THE ROLE OF TOLL-LIKE RECEPTOR 4 IN PAIN DUE TO GLUCURONIDATED METABOLITES AND NEUROPATHIC PAIN

by

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A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirement for the degree of

Doctor of Philosophy

Department of Psychology and Neuroscience

2011

This thesis entitled:
The role of toll-like receptor 4 in pain due to
glucuronidated metabolites and neuropathic pain
written by Susannah S. Lewis
has been approved for the Department of Psychology and Neuroscience

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Dr. Linda Watkins (chair)

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The final copy of this thesis has been examined by the signatories, and
we find that both the content and the form meet acceptable presentation
standards of scholarly work in the above mentioned discipline.

ABSTRACT

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The role of toll-like receptor 4 in pain due to glucuronidated metabolites and neuropathic pain.

Directed by Dr. Linda R. Watkins, Department of Psychology and Neuroscience, University of Colorado at Boulder

Toll-like receptor 4 (TLR4) has recently been implicated in both chronic neuropathic pain and in counter-regulating morphine analgesia. The studies undertaken here further show the potential for TLR4 signaling activation to produce pain states. Specifically, a group of metabolites, glucuronide metabolites, are shown to activate TLR4 signaling in an *in vitro* model and cause transient acute pain when injected intrathecally in small doses. The glucuronide metabolites tested included morphine-3-glucuronide, ethyl glucuronide, corticosterone-21-glucuronide, estradiol-3-glucuronide, and estradiol-17-glucuronide, as well as glucuronic acid itself. These metabolites were previously considered largely non-reactive without known biological activity or toxicity at clinically relevant doses. The current studies present the first evidence that they may cause TLR4-dependent enhanced pain, which could have influence in diverse conditions such as morphine withdrawal hyperalgesia, hangover headache and menstrual migraine. Additionally, a TLR4 signaling inhibitor, (+)-naloxone was tested in neuropathic pain and morphine-3-glucuronide induced pain. (+)-Naloxone does not act on opioid receptors, as the (-)-naloxone isomer does, but has been shown to reduce TLR4 signaling *in vitro* and *in vivo*. (+)-Naloxone blocked the development of pain following intrathecal

morphine-3-glucuronide injection, and reversed neuropathic pain in multiple models, up to 4 months following nerve injury. (+)-Naloxone is a candidate small molecule, blood-brain barrier permeable molecule for treating pain conditions where TLR4 is involved.

Dedication

For my mother, Huston Diehl.

Acknowledgements

This work has benefitted greatly from the intellectual insight, creative talents and collaborative atmosphere of the entire Maier-Watkins lab, in particular Steve Maier, Mark Hutchinson, Lisa Loram, Julie Wieseler and Khara Rhamos. Expert technical contributions from Yingning Zhang further enhanced this work, as did assistance from undergraduates Dana Hund, Hannah Goodman and Annie Grotheer. And finally I wish to thank my advisor, Dr. Linda Watkins, without whom this work simply would not have happened. Her high standards have challenged me to do my best work while her steadfast support made it all seem possible.

Financial support of this work came, in part, from NIH Grants DA023132, DA015642, DA024044, and DE017782. This work was also supported in part by the NIH Intramural Research Programs of National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism. This work was additionally supported by an International Association for the Study of Pain International Collaborative grant. We additionally thank Dr. Kirk Johnson from Avigen for the gift of the HEK293-TLR4 cell line.

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CHAPTER 1

INTRODUCTION

The conventional neuron-centric view of pain processing has been significantly enhanced in recent years by a growing understanding of the role of glial cells and proinflammatory molecules in the initiation and maintenance of pain states. This work has brought about novel targets for chronic pain treatment, as well as led to a better understanding of the mechanisms for a range of effects, including neuropathic pain and opioid drug action. The studies presented here propose to further characterize a particular mechanism, enhanced TLR4 signaling, for proinflammatory production in the central nervous system and consequent enhanced pain states.

PAIN PROCESSING

Pain Pathways

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage," (Merskey and Bogduk, 1994). The basic neural mechanisms leading to the sensation of acute pain have long been studied and are well understood. Pain sensation begins with peripheral nociceptors, found on sensory neurons in the skin and certain internal tissues. These peripheral nerve endings are stimulated by signals indicating potential tissue damage, such as hot, cold, or noxious chemicals. The peripheral neurons responding to noxious stimuli are either myelinated Aδ fibers, or slower, unmyelinated C fibers. Both fiber types have cell bodies in the dorsal root ganglia located just outside the spinal column. Following stimulation, the

sensory afferents send action potentials to the dorsal horn of the spinal cord where they first synapse. The dorsal horn is the first site of pain integration and modulation, and a focus of several of the studies presented here. From the dorsal horn, second order neurons send axons up the contralateral anterolateral funiculus to the ventrobasal and posterior thalamic nuclei, where third order neurons then relay pain information ipsilaterally to the sensory cortex and other cortical areas. Notably, the pain pathway in the CNS has numerous redundancies that make it very difficult to completely cut off pain sensation.

Within the dorsal horn of the spinal cord, a key region for the studies present here, integration and processing of pain information first takes place. The dense grey matter of the dorsal horn is organized into laminae, with nociceptive neuronal cell bodies that give rise to the spinothalamic tract being found principally in laminae I, IV, and V. Lamina I, the most superficial, receives primary sensory input from C and Aδ fibers. These fibers are all glutamatergic, with a sizeable subset also releasing substance P (Todd, 2010). Interneurons, both excitatory and inhibitory, from Laminae I and II project to the deeper layers (Todd, 2010). Laminae IV and V receive input not only from pain pathways, but from other modalities, including touch, conveyed by Aβ fibers, and integration between sensory modalities as well as top-down modulation from the brain occurs (Wall, 1967). The presence of first order neurons from the periphery and the integrative nature of the dorsal horn make it a prime location for both endogenous and experimental modulation of the pain response.

Modulation of pain processing

The degree of pain elicited by a given stimulus can be altered by several neuronal and non-neuronal mechanisms. Such alterations can cause an enhanced pain response to the same stimulus (hyperalgesia), or cause a previously nonpainful stimulus to elicit a pain response (allodynia). Such conditions occur following injury and often involve altered activity of neurons in the dorsal horn (Woolf and Salter, 2000). Several neuronal mechanisms influence the development of these states. The brain can influence the firing rate of spinal neurons through top-down inhibition or enhancement of the pain response (Wall, 1967). Additionally, the endogenous opioid system can reduce the amount of pain felt through the release of opioid peptides (Stefano et al., 2009). Finally, several mechanisms, including receptor upregulation and sensitization, can result in peripheral and central sensory neurons becoming more responsive to stimulation (Woolf and Salter, 2000).

Recently, the conventional, neuron-based view of pain modulation has been improved by a growing understanding of the role and function of proinflammatory molecules secreted by cells in the dorsal horn with innate immune abilities. These cells can become stimulated to secrete proinflammatory molecules by tissue damage in the CNS or remotely on the peripheral nerve, pathogen invasion or the presence of an immunogenic molecule, a process detailed in section 2.1. Many proinflammatory cytokines have neuroexcitatory effects. Interleukin-1β (IL-1), for instance, increases calcium conductance NMDA receptors, leading to enhanced glutamate signaling (Viviani et al., 2003). Astrocytes activated by proinflammatory molecules also down-regulate glutamate transporters, leaving a higher concentration of glutamate in the synapse, enhancing signaling (Tawfik et al., 2008). Both microglia and astrocytes secrete

prostaglandins and nitric oxide following proinflammatory activation, both of which can also enhance pain (Besson, 1999). Proinflammatory activation in the absence of tissue damage has also been shown to be sufficient for pain to develop. Intrathecal injection of IL-1, for instance, can induce pain when administered over the lumbar dorsal horn (Falchi et al., 2001). Another example is the HIV envelope protein, gp120. When injected intrathecally, gp120 causes an increase in dorsal lumbar spinal cord proinflammatory cytokine expression and a corresponding increase in pain sensitivity (Milligan et al., 2001; Ledeboer et al., 2005). These studies demonstrate that an immunogenic molecule, when injected intrathecally over the dorsal horn, can cause a sufficient spinal cord response to produce changes in pain behavior.

Pathological pain states

Although unpleasant, pain serves an important role in alerting an organism to situations with the potential to damage tissues, and encouraging rest while an injury heals. However, in certain situations pain continues even after tissues have healed, becoming a pathological condition itself. Chronic pain conditions, including neuropathic pain conditions, fibromyalgia, tempromandibular joint disorder, lower back pain and others affect millions of people. These conditions are often resistant to currently available treatments, making a better understanding of the mechanisms for chronic pain and the development of novel treatments an important priority. The powerful opioid analgesics, including morphine, fentanyl, and oxycontin, can provide temporary relief but the development of tolerance and dependence limits their long-term effectiveness.

The secretion of proinflammatory molecules in the CNS, principally by microglia and astrocytes, is a critical component of numerous pain conditions, including bone cancer pain (Lan et al., 2010), pain caused by an HIV envelope protein (Milligan et al., 2001), pain caused by the chemotherapy agent taxol (Ledeboer et al., 2007) and neuropathic pain following injury to peripheral nerve fibers. The subject of a series of studies in this dissertation, neuropathic pain is particularly well studied with regard to the role of proinflammatory glial activation. The microglial activation marker, CD11b, and the astrocyte activation marker, glial fibrilliary acidic protein (GFAP) are reliably upregulated in neuropathic pain conditions (Tsuda et al., 2003; Narita et al., 2006; Hutchinson et al., 2008b). Neuropathic pain is transiently reversed by glial activation inhibitors, such as minocycline (Raghavendra et al., 2003), propentofylline (Tawfik et al., 2007) and ibudilast (Ledeboer et al., 2006). Neuropathic pain is also transiently reversed by blocking cytokine action, including intrathecal injection of interleukin-1 receptor antagonist (IL-1ra) and tumor necrosis factor alpha (TNFα) soluble factor (Sweitzer et al., 2001). Given the pervasiveness of a CNS proinflammatory component to many different chronic pain states, developing chronic pain treatments that specifically target this response may prove fruitful.

Innate immune cell types in the CNS

Several cell types are capable of releasing proinflammatory molecules into the central nervous system. Astrocytes, microglia and CNS endothelial cells, while all having distinct functions in healthy tissue, have each been demonstrated to secrete proinflammatory molecules into the CNS following activation. The studies presented here focus on the proinflammatory response, utilizing study designs that are not able to

distinguish which of these cell type(s) are principally involved. It is nonetheless important to understand the function of these cell types, which are the candidates for mediating the effects to be examined.

Resident microglia in the CNS are bone-marrow derived cells similar to peripheral macrophages (McKercher et al., 1996). Microglia are the principal phagocytotic cell in the CNS that responds to damaged or apoptotic cells. They have many branches, each covered with receptors, including toll-like receptors, ATP receptors and neurotransmitter receptors that detect changes to their local environment in the CNS indicative of danger or pathogen invasion (Ransohoff and Perry, 2009). Following detection of a change to their environment, they change morphologically, biochemically and functionally. In most cases, they become activated to secrete proinflammatory molecules, which in turn activate other microglia and astrocytes nearby (Romero-Sandoval et al., 2008). In select circumstances, however, they can become alternatively activated, secreting instead anti-inflammatory molecules, such as IL-4 and IL-13 and/or reducing their secretion of proinflammatory cytokines (Ponomarev et al., 2007).

Astrocytes shelter nearly every synapse in the brain. They have glutamate receptors, which take up excess neurotransmitter following synaptic transmission, and in this respect have the ability to directly influence neuronal signaling (Parpura et al., 1994). Like microglia, they also have receptors that respond to changes in their microenvironment and can mount a proinflammatory response in addition to increasing the level of glutamate in the synaptic cleft (Dong and Benveniste, 2001).

Endothelial cells are a critical component to the blood-brain barrier. These polarized cells form tight junctions, creating the capillaries throughout the CNS. These

cells have one pole on the blood side and the other on the brain side and as such, have access to both the blood and the CNS (Persidsky et al., 2006). Endothelial cells also express several cell surface receptors for danger signals, including toll-like receptors, and can secrete proinflammatory cytokines following receptor signaling (Ransohoff and Perry, 2009; Nagyoszi et al., 2010). Additionally, following proinflammatory stimulation, endothelial cells can become 'leaky', allowing blood components, including limited numbers of monocytes, into the CNS (Persidsky et al., 1999).

TOLL-LIKE RECEPTOR 4 (TLR4) IN THE CENTRAL NERVOUS SYSTEM

TLR4

Many potential mechanisms for initiating proinflammatory glial cell activation have been proposed, including P2X4 ATP receptors (Tsuda et al., 2003), TLR3 (Obata et al., 2008), and fractalkine receptors (CX3CR1) (Milligan et al., 2004b). The mechanism of particular interest to the present body of work is the innate immune toll-like receptor 4 (TLR4). TLR4 is a highly conserved, transmembrane pattern recognition receptor that detects molecular patterns associated with pathogen invasion or tissue damage. TLR4 has been detected in the central nervous system on microglia (Bsibsi et al., 2002), CNS endothelial cells (Nagyoszi et al., 2010), astrocytes (Gorina et al., 2011) and, in particular circumstances following ischemic injury, neurons as well (Tang et al., 2007). Behaviorally, TLR4 signaling has been linked to such diverse conditions as enhanced pain (discussed in detail in section 2.3), tissue reperfusion injury following cortical ischemic insult (Tang et al., 2007; Arslan et al., 2010) and amyotrophic lateral sclerosis (Casula et al., 2011).

TLR4 signaling is best understood with regard to its classic agonist, lipopolysaccharide (LPS), a component of gram negative bacterial cell walls (for review, (Dauphinee and Karsan, 2006). In summary, TLR4 activation requires several co-receptors and extracellular factors, including CD14, MD-2, and LPS binding protein, to be brought together on a lipid raft in the cell membrane. Intracelluarly, two convergent signaling cascades are involved, the first, and most utilized, is the MyD88-dependent pathway and the second, the slower MyD88-independent pathway. Ultimately, these signaling pathways result in the activation of several kinases and transcription factors. The transcription factor NFκB is activated through either TRAF6 or PI3K/akt signaling. The mitogen-activated protein kinases, including ERK and JNK, are phosphorylated leading to the activation of the transcription factor AP-1. In a carefully regulated orchestration of these multiple pathways, TLR4 activation can lead to an increase in proinflammatory signaling, a pro- or anti-apoptotic state, or cell growth, depending on exact conditions. The complexity of the signaling pathways leads to many potential targets for manipulation.

TLR4 in neuropathic pain

TLR4 signaling has been linked to enhanced pain states. Either TLR4 (Tanga et al., 2005) or co-signaling molecule CD14 (Cao et al., 2009) knockout mice developed reduced neuropathic pain compared to wild type. Additionally, competitive TLR4 antagonists, including LPS from *Rhodobacter sphaeroides* and mutant LPS, have been shown to transiently reverse established neuropathic pain following chronic constriction injury (Hutchinson et al., 2008b). In a rat model of bone cancer pain, pain behavior was

attenuated when TLR4 expression was reduced with siRNA (Lan et al., 2010), and TLR4 siRNA has also been shown to reduce the development of neuropathic pain (Wu et al., 2010). A novel compound for TLR4 signaling inhibition is naloxone. Both the opioid-active (-)- and opioid-inactive (+)-naloxone isomers of naloxone have been shown to reduce TLR4 signaling in an *in vitro* model, and reduce glial activation markers and neuropathic pain *in vitro* (Hutchinson et al., 2008b). (+)-Naloxone is a novel, blood brain barrier permeable, TLR4 signaling inhibitor that will also be explored throughout this work.

TLR4 activation by exogenous drugs

Given that TLR4 responds to a range of molecules, and that TLR4 signaling has been linked to pain, the question arises of whether compounds that enhance TLR4 signaling will produce pain absent of any tissue damage. Evidence with the classic agonist LPS is mixed. Several groups have reported the development of allodynia following intrathecal injection of LPS (Clark et al., 2010; Saito et al., 2010). However, others had difficulty replicating these findings (Hutchinson et al., 2009b)). Given the complexity of the TLR4 receptor complex and intracellular signaling pathways, it may be that second signals are needed to bring all the necessary components together. Certain models and techniques (e.g. more invasive intrathecal injection protocols) may provide these second signals while others do not.

