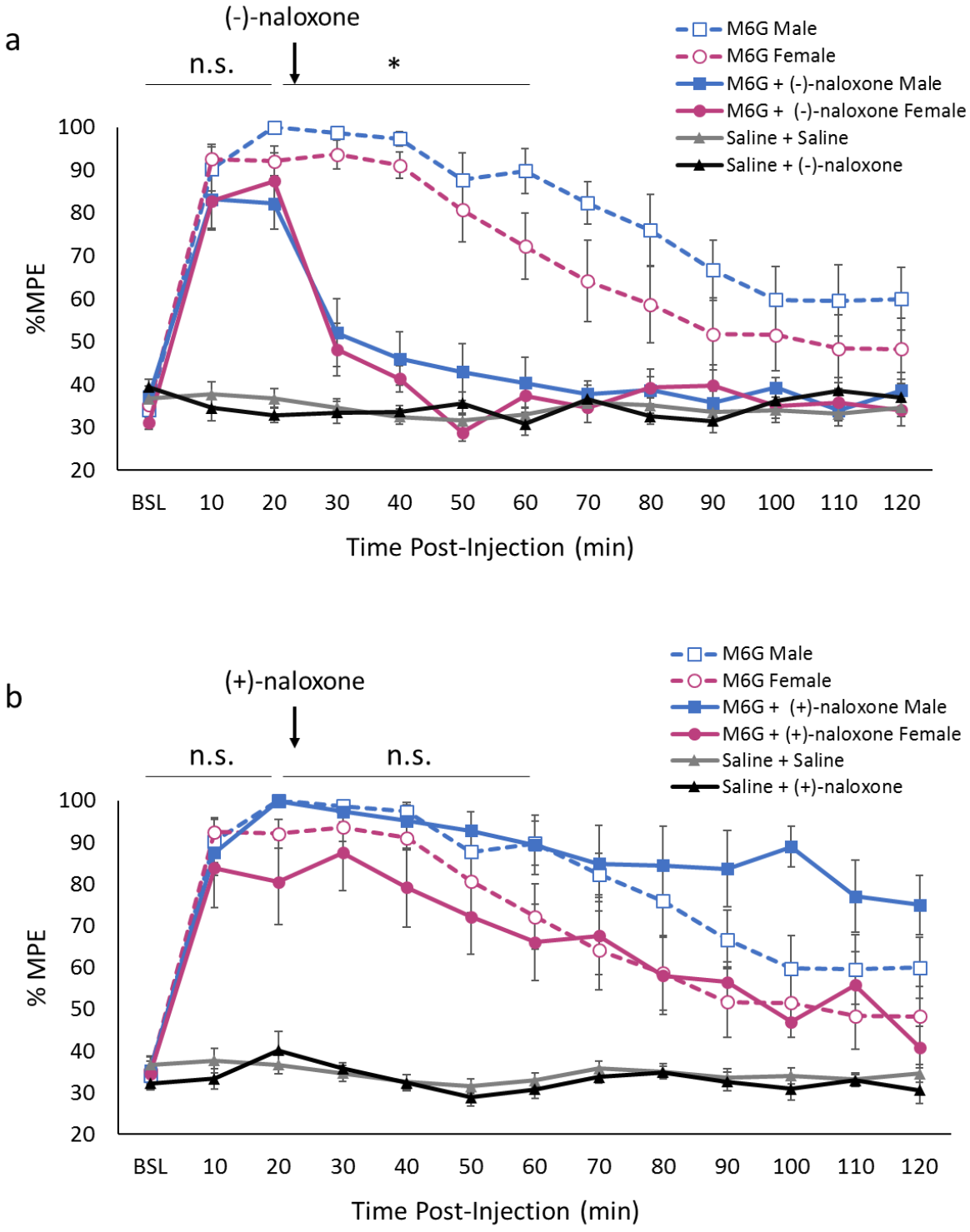

Figure 3.3.2. Receptor Mechanisms Underlying M6G Analgesia.
a) Inhibition of MOR with (-)-naloxone (s.c.) results in complete blockade of M6G analgesia, while b) inhibition of TLR4 with (+)-naloxone (s.c.) has no effect on PWL. Saline + (-)-naloxone (n= 6 males, 4 females), M6G + (-)-naloxone (n= 7 males, 5 females), Saline + (+)-naloxone (n= 5 males, 5 females), and M6G + (+)-naloxone (n= 7 males, 6 females).

3.3.3 *Experiment 1b – M6G does not alter cytokine profiles*

M6G has immunomodulatory effects on peripheral immune function, resulting in reduced cytokine production, decreased B cell and lymphocyte proliferation, and reduced natural killer cell activity (Thomas et al., 1995; Carrigan and Lysle, 2001). However, it is unknown if M6G is capable of altering central cytokine concentrations. Therefore, qPCR was used to determine cytokine expression in the vlPAG of morphine- and M6G-treated males and females from experiment 1a; Figure 3.3.3.

Significant treatment effects were found for IL-6 ($F_{(4,70)}$ =5.86, p<0.001) and IL-10 ($F_{(4,72)}$ =2.97, p=0.026), while significant interactions were found for IL-1β ($F_{(4,66)}$ =6.58, p<0.001), and TNF ($F_{(4,70)}$ =3.28, p=0.017). No significant main effects of sex were observed. In females, IL-1β expression was significantly increased with M6G relative to Saline (p<0.001), Morphine (p=0.002), and M6G + (+)-naloxone (p=0.002), Figure 3.3.3a. Morphine significantly increased IL-1β expression in males relative to saline (p=0.002). Morphine also increased expression of IL-6 and TNF in females relative to saline (p=0.004 and p=0.005, respectively). Intra-PAG M6G did not have a significant impact on cytokine expression of IL-6, TNF, or IL-10 within the PAG for either males or females.

Treatment with peripheral (-)-naloxone or (+)-naloxone following M6G administration had inconsistent effects on cytokine expression. Females receiving M6G+ (-)-naloxone showed significantly increased TNF relative to their male counterparts (p=0.009), as well as females treated with M6G alone (p=0.02), Figure 3.3.3c. Females receiving M6G + (+)-naloxone also showed significantly increased TNF

relative to their male counterparts (p=0.003), as well as increased IL-10 expression relative to females treated with M6G alone (p=0.04), Figure 3.3.3d.

