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Mini-Review

Sex Differences in Innate Immunity and Its Impact on Opioid Pharmacology

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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. In addition to binding to the neuronal μ-opioid receptor, morphine binds to the innate immune receptor toll-like receptor 4 (TLR4), located on glial cells. Activation of glial TLR4 initiates a neuroinflammatory response that directly opposes morphine analgesia. Females of many species have a more active immune system than males; however, few studies have investigated glial cells as a potential mechanism driving sexually dimorphic responses to morphine. This Mini-Review illustrates the involvement of glial cells in key processes underlying observed sex differences in morphine analgesia and suggests that targeting glia may improve current treatment strategies for pain.

Key words: opioids; analgesics; glia; pain

Chronic pain is one of the most commonly reported health problems in the United States, affecting approximately 25% of the population and increasing in prevalence with age (Elzahaf et al., 2012; Mogil, 2012; Kennedy et al., 2014). As clinicians advance toward more individualized treatment strategies for pain, the importance of biological sex is becoming increasingly clear. Indeed, women have a higher incidence rate of chronic pain conditions, in particular, those that include an inflammatory component, such as fibromyalgia, migraine, and osteoarthritis (Unruh, 1996; Fillingim et al., 2009; Mogil, 2012; Ruau et al., 2012; Buse et al., 2013; Kennedy et al., 2014). Descending pain modulatory circuits in the central nervous system, in particular the midbrain periaqueductal gray (PAG) and its descending projections to the rostral ventral medulla (RVM) and spinal cord, show innate sex differences in their anatomy and physiology that greatly influence pain management and the effectiveness of opioid drugs (Loyd and Murphy, 2006, 2014; Loyd et al., 2008a).

Morphine has been and continues to be one of the most effective and widely used drugs for the treatment of pain. However, preclinical studies using a variety of acute and persistent pain assays have repeatedly demonstrated that morphine is a more effective analgesic in males than in females (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., 2008b). Clinical studies examining sex differences in analgesia are more varied, with reports of decreased analgesic efficacy of morphine in females (Cepeda and Carr, 2003; Mehlisch, 2003; Miller and Ernst, 2004) as well as lower analgesia in males (Sarton et al., 2000; Niesters et al., 2010) and no sex difference at all (Fillingim et al., 2009). Despite discrepancies in absolute analgesia with morphine administration, women consistently experience a greater preponderance of the negative side effects associated with morphine consumption, including nausea, dysphoria, headache, and vomiting (Myles et al., 1997; Cepeda et al., 2003; Fillingim et al., 2005; Comer et al., 2010). Thus, development of novel nonopioid-based treatment strategies, or adjuvants to morphine that can improve analgesic quality in females, is clearly warranted.

SIGNIFICANCE

This Mini-Review focuses on glial cells as a potential mechanism underlying the observed sex differences in analgesic responses to morphine. These findings demonstrate that glia may be a powerful therapeutic target to improve the treatment of pain and the efficacy of opioid analgesics in both males and females. In addition, we illustrate the necessity of sex-specific research on pain and analgesia and individualized treatment strategies for the management of pain in men and women.

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MORPHINE AND NEUROINFLAMMATION

Glial cells, specifically microglia and astrocytes, are relatively new targets in the search for improved pain therapeutics (Tanga et al., 2005; Detloff et al., 2008; Milligan and Watkins, 2009; Nicotra et al., 2012). Glial cells become "activated" in the event of CNS trauma or infection, when pattern recognition receptors known as toll-like receptors (TLRs) bind pathogenic or damage-associated molecules and mount an immune response (Watkins and Maier, 2003; Bianchi, 2007; Watkins et al., 2009; Buchanan et al., 2010). Activation of glial TLR4 induces the expression of both pro- and anti-inflammatory molecules such as cytokines (interleukins [IL]-1, -6, and -10; tumor necrosis factor-α [TNFα]), chemokines, cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and reactive oxygen species (Bonizzi and Karin, 2004; Doyle and O'Neill, 2006).