Several classes of drugs can enhance TLR4 signaling, including opioids (Hutchinson et al., 2010b) and certain tricyclic antidepressents (Hutchinson et al., 2010a). The finding that morphine could activate TLR4 signaling raised an intriguing question:

what if TLR-4 signaling increased pain, the very condition morphine was trying to relieve? The finding that morphine could increase TLR4 signaling provided a novel hypothesis for certain negative side effects, including tolerance and dependence. Tolerance results in the need for increasing doses for the same analgesic effect, while dependence results in withdrawal symptoms upon drug cessation. Traditionally, morphine tolerance and dependence have been viewed as a purely neuronal mechanism. Mechanisms include mu opioid receptor desensitization or down regulation (Raehal and Bohn, 2005) and reduced endogenous opioid production (Stefano et al., 2009).

More recently, however, morphine has been shown to upregulate proinflammatory cytokines, which counteract morphine analgesia acutely (Hutchinson et al., 2008a), enhance the development of morphine tolerance (Johnston et al., 2004; Raghavendra et al., 2004b) and contribute to morphine dependence (Hutchinson et al., 2009a). The finding that morphine can activate TLR4, a receptor linked to enhanced pain states and proinflammatory cytokine release provides a novel mechanism for these counter-regulatory effects. Further, it suggests that other exogenous drugs that activate TLR4 may have similar side effects due to proinflammatory activation in the CNS. Such a finding would provide a mechanism for improving drug effect by blocking TLR4 or proinflammatory cytokines, much like blocking proinflammatory cytokines enhances morphine analgesia (Hutchinson et al., 2008a).

DRUG METABOLITES

Drug metabolism

Following drug administration, most compounds are absorbed into the bloodstream, distributed to the tissues, metabolized into chemically altered compounds and, finally, excreted. The studies here focus on the products of the metabolism portion of this process. In phase I metabolism, enzymes alter the chemical structure of the parent compound through oxidation, reduction or hydrolysis. For example, ethanol is oxidized by alcohol dehydrogenase to form acetaldehyde in phase I metabolism. In phase II metabolism, enzymes conjugate the parent drug with a subgroup that alters the functional properties of the parent compound. These subgroups include sulfate, glucuronide and methyl groups and change the properties of the parent drug, often making them more water soluble for urinary excretion. In many, but not all, cases, these conjugated compounds lose their activity at the site of action for the parent drug. Metabolites are typically of interest when they either remain active at the parent drug's site of action, or produce toxicity at clinically relevant dosages. More recently, more subtle effects of drug metabolites have begun to be recognized as potential contributors on- and off-target drug effects (Fura, 2006). These include the negative effects of the acyl glucuronides, discussed below.

Glucuronide metabolites

The conjugation of a glucuronide group to a parent drug is catalyzed by a family of enzymes, the uridine-diphosphate glucuronosyltransferases (UDP-GTs). These enzymes are primarily expressed in the liver, however central nervous system expression has also been reported in mouse (Inoue et al., 2007), rat (Leclerc et al., 2002) and human (Yamada et al., 2003). Numerous other classes of drugs are glucuronidated, including

opioids (Christrup, 1997), steroid hormones (Jantti et al., 2007), ethanol (Wurst et al., 2003) and non-steroidal anti-inflammatory drugs (NSAIDs) (Regan et al., 2010).

One intriguing finding from previous studies investigating opioid-induced TLR4 signaling *in vitro* was that one of two metabolites tested, morphine-3-glucuronide, appeared to activate TLR4 *in vitro*, even more so than morphine itself (Hutchinson et al., 2010b). This raised a question as to whether the conjugation of a glucuronide group to the morphine molecule actually enhanced its TLR4 signaling ability. Currently, the U.S. Food and Drug Administration (FDA) guidelines suggest that most glucuronide metabolites are inert and have a low risk of toxicity, and therefore are typically excluded from the safety testing required of other metabolite classes (Davis-Bruno and Atrakchi, 2006). However, such guidelines do not address the possibility of effects other than toxicity, which may impact the parent drug action or side effects. The survey here of glucuronidated metabolites seeks to determine if any of them possess the ability to enhance TLR4 signaling, proinflammatory signaling and/or increase pain behaviors.

One class of glucuronide metabolites specifically excluded from FDA guideline labeling most glucuronide metabolites as low risk are the acyl glucuronides. Acyl glucuronides are formed from the conjugation of numerous common, carboxylic acid-based drugs, including several NSAIDs, such as naproxen and ibuprofen, and the diuretic furosemide (Regan et al., 2010). These drugs are known to produce adverse drug effects in rare cases, including hypersensitivity and hepatitis (Regan et al., 2010). Although the mechanism of action for the adverse effects of acyl glucuronides is currently unknown, it has been proposed that protein adducts form, causing an immunogenic response (Regan et al., 2010). Unfortunately, none of these glucuronidated compounds were available

commercially and remain to be investigated. Nonetheless, the acyl glucuronides present a case of glucuronide metabolite that can cause an immunogenic response and adverse drug reactions. Other studies of glucuronide metabolites have viewed them as a deactivating step, as they commonly lose activity at the site of parent drug action. For instance, estradiol glucuronide conjugation is viewed as a positive step that reduces the estrogens available to estrogen-sensitive breast tumors (Guillemette et al., 2004). Notably, no systematic exploration of potential for enhanced pain or immune receptor activation of any of these metabolites has been undertaken previously.

PURPOSE OF STUDIES

The studies here explore the role of TLR4 in enhanced pain states. The potential of glucuronide metabolites to activate TLR4 and produce pain sensitization will be tested. Specifically, the potential of morphine-3-glucuronide, glucuronic acid, ethyl glucuronide, estradiol-3-glucuronide, estradiol-17-glucuronide and corticosterone-21-glucuronide will be investigated. Additionally, the role of TLR4 in ongoing neuropathic pain will be further investigated, expanding on the potential of (+)-naloxone as a TLR4 inhibitor, initially reported in Hutchinson et al. (2008). These studies aim to, in total, further explore the potential for TLR4 involvement in pain states.

CHAPTER 2

EVIDENCE THAT INTRATHECAL MORPHINE-3-GLUCURONIDE MAY CAUSE PAIN ENHANCEMENT VIA TOLL-LIKE RECEPTOR 4/MD-2 AND INTERLEUKIN-1β

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ABSTRACT

Morphine-3-glucoronide (M3G) is a major morphine metabolite detected in cerebrospinal fluid of humans receiving systemic morphine. M3G has little-to-no affinity for opioid receptors and induces pain by unknown mechanisms. The pain-enhancing effects of M3G have been proposed to significantly and progressively oppose morphine analgesia as metabolism ensues. We have recently documented that morphine activates toll-like receptor-4 (TLR4), beyond its classical actions on μ-opioid receptors. This suggests that M3G may similarly activate TLR4. This activation could provide a novel mechanism for M3G-mediated pain enhancement, as (a) TLR4 is predominantly expressed by microglia in spinal cord and (b) TLR4 activation releases pain-enhancing substances, including interleukin-1 (IL1). We present *in vitro* evidence that M3G activates TLR4, an effect blocked by TLR4 inhibitors, and that M3G activates microglia to produce IL1. *In vivo,* intrathecal M3G (0.75 μg) induced potent allodynia and

hyperalgesia, blocked or reversed by interleukin-1 receptor antagonist, minocycline (microglial inhibitor), and (+)- and (-)-naloxone. This latter study extends our prior demonstrations that TLR4 signaling is inhibited by naloxone nonstereoselectively. These results with (+)- and (-)-naloxone also demonstrate that the effects cannot be accounted for by actions at classical, stereoselective opioid receptors. Hyperalgesia (allodynia was not tested) and *in vitro* M3G-induced TLR4 signaling were both blocked by 17-DMAG, an inhibitor of heat shock protein 90 (HSP90) that can contribute to TLR4 signaling. Providing further evidence of proinflammatory activation, M3G upregulated TLR4 and CD11b (microglial/macrophage activation marker) mRNAs in dorsal spinal cord as well as IL1 protein in the lumbosacral cerebrospinal fluid. Finally, *in silico* and *in vivo* data support that the glucuronic acid moiety is capable of inducing TLR4/MD-2 activation and enhanced pain. These data provide the first evidence for a TLR4 and IL1 mediated component to M3G-induced effects, likely of at least microglial origin.

The neuroactive metabolites of morphine can contribute to its effects. Morphine is principally metabolized in the liver, but is also metabolized in the central nervous system (CNS) (King et al., 1999). In humans, ~44-55% of morphine is metabolized to morphine-3-glucuronide (M3G), 9-15% to morphine-6-glucuronide (M6G), 8-10% excreted as morphine and the remainder converted into numerous minor metabolites (Christrup, 1997; Andersen et al., 2003). Clinical studies have consistently found M3G and M6G in the CNS following systemic morphine, including in lumbosacral cerebrospinal fluid (CSF) (Sjogren et al., 1998; Dale et al., 2007).

One of the major metabolites of morphine, M6G, is a μ opioid receptor agonist and a more potent analgesic than morphine (Abbott and Palmour, 1988). In contrast to M6G, M3G has been reported to enhance pain, although the mechanisms are unknown. Unlike M6G, M3G has little-to-no affinity for μ, κ, or δ opioid receptors (LaBella et al., 1979). To date, all studies of M3G-induced allodynia and hyperalgesia have sought exclusively neuronal mechanisms to explain its effects. Behaviorally, inhibited NMDA receptor activity or increased GABA activity can reduce the effects of M3G (Bartlett et al., 1994b). However, M3G has no affinity for NMDA or GABA receptors (Bartlett et al., 1994a) and *in vitro* its effects on NMDA are not direct (Hemstapat et al., 2003).

We propose an alternative hypothesis for M3G-induced pain enhancement, through M3G-induced proinflammatory responses by immunocompetent cells including microglia. The proinflammatory molecules released by activated microglia and other immunocompetent cells increase neuronal excitability, which has been shown to increase pain sensitivity in various pain models (Milligan and Watkins, 2009). Studies of morphine indicate that a proinflammatory response opposes morphine analgesia, and

microglial and astrocyte activation markers are upregulated following repeated morphine (Song and Zhao, 2001; Hutchinson et al., 2009a). Behaviorally, blocking morphine-induced release of proinflammatory molecules prolongs acute morphine analgesia and attenuates morphine-induced hyperalgesia (Johnston et al., 2004; Hutchinson et al., 2008a). Given the structural similarity between morphine and M3G, we hypothesized that M3G may induce glial activation and release of proinflammatory molecules, and that this effect may be involved in enhanced pain in response to both M3G and morphine.

It has largely been assumed that morphine-mediated effects on microglia and astrocytes were due to μ opioid receptor activity, the same mechanism as the analgesic effects of morphine on neurons. However, a growing body of evidence suggests that a novel, non-classical opioid receptor is primarily responsible. In contrast to morphine's analgesic activity, glial activation following morphine is nonstereoselective, and there is a similar lack of stereoselectivity in naloxone inhibition of morphine-induced glial activation (Hutchinson et al., 2010b). One candidate receptor for nonstereoselective morphine-induced glial activation is toll-like receptor 4 (TLR4). TLR4 is expressed predominantly by microglia in the CNS and, when activated by a wide range of endogenous or exogenous molecules, leads to microglial activation and release of proinflammatory products including interleukin-1 (IL1) (Lehnardt et al., 2003). TLR4 is activated by opioids in a nonstereoselective manner (Hutchinson et al., 2010b), and inhibited by both (+)- and (-)-naloxone (Hutchinson et al., 2008b), consistent with observations of morphine-induced glial activation.

This study seeks to investigate a potential non-neuronal component to M3G-enhanced pain. We present evidence that inhibitors of TLR4 signaling can block M3G-

induced TLR4 activity *in vitro*. We then employ a range of pharmacological approaches to explore mechanisms underlying M3G-induced pain enhancement and define the effect of M3G on mRNA levels in the dorsal spinal cord reflective of a proinflammatory process.

MATERIALS AND METHODS

Animals

Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used for all *in vivo* experiments. Rats (350-400 g at time of experiments) were housed in temperature (23±3 ^{o}C) and light (12 h:12 h light:dark) controlled rooms with water and food given *ad libitum*. All habituation and behavioral testing procedures were performed during the light phase of the daily cycle. All procedures were approved by the University of Colorado-Boulder Institutional Animal Care and Use Committee. Unless otherwise noted, each experimental group was comprised of 6 rats.

Drugs

M3G, minocycline, (-)-naloxone and glucuronic acid were purchased from Sigma (St. Louis, MO). (+)-Naloxone was obtained from the National Institute on Drug Abuse. IL1 receptor antagonist (IL1ra) was purchased from Amgen (Thousand Oaks, CA). 17-DMAG was purchased from Tocris Biosciences (Ellisville, MO). Endotoxin-free, sterile 0.9% saline (Abbott Laboratories, North Chicago, IL) was used as a vehicle for all drugs in behavioral studies (Experiments 3-6, 8). M3G, saline, (+)-naloxone, (-)-naloxone, minocycline, 17-DMAG and IL1ra were confirmed to be endotoxin-free. Where appropriate, doses are reported as a free base concentration.

All drugs administered *in vivo* were given intrathecally over lumbosacral spinal cord in Experiments 3-6, 8 and 11. M3G was administered at a dose of 0.75 μg, based on pilot studies. Minocycline was administered as 2 successive doses (100 μg followed by 33.3 μg), with the first dose prior to M3G and the second dose concurrent with M3G, as described in the experimental procedures below. This dosing regimen was based on Ledeboer et al. (2005), which showed that minocycline is more effective at blocking microglial activation than reversing it. (+)-Naloxone and (-)-naloxone were given at a dose of 20 μg, consistent with Hutchinson et al. (2008b), which showed a significant reversal of neuropathic pain by this dose of (+)-naloxone. IL1ra was administered at a dose of 100 μg, a dose that potentiates morphine analgesia by reducing the proinflammatory response by glia and mitigating the neuroexcitatory effects of IL1 (Hutchinson et al., 2008a). In Experiment 4, a second dose of IL1ra was given 60 min into the 125 min time course to obviate the short half-life of IL1ra (Milligan et al., 2005). 17-DMAG was given at 4 μg, a dose which suppresses neuropathic pain (Hutchinson et al., 2009b). In Experiment 5, IL1ra and (+)-naloxone were each co-administered with M3G and readministered 1.5 h into the experiment due to their short half-lives. For the same reason, the second mechanical sensitivity testing time point was moved from 4 h to 3 h between Experiment 3a and Experiment 5. In Experiment 11, glucuronic acid was administered in equimolar concentration to the M3G dose, at a dose of 0.38 μg and (+)-naloxone was again administered at a dose of 20 μg.

Intrathecal catheter implantation and injections

Acute intrathecal injections were used to administer drugs in Experiment 3a. Acute indwelling intrathecal catheters were used to administer drugs in Experiments 3b, 4, 6 and 8. Chronic indwelling intrathecal catheters were used in Experiments 5 and 11. Catheter implantations via the L5/L6 intervertebral approach and drug microinjections were performed based on Milligan et al. (1999). For each of the 3 procedures, rats were briefly anaesthetized under isoflurane anesthesia. An 18-gauge needle was placed between L5 and L6 into the intrathecal space and served as a guide. Sterile polyethylene-10 tubing was threaded rostrally through the guide so to terminate over the lumbosacral enlargement.

For acute injections in Experiment 3a, the catheters were pre-loaded with drugs at the intrathecal end and the remainder filled with sterile saline. Upon insertion, the drug was injected with a 10.5 μl flush to ensure complete drug delivery, and the needle and catheter then removed.

For acute indwelling lumbosacral catheters, used in Experiment 3b, 4, 6 and 8 the 18-gauge needle was removed after catheter placement and the tubing secured to the superficial musculature of the lower back with 3-0 silk suture. The tubing was then threaded subcutaneously to exit the nape of the neck. Catheters were 90 cm in length, pre-loaded with drugs at the intrathecal end and the remainder filled with sterile saline. This allowed remote injection of drug during behavioral testing without disturbing the animal, and the injection of a small void volume (10.5 μl) that ensured delivery of drugs. Drug administration occurred 2 h after intrathecal catheter placement.

Chronic indwelling lumbosacral catheters used in Experiment 5 and 11 were 45 cm in length and heat sealed at the external end until used for drug injections. Rats were

allowed to recover from this catheter placement for 7 d before behavioral testing and drug administration. Catheters were filled with sterile saline and the appropriate drug dose in 1 μl, followed by 3 μl of saline. Injections had a total volume of 50 μl, due to the void volume of the catheter that preceded the drug into the intrathecal space.

Behavioral Testing

All testing was conducted by an experimenter blind to group assignment. Separate rats were used for assessing thermal and mechanical response thresholds. Rats received at least four 1 h habituations to the appropriate testing apparatus and environment prior to baseline testing, as in our previous studies (Hutchinson et al., 2008b). There were six rats in each experimental group.