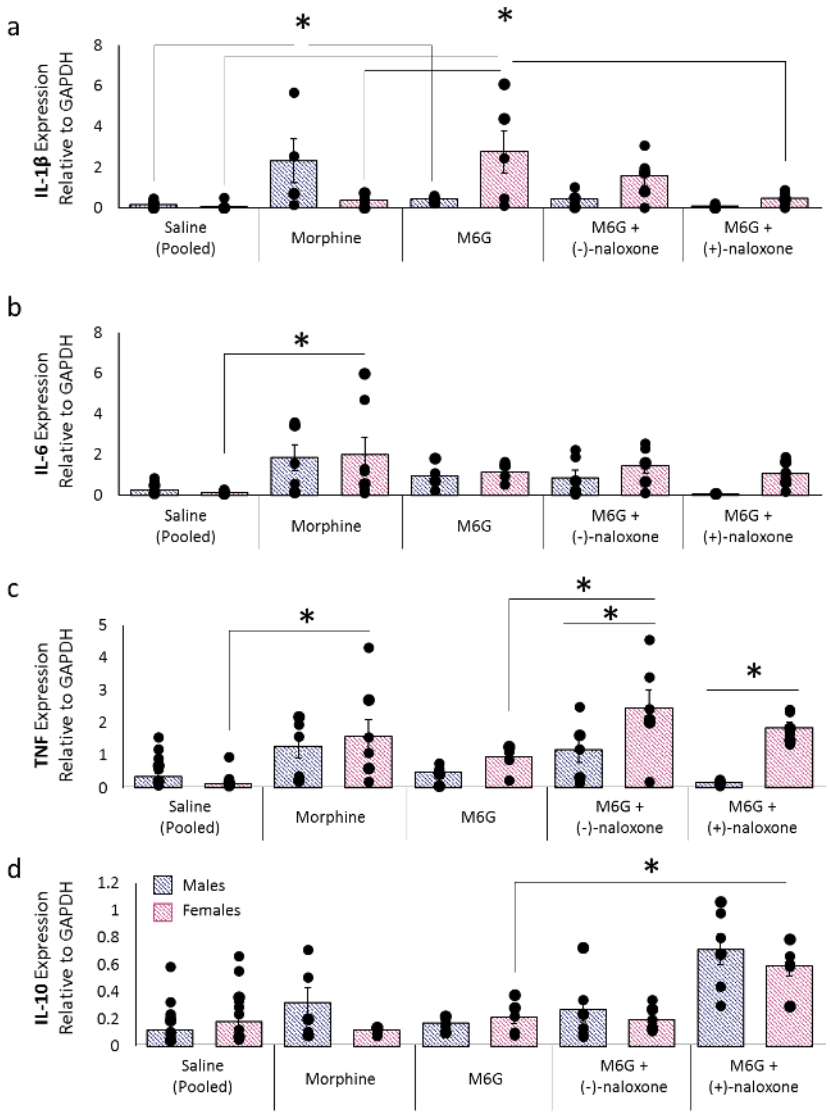

Figure 3.3.3. M6G Immunomodulation in the PAG.
a) IL-1β was significantly increased in M6G treated females relative to Saline and Morphine-treated females, M6G + (+)-naloxone females, and M6G males. b) IL-6 expression was significantly increased in morphine-treated females relative to saline females. C) TNF expression is significantly greater in Morphine females relative to Saline controls. TNF was also expressed significantly more in M6G + (-)-naloxone females relative to females treated with M6G only, as well as M6G + (-)-naloxone males. Sex differences were observed in the M6G + (+)-naloxone group. d) IL-10 expression was significantly increased in M6G + (+)-naloxone females relative to females treated with M6G alone. Saline (n=14 males, 12-13 females), Morphine (n= 4-6 males, 5-7 females), M6G Low-Dose (n=5 females), M6G High Dose (n=4-5 males), M6G+(-)-naloxone (n=5-6 males, 5-6 females), and M6G+(+)-naloxone (n=5 males, 6-7 females).

3.3.4 *Experiment 2a: M3G attenuates morphine analgesia in males only*

Previous studies reported that the morphine metabolite M3G, binds to TLR4, producing allodynia and hyperalgesia, actively opposing the analgesic effects of morphine and M6G (Yaksh et al., 1986; Smith et al., 1990; Ekblom et al., 1993; Bartlett et al., 1994; Smith and Smith, 1995; Angst and Clark, 2006). To determine if M3G induced hyperalgesia or morphine opposition in a sex-specific manner, a single injection of M3G (0.075 ug) or saline was administered into the vlPAG of male and female rats 45 min. prior to receiving cumulative injections of morphine or saline.

Repeated measures ANOVA of morphine-treated groups shows significant effect of treatment across time; $F_{(3,27)}$= 6.54, p=0.004; Figure 3.3.4. Consistent with our previous studies, morphine ED_{50} was significantly greater in females than males (ED_{50}= 7.81 and 5.00 respectively), indicating greater morphine efficacy in males (p<0.05). Interestingly, pre-treatment with M3G significantly attenuated morphine analgesia in males only (p<0.01), completely abolishing the sex difference in morphine response (ED_{50}= 10.28 and 8.82 in males and females, respectively p>0.05); Figure 3.3.4a.

We observed a significant effect of treatment, regardless of time in saline-treated groups; $F_{(7,245)}$=9.00, p<0.001; Figure 3.3.4b. No sex differences in baseline responses to the thermal stimulus were noted in our saline control groups (p=0.64). In addition, M3G alone did not significantly alter response latencies from saline control within male (p=0.99) or female (p=0.94) groups. M3G marginally increased PWL in males (AUC 61.7s vs 68.2s) and decreased it in females (AUC 57.9s vs 55.5s), such that a significant sex difference was observed in M3G-treated animals (p=0.003).

To determine if M3G was acting in a TLR4-dependent manner, the TLR4-specific antagonist (+)-naloxone was administered immediately following M3G. Male and female groups receiving only (+)-naloxone were significantly different from one another (p=0.04). Interestingly, (+)-naloxone had no effect on M3G-treated males (p=1.0) or females (p=0.67) compared with M3G alone, and no sex differences were observed between males and females treated with M3G + (+)-naloxone (p=0.17); Figure 3.3.4b.

Together, these data demonstrate that increased concentrations of M3G in the PAG are sufficient to attenuate morphine antinociception in males. However, no statistically significant effects of M3G alone or in combination with (+)-naloxone were observed.

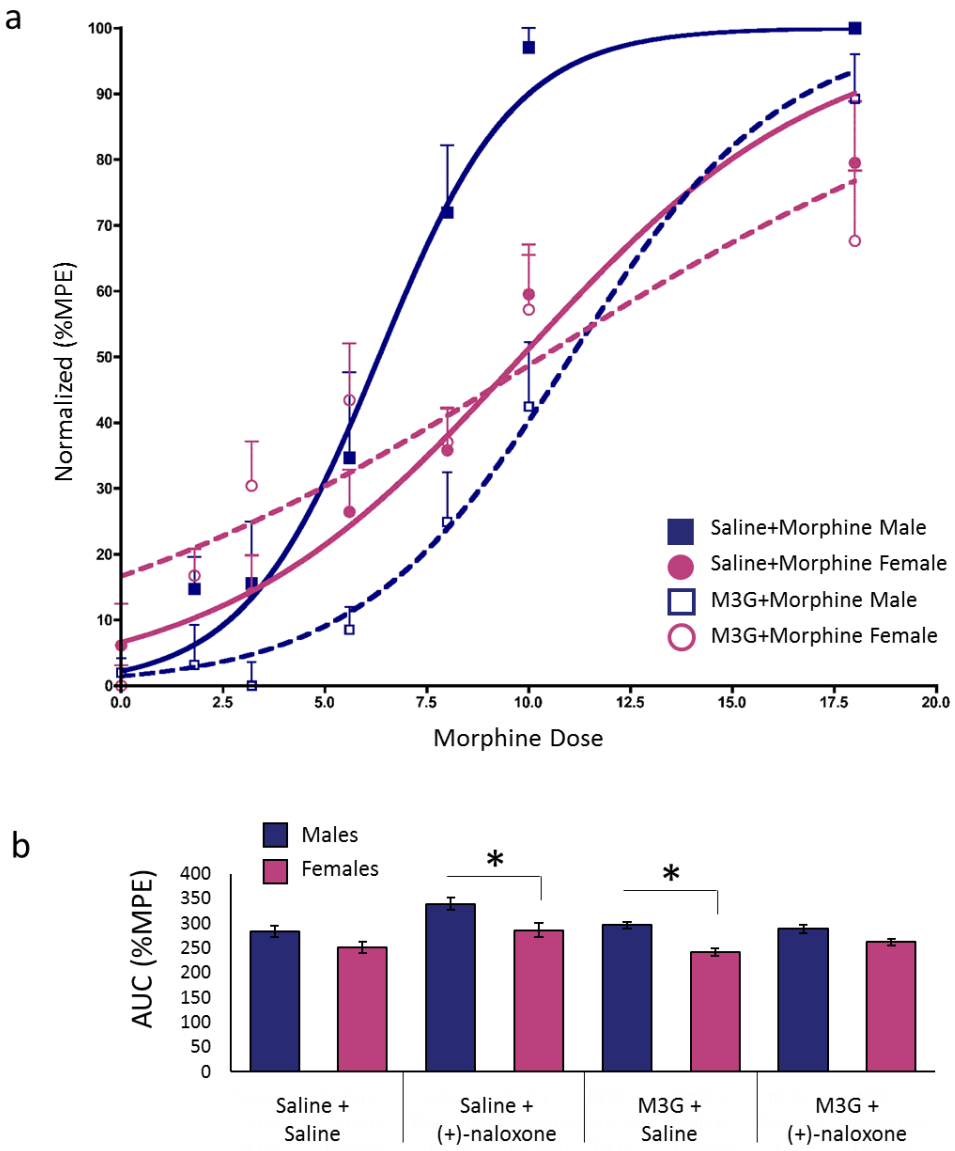

Figure 3.3.4. M3G Attenuates Morphine Analgesia in Male, but not Female, Rats.
a) Intra-PAG administration of M3G significantly attenuates morphine analgesia in males, but not females; Saline+Morphine (n=5 males, 7 females), and M3G+Morphine (n=9 males, 7 females). b) M3G alone was not sufficient to induce observable changes in PWL, and was not changed with (+)-naloxone; Saline+Saline (n=5 males, 5 females), M3G+Saline (n=8 males, 7 females), Saline+Naloxone (n=4 males, 4 females), and M3G+Naloxone (n=8 males, 8 females).