Similar to pathogenic molecules, morphine also binds to TLR4, in particular, the myeloid differentiation factor 2 (MD2) pocket of TLR4, to induce proinflammatory cytokine release and neuronal excitation that paradoxically reduces the analgesic efficacy of morphine (Stellwagen et al., 2005; Hutchinson et al., 2007, 2010; Franchi et al., 2012; Li, 2012; Eidson and Murphy, 2013a; Thomas et al., 2015). We have recently reported that chronic systemic administration of morphine in male rats activates TLR4 within the PAG, a brain region critical for opioid-induced analgesia, to induce local cytokine release, including TNFα (Eidson, 2016). Centrally, TNFα binds to the neuronal TNF receptor (TNFR1) to downregulate $GABA_A$ receptors, upregulate AMPA receptors, and increase extracellular concentrations of glutamate and K^+, producing a net increase in neural excitability (Ogoshi et al., 2005; Stellwagen et al., 2005; Watkins et al., 2005; Yan et al., 2014; for review see Tilleux and Hermans, 2007). This overall increase in neural excitability as a result of glial activation and cytokine release reduces opioid-mediated hyperpolarization and subsequent GABA release, resulting in an attenuation of morphine analgesia (Watkins et al., 2009; Hutchinson et al., 2010; Loram et al., 2012). Morphine opposition by glial activation is further supported by studies demonstrating that inhibition of microglia or TLR4 signaling or blockade of proinflammatory cytokine production results in a potentiation of morphine-induced analgesia (Raghavendra et al., 2004; Watkins et al., 2005; Hutchinson et al., 2008a,b,; Li, 2012; Eidson and Murphy, 2013a; Bai et al., 2014).

SEX DIFFERENCES IN INNATE IMMUNITY

Sex differences in immune responsiveness have been well demonstrated, with females mounting a more robust immune response than males (for reviews see Marriott and Huet-Hudson, 2006; Garcia-Segura and Melcangi, 2006; 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 proinflammatory molecules (i.e., cytokines) following immune challenge has been well documented, with females generally producing higher levels of proinflammatory cytokines than males, although the direction of the effect is not always consistent across experimental models (Drew and Chavis, 2000; Aulock et al., 2006; Calippe et al., 2010; Loram et al., 2012; Engler et al., 2016). Our laboratory has observed comparable effects in rats, with peripheral lipopolysaccharide (LPS) treatment disproportionately increasing glia activation and release of proinflammatory cytokines in females relative to males (Doyle and Murphy, unpublished observations). Similar results have been reported clinically, demonstrating that in healthy humans LPS immune challenge elicits a greater cytokine response in females (Karshikoff et al., 2015). This increase in cytokine release 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 pronociceptive cytokine response that is potentiated in females.

Although increased immune responsiveness in females is thought to be neuroprotective, overactivation 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 autoimmune disorders (Whitacre, 2001; Cooper and Stroehla, 2003) and chronic inflammatory conditions observed in females.

CURRENT HYPOTHESES UNDERLYING SEX DIFFERENCES IN MORPHINE ANALGESIA ARE LINKED TO GLIAL ACTIVITY

Several mechanisms have been identified as contributing to the dimorphic effects of morphine, including reproductive hormones (Craft, 2007), γ-aminobutyric acid (GABA), glutamate, and melanocortin-1 signaling (Mao, 1999; Lau and Vaughan, 2014; Tonsfeldt et al., 2016) and μ-opioid receptor (MOR) density and tone (Loyd and Murphy, 2014; see Table I). The fact that so many seemingly competitive theories exist to account for the dimorphic effects of morphine implies that a parallel and/or upstream mediator of these effects has yet to be identified. Given the inverse relationship between glial activation and analgesia (see Table II), we propose that sex differences in innate immune function may be a precursor of and/or a significant contributor to the sexually dimorphic actions of morphine. Therapeutic targeting of immune

TABLE I. Observed Mechanisms Underlying Sex Differences in Analgesia and Hyperalgesia*