Hargreaves test for thermal hyperalgesia

Thermal testing measured withdrawal latency to radiant heat applied separately to the tail and the plantar surface of each hind paw in a modified Hargreaves test (Hargreaves et al., 1988). Baseline latencies prior to drug administration were calculated as the average of two latencies, measured 15 min apart. Following drug administration, latencies to withdrawal from each paw and the tail were measured at 10 min intervals for 125 min. The intensity of the heat source was adjusted such that pre-drug latencies to withdrawal were 8-10 s, with a 20 s cut off to avoid tissue damage. This allowed both analgesia and hyperalgesia to be measured. The percent maximum possible effect (%MPE) was calculated for each testing point from each rat as: %MPE=[test latency-baseline latency]/[cut-off – baseline latency] x100.

von Frey test for low threshold mechanical allodynia

The response threshold to light touch on both hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as described in Milligan et al. (2000). Briefly, a logarithmic range of hairs from 0.406-15.136 g force were used, allowing both analgesia and allodynia to be measured, following the standard up-down procedures previously described (Chaplan et al., 1994). Baseline thresholds were taken as the average of two tests from each paw. Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described in Milligan et al. (2000).

Cellular and Molecular Analyses

In vitro assay for TLR4 signaling

A human embryonic kidney-293 (HEK 293) cell line was used that was stably transfected by Invivogen to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (293-htlr4a-md2cd14; here referred to as HEK-TLR4). In addition, these cells stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors, such as NF-κB and AP-1, activated as part of the TLR4 signaling cascade. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

HEK-TLR4 cells were grown at 37 ^{o}C (5% CO_2; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with

10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selection [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5 x 10^3 cells/well) with the same media. After 48 h, supernatants were removed and replaced with 180 µl artificial cerebrospinal fluid (sterile aCSF; 124 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2 \cdot 2H_20$, 3.2 mM $MgCl_2 \cdot 6H_20$, 25 mM $NaHCO_3$, 10 mM glucose, pH 7.4) to model *in vivo* CNS conditions. Drugs under test were added in concentrations indicated and incubated for 24 h. Supernatants (15 µl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 µl test samples were diluted in 45 µl of 1x dilution buffer, transferred to 96-well plates (Thermo, Walthma, MA, USA), and heated at 65^{o}C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 µl/well) was added and, 5 min later, reaction buffer (50 µl/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

In vitro assay for microglial cell IL1 production

A murine microglial cell line, BV-2 (gifted by Rona Giffard, Stanford University) were grown in macrophage serum free media (Invitrogen, Carlsbad, CA) in 75 cm^2

Primaria-treated flasks (Falcon, BD Biosciences, San Jose, CA) with no supplementation at 5% CO_2 and 37 ^{o}C. 200 000 cells/well were plated in 6-well Primaria-treated plates (Falcon, BD Biosciences, San Jose, CA) for 48 h, in 1.6 ml of the same media, before drug administration.

Enzyme-linked Immunosorbant Assay (ELISA) for IL1 protein

Cell lysates from BV-2 cells and CSF samples were assayed for IL1 protein content using commercially available mouse (BV-2 cell lysates) and rat (CSF samples) IL1β kits (R&D Systems, Minneapolis, MN). Procedures were conducted according to manufacturer's directions. CSF samples were diluted 1:10 due to small sample volumes, as described in O'Connor et al. (2004). The rat IL1 kit has a minimum detection threshold of <5 pg/ml and an inter- and intra-assay variability of <10%. The mouse IL1 kit had a minimum detection threshold of 6 pg/ml.

mRNA isolation and real-time polymerase chain reaction (RT-PCR) quantification

Total RNA was isolated as described in Johnston et al. (2004). Samples were DNase treated (DNA-free kit; Ambion), followed by cDNA synthesis. Amplification of cDNA was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) in iCycler iQ 96 well PCR plates (Bio-Rad) on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad), as previously described (Johnston et al., 2004). SYBR Green 1 fluorescence (PCR product formation) was monitored in real time using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log–linear phase of amplification and the threshold cycle (CT, the number of cycles to reach threshold of detection) was determined for each

reaction. The levels of the target mRNAs were quantified, using blinded procedures, relative to the level of the housekeeping gene β-actin using the comparative ΔΔCT method. In the event that an individual mRNA level was more than two standard deviations above the group when not included, it was considered an outlier and excluded.

The following targets were investigated and expressed relative to the levels of the housekeeping gene β-actin (BC063166; forward: AGAGGCATCCTGACCCTGAA; reverse: GCTCATTGTAGAAAGTGTGGT): IL1 (M98820; forward: GAAGTCAAGACCAAAGTGG; reverse: TGAAGTCAACTATGTCCCG), Tumor Necrosis Factor-α (TNF) (D00475; forward: CTTCAAGGGACAAGGCTG; reverse: GAGGCTGACTTTCTCCTG), TLR4 (NM_019178; forward: CAGAGGAAGAACAAGAAGC; reverse: CCAGATGAACTGTAGCATTC), and CD11b (NM_012711, forward: CTGGGAGATGTGAATGGAG, reverse: ACTGATGCTGGCTACTGATG). Primers were purchased from Proligo (Boulder, CO, USA).

in silico Docking simulations

in silico Docking simulation methods previously outlined were employed to examine the docking of M3G and (+)-naloxone to TLR4 and MD-2 (Hutchinson et al., 2010b). Briefly, the complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). Modified pdb files were inputted into AutoDock 4.0 (http://autodock.scripps.edu), hydrogens added, and resaved in pdbqt format. M3G and (+)-naloxone structures were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator

(http://cactus.nci.nih.gov/services/translate/). All dockings were executed with Lamarkian genetic algorithms.

Statistics

GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all statistical analyses. Two-way repeated-measures ANOVAs with Bonferroni post-hoc tests when appropriate were used to determine statistical significance between behavioral measures. One-way ANOVAs with appropriate Bonferroni post hocs were used to confirm that there were no baseline differences on behavioral measures. Independent sample t-tests were used to determine statistical significance on the mRNA tests. For all analyses, $p<0.05$ was considered significant.

Experimental Procedures

Experiment 1: Can the *in vitro* increase in TLR4 signaling seen with M3G be blocked by a TLR4 antagonist?

M3G (10 μM) was applied to HEK-TLR4 cells as described above. This concentration was determined by pilot studies to produce a robust effect without cytotoxicity. Lipopolysaccharide from Rhodobacter sphaeroides (LPS-RS, Invivogen, San Diego, CA) lacks functional TLR4 activity and acts as a competitive TLR4 antagonist. LPS-RS was applied to the HEK-TLR4 cells at the same time as the M3G at concentrations of 0.0, 0.01, 0.1, 1.0 ng/ml. Cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4 activity.

Experiment 2: Does M3G induce IL1 protein in mouse BV-2 microglial cells?

BV-2 cells were plated in 6-well Primaria-treated plates with 200,000 cells per well. After 48 h, fresh media and M3G (final concentrations of: 10 nM, 100 nM, 1 μM, 10 μM in the well) or vehicle was added (n=6 per condition). 24 h after drug administration the supernatants were collected and the cells lysed in 200 μl of sonication buffer (cold Iscove's culture medium containing 5% fetal calf serum and a cocktail enzyme inhibitor: 100 mM amino-*n*-caproic acid, 10 mM EDTA, 5 mM benzamidine–HCl, and 0.2 mM phenylmethylsulfonyl fluoride). Sonicated samples were centrifuged at 14,000 rpm at 4 °C for 10 min. Supernatants of the cell lysate and the cell culture supernatants were immediately measured for IL1 protein using the mouse ELISA described above.

Experiment 3: Does M3G produce enhanced pain responses?

Experiment 3a: Rats received either 0.75 μg M3G or equivolume (1 μl) saline vehicle, via an acute intrathecal injection, as described above. Both injections had a saline flush for a total injection volume of 12.5 μl. Rats were tested for mechanical allodynia 1 d prior to injection (baseline; BL) and again 0.75 and 4 h post-injection.

Experiment 3b: Drug dosing was identical to that described in Experiment 3a, above. Rats were tested for thermal hyperalgesia just prior to injection (BL) and again from 5-125 min post injection.

Experiment 4: Is M3G induced hyperalgesia blocked by inflammatory and glial modulators?

All rats received 0.75 μg M3G (in 1 μl) coadministered with one of four glial or inflammatory modulators or equivolume (1 μl) saline vehicle. All injections were followed by a saline flush and had a total injection volume of 12.5 μl. Behavioral testing for thermal sensitivity occurred prior to (BL) and 5-125 min post-M3G and coadministered drug. Drugs separately coadministered with M3G were: 100 μg IL1ra, 20 μg (+)-naloxone, 20 μg (-)-naloxone, or 100 μg minocycline administered 2 h prior to 33.3 μg minocycline coadministered with M3G as discussed above.

Experiment 5: Is M3G induced allodynia blocked by inflammatory and glial modulators?

All rats received 0.75 μg M3G (in 1 μl) coadministered with one of 4 glial or inflammatory modulators or equivolume (1 μl) saline vehicle. An additional 6 rats received only equivolume vehicle injections. All injections were followed by a saline flush for a total volume of 50 μl. Behavioral testing for mechanical allodynia occurred prior to (BL), 0.75 and 3 h post-M3G and coadministered drug. IL1ra (100 μg) was coadministered with M3G and again (100 μg) 1.5 h post-M3G administration due to its shorter half-life. Similarly, (+)-naloxone (20 μg) and (-)-naloxone (20 μg) were both coadministered with M3G and administered again 1.5 h after M3G administration due to short half lives. Minocycline was given 0.75 h (100 μg) prior to M3G and coadministered (33.3 μg) with M3G, as discussed above.

Experiment 6: Is M3G induced hyperalgesia reversed by IL1ra?

Rats received either 0.75 μg M3G (in 1 μl) or equivolume saline vehicle, via an acute indwelling intrathecal injection. Both injections had saline flush for a total

injection volume of 12.5 μl. Behavioral testing occurred prior to (BL) and then 5-55 min after injection. At this time, either IL1ra or equivolume (12.5 μl) saline vehicle was administered, and response thresholds recorded for an additional 5-65 min post-IL1ra.

Experiment 7: Does the HSP90 inhibitor 17-DMAG block M3G-induced TLR4 signaling *in vitro*?

M3G (10 μM) was applied to HEK-TLR4 cells as described above and identical to the procedure for Experiment 1. This concentration of M3G was determined by pilot studies to produce a robust effect without cytotoxicity. 17-DMAG, a HSP90 inhibitor, was applied to the HEK-TLR4 cells at the same time as the M3G at concentrations of 0.0, 0.01, 0.1, 1.0 ng/ml. Cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4 activation.

Experiment 8: Does the HSP90 inhibitor 17-DMAG block M3G-induced hyperalgesia?

Rats received 0.75 μg M3G (in 1 μl) with either 4 μg 17-DMAG or equivolume (1 μl) saline vehicle. All injections were followed by a saline flush and had a total injection volume of 12.5 μl. Behavioral testing for thermal sensitivity occurred prior to (BL) and 5-125 min post-M3G and coadministered drug.

Experiment 9: Is there an increase in IL1 CSF protein level or transcription of glial activation markers in the dorsal spinal cord following intrathecal M3G?

M3G (0.75 μg, n=6) or equivolume saline vehicle (n=5) was administered via acute intrathecal injection, as in Experiment 3a. While cell surface marker evidence of

glial activation can take 6-8 h to develop (Milligan et al., 2001) mRNA and proinflammatory cytokine protein expression changes can be detected much more rapidly, in a time frame consistent with M3G's *in vivo* half-life following a single injection. Sixty min after acute intrathecal M3G injection, rats were deeply anaesthetized with sodium pentobarbital (50 mg/kg) and lumbrosacral CSF was collected via acute intrathecal puncture and immediately flash frozen in liquid nitrogen. Following CSF collection, rats were transcardially perfused with chilled saline. The lumbrosacral enlargement of the spinal cord was exposed by laminectomy and a 1 cm section dissected and moved to an ice chilled glass plate. The meninges were removed and the dorsal half of the spinal cord dissected. The dorsal section was then flash frozen in liquid nitrogen. Samples were stored at -80 °C until RT-PCR or ELISA analysis.

Experiment 10: Does M3G dock *in silico* the TLR4/MD-2 complex?

Initially, the *in silico* docking of ligands to the entire TLR4 MD-2 dimer complex was conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; GA running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). These data demonstrated that the two ligands docked with human MD-2 independent of human TLR4 interactions. Therefore, all the ligands were docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; GA running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized for (+)-naloxone and M3G. The (+)-naloxone conformation was then saved as a combined MD-2/(+)-naloxone pdf file and the M3G *in silico* docking was repeated on the new combined

MD-2/(+)-naloxone complex to determine the change in docking owing to the presence of already docked (+)-naloxone.

Experiment 11: Does glucuronic acid alone induce pain behaviors and is this a TLR4-dependent mechanism?

Rats received 0.38 μg glucuronic acid (in 1 μl) intrathecally, a dose equimolar to the M3G dose used previously, coadministered with either (+)-naloxone (20 μg in 1 μl) or equivolume saline. All injections were followed by a saline flush for a total volume of 50 μl. Behavioral testing for mechanical allodynia occurred prior to (BL), 0.75 and 3 h following glucuronic acid administration. (+)-Naloxone (20 μg) was coadministered with glucuronic acid and administered again (20 μg) 1.5 h post-M3G administration due to its shorter half-life.

RESULTS

In all studies employing the von Frey test, no differences between right and left hind paws were detected. Thus, results are reported as the average of the response thresholds recorded for both paws. In response to the Hargreaves test, both hind paws and tail latencies to withdrawal showed identical patterns of results. As consistent data were obtained across both hind paws and the tail, only tail flick responses are reported for simplicity.

Experiment 1: M3G-induced TLR4 activation is blocked by a TLR4 antagonist.

Previous studies have shown that M3G increases TLR4-dependent SEAP expression in HEK-TLR4 cells compared to media control, an effect dose-dependently blocked by (+)-naloxone (Hutchinson et al., 2010b). HEK cells with the SEAP reporter gene but without TLR4 overexpression did not increase SEAP expression (Hutchinson et al., 2010b). Here, the M3G-induced TLR4-dependent SEAP expression was dose-dependently suppressed by LPS-RS, a classical TLR4 competitive antagonist ($F_{5,6}$= 110, p<0.05, Figure 2.1), providing further evidence that M3G can signal through TLR4. Post-hoc analyses showed that LPS-RS significantly reduced SEAP TLR4 signaling at concentrations of 0.01, 0.1 and 1.0 ng/ml. LPS-RS alone, without coadministration of M3G, did not produce any increase in SEAP expression above media control.

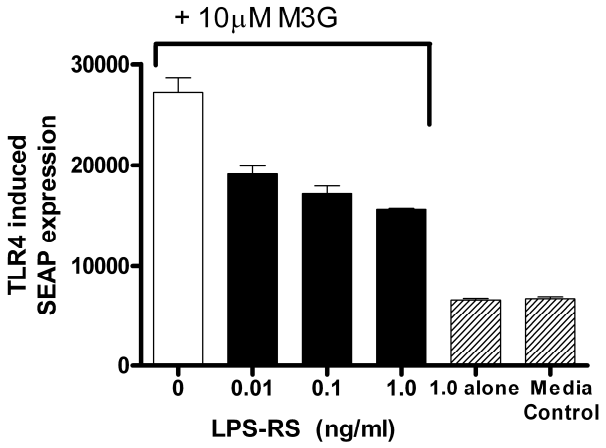


Figure 2.1. M3G causes a significant increase in HEK-TLR SEAP reporter protein which is significantly reduced by the TLR4 antagonist LPS-RS at all three concentrations ($F_{(5,6)}$= 110, p<0.05). Error bars represent standard error of the mean.

Experiment 2: Mouse BV-2 microglial cells produce IL1 in response to M3G *in vitro*

Given that TLR4 is expressed on microglial cells, IL1 is an end product of TLR4 activation, and that both activated microglial cells and IL1 can each contribute to

enhanced pain, we explored whether M3G could produce a proinflammatory response in mouse BV-2 microglial cells. These cells were stimulated with 10 μM, 1 μM, 100 nM, 10 nm or 0 μM M3G for 24 h. M3G dose dependently increase IL1 protein (Figure 2.2, $F_{(4,25)}=28.9$, $p<0.05$). Post-hoc test showed significant increases in IL1 protein in response to 10 μM (t=9.53, $p<0.05$) and 1 μM M3G (t=3.57, $p<0.05$) compared to vehicle. There was no increase in IL1 protein in the cell culture supernatants, suggesting that IL1 is produced following M3G stimulation *in vitro* but not released under these experimental conditions.