3.3.5 *Experiment 2b: M3G alters cytokine profiles*

M3G is known to induce pro-inflammatory responses that oppose the analgesic effects of morphine (Lewis et al., 2010; Due et al., 2012; Grace et al., 2014; Xie et al., 2017). To determine if M3G induced comparable levels of neuroinflammation in males and females *in vivo*, PAG punches were collected in experiment 2a following behavior testing, and cytokines were measured using qPCR.

A significant interaction effect was observed for IL-1β ($F_{(4,61)}$ =3.01, p=0.026). Females treated with M3G+Morphine had significantly higher levels of IL-1β expression than females receiving morphine alone (p=0.009), or males receiving M3G+Morphine (p=0.04); Figure 3.3.5a. A main effect of treatment was observed for IL-6 ($F_{(4,63)}$ =5.23, p<0.001). Regardless of sex, M3G+Morphine treated animals had greater IL-6 expression relative to groups treated with morphine alone (p=0.019); Figure 3.3.5b. Main effects of both sex and treatment were observed for TNF ($F_{(4,65)}$ =10.24, p=0.002 and $F_{(1,65)}$ =4.99, p=0.002, respectively), however no interaction effects were observed. Females treated with M3G alone had significantly more TNF expression than females treated with morphine (p=0.005), or M3G-treated males (p=0.02); Figure 3.3.5c. No significant main effects or interactions were detected for IL-10; Figure 3.3.5d. Indeed, IL-10 levels were remarkably stable regardless of treatment or sex. Surprisingly, (+)-naloxone administration did not block M3G-induced increases in IL-1β, IL-6, or TNF.

Overall, neither subcutaneous morphine nor intra-PAG M3G administration were sufficient to alter cytokine expression in the vlPAG relative to Saline groups. However, the combination of M3G+Morphine significantly increased IL-1β in females and IL-6 in both sexes. Although M3G has been previously shown to augment cytokine levels in a

TLR4-dependent manner *in vitro*, co-administration of the TLR4 antagonist (+)-naloxone did not significantly impact M3G-induced increases in IL-1β, IL-6, and TNF.

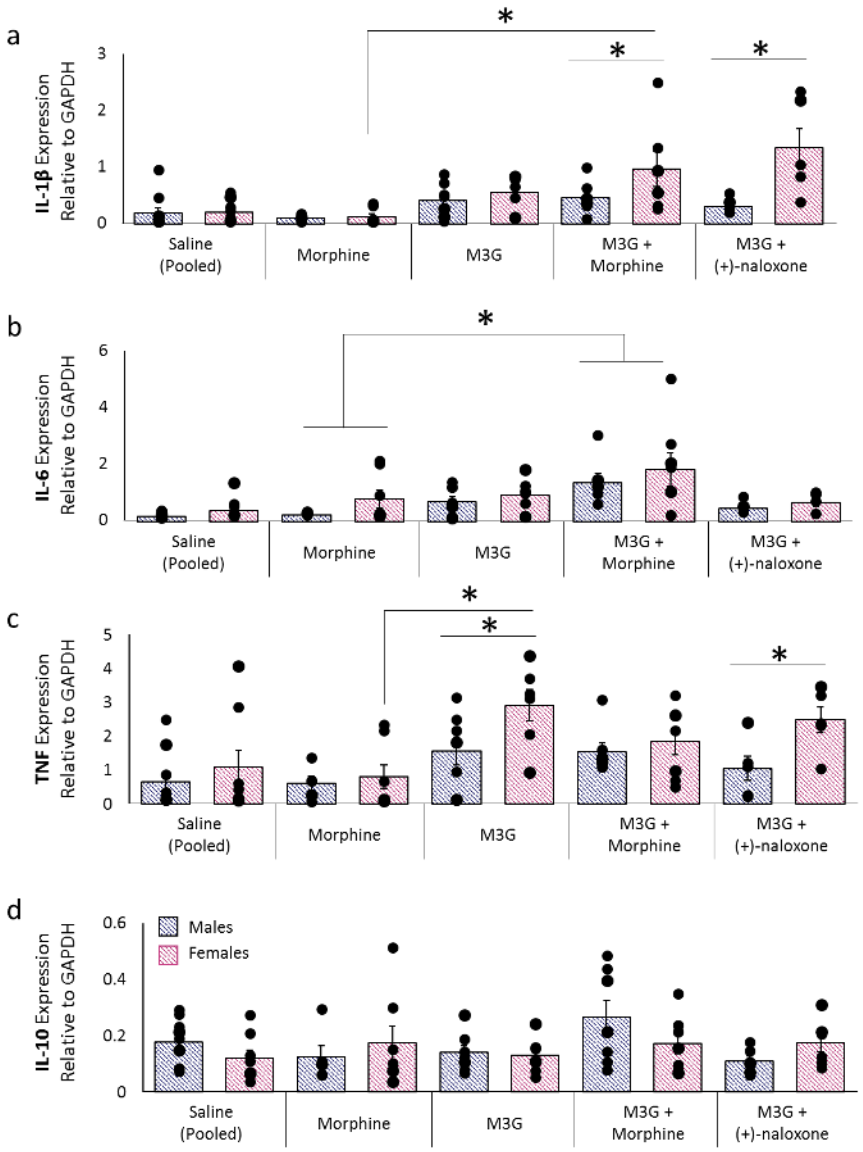

Figure 3.3.5. M3G Immunomodulation in the PAG.
a) IL-1β and b) IL-6 expression significantly increased in M3G+Morphine treated females relative to morphine-only controls. Sex differences in IL-1β expression were observed between males and females treated with M3G + (+)-naloxone. c) TNF expression significantly increased in M3G females relative to morphine females as well as M3G males. Significant sex differences were observed in TNF expression between males and females treated with M3G + (+)-naloxone. d) No changes in IL-10 expression were observed between any treatment groups. Saline (n=9 males, 8 females), M3G+Saline (n=5-7 males, 5-6 females), Saline+Morphine (n=5 males, 6-7 females), M3G+Morphine (n=6-7 males, 6-7 females), and M3G+Naloxone (n=5-6 males, 5-6 females).

3.4 Discussion

The present study examined both the behavioral and immunomodulatory effects of the morphine metabolites, M3G and M6G. Intra-PAG administration of M6G results in significantly greater analgesia in females than in males. Importantly, M6G resulted in near maximal analgesia in females, a 26% increase from maximal morphine analgesia.