Manipulation	Sex/species	Drug/analgesic treatment	Nociceptive test	Effect on analgesia (relative to control group)	Procedural comments	Reference
Reproductive hormones						
Adult: GDX Early life: Males + GDX or females + T	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 4 hr ↓ At 24 hr and 48 hr	24 hr for tail-withdrawal test only	Craft et al., 2008
GDX	M/F; albino rat	Morphine, five doses of 1–40 μg 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, five doses of 1–10 μg intra-PAG	Hotplate	− Males ↑ Females	Analgesia increased at high but not low doses	Krzanowska and Bodnar, 1999
E2, P4, E2 + P4 chronic)	OVX F; SD rat	Morphine, 5 mg/kg s.c.	Hotplate	↓ With E2, P4, or E2 + P4		Ratka and Simpkins, 1991
NMDA						
NMDA antagonism (DXMP, ketamine, MK-801)	M/F; SD rat	Morphine, 3 mg/kg s.c.	Tail flick	DXMP: ↑ Males ↑ Females Ketamine: − Males ↑ Females MK-801: − Males ↑ Females	Antagonist increased female analgesia at high doses only	Holtman et al., 2003
NMDA antagonism (MK-801)	M/F deer mice	Morphine, 1 mg/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 (DXMP, 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	Results show effects of antagonists on low (15, 25 mg/kg)/high (35, 45 mg/kg) doses of morphine analgesia	Nemmani et al., 2004

TABLE I. *Continued*

Manipulation	Sex/species	Drug/analgesic treatment	Nociceptive test	Effect on analgesia (relative to control group)	Procedural comments	Reference
				−/↓ Females		
NMDA antagonism (MK-801 ± P)	M/OVXF; CD1 mice	Morphine, 40 mg/kg	Tail withdrawal	↑ Intact males – Intact males + P – Intact females ↑ OVX females – OVX females + P	Study 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 10 μg s.c.	Tail withdrawal	↓ Males ↓ Females	Antagonist more potent in females	Bernal et al., 2007
MOR antagonism	M/F; SD rat	Fentanyl, morphine, buprenorphine, s.c.	Hotplate	↓ Males ↓ Females	Antagonist more potent in females	Craft et al., 2001
MOR lesions ± inflammatory CFA	M/F; SD rat	Morphine, cumulative up to 18 mg/kg s.c.	Hotplate	↓ Males – Females		Loyd et al., 2008b
MOR antagonism	M/F; SD rat	Morphine, cumulative up to 10 mg/kg s.c.	Tail withdrawal	↓ Males ↓ Females	Antagonist more potent in females	Peckham et al., 2005

*GDX, gonadectomized; OVX, ovariectomized; T, testosterone; E2, estradiol-2; P4, progesterone-4; SD, Sprague-Dawley; DXMP, dextromethorphan; DXTP, dextrorphan.

cells may greatly improve pain management in both men and women.

Estradiol

Studies employing a variety of techniques and pain modalities have assessed the role of steroid hormones, in particular, estradiol, in modulating pain and opiate analgesia. In terms of basal sensitivity, most preclinical studies report no differences in somatosensory thresholds across the estrous cycle, although pro- and antinociceptive 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 in rodents (for review see Craft et al., 2004): 1) morphine is most efficacious in gonadally intact male 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 are peaking, relative to diestrus. Together this body of literature suggests that female hormones, specifically estradiol, increase hyperalgesia and decrease the antinociceptive potency of morphine.

Estradiol has also been implicated as 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-α (ERα), making them susceptible to changes in estradiol across the estrous/menstrual cycle (Sierra et al., 2008). Estradiol also 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 on 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; Chakrabarti et al., 2014). 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; chronic but not acute estradiol administration increases TLR4-mediated proinflammatory responses in immune cells of females compared with males (Soucy et al., 2005; Calippe et al., 2008, 2010) and potentiates LPS-evoked TLR4 immune responses in vitro (Loram et al., 2012). Estradiol's effects may also be dependent on the setting because

TABLE II. Mechanisms Attributed to Sex Differences in Morphine Analgesia Share an Inverse Relation With Inflammatory Markers*

Manipulation	Model/tissue	Immune challenge	Effect on proinflammatory markers (relative to control group)	Procedural comments	Reference
Reproductive hormones					
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	↓ 24 hr post-LPS − 3 days, 7 days 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; 1-day-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., 2008b
MOR knockout	Sex not specified; B6 background	Restraint stress + allergen	−	Suggests thast, 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 directly linked with decreases in analgesia and increases in morphine tolerance	Raghavendra et al., 2002

*Note: Given the known discrepancies between in vitro and in vivo application of steroid hormones, studies included here are limited to in vivo application of hormones. OVX, ovariectomized; E2, estradiol-2; P4, progesterone-4; SD, Sprague-Dawley; DXMP, dextromethorphan.