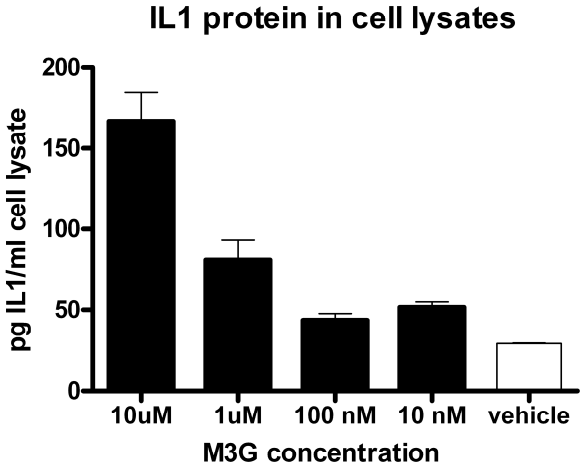


Figure 2.2. M3G induces a significant increase in IL1 protein production in the mouse BV2 microglial cell line at concentrations of 10 μM and 1 μM ($F_{(4,25)}=28.89$, $p<0.05$). Error bars represent standard error of the mean.

Experiment 3: Intrathecal M3G induces thermal hyperalgesia & mechanical allodynia

To confirm that M3G induces exaggerated pain responses under our laboratory conditions, we examined both thermal (Hargreaves test) and mechanical (von Frey test) sensitivities following acute intrathecal injection of M3G compared to an equivolume saline injection. On the Hargreaves test, there was no difference on baseline latencies to

withdrawal from radiant heat prior to drug administration (t_{10}=0.53, p>0.05). Following M3G administration, there was a rapid and reliable decrease in latencies compared to saline controls (Figure 2.3A, $F_{(1,10)}$=43.3, p<0.05).

On the von Frey test, there was no difference between groups on baseline response to calibrated pressure stimuli prior to intrathecal injection (t_{10}=0.03, p>0.05), but a robust decrease in tactile thresholds in rats given M3G compared to those receiving saline (Figure 2.3B, $F_{(1,10)}$=26.2, p<0.05). Post-hoc analysis revealed a significant decrease in response threshold in rats receiving M3G compared to saline 4 h post-M3G administration (t_{10}=5.78, p<0.05).

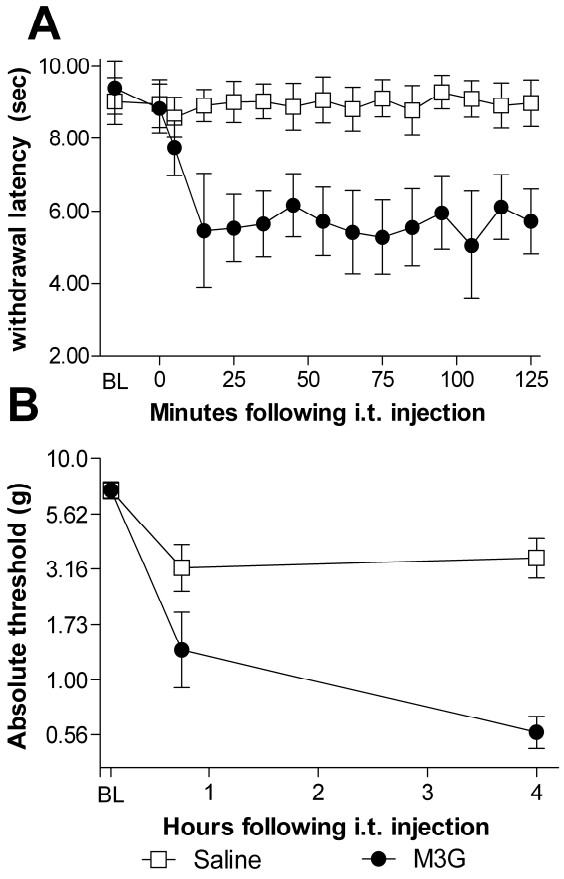


Figure 2.3. Intrathecal M3G causes the rapid onset of robust thermal hyperalgesia (A) and tactile allodynia (B). Intrathecal M3G (0.75 μg) induced significant decrease in

withdrawal latencies from a radiant heat source (main effect of drug treatment $F_{(1,10)}=43.3$, $p<0.05$, A). The M3G group had a baseline withdrawal latency mean of 9.4 s and the saline group had a baseline withdrawal mean of 8.9 s. The same dose of M3G produced a significant increase in sensitivity to calibrated pressure stimuli 4 h following injection (main effect of drug treatment $F_{(1,10)}=26.2$, $p<0.05$, B). Error bars represent standard error of the mean.

Experiment 4: M3G induced hyperalgesia is blocked by glial and inflammatory inhibitors.

As Experiment 3 clearly showed that intrathecal M3G rapidly and potently produced hyperalgesia, the question arises as to the mechanisms underlying that effect. The induction of IL1 production in microglial cells in Experiment 2 suggests that proinflammatory microglial activation may be involved. Here, we explore whether modulating glial activation or proinflammatory molecules will affect this M3G-induced hyperalgesia. To investigate this, we tested a range of glial and inflammatory modulators to determine if they could block M3G hyperalgesia. As detailed below, all glial activation and inflammatory inhibitors tested blocked M3G hyperalgesia when coadministered with M3G. There were no baseline differences between any of the groups tested ($F_{(5, 30)}=0.67$, $p>0.05$). When all M3G, saline, and coadministered blocker groups were analyzed, there was a significant main effect of drug treatment on behavioral responses ($F_{(5,30)}=13.04$ $p<0.05$). Post-hoc analyses were used to delineate what treatment and time points were significantly different, as reported below.

IL1 is a proinflammatory cytokine known to be induced by TLR4 signaling and upregulated in the CNS following morphine administration (Johnston et al., 2004; Hutchinson et al., 2008a). Blocking the effect of IL1 with its receptor antagonist (IL1ra), while having no effect in the absence of morphine, increases the duration of morphine

analgesia, suggesting that IL1-induced neuroexcitation opposes the pain-suppressive effects of morphine (Hutchinson et al., 2008a). Coadministration of M3G + IL1ra suppressed M3G-induced hyperalgesia (Figure 2.4A). The area under the curve was significantly smaller for M3G + IL1ra than M3G + saline, indicative of reduced hyperalgesia when IL1ra was also administered (Figure 2.4E). Post hoc analysis showed the M3G + saline group had significantly faster ($p<0.05$) withdrawal latencies than M3G + IL1ra at 15, 25, 35, and 75 min following intrathecal injection. There were no significant differences between M3G + IL1ra and the saline + saline control group, indicative of a complete blockade of M3G induced hyperalgesia by IL1ra.

Minocycline has been shown to reduce microglial activation, proinflammatory cytokines and neuropathic pain following inflammatory nerve injury and gp120-induced spinal glial activation (Ledeboer et al., 2005). Minocycline alone has no effect on basal pain thresholds (Ledeboer et al., 2005). Coadministration of minocycline (100 μg) with M3G did not significantly attenuate pain behaviors (n=4, data not shown). However, a dosing paradigm where minocycline (100 μg) was given 2 h prior to the M3G and an additional 33.3 μg of minocycline successfully suppressed M3G-induced hyperalgesia (Figure 2.4B). This result is consistent with prior work showing that minocycline is more effective when given prior to the inflammatory stimulus (Ledeboer et al., 2005). The area under the curve was significantly smaller for M3G + minocycline than M3G + saline, indicating reduced hyperalgesia when minocycline was administered along with the M3G (Figure 2.4E). Post-hoc analyses showed a significant decrease in withdrawal latencies between M3G + saline relative to M3G + minocycline groups at every time point from 15-125 min following intrathecal injection. There were no significant

differences in withdrawal latencies between M3G + minocycline and saline + saline groups, indicative of a complete blockade of M3G induced hyperalgesia by minocycline.

(+)-Naloxone has been shown to block TLR4 signaling by the classic TLR4 agonist LPS and by morphine in both HEK-TLR4 and RAW macrophages (Hutchinson et al., 2008b; Hutchinson et al., 2010b), reduce proinflammatory cytokine induction and glial activation following nerve injury, and reverse TLR4-dependent neuropathic pain (Hutchinson et al., 2008b). Coadministration of (+)-naloxone with M3G significantly reduced M3G-induced hyperalgesia (Figure 2.4C). The area under the curve was significantly smaller for M3G + (+)-naloxone than M3G + saline (Figure 2.4E). Post-hoc analysis showed a significant decrease in withdrawal latencies in the group administered M3G + vehicle relative to M3G + (+)-naloxone at 25, 35, 95 and 105 min following intrathecal injection. No significant differences were observed between M3G coadministered with (+)-naloxone and saline controls, indicative of a complete blockade of M3G induced hyperalgesia by minocycline.

(-)-Naloxone is the opioid-active, clinically employed isomer of naloxone, which blocks the effect of opioids on classical opioid receptors. However, M3G does not have any activity at those receptors (LaBella et al., 1979), so its hyperalgesic effects are not accounted for via such actions. In contrast, both the (+) and (-) isomers of naloxone are able to block TLR4 activation and subsequent proinflammatory response with similar efficacy (Hutchinson et al., 2008b). We predicted that (-)-naloxone would be similarly effective to (+)-naloxone in blocking M3G-induced hyperalgesia. Coadministration of M3G + (-)-naloxone blocked M3G-induced hyperalgesia (Figure 2.4D). The area under the curve was significantly smaller for M3G + (-)-naloxone than M3G + saline (Figure

2.4E). Post-hoc analysis showed a significant decrease in withdrawal latencies in the group administered M3G + vehicle relative to M3G + (-)-naloxone at 15, 25, 35, 45, 55, 65, 75, 115 and 125 min following intrathecal injection. There were no significant differences between M3G coadministered with (-)-naloxone and saline controls, indicative of a complete blockade of M3G induced hyperalgesia by (-)-naloxone.

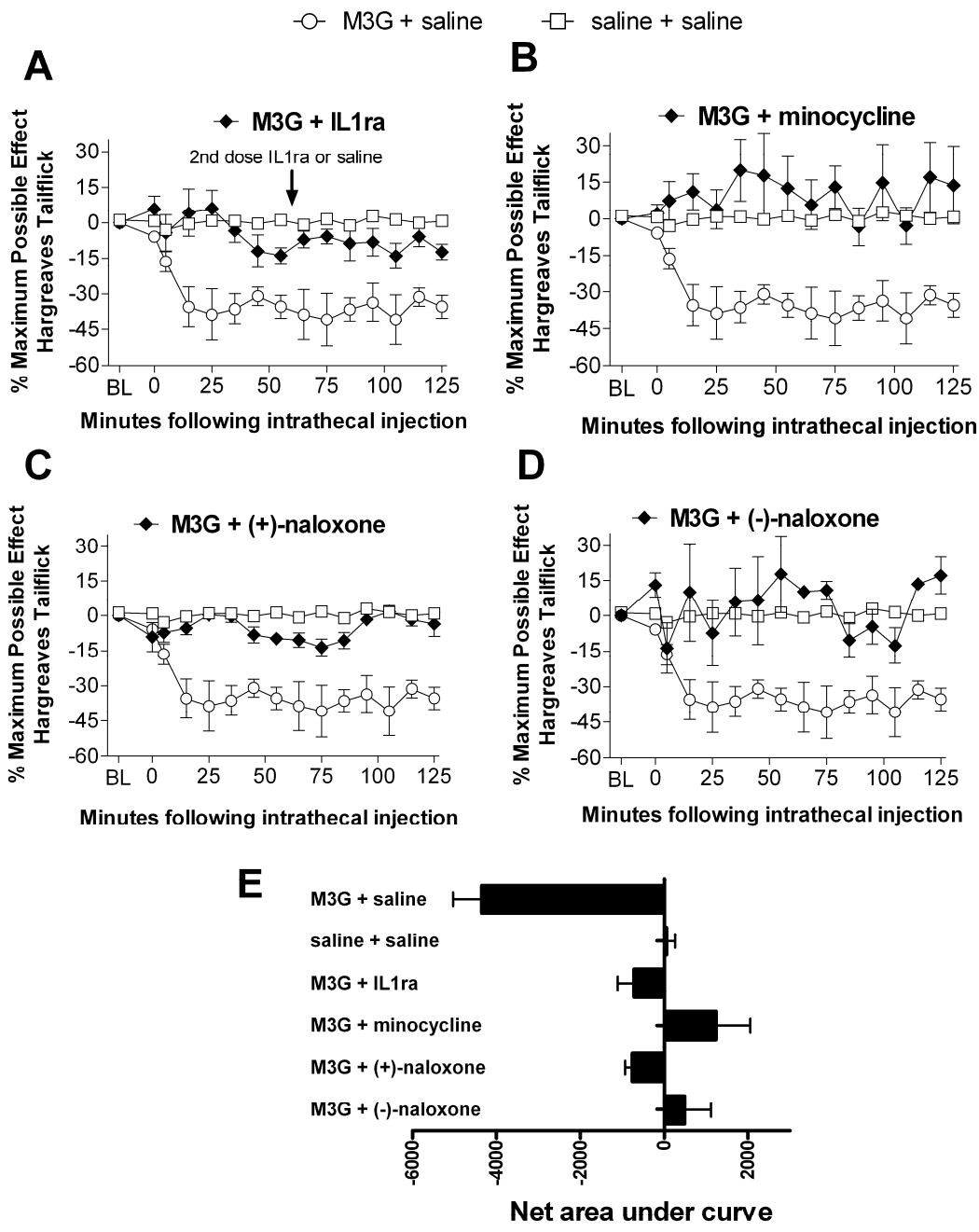


Figure 2.4. M3G-induced hyperalgesia is blocked by several inflammatory and glial mediators. Acute intrathecal M3G (0.75) coadministered with one of: IL1ra (100 μg coadministered with M3G, 100 μg at 65 min, baseline latency of 8.1 ± 0.33 s, A), minocycline (100 μg 120 min prior to M3G and 33.3 μg with M3G, baseline latency of 8.8 ± 0.69 s, B), (+)-naloxone (20 μg, baseline latency of 8.8 ± .51 s, C), (-)-naloxone (20 μg, baseline latency of 9.7 ± 0.90 s, D), blocks the rapid onset of M3G-induced hyperalgesia (main effect of drug $F_{(5,30)}$=13.04 p<0.05). Area under the curve analyses of these time courses showed M3G + saline had a significantly larger area under the curve

than saline + saline, M3G + IL1ra, M3G + minocycline, M3G + (+)-naloxone, M3G + (-)-naloxone ($F_{(5, 30)}$=13.1, p<0.05, E). Saline and M3G groups are repeated on each graph to facilitate visual comparisons. Error bars and ranges represent standard error of the mean.

Experiment 5: M3G-induced allodynia is blocked by glial and inflammatory inhibitors.

The mechanisms of allodynia and hyperalgesia are not identical (Zimmermann, 2001). Given the success of glial and inflammatory modulators in reducing M3G induced hyperalgesia, we investigated these molecules on M3G induced allodynia. Rationales for the drugs selected for test are as described in Experiment 4, above. Coadministered IL1ra, minocycline, (+)-naloxone and (-)-naloxone were all able to block M3G-induced allodynia. There were no baseline differences between any of the experimental groups ($F_{(5, 28)}$=.33, p>0.05). When all M3G, saline, and coadministered blocker groups were analyzed there was a significant main effect of drug treatment on behavioral responses ($F_{(5,28)}$=2.73 p<0.05, Figure 2.5). Post-hoc analyses were used to determine what treatments and time points were significantly different from M3G + saline controls, and are summarized below.

Coadministration of IL1ra with M3G resulted in the blockade of M3G-induced allodynia. Post-hoc analysis showed that M3G + IL1ra group had significantly decreased sensitivity to mechanical stimuli compared to M3G + saline 3 h following intrathecal administration, but not at 45 min following injection (Figure 2.5A). There were no significant differences between M3G coadministered with IL1ra and saline controls.

Coadministration of minocycline with M3G resulted in the blockade of M3G-induced allodynia. Post-hoc analysis showed that M3G + minocycline group had significantly decreased sensitivity to mechanical stimuli compared to M3G + saline 3 h

following intrathecal administration, but not at 45 min following injection (Figure 2.5B). There were no significant differences between M3G coadministered with minocycline and saline controls.

Coadministration of (+)-naloxone with M3G resulted in the blockade of M3G-induced allodynia. Post-hoc analysis showed that M3G + (+)-naloxone group had significantly decreased sensitivity to mechanical stimuli compared to M3G + saline 3 h following intrathecal administration, but not at 45 min following injection (Figure 2.5C). There were no significant differences between M3G coadministered with (+)-naloxone and saline controls, indicative of a complete blockade of M3G hyperalgesia.