In both males and females, M6G analgesia was reversed with (-)-naloxone but was unchanged with (+)-naloxone, consistent with previous studies showing that M6G acts at MOR, but not glial TLR4 (Abbott and Palmour, 1988; Wittwer and Kern, 2006; Hutchinson et al., 2010). M3G administration resulted in a significant rightward shift in the morphine dose response curve, in males only. In contrast, pretreatment with M3G in females did not alter the response to morphine, suggesting a ceiling effect of M3G. Despite the large and significant shifts in pain sensitivity observed following M6G administration in males and females, M6G did not reliably alter vlPAG proinflammatory cytokine concentrations, and were also not consistently changed with either (-)-naloxone or (+)-naloxone. In contrast, concentrations of pro-inflammatory molecules in females treated with M3G alone or in combination with morphine were significantly increased, although these effects were not reversible with (+)-naloxone in either sex. Together, these data indicate that M3G may contribute to the attenuation of morphine analgesia observed in females, as administration of M6G, but not M3G and/or morphine results in significantly greater analgesia relative to males. These studies suggest that the immunomodulatory effects of morphine and its metabolites result in sex-dependent effects on pain modulation, and have far-reaching implications for the use of opioids to treat pain in women.

3.4.1 *Sex differences in pharmacokinetics*

Previous dogma held that there were no sex differences in morphine metabolism, likely due to a history of conflicting HPLC results in human studies showing both the presence (Murthy et al., 2002) and absence (Sarton et al., 2000; Romberg et al., 2004) of sex differences in M3G or M6G concentrations following morphine treatment. However, pre-clinical studies in rats have consistently reported significant sex differences in metabolite concentrations using HPLC (South et al., 2001; Baker and Ratka, 2002; South et al., 2009), with a single exception, see (Cicero et al., 1997). To date, few studies have examined the effect of endogenous morphine metabolites on pain modulation—and to our knowledge, the present experiments are the first to demonstrate sex-specific causal relationships.

Results from pharmacokinetic studies also support sex-specific differences in morphine metabolism. In humans, morphine is metabolized by isozymes in the uridine 5'-diphospho-glucuronosyltransferase (UGT) 1 and 2 subfamilies, almost all preferentially synthesizing M3G over M6G (~45-55% and 15% of metabolized product, respectively; (De Gregori et al., 2012)). Importantly, sex differences in the expression of UGT1 and 2 subclasses of enzymes have been reported in humans (Gallagher et al., 2010) and rats (Iwano et al., 2012). Further, the enzymes metabolize, and are directly influenced by, steroid hormones (Strasser et al., 1997). In rats, gonadectomy significantly decreases the M3G:morphine ratio in females only, suggesting the involvement of steroid hormones in mediating sex differences in morphine metabolism (Baker and Ratka, 2002). To date, no studies have examined the effects of sex or gonadal hormones on the expression of these isozymes in brain tissue.

3.4.2 *Immunomodulatory effects of morphine metabolites*

M6G is a known immunomodulator, with predominantly anti-inflammatory effects (Thomas et al., 1995; Carrigan and Lysle, 2001), although the exact mechanism through which M6G exerts its effects on immune function is uncertain. Here, M6G increased expression of IL-1β, but did not change expression of IL-6, TNF, and IL-10 relative to saline controls. Antinociceptive and immunomodulatory effects of M6G have been classically attributed to neuronal MOR (Carrigan and Lysle, 2001; Lysle and Carrigan, 2001); however, no studies have investigated the possibility of M6G exerting its effects via MOR on CNS glial cells, which may account for the discrepancies in immune modulation between previous and present studies (Gessi et al., 2016).

Inflammatory mechanisms of M3G have been established, as M3G significantly increases pro-inflammatory IL-1β mRNA in BV-2 microglia cultures (Lewis et al., 2010). TLR4 activation by M3G is modest relative to TLR4's natural agonist lipopolysaccharide (LPS; (Hutchinson et al., 2010)), and may not induce robust increases cytokine concentrations *in vivo* (Lewis et al., 2010; Xie et al., 2017). In the present study, we report that M3G, administered in combination with morphine, results in increased expression of IL-1β in females and IL-6 in both sexes relative to saline-treated controls. However, M3G alone was not sufficient to alter cytokine expression in the PAG, suggesting that, *in vivo,* central M3G does not produce measurable immune activation relative to peripheral LPS, which has robust sex-specific effects on cytokine expression in the PAG (Doyle et al., 2017).

Overall, patterns of cytokine expression observed following M3G or M6G treatment were equivocal, and not consistently reversed with (+)-naloxone or (-)-naloxone. Similar inconsistencies have been reported, suggesting a complicated role for morphine metabolites and their relative contributions to immune modulation following morphine (Thomas et al., 1995; Hashiguchi et al., 2005). Estrus cycle was monitored in the present experiments, however, stage of estrus did not correlate with the observed variability in qPCR results. A number of other factors may play a role; for example, route of drug administration [intracerebroventricular vs. subcutaneous (Hashiguchi et al., 1998)], and duration [acute vs. chronic administration; (Eckhardt et al., 2000)] have been shown to alter metabolite-induced, immune-related activity. Immune modulation by M3G and M6G remains *vastly* understudied, and further experiments comparing brain cytokine concentrations using various doses, time-points, and routes of administration will be useful to understand how M3G and M6G each contribute to immune modulation.

3.4.3 *Behavioral effects of morphine-6-glucuronide*

In the present study, we hypothesized that in the absence of immune activation by M3G or morphine, M6G would produce equipotent analgesia in males and females. This is supported by a study in healthy human subjects demonstrating no sex differences in analgesic responses to M6G between males and females (Romberg et al., 2004). Surprisingly, we found that exogenous administration of M6G produces significantly greater analgesia in females than in males. Along with our previous data, this finding provides a new converging line of evidence to support our hypothesis that TLR4 is a primary contributor to sex differences in morphine action. It also initiates exciting and important questions regarding the mechanisms of opioid analgesia;

specifically, why and how does M6G produce robust analgesia in females compared with many other opioids that produce more potent analgesia in males (Barrett et al., 2002; Terner et al., 2003; Stoffel et al., 2005; Peckham and Traynor, 2006; Bai et al., 2015)?

One possible explanation for the reversal of sex differences observed with M6G, is that M6G is able to better utilize MOR than morphine. The PAG sends dense projections to the rostral ventromedial medulla (RVM), which together with descending projections to the spinal cord dorsal horn, constitute the endogenous descending analgesia circuit. Previous analtomical studies in our lab have reported that the density of PAG-RVM output neurons is significantly greter in females frompared with males. However, despite this difference in the number of projection neruons, the percent of PAG-RVM neurons activated by morphine is significantly greater in males (20% vs 50%) (Loyd and Murphy, 2006; Loyd et al., 2008b). Based on the resuls of the present study, we would predict that M6G activates a greater proportion of PAG-RVM neurons than morphine in females than in males, resulting in improved analgesia. Further investigation of the binding properties of M6G in males and females is clearly warranted.

3.4.4 *Behavioral effects of morphine-3-glucuronide*

Interestingly, the present study shows that injection of M3G into the vlPAG prior to morphine administration causes a significant attenuation of morphine analgesia in males only. It has been previously reported that female rats metabolize approximately 2-3 times more M3G than their male counterparts following a single systemic injection of morphine (Baker and Ratka, 2002; South et al., 2009). M3G levels are significantly

higher in females following morphine; therefore, we suspect that the lack of behavioral effect observed in females is due to saturation of M3G at TLR4 (i.e., a ceiling effect of M3G). This interpretation is consistent with our hypothesis that increased M3G reduces morphine's effects, and may contribute to sexually dimorphic responses to morphine.

We did not observe changes in PWL with administration of M3G alone. This is likely due to discrepancies in testing procedures, as previous studies examining M3G adjusted baseline PWL to ~10s in order to observe hyperalgesia (Lewis et al., 2010). However, the present experiments used a lower baseline threshold (approx. 4-7s; consistent with our previous studies (Loyd et al., 2008a; Eidson and Murphy, 2013a)) to accommodate increases in PWL as a result of morphine administration. This may have created a behavioral floor effect for animals treated with M3G alone, and decreases in PWL due to hyperalgesia may not be statistically observable at the current threshold.