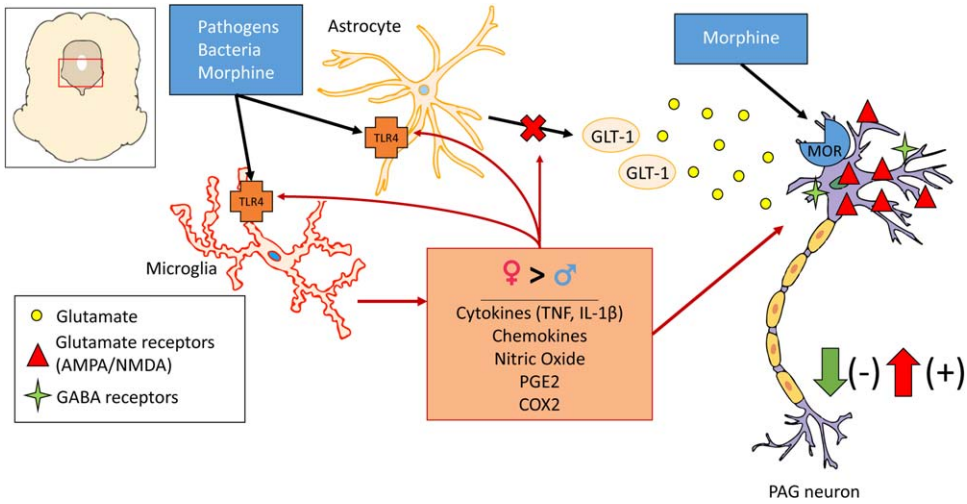

Fig. 1. Impact of TLR4 activation. Microglial and astrocytic TLR4 in the periaqueductal gray respond to immune challenge or morphine activation, and increase the expression of pro- and anti-inflammatory mediators. Pro-inflammatory cytokines (specifically TNFa), decrease GABAR, upregulate AMPAR, and decrease astrocytic glutamate transporters (GLT-1), resulting in excess glutamate in the synapse and net neuro-excitation associated with neuropathic pain. Pro-inflammatory conditions appear to be exacerbated in females relative to males, and likely contributes to decreased opioid efficacy in females.

in vitro administration is often anti-inflammatory (Drew and Chavis, 2000), but estradiol administered in vivo results in proinflammatory responses (Soucy et al., 2005; Calippe et al., 2008; Rettew et al., 2009; Loram et al., 2012).

Gutamate, Melanocortin, and GABA Signaling

The antinociceptive effects of opiates are mediated, in part, through removal of $GABA_A$-mediated inhibition on excitatory glutamatergic ventrolateral PAG (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 MOR agonists, hyperpolarizes GABAergic interneurons to decrease (or disinhibit) GABA signaling and facilitate opioid analgesia (Vaughan et al., 1997). Dimorphic glutamatergic and GABAergic signaling within the PAG and RVM have been identified as a contributing factor to sex differences in opiate analgesia. For example, blockade of N-methyl-D-aspartate (NMDA) receptor signaling in male mice via peripheral antagonist administration results in a complete attenuation in both the analgesic and the 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 in, or has no effect on, morphine analgesia in female mice (Lipa and Kavaliers, 1990; Mogil et al., 1993; Holtman et al., 2003; Nemmani et al., 2004; Craft and Lee, 2005; Waxman et al., 2010). These data suggest that NMDA receptor signaling is necessary to facilitate morphine antinociception in males but not females. With 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 MOR-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). In addition to NMDA/MCR1, 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). Furthermore, 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). These findings together suggest that NMDA and $GABA_A$ receptor signaling may be innately 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 proinflammatory cytokine release, including TNFα, 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, See Figure 1). Morphine-induced TNF release has also been shown to site specifically

downregulate glial GLAST and GLT-1 glutamate transporter expression in the vlPAG (Eidson, 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). 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) further supports the idea that downstream sex differences in the neuroexcitatory effects of glial activation on glutamate and GABA signaling contribute to sex differences in opioid analgesia.