Coadministration of (-)-naloxone with M3G resulted in the blockade of M3G-induced allodynia. Post-hoc analysis showed that M3G + (-)-naloxone group had significantly decreased sensitivity to mechanical stimuli compared to M3G + saline 3 h following intrathecal administration, but not at 45 min following injection (Figure 2.5D). There were no significant differences between M3G coadministered with (-)-naloxone and saline controls, indicative of a complete blockade of M3G hyperalgesia.

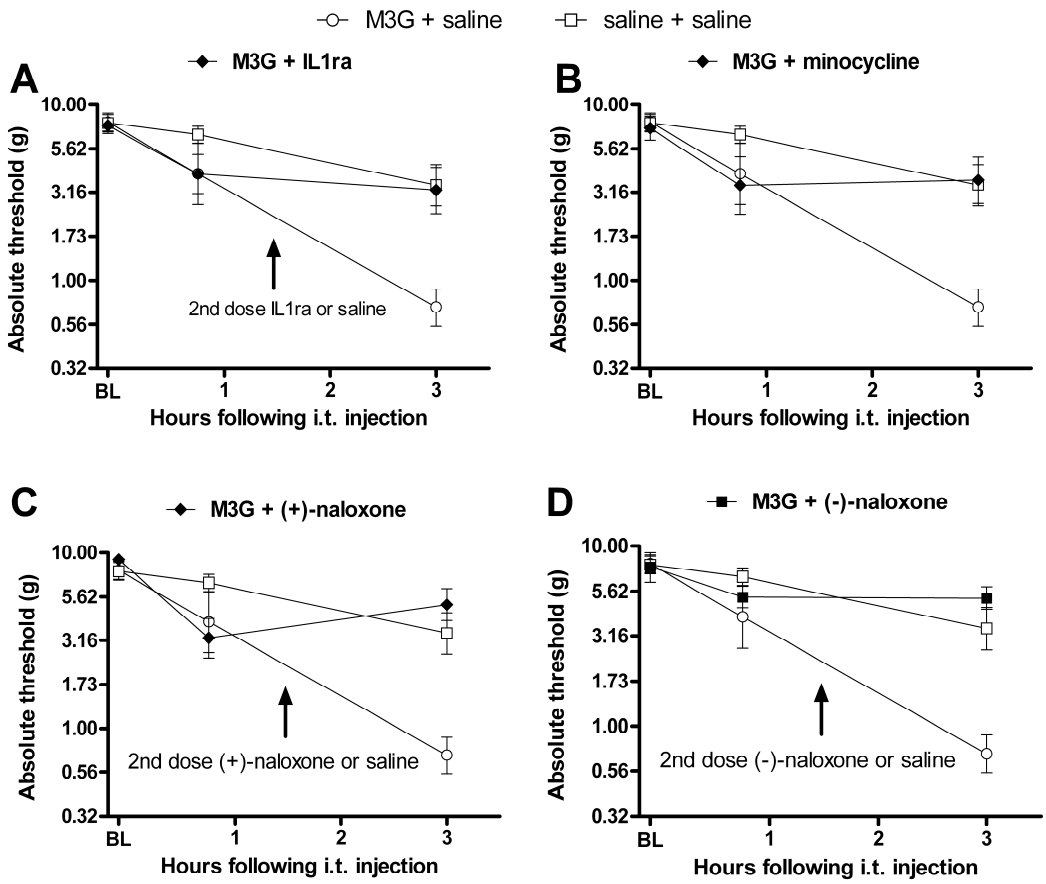


Figure 2.5. M3G-induced allodynia is blocked by glial and immune mediators ($F_{(5,28)}$=2.73 p<0.05). Acute intrathecal M3G (0.75 μg) produced allodynia which was blocked by any one of: IL1ra (100 μg coadministered with M3G, 100 μg at 1.5 h, A), minocycline (100 μg 1 h prior to M3G, 33.3 μg with M3G, B), (+)-naloxone (20 μg coadministered with M3G, 20 μg at 1.5 h, C) or (-)-naloxone (20 μg coadministered with M3G, 20 μg at 1.5 h, D). Saline and M3G groups are repeated on each graph to facilitate visual comparisons. Error bars represent standard error of the mean.

Experiment 6: IL1ra reverses established M3G hyperalgesia.

Given the effectiveness of IL1ra in blocking M3G induced hyperalgesia and allodynia, we investigated whether continuing IL1 release was necessary for the maintenance of M3G induced hyperalgesia. IL1ra completely reversed established M3G hyperalgesia. There were no baseline differences between M3G + vehicle, M3G + IL1ra, and vehicle + vehicle groups ($F_{(2,14)}$=0.14, p>0.05). There was a significant main effect

of drug treatment ($F_{(2,14)} = 14.81$, $p<0.05$, Figure 2.6) and a significant interaction between drug treatment and time ($F_{(2,14)}= 4.71$ $p<0.05$). Post-hocs test showed that there were no significant differences between the M3G + later IL1ra and M3G + saline groups prior to IL1ra administration. The M3G + later IL1ra group had significantly shorter withdrawal latencies prior to IL1ra administration when compared to saline at 25, 45, 55 min following intrathecal M3G, prior to intrathecal IL1ra. Following IL1ra administration 60 min after M3G administration, the M3G + IL1ra group's withdrawal latencies rapidly increased to become significantly longer than M3G+saline at 65- 85 and 105-125 min following M3G administration. The withdrawal latencies for the M3G + IL1ra groups following IL1ra were indistinguishable from saline + saline withdrawal latencies, indicating that IL1ra was able to fully reverse M3G-induced allodynia.

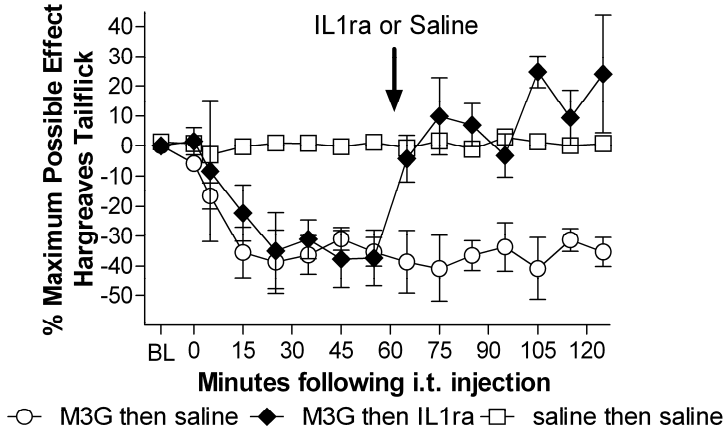


Figure 2.6. Established M3G-induced hyperalgesia is reversed by IL1ra. Intrathecal M3G (0.75 μg) produced hyperalgesia that was significantly reversed by intrathecal IL1ra (100 μg, baseline latency 9.0 ± 0.82 s) given 60 min following M3G (interaction $F_{(2,14)}= 4.71$ $p<0.05$). Reversal was maintained for the remainder of the test. Error bars and ranges represent the standard error of the mean.

Experiment 7: M3G-induced TLR4 activity is blocked by the HSP90 inhibitor 17-DMAG *in vitro*.

TLR4 is activated in models of spinally mediated pain, but producing pain with TLR4 activation alone using LPS has been unexpectedly difficult. Recent evidence suggests that HSP90 is able to function as a second signal to enhance TLR4 activation (Hutchinson et al., 2009b). We tested the HSP90 inhibitor, 17-DMAG, to determine if it would significantly reduce M3G-induced TLR4 activity *in vitro*. The M3G-induced SEAP signal was significantly blocked by 17-DMAG, ($F_{(3,8)}$= 94.6, p<0.05, Figure 2.7A). Post-hoc analyses showed that 17-DMAG reduced M3G-induced TLR4 activity, as measured by SEAP expression, at concentrations of 0.1 (t=6.34, p<0.05) and 1 ng/ml (t=17.8 p<0.05). 17-DMAG without M3G also reduced SEAP expression compared to media control (t=4.64, p<0.05), but a 2 (0 vs 1.0 ng/ml 17-DMAG) x 2 (0 vs 10 μM M3G) ANOVA indicated that the efficacy of 17-DMAG in reducing SEAP expression was greater when M3G was present. This finding suggests that hsp90 may be involved in TLR4 signaling under basal conditions as well as during TLR4 activation.

Experiment 8: M3G-induced hyperalgesia is blocked by the HSP90 inhibitor 17-DMAG

Experiment 7 documented that the HSP90 inhibitor 17-DMAG was able to block M3G-induced TLR4 signaling *in vitro*. To determine if HSP90 was involved in M3G-induced hyperalgesia, we tested whether 17-DMAG could block M3G-induced hyperalgesia. 17-DMAG at this intrathecal dose was able to enhance morphine analgesia, presumably by removing anti-nociceptive morphine-induced glial activation, while having no effect on pain thresholds in the absence of morphine (Hutchinson et al., 2009b). Experiment 8 was run concurrently with Experiment 4 so the same control groups are used here. There were no significant baseline differences between M3G+ saline, M3G+17-DMAG and saline+saline groups prior to drug administration

($F_{(2,14)}$=.215, p>.05). Coadministration of the HSP90 inhibitor 17-DMAG with M3G resulted in a blockade of M3G-induced hyperalgesia ($F_{(2,14)}$=24.28, p<0.05, Figure 2.7B). The area under the curve was significantly smaller for M3G + 17-DMAG than M3G + saline (F=23.5, p<0.05). Post-hoc analysis showed a significant decrease in withdrawal latencies in the group administered M3G + vehicle compared to M3G + 17-DMAG at 15, 25, 35, 45, 55, 65, 75, 85, 95, 105, 115 and 125 min following intrathecal injection. There were no significant differences between M3G coadministered with 17-DMAG and saline controls. The relatively complete blockade here, compared to the partial blockade seen in *in vitro* Experiment 7 may be, at least in part, explained by the short 2-hour time course of behavioral measurement, rather than the 24 time point for the SEAP assay.

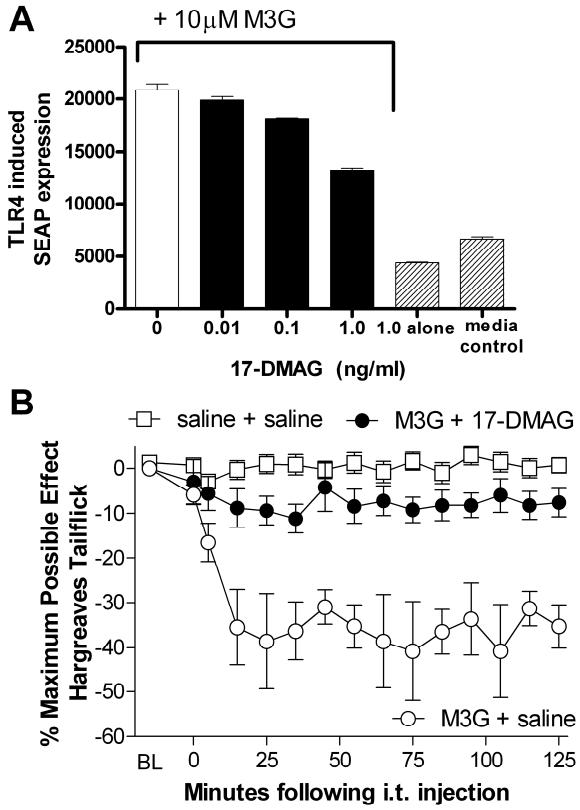


Figure 2.7. The HSP90 inhibitor 17-DMAG significantly reduces M3G-induced TLR4 activation, measured by SEAP activity in TLR4-HEK cells (main effect of 17-DMAG:

$F_{(3,8)}$= 94.6, p<0.05, A). 17-DMAG alone reduced TLR4-induced SEAP signal, but the effect of 17-DMAG depended on whether M3G was present or absent ($F_{(1,7)}$ =57.1, p<0.05). Intrathecal M3G (0.75 μg)-induced hyperalgesia is blocked by coadministration of 17-DMAG (4 μg, $F_{(2,14)}$=24.28, p<0.05, baseline latency of 8.9 ± 0.36 s, B). Error bars represent standard error of the mean.

Experiment 9: M3G increases the levels of glial activation marker mRNAs and proinflammatory molecules.

Given the success of microglial and inflammatory inhibition in blocking M3G-induced allodynia and hyperalgesia, we examined the changes in IL1 protein expression in the CSF and proinflammatory and glial activation marker gene transcription in the dorsal spinal cord. Our behavioral data showed a rapid onset of allodynia that can be blocked with IL1ra, indicative of rapid IL1 release following M3G administration. In support of this, intrathecal M3G (0.75 μg) caused an increase in IL1 protein in the lumbrosacral CSF in rats given M3G compared to saline vehicle (t_9=2.461, p<0.05, Figure 2.8A).

We then analyzed dorsal spinal cord (meninges removed) 60 min after 0.75 μg intrathecal M3G administration for changes in mRNA expression relative to the housekeeping gene β-actin using RT-PCR. There was no difference between treatment groups in the level of β-actin mRNA (t_9= 0.66, p>0.05)

Our behavioral data suggested that microglia were activated by M3G, and previous research has shown that the microglial activation marker CD11b is upregulated following chronic morphine administration (Hutchinson et al., 2009a). Supporting and extending those results, we found a significant upregulation of CD11b mRNA 60 min following intrathecal M3G (t_8= 2.45, p<0.05, Figure 2.8B). TLR4 mRNA is increased following glial activation in both acute (Raghavendra et al., 2004a) and chronic pain

models (Tanga et al., 2005). Again supporting and extending those results, here we found a significant increase in TLR4 mRNA 60 min following intrathecal M3G (t_8= 5.44, p<0.05, Figure 2.8C), consistent with an increase in proinflammatory activation of glia. At the time point sampled, the proinflammatory cytokines IL1 and TNF showed no significant changes at an mRNA level between rats administered intrathecal M3G and those administered intrathecal saline.

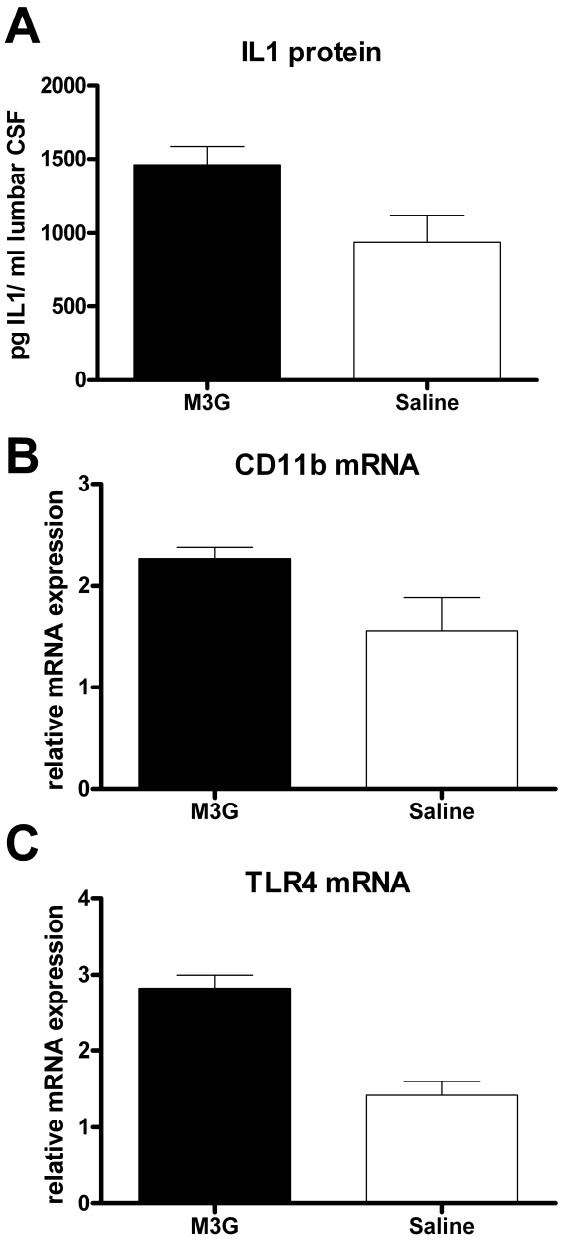
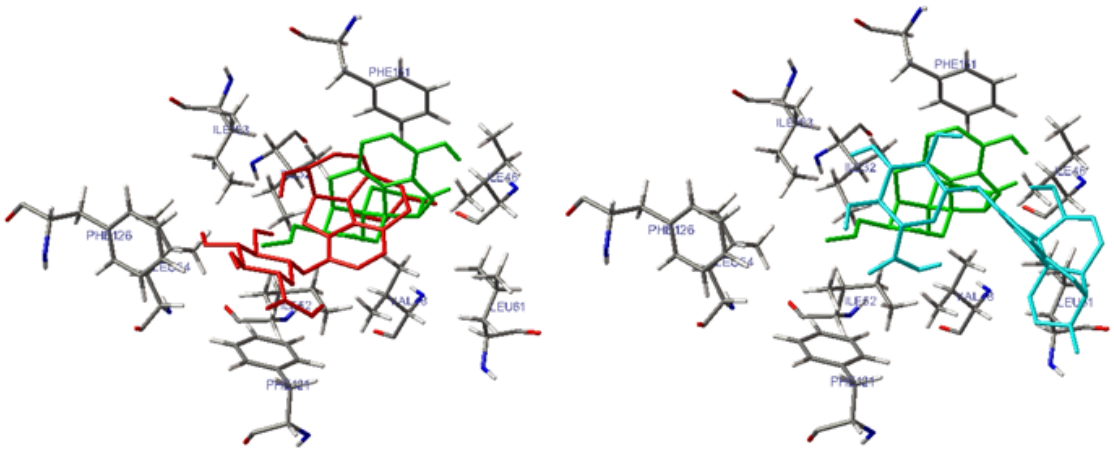


Figure 2.8. M3G significantly upregulates IL1 protein in the CSF and the mRNA levels of glial activation markers in the dorsal spinal cord. Intrathecal M3G (0.75 μg) significantly increased IL1 protein in the CSF (t_9=2.461, p<0.05, A) and mRNA levels of CD11b (t_8= 2.45, p<0.05, B) and TLR4 (t_8= 5.44, p<0.05, C) compared to equivolume saline vehicle controls. Error bars represent standard error of the mean.