The sex-specific effects we observe here with M3G have broad implications that apply to other opioids that create 3-glucuronide metabolites. Glucuronidation at the 3-site of the substrate molecule is associated with glial activation and neuronal excitability; for example, morphine-3-glucuronide (Lewis et al., 2010) and estradiol-3-glucuronide (Lewis et al., 2015), activate glial cells in a TLR4-dependent manner, increasing the release of pro-inflammatory mediators, ultimately resulting in increased neuronal excitation. Hydromorphone-3-glucuronide (Smith, 2000) and normorphine-3-glucuronide (Smith et al., 1997) also have been shown to increase neuronal excitability, likely through the same mechanisms. Interestingly, exogenous opioids with the greatest sex difference in ED_{50} in rats (oxymorphone, hydromorphone, and morphine (Peckham and Traynor, 2006)) all produce 3-glucuronide metabolites by Phase II metabolism via

UGTs. In contrast, drugs producing comparable ED_{50}'s in males and females (codeine, oxycodone, fentanyl) undergo Phase I metabolism by cytochrome P450 (CYP) enzymes, and therefore do not produce 3-glucuronide metabolites on their first pass (Holtman and Wala, 2006; Chan et al., 2008; Smith, 2009). More research is needed to understand how metabolism and elimination of these drugs may differ in males and females, and how 3-glucuronide metabolites impact analgesia.

Together, these data demonstrate an important proof of principle: that in the absence of TLR4 signaling, opioid analgesia is equally effective—if not more effective—in females than in males. Historically, M6G has not been used for the treatment of clinical pain in humans. This is perhaps due to its "low and slow" blood brain barrier permeability, high variability in the doses of M6G required to induce analgesia (depending on the type of pain and method of administration), and tendency to accumulate in plasma in patients with impaired renal function [see (Lotsch and Geisslinger, 2001; Kilpatrick and Smith, 2005) for review]. However, clinical trials of M6G demonstrate comparable analgesia to morphine at appropriate doses, while reducing the negative side effects typically associated with morphine, such as nausea and sedation, in *both men and women* (Cann et al., 2002; Romberg et al., 2004; Hanna et al., 2005; Dahan et al., 2008). Clearly, further research is required to address the relevance of treatment with M6G, as these studies may provide insight into improved treatment strategies for pain management in females.

3.5 Acknowledgements

National Institutes of Health Grant DA16272 awarded to A.Z.M. supported this work. (-)-Morphine sulfate, Morphine-3-β-D-glucuronide, Morphine-6-β-D-glucuronide, (-)-naloxone and (+)-naloxone were kindly provided by the National Institute on Drug Abuse drug supply program.

4 GENERAL DISCUSSION

4.1 Summary: Sex differences in vlPAG glial activity significantly contribute to morphine efficacy via TLR4

Opioids are the most commonly used prescription medications for severe pain, with approximately 1 in 5 Americans prescribed opioids each year (Daubresse et al., 2013). Women are more likely than men to suffer from chronic inflammatory conditions, and to be prescribed opioid drugs for the treatment of pain (Frenk et al., 2015). Unfortunately, women experience increased negative side effects of opiate use (Fillingim et al., 2009), and decreased analgesic efficacy relative to men (Cepeda and Carr, 2003). Rodent studies from our lab and others demonstrate that females require approximately two times as much morphine to achieve analgesia comparable to males (Craft et al., 1999; Ji et al., 2006; Wang et al., 2006; Loyd et al., 2008b).

Many mechanisms have been identified as contributing to the dimorphic effects of morphine, including reproductive hormones (Craft, 2007), GABA, glutamate and melanocortin-1 signaling (Mao, 1999; Tonsfeldt et al., 2016), and MOR density and tone (Loyd and Murphy, 2014) [see Table 3]. The fact that so many seemingly competitive theories exist to account for the dimorphic response to morphine implies a parallel and/or upstream mediator of these effects. Given the inverse relationship between glial activation and analgesia [see Table 4], and the known contribution of glial TLR4 to modulate morphine analgesia in male rats (Eidson and Murphy, 2013a) we hypothesized that sex differences in innate immune function are a likely precursor and/or a significant contributor to the sexually dimorphic actions of morphine.

The data presented in Chapters 2 and 3 demonstrate that innate immune glial cells, via TLR4 signaling, contribute to the sexually dimorphic effects of morphine in the rat. Specifically, sex differences in microglial activation were found in the PAG, as females have a significantly larger proportion of microglia in the PAG with an "activated" morphology, both at baseline and following immune challenge with LPS. Although acute morphine treatment is not sufficient to alter the morphological state of microglia in the PAG, the percentage of endogenously activated glia in this region significantly correlates with morphine ED_{50}. This indicates that the baseline level of microglia activation in the PAG can predict the dose of morphine required to elicit analgesia; specifically, as the proportion of activated glia in the PAG increases, morphine analgesia decreases. Importantly, we demonstrate that PAG TLR4 is both sufficient and necessary to drive sex differences in morphine response. Activation of PAG glia with LPS prior to morphine administration reduces male analgesia to "female-typical" levels, and reduces or abolishes analgesia in a subset of females. On the other hand, inhibition of PAG TLR4 with (+)-naloxone increases morphine analgesia in females, abolishing observed sex differences in morphine response.

Further, we demonstrate that morphine metabolites have sex specific effects on analgesia that may contribute to the sexually dimorphic response to morphine, as increased concentrations of M3G in the PAG prior to morphine administration decrease male analgesia to "female-typical" levels, similar to LPS, abolishing sex differences in analgesia. With administration of M6G, female analgesia not only matches, but *surpasses* male analgesia—suggesting that in the absence of TLR4-induced signaling, sex differences in response to opioids are reversed.

4.2 Current hypotheses underlying sex differences in morphine analgesia are linked with glial activity

Together, the present body of work strongly implicates PAG TLR4 in driving the sex differences in morphine analgesia, and suggests that opioid efficacy is dependent upon glial activation in the PAG. Indeed, previous hypotheses that have been proposed to underlie sex differences in morphine analgesia are all directly linked with glial activity:

4.2.1 *Estradiol*

Studies employing a variety of techniques and pain modalities have assessed the role of steroid hormones, and in particular estradiol, in modulating pain and opiate analgesia. In terms of basal sensitivity, the majority of preclinical studies report no differences in somatosensory thresholds across the estrus cycle, although pro- and anti-nociceptive effects of estradiol have also been reported (Craft, 2007; Craft et al., 2008). In terms of opiate analgesia, several general claims can be made regarding the role of hormones in preclinical studies using rodents [for review, see (Craft et al., 2004)]: (1) morphine is most efficacious in intact males and gonadectomized males supplemented with testosterone; (2) morphine is least efficacious in females supplemented with estradiol; and (3) in normally cycling females, morphine responses are decreased in proestrus and estrus stages, when circulating hormones peak, relative to diestrus. Together, this body of literature suggests that female hormones, specifically estradiol, decreases the analgesic potency of morphine.

Estradiol is a contributing factor in sex differences in immune function. CNS immune cells, specifically astrocytes, express the enzymes 5a-reductase and 3a-

hydroxysteroid dehydrogenase) and are implicated in both progesterone and testosterone metabolism (Garcia-Segura and Melcangi, 2006). Glial cells do not express aromatase for the conversion of testosterone into estradiol under normal conditions (Garcia-Segura and Melcangi, 2006); however, astrocytes and microglia possess steroid hormone receptors, including estrogen receptor alpha (ERα), making them susceptible to changes in estradiol across the estrus/menstrual cycle (Sierra et al., 2008).