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, 2009; Bernal et al., 2007; Loyd et al., 2008a). Specifically, we have previously reported 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, 2009; Loyd et al., 2008b). Indeed, MOR levels are 40% lower in proestrus females compared with males; this corresponds to the stage of estrous when intra-PAG morphine is least effective in modulating pain (Loyd et al., 2008b). Furthermore, 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., 2008b).

Not all MOR agonists produce sexually dimorphic responses, suggesting that factors in addition to opioid receptor expression contribute. For example, sufentanil, a potent MOR agonist, produces comparable levels of analgesia in both males and females (Kumar et al., 2015a). This lack of sex difference is not due to receptor affinity because the analgesic response to [D-Ala2-N-Phe 4, Gly-ol]-enkephalin (DAMGO), which has equal efficacy for MOR (Saeki and Yaksh, 1993; Emmerson et al., 1996), is highly sexually dimorphic (Bobeck et al., 2009; Bai et al., 2015). A thorough investigation of nine commonly used opioids further indicates that MOR binding affinity has little or no relationship to sex differences in response (Peckham and Traynor, 2006). Together these results suggest that, despite physiological differences in MOR expression and affinity, these factors alone do not account for the attenuated response to morphine observed in females.

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 (μ, κ, and δ) 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), whereas application of proinflammatory IL-1β or TNFα increases proenkephalin expression in astrocytes (Spruce et al., 1990). Similarly, the potent endogenous MOR agonists endomorphins 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 (Labuz et al., 2006; Liu and Gintzler, 2013; Kumar et al., 2015a,b).

Neuronal MOR tone is also modified by increased glial activity such that in males 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). Neuronal MOR tone is also modified by increased glial activity, such that the release of proinflammatory cytokines upregulates MOR expression in males but not females (Zhang et al., 2014). Increased release of pronociceptive messengers from glia, sex differences in anti-inflammatory EM2 activity, and a lack of MOR upregulation upon glial cell activation may further contribute to the attenuated response to morphine observed in females.

SEX DIFFERENCES IN MICROGLIA AND TLR4 AFFECT PAIN AND ANALGESIA IN RODENTS

Given the robust evidence that microglial TLR4 activation directly opposes the analgesic response to morphine (Raghavendra et al., 2004; Watkins et al., 2005; Hutchinson et al., 2008a,b,; Li, 2012; Eidson and Murphy, 2013a,b; Bai et al., 2014), our group is now investigating the contribution of TLR4 signaling in the attenuated response to morphine observed in females. Our recent data demonstrate that blockade of TLR4 signaling with intra-PAG administration of (+)-naloxone, a selective TLR4 antagonist, results in a significant, twofold leftward shift in the morphine dose–response curve for females, such that no sex differences in morphine ED_{50} values are observed (male $ED_{50} = 3.04$ mg/kg, female ED_{50} decreased from 7.9 mg/kg to 3.16 mg/kg). Similarly, activation of PAG microglia with site-specific administration of the TLR4 agonist LPS results in a twofold rightward shift in the morphine dose–response curve for males

(ED_{50} morphine = 3.04 mg/kg; ED_{50} with LPS = 10.69 mg/kg), such that the response to cumulative doses of morphine are comparable between the sexes (ED_{50} morphine = 7.9 mg/kg). These data suggest that TLR4 signaling in the vlPAG is both necessary and sufficient to induce sex-specific responses to morphine.

The impact of TLR4 signaling on sex differences in response to morphine may be site specific (Sorge et al., 2011, 2015). Spinal cord blockade of TLR4 attenuates the inflammation and allodynia induced by either intrathecal LPS or intraplantar complete Freund's adjuvant (CFA), as well as sparing nerve injury, but in males only (Sorge et al., 2011). This suggests that in females chronic inflammatory pain signaling in the spinal cord utilizes a TLR4-independent pathway. Indeed, adaptive immune T cells are used in females as an analogous method of producing allodynia in response to chronic pain. In the presence of testosterone, or in the absence of adaptive immune cells, females switch to the male-like TLR4-dependent pathway (Sorge et al., 2015). This sex difference in TLR4 function illustrates the clear need for consideration of sex in studies involving pain and immunity because drugs targeting the immune system may not be equally efficacious in alleviating pain in males and females.