Experiment 10: M3G docks *in silico* to MD-2 in a (+)-naloxone sensitive manner.

Experiments 1-9 provide substantial evidence for a TLR4-dependent pro-inflammatory response to M3G in the central nervous system. Therefore, to further validate these *in vitro* and *in vivo* experimental data, an *in silico* docking analysis was conducted using the recently published high-resolution crystalline structure of the dimer of human TLR4 and MD-2 (Park et al., 2009) and the *in silico* docking software suite AutoDock 4. In addition, the possible interaction of (+)-naloxone with M3G *in silico* was examined. Using AutoDock 4, 100 independent docking simulations were run for each ligand with the entire dimer of human TLR4 and MD-2. M3G and (+)-naloxone both docked with the LPS binding cleft of MD-2, independent of interactions with TLR4. Current docking simulations have higher confidence in predicting the binding site than calculating the binding energies. Therefore, the *in silico* docking was repeated with MD-2 alone at higher resolution (0.375 Å), M3G and (+)-naloxone docked to the same pocket of MD-2 (Figure 2.9). Interestingly, the glucuronic acid portion of M3G interacted closely with MD-2 residues F121 and F126 previously identified as important for TLR4 activation by LPS (Teghanemt et al., 2008). When M3G was docked *in silico* again to MD-2 with (+)-naloxone already in place, M3G docking was modified substantially such that it no longer interacted with these pivotal MD-2 residues (Figure 2.9).



A: M3G (red) and (+)-naloxone (green) bound to MD-2

B: M3G (teal) interacting with MD-2/(+)-naloxone complex

Figure 2.9 M3G (red) and (+)-naloxone (green) both bind to the LPS-binding pocket on MD-2 in an *in silico* model (A). When M3G (teal) binding was modeled to a MD-2/(+)-naloxone complex, M3G no longer interacted with the critical LPS-binding residues in MD-2 (B).

Experiment 11: Glucuronic acid produced enhanced pain sensitivity, and that change is blocked by a TLR4 inhibitor.

Based on the results from Experiment 10, we would predict that glucuronic acid alone may be capable of inducing TLR4 activation, and that (+)-naloxone could block this effect. To test whether the glucuronide portion of the molecule is capable of producing enhanced pain *in vivo* through TLR4-dependent processes, glucuronic acid (0.38 μg) was given intrathecally, coadministered with either (+)-naloxone (20 μg) or saline. As discussed in Experiment 3, (+)-naloxone is the opioid-inactive isomer of naloxone which has been shown to block TLR4 activation. Glucuronic acid produced significant allodynia 0.75 and 3 h following intrathecal injection (Figure 2.10, $F_{(3,20)}=4.23$, $p<0.05$). This allodynia was blocked by (+)-naloxone (20 μg) 3 h following glucuronic acid administration.

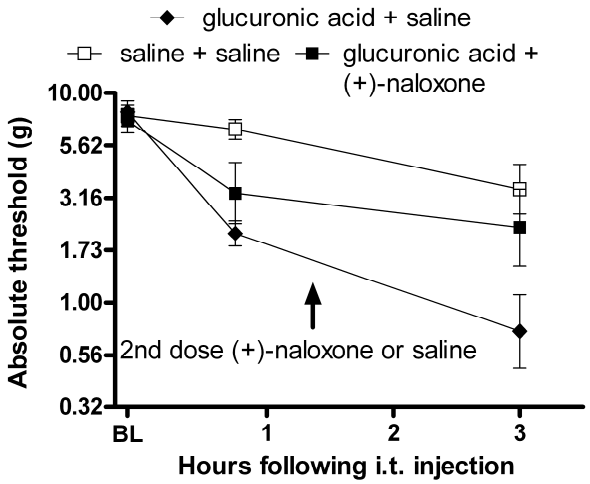


Figure 2.10. Glucuronic acid causes enhanced pain which is blocked by the TLR4 antagonist (+)-naloxone. Intrathecal glucuronic acid (0.38 μg) produced robust allodynia 0.75 and 3 h following administration (main effect of drug treatment $F_{(3,20)}$=4.23, p<0.05). Coadministered (+)-naloxone blocked that allodynia 3 h after administration.

DISCUSSION

The present series of studies explored the mechanistic basis of hyperalgesia and allodynia induced by intrathecal administration of the morphine metabolite M3G. We present the first evidence that M3G can activate microglial cells *in vitro* to induce IL1 release, and that M3G induced pain enhancement is mediated by, at minimum, the proinflammatory cytokine, IL1, as IL1ra prevented and/or reversed M3G-induced hyperalgesia and allodynia and was increased in the CSF 60 min following intrathecal M3G. Additionally, intrathecal minocycline, a compound known to inhibit activation of microglia and other monocyte-derived cells, prevented M3G-induced hyperalgesia and allodynia. TLR4, a receptor in the spinal cord principally expressed on microglia in the central nervous system, appears to be an important link. First, M3G induced TLR4 signaling *in vitro,* an effect which was blocked both by the TLR4 competitive antagonist, LPS-RS, and by inhibition of the TLR4 cosignaling molecule, HSP90 with 17-DMAG. Behaviorally, the recently discovered inhibitors of TLR4 signaling , (+)- and (-)-naloxone

(Hutchinson et al., 2008b; Hutchinson et al., 2010b), were each able to reduce M3G-induced pain enhancement, an effect duplicated by the HSP90 inhibitor 17-DMAG. Providing further evidence for a proinflammatory response to M3G, mRNA for TLR4 and the microglial/macrophage activation marker CD11b were both upregulated 60 min following intrathecal M3G. Finally, *in silico* modeling confirmed the *in vitro* and *in vivo* inhibition of M3G effects by (+)-naloxone, demonstrating M3G docked to MD-2 and interacted closely with pivotal residues on MD-2 which may facilitate TLR4/MD-2 signaling. Importantly, the *in silico* docking was (+)-naloxone sensitive agreeing with *in vitro* and *in vivo* data. The action of glucuronic acid alone was tested *in vivo* and found to produce marked allodynia which was also blocked by (+)-naloxone, again suggesting a role for TLR4.

All previous studies of M3G have sought a direct action of M3G on neurons to explain its observed effects. The present study provides the first evidence that spinal microglia/macrophages likely contribute to the effects of M3G. Because M3G has no affinity for classical opioid receptors (LaBella et al., 1979), M3G must act via a different receptor to create pain enhancement. Our data support TLR4 as a receptor activated by M3G. TLR4 is a pattern-recognition receptor that is expressed predominantly by peripheral and central immunocompetent cells of monocyte origin, including macrophages and microglia. There is little to no evidence of TLR4 expression by spinal cord astrocytes, neurons, or endothelial cells under basal conditions (Miyake, 2007). Further, fluorescently tagged LPS binds to microglia but not to neurons (Lehnardt et al., 2003), although neurons can express and up-regulate TLR4 in response to ischemia (Tang et al., 2007). TLR4 signaling activation following complexing of the required

accessory molecules CD14 and MD-2, induces proinflammatory responses by macrophages following exposure to bacterial lipopolysaccharide (LPS) and other 'danger signals'. Like macrophages, TLR4 activation on microglia induces a proinflammatory state, including the release of proinflammatory cytokines, such as IL1, that are well-documented to enhance pain and neuroexcitation (Lehnardt et al., 2003; Milligan and Watkins, 2009).

The present studies expand on prior work indicative of TLR4 signaling activation by both morphine and M3G, but not by the second major metabolite of morphine, M6G. *In vitro* studies of HEK-TLR4 cells found significant increases in TLR4-dependent reporter protein expression in response to (+)-morphine or (-)-morphine, compared to media control (Hutchinson et al., 2010b). Intriguingly, M3G, but not M6G, also increased TLR4 signaling by these cells (Hutchinson et al., 2010b). These HEK-TLR4 signaling data are consistent with *in silico* TLR4/MD-2 *in silico* docking data which indicate that M3G and morphine both optimally interact with the same "pocket" on the 3-dimensional TLR4/MD-2 complex, whereas M6G does not bind there in the same fashion (Hutchinson et al., 2010b). Indeed, TLR4 signaling induced by M3G in HEK-TLR4 cells was significantly greater than that induced by equimolar morphine (Hutchinson et al., 2010b), suggestive that *in vivo* TLR4 signaling following morphine administration may increase as morphine is metabolized to M3G.

The *in silico* and *in vivo* data strongly support a role for the glucuronide group in producing enhanced pain following M3G administration owing to the close interaction with two MD-2 residues previously found to be pivotal to LPS TLR4 signaling LPS (Teghanemt et al., 2008). The implications of TLR4 signaling activation by M3G's

interaction with these and other residues in the absence of LPS deserves further investigation as it has implications for TLR4 signaling activation by other endogenous small molecules, environmental or pharmacological xenobiotics. For example, other 3-glucuronide opioid metabolites have been shown to neuroexcitatory or pain enhancing properties, including hydromorphone-3-glucuronide (Wright et al., 1998) and normorphone-3-glucuronide (Smith and Smith, 1998). The studies presented here would predict that glial and inflammatory activity could contribute to these responses. However, there may also be a contribution of the morphine moiety to the pain response, as other morphine-3 conjugates can also produce hyperactive motor behavior (LaBella et al., 1979) and tactile sensitivity (Yaksh et al., 1986; Yaksh and Harty, 1988). Further studies are necessary to fully elucidate the structure-function relationship of these molecules with TLR4/MD-2.

The use of (+)-naloxone and (-)-naloxone as inhibitors of TLR4 signaling in the present studies is based on recent *in vitro*, *in vivo*, and *in silico* studies. *In silico* receptor/ligand computer modeling studies reveal that both (+)-naloxone and (-)-naloxone dock in the same TLR2/MD-2 binding pocket as does morphine (Hutchinson et al., 2010b). In such computer modeling studies, activators and inhibitors of TLR4 signaling are predicted to dock similarly, as the software does not allow for global conformation change. Conformational change upon agonist binding is necessary to facilitate signal transduction (agonism) versus inhibition (antagonism). Notably, a recently solved TLR4/ligand complex crystal structure showed that activators and inhibitors of TLR4 signaling occupy the same binding site in the TLR4/MD-2 association (Park et al., 2009). *In vitro* studies support that naloxone inhibits TLR4 signaling

nonstereoselectively as: (a) (+)-naloxone and (-)-naloxone each block LPS signaling both in HEK-TLR4 cells and in RAW274.7 macrophages, as does the classic TLR4 antagonist, LPS-RS; (b) (+)-naloxone blocks morphine signaling both in HEK-TLR4 cells and in RAW274.7 macrophages, as does the classic TLR4 antagonist, LPS-RS; and (c) while (+)-naloxone potentiates morphine analgesia in wild-type mice, it fails to potentiate morphine analgesia in TLR4 knockout mice (which do not express opioid receptors) (Hutchinson et al., 2008a; Hutchinson et al., 2010b). This same lack of stereoselectivity for naloxone actions was also observed in the blockade of M3G-induced hyperalgesia in the present study, consistent with a TLR4-dependent mechanism. Were M3G action to involve classical opioid receptors, only (-)-naloxone would antagonize the effect while (+)-naloxone would be ineffective, which was not the results observed. Thus, our findings support M3G action on a non-classical opioid receptor, found principally on microglia and blocked nonstereoselectively by naloxone, all of which is consistent with M3G action on TLR4/MD-2.

TLR4 proinflammatory activation of microglia, however, appears to require a second signal *in vitro* and *in vivo*. Very recent evidence suggests that heat shock protein 90 (HSP90) can act as a TLR4 co-signaling molecule *in vitro* and *in vivo*, and blocking HSP90 function significantly increases and prolongs morphine analgesia consistent with the effects observed by direct TLR4 antagonism (Triantafilou et al., 2001; Hutchinson et al., 2009b). To ascertain if HSP90 was needed as a second signal to facilitate M3G-induced TLR4 activation, 17-DMAG, an HSP90 inhibitor, was tested both *in vitro* and *in vivo*. 17-DMAG did significantly attenuate M3G-induced TLR4 activity *in vitro* and was able to block M3G-induced hyperalgesia, suggestive of HSP90 involvement. While 17-

DMAG can also apparently enhance HSP70 following chronic administration, the direction of effects observed here following acute administration are not congruent with such a mechanism. Given previous evidence for a role of HSP90 in TLR4-dependent signaling *in vitro* and *in vivo*, this provides another line of evidence for TLR4 involvement in M3G-induced hyperalgesia.

M3G induces other behaviors in addition to enhanced pain, including wet-dog shakes, tonic-clonic seizures, myoclonus and ataxia in rats (Bartlett et al., 1994a). The mechanism(s) by which M3G induces such behaviors is not known, although several neuronally based mechanisms have been investigated without resolving the question. All of the reported M3G-induced behaviors, including enhanced pain, are indicative of increased neuronal excitation. Our data are supportive of a TLR4, IL1 and, likely, microglial component to the neuroexcitation leading to enhanced pain, and suggest that parallel investigations for other M3G-induced behaviors is warranted especially in light of recent advances in the epilepsy seizure literature implicating TLR4 and IL1 in epileptogenesis (Rodgers et al., 2009).

M3G effects are very potent, requiring only 0.75 μg to markedly enhance pain, while greater doses of morphine are needed to produce analgesia (Hutchinson et al., 2008a). Given the presence of M3G in the CNS following morphine administration and the potency of M3G, the natural metabolic formation of M3G may be a significant endogenous counter-regulator, or opponent process, to morphine analgesia. Recent research supports this possibility. In rats, the decline in morphine analgesia following a single acute injection can be prolonged by an injection of IL1ra, evidence that proinflammatory mechanisms may reduce the analgesic actions of morphine (Hutchinson

et al., 2008a). In the same study, inhibiting other proinflammatory cytokines with soluble TNF receptor and interleukin-6 neutralizing antibody, as well as inhibiting microglia with minocycline enhanced and prolonged morphine analgesia (Hutchinson et al., 2008a). Further, blocking TLR4 activation enhances morphine analgesia in rats (Hutchinson et al., 2010b). Given the evidence for M3G-induced inflammatory molecules presented here, it is reasonable to suspect that M3G-induced inflammation could contribute to the inflammatory opposition of morphine analgesia.

Such a conclusion would support prior work, using pharmacological modeling, which concluded that the time course of morphine analgesia was optimally accounted for only when including M3G concentrations as an anti-analgesic component (Mazoit et al., 2007). This study was based on the finding in humans that the analgesic profile of morphine does not parallel its CSF or plasma concentrations (Mazoit et al., 2007). This provided further evidence that active metabolites of morphine may play an important role in modulating morphine analgesia. In this model, based on the results of a study of 50 pain patients, the CSF time to maximum concentration of morphine was 30 min, while M6G had a time to maximum concentration of 4 h and M3G 10 h. Analgesia was maximal around 4 h following i.v. morphine administration, such that analgesia is waning as M3G levels continue to rise (Mazoit et al., 2007). Resolving the anti-analgesic effects of M3G could improve the analgesic efficacy of morphine. As presented here, M3G and morphine both activate TLR4, M3G more potently, and produce proinflammatory glial activation that may oppose the analgesic effects of morphine. This proinflammatory glial activation is effectively blocked by (+)-naloxone in rats. Because (+)-naloxone does not reduce morphine's analgesic effects, it may be an effective way to reduce TLR4 signaling

and consequent proinflammatory glial activation and anti-analgesia following morphine administration.