Estradiol has a well-documented biphasic effect on immune function in both preclinical and clinical studies (Whitacre et al., 1999; Nilsson, 2007; Straub, 2007), and its pro- or anti-inflammatory effects are dependent upon dose, time, and method of testing *(in vitro* vs. *in vivo*). For example, high levels of estradiol (typical of pregnancy) decrease proinflammatory cytokine production and attenuate inflammatory responses to LPS (Vegeto et al., 2003; Dimayuga et al., 2005; Lewis et al., 2008). In contrast, low doses of estradiol, comparable to normal circulating levels, increase peripheral concentrations of proinflammatory cytokines (Correale et al., 1998). Removal of endogenous estrogens decreases cytokine production and cell-surface expression of TLR4 (Rettew et al., 2009). Estradiol also influences cytokine release in a time-dependent fashion, as chronic but not acute estradiol administration increases TLR4-mediated pro-inflammatory responses in immune cells of females compared with males (Soucy et al., 2005; Calippe et al., 2008; Calippe et al., 2010), and potentiates LPS-evoked TLR4 immune responses *in vitro* (Loram et al., 2012). Estradiol effects may also be dependent upon the setting, as *in vitro* administration is often anti-inflammatory (Drew and Chavis, 2000), but estradiol administered *in vivo* results in pro-inflammatory

responses (Soucy et al., 2005; Calippe et al., 2008; Rettew et al., 2009; Loram et al., 2012). Overall, these data suggest that 1) glial cells are responsive to gonadal hormones, and 2) low-dose, chronic estradiol may create a more pro-inflammatory environment *in vivo*.

These conclusions are consistent with the present studies, as increased glial activation was observed in intact females in the PAG at baseline, suggesting that glial cells are innately more activated in females than in males. In addition, LPS immune challenge had a greater effect on females, increasing pro-inflammatory IL-1β, 'activated' microglial morphological subtypes, and completely abolishing morphine analgesia in a subset of females. To examine the system in its most physiologically relevant state, estrus cycle was monitored but not controlled throughout our experiments. Estrus cycle did not correlate with measures of glial activation in the present data, reinforcing the notion that the interaction between estradiol and immune function is context dependent. Future studies examining gonadectomized vs. intact animals may elucidate the role of estradiol on TLR4 in the observed effects on morphine analgesia.

4.2.2 *GABA and glutamate signaling*

The antinociceptive effects of opiates are mediated, in part, through removal of $GABA_A$-mediated inhibition on excitatory glutamatergic vlPAG neurons that project to the RVM and spinal cord [for review, see (Lau and Vaughan, 2014)]. Blocking $GABA_A$ receptor signaling, or administration of μ opioid receptor agonists, hyperpolarizes GABAergic interneurons to decrease (or disinhibit) GABA signaling and facilitate opioid analgesia (Vaughan et al., 1997). Dimorphic GABAergic and glutamatergic signaling

within the PAG and RVM have been identified as contributing factors to sex differences in opiate analgesia. GABA has been shown to influence the antinociceptive effects of morphine in a sex-dependent manner. Consistent with the dimorphic effects observed following intra-PAG morphine (Krzanowska and Bodnar, 1999; Loyd et al., 2008b), intra-PAG administration of the GABA antagonist bicuculline produces greater analgesia in male rats (Bobeck et al., 2009). Further, chronic peripheral inflammatory pain decreases tonic $GABA_A$-mediated currents and increases the efficacy of systemic morphine in females, an effect reversed by potentiation of $GABA_A$ receptor currents (Tonsfeldt et al., 2016).

Glutamatergic NMDA receptors have also been implicated in the sexually dimorphic response to opioids. In rats, NMDA antagonism increases morphine analgesia in both males and females; however, this effect is highly variable and is dependent upon the drug, dose, and nociceptive test being used (Craft and Lee, 2005; Holtman and Wala, 2006). In male mice, blockade of NMDA receptor signaling via systemic antagonist administration results in a complete attenuation in both the analgesic and hyperalgesic responses to high (but not low) doses of morphine [for review see (Mao, 1999)]. In contrast, NMDA receptor blockade results in a partial reduction, or has no effect on, morphine analgesia in female mice (Lipa and Kavaliers, 1990; Nemmani et al., 2004; Waxman et al., 2010). These data suggest that NMDA receptor signaling is necessary to facilitate morphine antinociception in male, but not female mice. Using quantitative trait locus mapping in male and female mice, the melanocortin-1 receptor (MC1R) gene was identified as a potential ‘female counterpart’ to NMDA. Genetic knockout of the *Mc1r* gene, or pharmacological antagonism of

functional MC1R, produces increased μ-opioid receptor mediated analgesia and decreased μ-opioid hyperalgesia in females, but is without effect in males (Mogil et al., 2003; Juni et al., 2010; Arout et al., 2015). These findings, together, suggest that NMDA and $GABA_A$ receptor signaling may be inherently different in males and females, and contribute to the dimorphic effects of morphine.

Sex differences in glial activation likely contribute to the observed differences in NMDA and GABA signaling [for review see (Ji et al., 2013)]. Morphine-induced activation of microglia and astrocytes induces pro-inflammatory cytokine release, which rapidly upregulates the expression of neuronal NMDA (Wei et al., 2008; Olmos and Llado, 2014), and decreases cell surface expression of $GABA_A$ receptors *in vitro* (Ogoshi et al., 2005; Stellwagen et al., 2005; Tilleux and Hermans, 2007; Yan et al., 2014). Morphine-induced TNF release has also been shown to site specifically downregulate glial GLAST and GLT-1 glutamate transporter expression in the vlPAG (Eidson et al., 2016), leading to increased glutamate in the synapse, and increased neuronal excitation (Mao et al., 2002). Conversely, inhibition of glial activation within the RVM attenuates the enhanced neuronal NMDA signaling normally observed following nerve-injury (Wei et al., 2008). Interestingly, several NMDA antagonists that reportedly potentiate morphine analgesia (including MK-801 and ketamine) (Johnston and Westbrook, 2005) also block microglia activation, both *in vivo* and *in vitro* (Ma et al., 2002; Thomas and Kuhn, 2005; Murugan et al., 2011).

The sexually dimorphic response of glia to perturbation models (Drew and Chavis, 2000; Aulock et al., 2006; Calippe et al., 2010; Loram et al., 2012; Engler et al., 2016), in combination with the present studies, supports the possibility of downstream

sex differences in glutamate and GABA signaling that ultimately contribute to sex differences in opioid analgesia. Previous studies in our lab indicate that chronic morphine is capable of altering glutamate transporters within the PAG, however these studies were conducted exclusively in males (Eidson et al., 2016). Similarly, the experiments described above demonstrating a link between glial activation and glutamate/GABA signaling, when performed *in vivo*, were also conducted only in males (Wei et al., 2008; Yan et al., 2014). Thus, a clear relation between glial activation and glutamate/GABA signaling has never been investigated in females and additional studies are clearly warranted.

4.2.3 *Opioids and opioid receptors*

Sex differences in opioidergic signaling have also been reported (Zubieta et al., 2002; Peckham et al., 2005; Loyd and Murphy, 2006; Bernal et al., 2007; Loyd et al., 2008b; Loyd et al., 2008a). Specifically, we have previously demonstrated that male rats have significantly higher levels of MOR protein and radioligand binding in the vlPAG, and respond more robustly to morphine, than females (Zubieta et al., 2002; Loyd and Murphy, 2006; Loyd et al., 2008a; Loyd and Murphy, 2009). Indeed, MOR levels are 40% lower in proestrus females compared with males; this corresponds to the stage of estrus when intra-PAG morphine is least effective in modulating pain (Loyd et al., 2008a). Further, selective ablation of vlPAG MOR-expressing neurons significantly attenuates the response to morphine in males but not females, indicating that the density of PAG MOR expression is significantly correlated with the degree of opioid analgesia in male, but not female, rats (Loyd et al., 2008a).

Given that the opioid system is inextricably linked with immune function, it is highly likely that microglia play a role in the development of sex differences in MOR expression and signaling. Opioid receptors and their endogenous ligands communicate bidirectionally with immune cells of the CNS, and all three opioid receptor subtypes (mu, kappa, and delta) have been localized on immune cells, including T-cells, B-cells, lymphocytes and macrophages [for review, see (Bidlack et al., 2006)]. Application of the endogenous opioid met-enkephalin increases LPS-induced release of proinflammatory IL-1β in primary brain cultures (Kowalski et al., 2002), while application of proinflammatory IL-1β or TNFα increases pro-enkephalin expression in astrocytes (Spruce et al., 1990). Similarly, the potent endogenous MOR agonists endomorphin 1 and 2 (EM1 and EM2) are expressed in immune cells and upregulated in response to peripheral inflammation (Jessop et al., 2000; Mousa et al., 2002). EM2 has been shown to modulate cytokine production (decreased TNFα and IL-10, increased IL-1β; (Azuma and Ohura, 2002), although the mechanism whereby EM2 and immune function contribute to the sexually dimorphic effects observed following endomorphin administration is not clear (Liu and Gintzler, 2013; Kumar et al., 2015b; Kumar et al., 2015a).