CLINICAL FINDINGS SUPPORT AN IMMUNE-BASED TREATMENT FOR WOMEN

The lack of preclinical studies on immune-based treatments for pain in females is surprising, particularly given that clinical studies have been indirectly investigating sex differences in immune-derived pain and analgesia for quite some time. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in a clinical setting to reduce inflammation, improve the efficacy of morphine, and reduce the negative side effects associated with morphine consumption (Elia et al., 2005). Although it is not known whether the response to NSAIDs is sexually dimorphic, robust sex differences have been observed in studies examining the prevalence of 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). 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). COX-2 is a proinflammatory 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). Both preclinical and clinical studies indicate that coadministration of COX-2 inhibitors (e.g., celecoxib) with morphine significantly potentiates the resulting pain relief in both sexes (Vaughan et al., 1997; Deciga-Campos et al., 2003; Pinardi et al., 2005; Reuben and Ekman, 2005; Aynehchi et al., 2014). Similarly, in studies of women after gynecological surgery, treatment with the COX-2-selective inhibitor rofecoxib significantly attenuated both surgical pain and opioid consumption (Sinatra et al., 2006), whereas treatment with parecoxib was associated with a lower incidence of postoperative headache and greater overall satisfaction compared with placebo (Luscombe et al., 2010).

Postmarketing studies show that women are the greatest consumers of COX-2 selective inhibitors (approximately 85%; Solomon et al., 2006), suggesting that there is a greater demand from women for drugs that inhibit inflammation and/or improve opioid effectiveness. Although both men and women are included in clinical trials of COX-2 inhibitors, studies that include sex as a factor for analysis are extremely limited. A meta-analysis of the COX-2 inhibitor rofecoxib shows that, among the 28 clinical studies, only 20% specifically address sex differences in effectiveness, 8% considers hormonal effects on the observed results, and only one of these studies discusses sex-specific negative side effects (Cascales Perez et al., 2003). A similar meta-analysis of the 58 clinical studies of etoricoxib indicated that only 14% stratified data by sex, 7% analyzed the adverse effects by sex, and a shocking 4% discussed the results by sex (Chilet-Rosell et al., 2009). Sex-specific (i.e., hormonally derived) pharmacokinetics were not discussed in any clinical trial examined in these meta-analyses. Despite the evidence described above, sex differences in the efficacy of COX-2 inhibitors is severely (and disappointingly) underrepresented in clinical trials (Demyanets and Wojta, 2012).

CONCLUSIONS

This Mini-Review addresses current gaps in the preclinical and clinical literature investigating sex differences in the effectiveness of opioids. The studies reviewed here demonstrate an intimate relationship among pain, analgesia, and inflammation and suggest a prominent role for glial activation of TLR4 in the mediation of sexually dimorphic responses to morphine. Although several contributing factors have been identified, including sex differences in gonadal steroids and opioidergic signaling, these mechanisms likely occur in tandem with, or as a result of, sex differences in glial activation. This Mini-Review also highlights the fact that sex differences are continuously underacknowledged in preclinical and clinical studies of immune function and drug administration, despite evidence that females may be especially at risk for developing inflammatory pathologies compared with their male counterparts (Whitacre et al., 1999; Whitacre, 2001; Kivity and Ehrenfeld, 2010) and that females are more likely than males to be taking anti-inflammatory drugs that improve opiate action. Future studies addressing the role of glia in morphine modulation of pain will facilitate the development of novel treatment strategies. Specifically, preclinical immune studies on pain modulation should include both sexes and include sex as an independent variable. 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.

CONFLICTS OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

Both authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the analysis. Concept and design: AZM, HHD. Drafting of the manuscript: HHD. Critical revision of the manuscript for important intellectual content: AZM. Study supervision: AZM.

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