In summary, converging lines of evidence support glial activation and release of proinflammatory molecules as necessary for M3G-induced pain behaviors. Blocking the proinflammatory cytokine IL1, microglial activation, or TLR4 activity were each able to completely block M3G-induced allodynia and/or hyperalgesia. Increases in mRNA levels of proinflammatory activation markers and production and release of IL1 by M3G both *in vitro* and *in vivo* further support this hypothesis. Taken together, these data provide evidence for a substantial TLR4-dependent glial component to M3G-induced effects including enhanced pain and gene activation and suggest that the clinical efficacy of opioids for pain control could be enhanced by selectively blocking TLR4 activation by drugs like (+)-naloxone.

CHAPTER 3

GLUCURONIC ACID AND THE ETHANOL METABOLITES ETHYL-GLUCURONIDE CAUSE TOLL-LIKE RECEPTOR 4 ACTIVATION AND ENHANCED PAIN

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ABSTRACT

We have previously observed that the non-opioid morphine metabolite, morphine-3-glucuronide, enhances pain via a toll-like receptor 4 (TLR4) dependent mechanism. The present studies were undertaken to determine whether TLR4-dependent pain enhancement generalizes to other classes of glucuronide metabolites. *In silico* modeling predicted that glucuronic acid alone and ethyl glucuronide, a minor but long-lasting ethanol metabolite, would dock to the same MD-2 portion of the TLR4 receptor complex previously characterized as the docking site for morphine-3-glucuronide. Glucuronic acid, ethyl glucuronide and ethanol all caused an increase in TLR4-dependent reporter protein expression in a cell line transfected with TLR4 and associated co-signaling molecules. Glucuronic acid-, ethyl glucuronide-, and ethanol-induced increases in TLR4 signaling were blocked by the TLR4 antagonists LPS-RS and (+)-naloxone. Glucuronic acid and ethyl glucuronide both caused allodynia following intrathecal injection in rats,

which was blocked by intrathecal co-administration of the TLR4 antagonist LPS-RS. The finding that ethyl glucuronide can cause TLR4-dependent pain could have implications for human conditions such as hangover headache and alcohol withdrawal hyperalgesia, as well as suggesting that other classes of glucuronide metabolites could have similar effects.

Many drug metabolites are considered inert byproducts, but increasing evidence suggests certain metabolites can have more complex effects. For instance, morphine-3-glucuronide, a morphine metabolite, causes pro-inflammatory responses in the CNS (Komatsu et al., 2009; Lewis et al., 2010a). This response induces pain enhancement blocked by broad-spectrum immune inhibitors (e.g. minocycline) as well as the toll-like receptor 4 (TLR4) signaling inhibitor, (+)-naloxone (Lewis et al., 2010a). These data raise the question of whether this TLR4-dependent pain-sensitizing effect is unique to morphine-3-glucuronide, or found more broadly in glucuronidated metabolites.

The pattern recognition receptor TLR4 is present on many immune cells, including at least microglia in the CNS (Bsibsi et al., 2002). TLR4 activation initiates a proinflammatory cascade leading to proinflammatory cytokine release (Lehnardt et al., 2003). TLR4 is linked to enhanced pain, including neuropathic pain (Hutchinson et al., 2008b), and decreased morphine analgesia (Hutchinson et al., 2010b), in addition to pain induced by morphine-3-glucuronide (Lewis et al., 2010a). TLR4 signaling can be blocked nonstereoselectively by (+)- and (-)-naloxone as well as by TLR4-inactive lipopolysaccharide made by Rhodobacter sphaeroides (Hutchinson et al., 2008b; Hutchinson et al., 2010b).

Several classes of commonly abused drugs have also been linked to increased TLR4 activity. TLR4 inhibition reduces the rewarding affects of morphine and cocaine (Northcutt et al., 2011), and reduces sedative and ataxic effects of ethanol (EtOH) (Wu et al., 2011). Additionally, TLR4 knockout mice display reduced ethanol-induced neuroinflammatory damage following chronic alcohol feeding (Pascual et al., 2011) and cultured microglia showed TLR4-dependent increases in pro-inflammatory cytokines in

response to EtOH (Fernandez-Lizarbe et al., 2009). Intriguingly, none of these studies considered the metabolism of the drugs, leaving the potential that glucuronide or other metabolites could have contributed.

EtOH is primarily metabolized by conversion to acetaldehyde in the liver. Ethyl-β-D-glucuronide (EtG) is a minor ethanol metabolite with about 0.04% of an EtOH dose excreted as EtG (Goll et al., 2002). However, EtG has a much longer half-life than EtOH, and can be detected for up to 4 days after the elimination of EtOH from the body (Wurst et al., 2003). EtG has also been measured in the brain tissue and cerebrospinal fluid in a human who died due to acute alcohol intoxication (Wurst et al., 1999). Notably, a standard alcoholic drink has about 13 g EtOH, so even minor metabolites such as EtG can be present at physiologically relevant concentrations following heavy drinking. The inflammatory potential of EtG has not been previously investigated. However, the glucuronide component of the molecule, glucuronic acid (GA) alone can cause pain when injected intrathecally, which is blocked by the TLR4 antagonist (+)-naloxone (Lewis et al., 2010a). EtG is structurally quite similar to GA, leading to the prediction that it will have similar TLR4 agonist effects.

We hypothesize that the TLR4 agonist, nociceptive response to morphine-3-glucuronide described in Lewis et al. (2010a) will generalize to other glucuronide molecules, namely GA and EtG. Further, we propose that EtOH will also have TLR4 agonist potential, as suggested by prior studies.

MATERIALS AND METHODS

Drugs

Ethyl glucuronide (EtG) was purchased from Aurora Analytics (Baltimore, MD) and ethyl alcohol (EtOH) from Pharmco (Brookfield, CT). Sodium glucuronic acid (GA) was purchased from Sigma (St. Louis, MO). LPS-RS was purchased from Invivogen (Thousand Oaks, CA) and (+)-naloxone was obtained from the National Institute on Drug Abuse. Endotoxin-free sterile water (Hospira, Lake Forest, IL) was used as a vehicle for all drugs *in vitro* (Experiment 2). Endotoxin-free, sterile 0.9% saline (Hospira, Lake Forest, IL) was used as a vehicle for all drugs in behavioral studies (Experiment 3). GA, EtG, EtOH, (+)-naloxone, saline and water were confirmed to be endotoxin-free by the limulus amebocyte lysate assay (Lonza, Walkersville, MD) conducted per manufacturers instructions. Where appropriate, doses are reported as a free base concentration.

In silico docking simulations

In silico docking simulation methods were identical to those previously described in detail (Hutchinson et al., 2010b). These methods were employed to examine the docking of glucuonic acid (GA), ethyl glucuronide (EtG), and ethyl alcohol (EtOH) to the TLR4/MD-2 complex. The *in silico* docking analysis conducted in Experiment 1 used the recently published high-resolution crystalline structure of the dimer of human TLR4 and MD-2 (Park et al., 2009) and the *in silico* docking software suite AutoDock 4. Briefly, the complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). Modified pdb files were inputted into AutoDock 4.0 (http://autodock.scripps.edu), hydrogens added, and resaved in pdbqt format. GA and EtG structures were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator

(http://cactus.nci.nih.gov/services/translate/). All dockings were executed with Lamarkian genetic algorithms.

In vitro assay for TLR4 signaling

A human embryonic kidney-293 (HEK 293) cell line was used in Experiment 2. This cell line was stably transfected by Invivogen (San Deigo, CA) to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (293-htlr4a-md2cd14; referred to here as HEK-TLR4). In addition, these cells stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors, such as NFκB and AP-1, activated as part of the TLR4 signaling cascade. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

HEK-TLR4 cells were grown at 37^{o}C (5% CO_2; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selection [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5 x 10^3 cells/well) with the same media. After 48 h, supernatants were removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 μl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 μl test samples were diluted in 45 μl of 1x dilution buffer, transferred to 96-well plates (Thermo, Walthma, MA, USA), and heated at 65^{o}C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 μl/well) was added and, 5 min later, reaction buffer (50 μl/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

In vivo behavioral assessments

Animals. Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used for all *in vivo* experiments. Rats (325-375 g at time of experiments) were housed in temperature (23±3 ^{o}C) and light (12 h:12 h light:dark) controlled rooms with water and food given *ad libitum*. All habituation and behavioral testing procedures were performed during the light phase of the daily cycle. All procedures were approved by the University of Colorado-Boulder Institutional Animal Care and Use Committee. Each experimental group contains 6-8 rats.

Intrathecal catheter implantation and injections. All drugs administered *in vivo* in Experiment 3 were given intrathecally over lumbosacral spinal cord with acute intrathecal injections. Catheter placements via the L5/L6 intervertebral approach and drug microinjections were performed based on Milligan et al. (1999). Rats were briefly

anaesthetized under isoflurane anesthesia. An 18-gauge needle was placed between L5 and L6 into the intrathecal space and served as a guide. Polyethylene-10 tubing was threaded rostrally through the guide so to terminate over the lumbosacral enlargement. The catheters were pre-loaded with drugs at the intrathecal end and the remainder filled with sterile saline. Upon insertion, the drug was injected with a 8.5 μl flush to ensure complete drug delivery, and the needle and catheter then removed. The rats were anaesthetized for, on average, 5 minutes and had no skin incision or implantations upon awakening.

GA was given at a dose of 0.32 μg, consistent with previously published data showing that dose was capable of inducing pain following intrathecal injection (Lewis et al., 2010a). EtG was given at a dose of 0.38 μg, equimolar to the GA dose and also equimolar to a previously published dose of morphine-3-glucuronide that induced pain behaviors (Lewis et al., 2010a). EtOH was also given equimolar to GA and EtG at 0.074 μg. LPS-RS was given at an intrathecal dose of 40 μg, consistent with previous literature showing this dose was capable of reversing neuropathic pain (Hutchinson et al., 2008b).

von Frey test for tactile allodynia. All testing was conducted by an experimenter blind to group assignment. Rats received at least four 1 h habituations to the appropriate testing apparatus and environment prior to baseline testing, as in our previous studies (Lewis et al., 2010a).

The response threshold to light touch on both plantar hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as described in Milligan et al. (2001). Briefly, a logarithmic range of hairs from 0.406-15.136 g force were used, allowing both analgesia and allodynia to be measured,

following the standard up-down procedures previously described (Chaplan et al., 1994). Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described in Milligan et al. (2001).

Statistics

GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all statistical analyses. One-way ANOVAs with appropriate Bonferroni post hocs were used to compare experimental groups on *in vitro* experiments and to confirm that there were no baseline differences on behavioral measures. Two-way repeated-measures ANOVAs with Bonferroni post-hoc tests when appropriate were used to determine statistical significance between behavioral measures. For all analyses, $p<0.05$ was considered significant.

Experimental Procedures

<u>Experiment 1:</u> Does *in silico* docking predict TLR4-MD2 binding of GA, EtG and/or EtOH?

Initially, the *in silico* docking of GA and EtG to the entire TLR4 MD-2 dimer complex was conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). Additionally, all the ligands were docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 0.375 Å

spacing). The lowest energy and highest interaction docking conformation was visualized and the residues on MD-2 that interacted with that conformation determined for each of the experimental drugs.

<u>Experiment 1B:</u> For drugs predicted to dock to the TLR4/MD-2 complex in Experiment 1A, would previously docked (+)-naloxone reduce the likelihood of their *in silico* docking?

The (+)-naloxone conformation interacting with MD-2 from a previous study (Lewis et al., 2010a) was saved as a combined MD-2/(+)-naloxone pdb file and the *in silico* docking was repeated on the combined MD-2/(+)-naloxone complex to determine the change in docking due to the presence of already docked (+)-naloxone.

<u>Experiment 2A:</u> Does *in vitro* GA, EtG or EtOH cause an increase in TLR4-dependent SEAP expression in HEK-TLR4 cells?

The experimental drugs, GA, EtG and EtOH, were each applied to HEK-TLR4 cells at concentrations of 0, 1, 10 or 100 μM as described above. Cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4-induced NFκB activity.

<u>Experiment 2B:</u> Is the *in vitro* increase in TLR4-dependent SEAP expression seen in Experiment 2A blocked by the TLR4 antagonists LPS-RS and/or (+)-naloxone?

For drugs which increase HEK-TLR4 SEAP production in Experiment 2A, the most effective dose (100 μM for each) was applied to HEK-TLR4 cells as described

above. Additionally, one of two TLR4 antagonists was coadministered. One, lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS, Invivogen, San Diego, CA), lacks functional TLR4 activity and acts as a competitive TLR4 antagonist. LPS-RS was applied to the HEK-TLR4 cells at the same time as the GA, EtG or EtOH at concentrations of 0, 0.1, 1 or 10 ng/ml. The other, (+)-naloxone, is the opioid-inactive isomer of naloxone and has been shown to inhibit LPS-induced increases in HEK-TLR4 signaling (Hutchinson et al., 2010b). (+)-Naloxone was applied to the HEK-TLR4 cells at the same time as the GA, EtG or EtOH at concentrations of 0, 1, 10 or 100 μM. For all assays, cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4 activity.

<u>Experiment 3:</u> Does intrathecal GA, EtG and/or EtOH cause tactile allodynia? Is that allodynia TLR4 dependent?

Rats received either 0.32 μg GA, 0.38 μg EtG or 0.074 μg EtOH or equivolume (1 μl) saline vehicle via an acute intrathecal injection, as described above. Both injections had a saline flush for a total injection volume of 9.5 μl. Rats were tested for tactile allodynia 1 d prior to injection (baseline; BL) and again 1 and 3 h post-injection.

The drugs that produced significant allodynia (in this case, GA and EtG) were coadministered with the TLR4 antagonist LPS-RS. Rats received either 0.32 μg GA or 0.38 μg EtG or equivolume (1 μl) saline with 40 μg LPS-RS via an acute intrathecal injection.

RESULTS

Experiment 1A: ***In silico*** **modeling predicts that GA and EtG will bind to MD-2.**

An *in silico* docking study was done to determine if GA, EtG and/or EtOH would be predicted to dock to the TLR4/MD-2 complex and whether they interacted at the same residues as the TLR4 antagonist (+)-naloxone. This *in silico* technique has been successfully used previously to model the docking of compounds, including opioids, morphine-3-glucuronide, and tricyclic antidepressants and to predict whether the compounds would act as TLR4 agonists *in vitro* and *in vivo* (Hutchinson et al., 2010a; Hutchinson et al., 2010b; Lewis et al., 2010a).

The EtOH molecule was too small to be effectively modeled in this paradigm. The *in silico* docking of GA or EtG to the entire human TLR4 MD-2 dimer complex was initially conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). These data demonstrated that the two ligands docked with human MD-2 independent of human TLR4 interactions. Therefore, the ligands were then docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized for GA and EtG and the residues that interacted with each compound determined (Table 3.1). The residues at which lipid A chains interacted in previously published crystallography studies (Park et al., 2009) are also included for comparison on Table 3.1.

Both GA and EtG were predicted to dock to MD-2, as demonstrated by negative predicted binding energies. EtG interacted at 7 residues, 6 of which were residues also

known to interact with the lipid A moiety of lipopolysaccharide. GA interacted at 8 residues on MD-2, only 1 of which was in common with lipid A interactions. GA largely interacted with MD-2 at residues distant from the pockets where lipid A interacts.