Neuronal MOR tone is also modified by increased glial activity in males such that the release of proinflammatory cytokines upregulates MOR expression both *in vivo* (Ji et al., 1995; Ruzicka and Akil, 1997; Mousa, 2003; Puehler et al., 2004) and *in vitro* (Ruzicka et al., 1996; Borner et al., 2004). In females however, glial activation does not similarly upregulate MOR expression (Zhang et al., 2014), suggesting that analgesia may be buffered by an increase in MOR following immune challenge in males but not

females. Together, these data support a bidirectional link between MOR and glia, suggesting that increased glial cells activity may influence MOR activity, contributing to the attenuated response to morphine observed in females. Our present data indicate increased activity of glial cells within the PAG, which may influence MOR tone and contribute to the dimorphic expression of MOR observed in our earlier studies (Loyd et al., 2008a).

Our novel studies examining M6G demonstrate that in the absence of TLR4 signaling, MOR-induced analgesia is more effective in females than in males. The reversal of sex differences in analgesic response with M6G suggests unique and sex-specific binding properties not shared by other MOR agonists (*e.g.* longer time at the receptor, or increased affinity in females). M6G, though more efficacious at MOR than morphine, has decreased affinity at the MOR receptor [see (Kilpatrick and Smith, 2005) for review], however, the binding properties of M6G have never been compared directly between males and females. Future studies examining MOR activation and binding by M6G are necessary, and may reveal novel pharmacokinetic mechanisms that can be applied to the development of new therapeutic drugs that improve analgesia in females relative to current opioid treatments.

4.3 Alternative pain treatments further suggest a role for glia

The data presented in this dissertation suggest a clear relation between analgesic efficacy and immune activity in females. Although a formal examination of our hypothesis has not been performed in the clinic, human studies have been inadvertently investigating sex differences in immune-derived pain and analgesia for quite some time.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) as an alternative treatment in women is perhaps the most clinically relevant example of this conclusion.

NSAIDs inhibit the enzyme cyclooxygenase (COX), which is responsible for production of inflammatory prostanoids. COX exists in two isoforms: COX-1, which is constitutively expressed in most cells, and COX-2 which is expressed more selectively in immune macrophages and is upregulated in response to inflammation (Hawkey, 2001; Bertolini et al., 2002). Importantly, COX-2 is a pro-inflammatory product of TLR4-mediated immune signaling (Cao et al., 1997; Zhang et al., 2008; Czapski et al., 2010; Tse et al., 2014; Gaikwad and Agrawal-Rajput, 2015). NSAIDs are commonly used in a clinical setting to reduce inflammation, and also as an opioid adjuvant (a non-opioid drug used to improve the efficacy of opioids, and reduce the negative side effects associated with opioid consumption) in both men and women (Elia et al., 2005).

Despite the prevalence of NSAID use, to our knowledge only two studies have directly investigated sex differences in the analgesic response of NSAIDs alone. Both studies were conducted by the same laboratory, and demonstrate greater analgesic efficacy of ibuprofen in males relative to females (Walker and Carmody, 1998; Butcher and Carmody, 2012). Studies of sex differences in analgesic efficacy of other commonly used NSAIDs have not been performed, however, robust sex differences have been observed in studies examining the prevalence of overall NSAID use (Dominick et al., 2003; Fosbol et al., 2008). Indeed, disparate incidence rates of 57% female vs. 17% male for NSAID prescriptions for cancer pain have been reported (Shinde et al., 2015). Interestingly, post-marketing studies show that women are the greatest consumers of COX-2 selective inhibitors [approximately 85%; (Solomon et al., 2006)], suggesting that

there is a much greater demand from women for drugs that reduce pain and/or improve opioid effectiveness by decreasing inflammation.

Both preclinical and clinical studies report that co-administration of COX-2 inhibitors with morphine significantly potentiates pain-relief in both sexes (Deciga-Campos et al., 2003; Pinardi et al., 2005; Reuben and Ekman, 2005; Aynehchi et al., 2014). In studies of women following gynecological surgery, treatment with the COX-2 selective inhibitor Rofecoxib significantly attenuates both surgical pain and opioid consumption (Sinatra et al., 2006), while treatment with parecoxib was associated with a lower incidence of post-operative headache and greater overall satisfaction compared with placebo (Luscombe et al., 2010). These studies are consistent with the present data demonstrating increased analgesic efficacy of opioids in females treated with immune-inhibiting drugs.

In further support of this conclusion, preclinical studies show that several other non-opioid analgesics used to improve opioid efficacy in men and women are also glial inhibitors. For example, tri-cyclic antidepressants are commonly used to improve pain treatment (McQuay et al., 1996). The tri-cyclic paroxetine inhibits microglia activation (Liu et al., 2014), while the tri-cyclic amitriptyline not only inhibits microglia activation, but has been shown to directly improve morphine analgesia in male rats by reversing morphine-induced decreases in glutamate transporter expression, and suppression of pro-inflammatory cytokines (Tai et al., 2006). Perhaps the most relevant and highly studied opioid adjuvant, the anti-seizure medication gabapentin, is commonly used to improve opioid analgesia, reduce the negative side effects of opioids, and reduce opioid consumption (Bennett, 2011; Shinde et al., 2015). Gabapentin's primary mechanism of

action is unclear, however, gabapentin is thought to modulate neuronal GABA synthesis and affect calcium gated ion channels on neurons (Taylor, 1997). In addition, gabapentin also inhibits the release of pro-inflammatory mediators (Yang et al., 2012) and promotes the release of anti-inflammatory mediators (Dambach et al., 2014) from microglia. Consistent with the studies presented in Chapter 2, Figure 2.3.5, gabapentin reverses sex differences in anti-nociception induced by tramadol in mice (Dai et al., 2008). Interestingly, when co-administered with morphine, gabapentin also reduces M3G formation by ~33% in male rats (Papathanasiou et al., 2016).

Overall, the literature suggests a pattern, that with the absence or inhibition of glial signaling, sex differences in anti-nociception are reduced or abolished. Inhibiting COX-2, a pro-inflammatory product of glia, improves opioid analgesia in men and women. Despite having vastly different effects on neurons, drugs such as gabapentin and amitriptyline are used clinically to improve analgesia in both sexes, likely through their similar actions on glial cells. The data presented in this dissertation directly test and support this theme, demonstrating that direct inhibition of glial TLR4 with (+)-naloxone abolishes sex differences in morphine anti-nociception, resulting in equivalent analgesia in males and females. Further, MOR agonism in the absence of glial activation, as is the case with M6G, results in the complete reversal of sex differences in anti-nociception. Together, these studies outline a clear strategy for pain management: improvements in opioid analgesia can be made by inhibiting or preventing the activation of glia, and this may be especially beneficial for pain management in women.

4.4 Future directions of pain treatment: Focus on the individual

Pain treatment has not changed drastically over the past two hundred years, and opioids remain the most prevalent and highly prescribed analgesic drugs. Opioids themselves have been altered only slightly to benefit unique types of pain, changing in their analgesic efficacy relative to morphine; for example, less painful conditions may be treated with the weak analgesic, codeine, and more painful conditions may be treated with the more potent analgesic, fentanyl. However, well-known issues with opioid use, such as the high risk of tolerance and dependence in both sexes—as well as decreased analgesic efficacy and increased negative side-effects in women—have not been addressed with these 'novel' opioids. The use of glial inhibitors to treat pain is finally beginning to address unique mechanisms to improve the analgesic efficacy of opioids in both sexes, and more directly reduce the negative side effects of opiate use such as hyperalgesia, tolerance, and dependence.

As we move forward, one thing is clear: it is important to consider sex differences in all pain research, both preclinical and clinical. It is not enough to include women in clinical studies and ignore them during data analysis. For example, although both men and women were included in recent clinical trials of COX-2 inhibitors, only 20% of the 28 clinical studies on rofecoxib (Cascales Perez et al., 2003), and 14% of the clinical studies of etoricoxib (Chilet-Rosell et al., 2009) stratified or analyzed their data by sex. Currently, examination of sex differences in the efficacy of analgesics is severely (and disappointingly) underrepresented in both preclinical and clinical studies.

The overall message of this dissertation is not that females should be given special treatment in pain research, rather, that men and women require different

treatment in order to achieve equal health outcomes. Furthermore, this body of work advocates for the study pain and immune function in *both men and women* because it uniquely effects the daily lives of so many individuals suffering from pain. Preclinical studies investigating pain and pain management should carefully consider biological sex when planning experiments, and it should go without saying that clinical studies need to analyze all possible aspects of their data—not just sex, but age and race as well. Careful investigation of sex-specific effects will not only provide a more complete understanding of the biological system but will facilitate new options for *individualized* treatment in both men and women.

Table 3. Observed Mechanisms Underlying Sex Differences in Analgesia and Hyperalgesia.

Manipulation	Sex / Species	Drug / Analgesic Treatment	Nociceptive Test	Effect on Analgesia (relative to control)	Procedural Comments	Reference
Reproductive hormones						
Adult: GDX Early life: Females+T or Males+GDX	M/F; SD rat	Morphine, s.c.	Hotplate	− Adult Males/Females ↓ Young Males + GDX ↑ Young Females + T	Indicates organizational but not activational effects of hormones	(Cicero et al., 2002)
E2 (chronic)	F; SD rat	Morphine, Cumulative (total 3.2 mg/kg; s.c.)	Hotplate Warm water tail-withdrawal	↑ at 4h; ↓ at 24h* and 48h.	*24h for tail-withdrawal test only	(Craft et al., 2008)
GDX	M/F; albino rat	Morphine (5 doses 1-40ug; i.c.v.)	Tail-flick, shock	− Males/Females	Analgesia ↓ at proestrus but not estrus or diestrus in intact females	(Kepler et al., 1989)
GDX	M/F; albino rat	Morphine (5 doses 1-10ug; intra-PAG)	Hotplate	− Males ↑ Females*	*Analgesia increased at high but not low doses	(Krzanowska and Bodnar, 1999)
E2, P, E2+P4 (chronic)	OVX F; SD rat	Morphine, (5mg/kg s.c.)	Hotplate	↓ with E2, P4, or E2+P4		(Ratka and Simpkins, 1991)
NMDA						
NMDA antagonism (Dextromethorphan [DXMP], ketamine, MK-801)	M/F; SD rat	Morphine (3mg/kg; s.c.)	Tail-flick	*DXMP:* ↑ Males ↑ Females Ketamine: − Males ↑ Females *MK-801:* − Males ↑ Females	Antagonist increased F analgesia at high doses only	(Holtman et al., 2003)
NMDA antagonism (MK-801)	M/F deer mice	Morphine (1mg/kg; i.p.)	Hotplate	↓ Males ↓ Females	Complete attenuation in males, partial attenuation in females	(Lipa and Kavaliers, 1990)
NMDA antagonism (Mk-801) ±GDX	M/F; SW mice	Forced swim	Hotplate	↓ Intact Males − Intact Females ↓ GDX Males ↓ GDX Females		(Mogil et al., 1993)

NMDA antagonism (Dextromethorphan [DXMP), Dextrorphan [DXTP], MK-801, LY235959, L-701324, Ro25-6981)	M/F; CD1 mice	Morphine (15, 25, 35 and 45 mg/kg; i.p.)	Tail-flick	*DXMP:* ↑/↓ Males −/− Females *DXTP*: ↑/− Males −/− Females *MK-801:* ↑/− Males −/− Females *LY235959:* ↑/↑ Males ↑/↑Females *L-70132:* ↑/↑ Males ↑/↑Females *Ro25-6981:* −/↓ Males −/↓Females	*Results show effects of antagonists on LOW (15, 25mg/kg) / HIGH (35, 45 mg/kg) doses of morphine analgesia	(Nemmani et al., 2004)
NMDA antagonism (MK-801); ± Progesterone	M/ OVXF; CD1 mice	Morphine (40 mg/kg)*	Tail-withdrawal	↑ Intact Males − Intact Males + P − Intact Females ↑ OVX Females − OVX Females + P	*Measures morphine hyperalgesia. Here, increased analgesia reflects decreased hyperalgesia	(Waxman et al., 2010)
MOR						
MOR antagonism	M/F; SD rat	Morphine (0.3, 1.0, 3.0, or 10ug); s.c.	Tail withdrawal	↓ Males ↓ Females*	*Antagonist more potent in females	(Bernal et al., 2007)
MOR antagonism	M/F; SD rat	Fentanyl, morphine, buprenorphine	Hotplate	↓ Males ↓ Females*	*Antagonist more potent in females	(Craft et al., 2001)
MOR lesions ± inflammatory CFA	M/F; SD rat	Morphine Cumulative (up to 18mg/kg); s.c.	Hotplate PWL	↓ Males − Females		(Loyd et al., 2008a)
MOR antagonism	M/F; SD rat	Morphine, (cumulative up to 10mg/kg);s.c.	Wail withdrawal	↓ Males ↓ Females*	*Antagonist more potent in females	(Peckham et al., 2005)

Table 4. Mechanisms Attributed to Sex Differences in Morphine Analgesia Share an Inverse Relation with Inflammatory Markers.

Manipulation	Model / Tissue	Immune challenge	Effect on Pro-Inflammatory Markers	Procedural comments	Reference
Reproductive hormones Note: Given known discrepancies between *in vitro* and *in vivo* application of steroid hormones, studies included in this table are limited to *in vivo* application of hormones only.					
E2	OVX Female C57BL/6J mice	LPS *ex vivo*	↑	E2 for 4 weeks before testing	(Calippe et al., 2008)
E2	Intact M/ OVX F; SD rat	LPS *ex vivo*	− Male ↑ Female	E2 for 12 days before testing	(Loram et al., 2012)
E2, P4, E2+P4	OVX Female C57BL/6 mice	LPS *in vivo*	− P4 ↑ E2 ↑ E2+P4	E2, P4 for 5 weeks before testing	(Rettew et al., 2009)
OVX	Female CD1 and C57BL/6J mice	LPS *in vivo*	↓ 24h post-LPS − 3d, 7d post-LPS		(Soucy et al., 2005)
NMDA					
NMDA antagonism (DXMP)	BV2 microglia cell line	LPS in vitro	↓		(Cheng et al., 2015)
NMDA antagonism (MK-801)	Sex not specified; 1d old Wistar rat	Hypoxia *in vivo*	↓		(Murugan et al., 2011)
NMDA antagonism (MK-801)	M/F; rat	None	− Male ↑ Female		(Nieto-Sampedro et al., 1991)
NMDA antagonism (MK-801, DXMP)	BV2 microglia cell line	LPS in vitro	↓		(Thomas and Kuhn, 2005)
MOR					
MOR antagonist [(-)-Naloxone]	Male; SD rat	Chronic constriction injury in vivo	↓	*CNS inflammation	(Hutchinson et al., 2008a)
MOR-KO	Sex not specified; B6 background	Restraint stress + allergen	−	*Suggests in the absence of MOR, there is no challenge-induced increase in inflammation	(Okuyama et al., 2010)
MOR agonism (morphine)	Male; SD rat	L5 spinal nerve injury	↑	*CNS inflammation; Increases in immune activation linked with decreases in analgesia and increases in morphine tolerance	(Raghavendra et al., 2002)

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