<u>Experiment 1B</u>: (+)-naloxone alters the predicted MD-2 docking conformations of EtG and GA *in silico.*

Given prior evidence that (+)-naloxone was able to block GA-induced pain (Lewis et al., 2010a), we hypothesized that GA and the structurally similar EtG would dock such that when (+)-naloxone was docked to MD-2, there would be a lower probability of GA or EtG docking. The *in silico* docking analysis performed in Experiment 1A was repeated using a saved combined MD-2/(+)-naloxone pdb file to determine the change in docking due to the presence of already docked (+)-naloxone. The residues at which the lowest binding energy conformation of EtG docked to the MD-2 were altered when (+)-naloxone was bound (Table 3.1). In contrast, the residues at which the lowest binding energy conformation of GA docked were not altered by (+)-naloxone. Given prior evidence that (+)-naloxone was able to reverse GA-induced allodynia (Lewis et al., 2010a), the *in silico* data here does not predict, an any obvious manner, the physiological effect observed. This discrepancy may be explained by a second conformation, other than the lowest binding energy conformation, being responsible for the physiological effect, an allosteric effect on MD-2/TLR4 interactions, differences in human and rat MD-2/TLR4 structure, or inverse agonist actions of (+)-naloxone at MD-2/TLR4, but such a distinction is beyond the capabilities of this *in silico* analysis.

residue	lipid A	(+)-Naloxone	GA	EtG
24	X	X		***X***
29			X	
32	X			
44				**G**
46	X	X		X
48	X	X		
52	X	X		
54	X	X	X	
55			X	
61	X			***X***
63	X			X
76	X			**G**
94	X			**G**
125			X	
126	X		X	
133				
135	X			***X***
147	X			**G**
149				X
151	X	X		X
153	X	X		
155			X	
157			X	
158			X	

Table 3.1. *In silico* docking studies predict that GA and EtG will bind to the MD2 portion of the TLR4-MD2 complex. The residues on MD-2 at which GA and EtG interact with MD-2 are denoted with an X. Residues that interacted with lipid A chains of lipopolysaccharide in the crystal structure determined by Park et al., (2009) are included for reference. When the docking simulations were repeated with (+)-naloxone ((+)-Nal) already bound to the MD2 molecule, the residues at which EtG interacted were altered, but the residues that GA interacted with did not change. The residues that lost interaction with EtG when (+)-naloxone was already docked are denoted with a ***X.*** Residues that gained interaction when (+)-naloxone was already docked are denoted with a **G**.

<u>Experiment 2A:</u> *In vitro* TLR4-dependent SEAP expression in HEK-TLR4 cells is increased by GA, EtG and EtOH.

Experiment 1 provided initial evidence suggesting that EtG and GA may have effects on TLR4. In order to determine if these effects were present in a cellular system, HEK-TLR4 cells were utilized. As detailed in the methods, HEK-TLR4 cells have been transfected with TLR4 and a NFκB-dependent SEAP reporter gene. Thus, an increase in TLR4 signaling will lead to an increase in NFκB transcription and subsequent increase in SEAP levels measured. Previous studies have confirmed that HEK cells with the SEAP reporter gene but without TLR4 overexpression did not increase SEAP expression due to the classic TLR4 agonist LPS (Hutchinson et al., 2010b). A dose-response function of TLR4 signaling changes for GA, EtG and EtOH was performed.

GA caused a significant increase in SEAP production in the HEK-TLR4 cells ($F=19.16$, $p<0.05$). Post-hoc tests showed that GA increased SEAP expression relative to vehicle at the 10 μM and 100 μM concentrations, but was not significantly different from vehicle at lower concentrations (Figure 3.1A). Similarly, EtG caused a significant increase in SEAP expression in HEK-TLR4 cells ($F=19.44$, $p<0.05$). Post-hoc tests showed that EtG increased SEAP expression relative to vehicle at the 10 and 100 μM concentrations, but caused no significant changes at the lower concentrations (Figure 3.1B). Finally, EtOH also caused an increase in SEAP production in the HEK-TLR4 cells ($F=22.18$, $p<0.05$). However, Post-hoc tests showed that only the 100 μM

concentration of EtOH was significantly increased relative to vehicle, while the 0.01, 0.1, 1 and 10 μM concentrations were not significantly different from the vehicle.

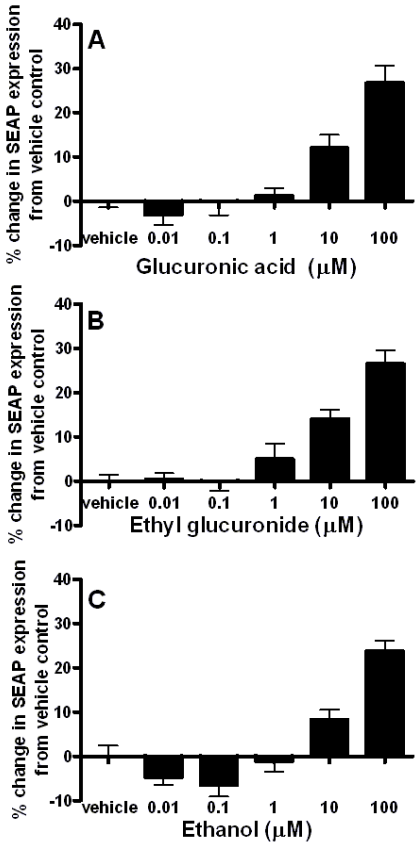

Figure 3.1. GA, EtG and EtOH all cause a significant increase HEK-TLR4 cell SEAP reporter expression. GA caused a significant increase relative to vehicle at 10 and 100 μM concentrations ($F=19.16$ $p<0.05$, A). EtG caused a significant increase relative to the vehicle at 10 and 100 μM concentrations ($F=19.44$, $p<0.05$, B). EtOH caused a significant increase relative to vehicle at the 100 μM concentration ($F=22.18$, $p<0.05$).

<u>Experiment 2B:</u> ***In vitro*** **GA, EtG and EtOH induced increases in SEAP expression are blocked by the TLR4 antagonists LPS-RS and (+)-naloxone.**

Experiment 2A provides evidence that GA, EtG and EtOH were able to increase NFkB-dependent SEAP expression in HEK-TLR4 cells. Here, the specificity of that response was tested by blocking the GA, EtG and EtOH-induced SEAP expression with

one of two TLR4 antagonists. The first, LPS-RS, is structurally similar to LPS but fails to produce a TLR4-dependent proinflammatory response. It acts as a competitive antagonist of the LPS effects on TLR4 complex and has been shown to reduce the increase in SEAP expression caused by another glucuronidated metabolite, morphine-3-glucuronide (Lewis et al., 2010a). The second, (+)-naloxone, has also been shown to inhibit the LPS response *in vitro* (Hutchinson et al., 2008b; Hutchinson et al., 2010b) . Further, (+)-naloxone was able to block the increase in HEK-TLR4 SEAP expression caused by morphine-3-glucuronide (Hutchinson et al., 2010b) as well as the *in vivo* pain responses caused by both morphine-3-glucuronide and GA (Lewis et al., 2010a). The *in silico* results of Experiment 1 further predict that (+)-naloxone would be able to block EtG-induced HEK-TLR4 cell SEAP expression.

LPS-RS effectively blocked the increase in HEK-TLR4 SEAP expression caused by GA ($F=7.51$, $p<0.05$, Figure 3.2A), EtG ($F=7.99$, $p<0.05$, Figure 3.2C), and EtOH ($F=30.0$, $p<0.05$, Figure 3.2E). Post-hoc tests showed that when 100 μM GA was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to 100 μM GA alone. Similarly, when 100 μM EtG was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to100 μM EtG alone. Finally, when 100 μM EtOH was incubated with either 0.1, 1 or 10 ng/ml of LPS-RS, SEAP expression was significantly decreased compared to 100 μM EtOH alone.

(+)-Naloxone was also able to block the increase in HEK-TLR4 SEAP expression caused by 100 μM GA ($F=5.36$, $p<0.05$, Figure 3.2B), EtG ($F= 4.92$, $p<0.05$, Figure 3.2D) or EtOH ($F=8.36$, $p<0.05$, Figure 3.2F). Post-hoc tests showed that when 100 μM

GA was incubated with 1, 10 and 100 μM (+)-naloxone, SEAP expression was significantly decreased compared to 100 μM GA alone. Similarly, when 100 μM EtG was incubated with 1 or 10 μM (+)-naloxone, SEAP expression was significantly decreased compared to 100 μM EtG alone. Finally, when 100 μM EtOH was incubated with 1 and 10 μM (+)-naloxone, SEAP expression was significantly decreased compared to 100 μM EtOH alone.

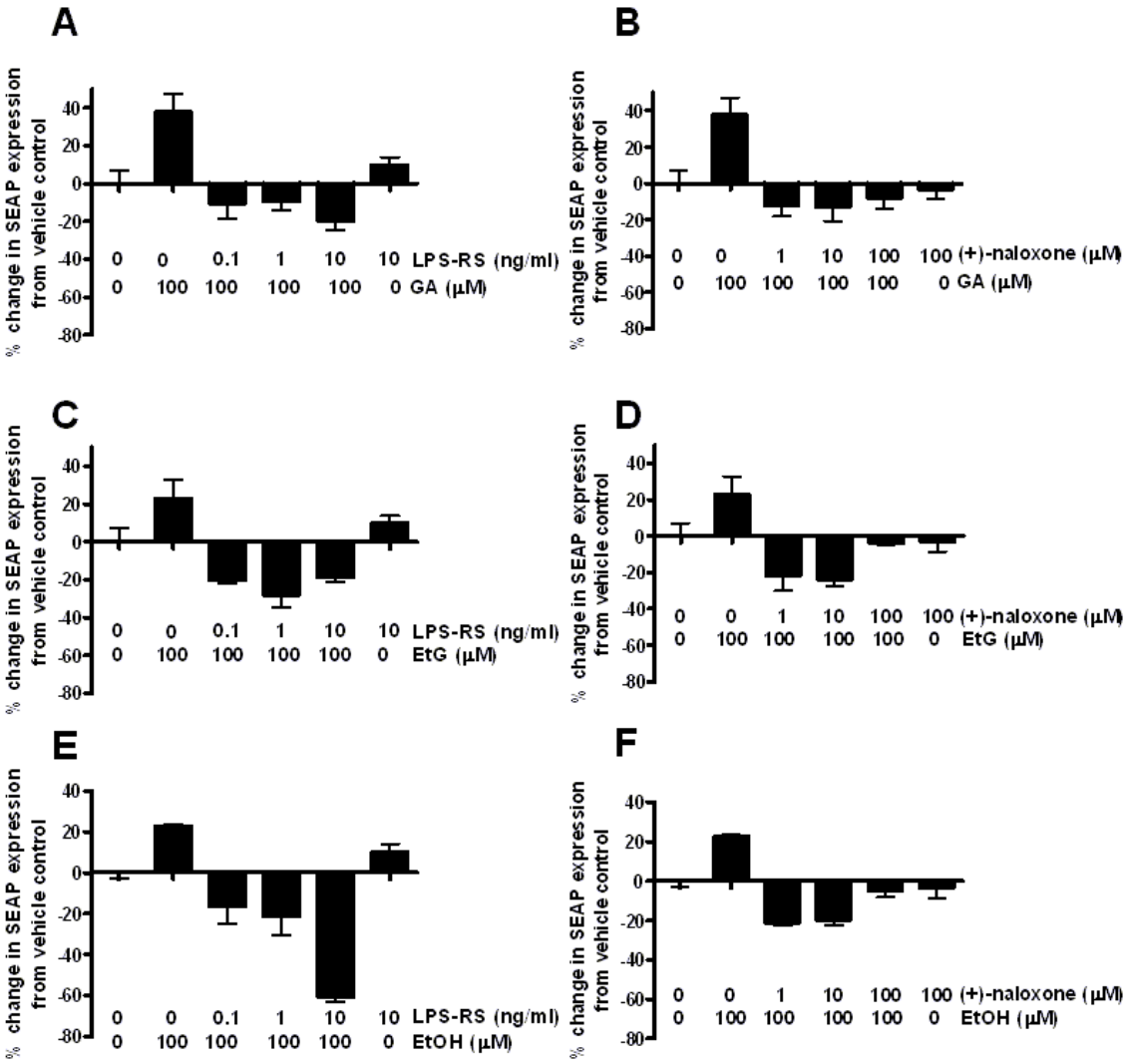

Figure 3.2. The TLR4 antagonist LPS-RS significantly attenuated the HEK-TLR4 cell SEAP increases seen with GA ($F=7.51$, $p<0.05$, A), EtG ($F=7.99$, $p<0.05$, C) and EtOH ($F=30.0$, $p<0.05$, E). Similarly, the TLR4 antagonist (+)-naloxone significantly

attenuated the HEK-TLR4 cell SEAP increases seen with GA ($F=5.36$, $p<0.05$, B), EtG ($F= 4.92$, $p<0.05$, D) and EtOH ($F=8.36$, $p<0.05$, F).

<u>Experiment 3:</u> Intrathecal GA and EtG cause tactile allodynia.

The *in silico* and *in vitro* studies, above, strongly suggest that GA, EtG and EtOH are capable of activating TLR4. Previous studies have shown that other glucuronide metabolites that can act as TLR4 agonists are able to produce pain *in vivo* (Lewis et al., 2010a). Additionally, previous studies have shown that GA was able to cause pain *in vivo* through TLR4-dependent mechanisms (Lewis et al., 2010a). To confirm the nociceptive capability of GA and to determine if EtG and/or EtOH are able to cause allodynia, GA, EtG , EtOH or vehicle were injected intrathecally at equimolar doses and allodynia tested 1 and 3 h following injection. To test whether this TLR4 signaling activation was necessary for the development of GA and EtG-induced pain, LPS-RS was coadministered intrathecally with those drugs that caused a significant allodynia to develop. Intrathecal LPS-RS has been shown to block neuropathic pain for a minimum of 3 h after an intrathecal injection (Hutchinson et al., 2008b). Experiment 2B further indicated that LPS-RS was able to block the TLR4 signaling caused by both GA and EtG *in vitro*.

In Experiment 3, no differences between right and left hind paws were detected. Thus, results are reported as the average of the response thresholds recorded for both paws. There were also no baseline differences between any groups ($F=0.458$, $p>0.05$).

Following intrathecal injection, GA caused significant allodynia ($F=17.1$, $p<0.05$, Figure 3.3A). Post-hoc test showed that 0.32 μg GA i.t. produced a robust decrease in tactile threshold relative to vehicle 1 and 3 h following injection, replicating the results of

Lewis et al. (2010a). The TLR4 antagonist LPS-RS was able to block the development of allodynia at the 3 h time point.

EtG (0.38 μg, i.t.) also produced a robust decrease in tactile threshold ($F=16.1$, $p<0.05$, Figure 3.3B). Post-hoc tests showed significant allodynia compared to vehicle at both 1 and 3 h following intrathecal injection. This allodynia was blocked by LPS-RS at 3 h post injection. EtOH (0.07 μg, i.t.) failed to produce a significant change in tactile threshold relative to vehicle ($F=0.204$, $p>0.05$, Figure 3.3C).

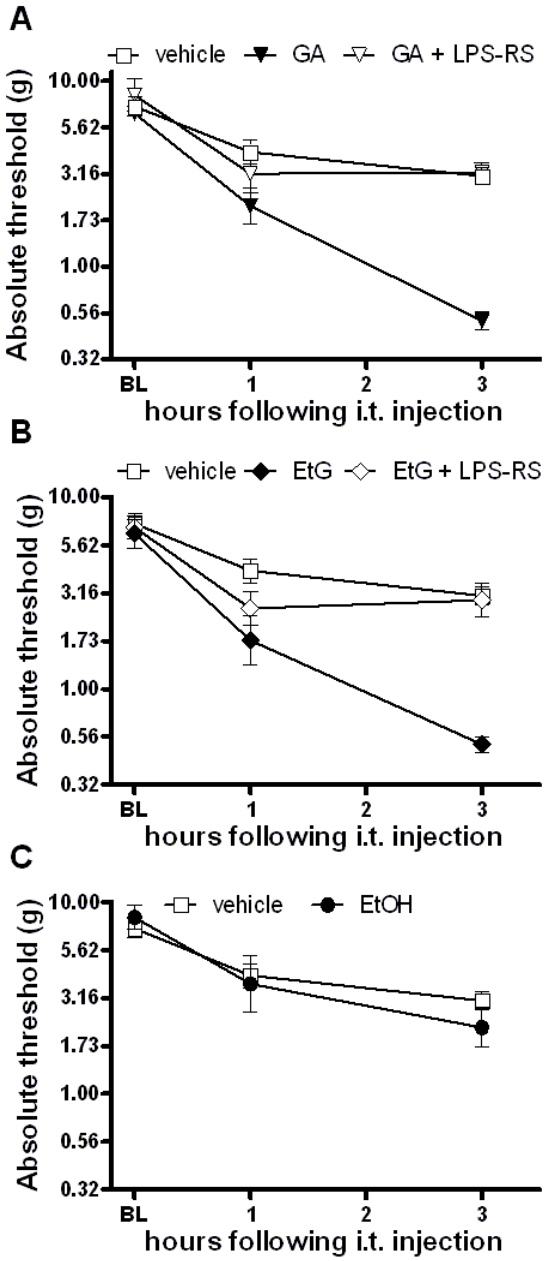

Figure 3.3. GA (0.34 μg, i.t.) caused a significant allodynia 3 hours post injection relative to vehicle injected rats, which was blocked by coadministered LPS-RS (40 μg, i.t.). EtG (0.38 μg, i.t.) caused significant allodynia 1 and 3 hours post injection compared to vehicle injected rats at both time points which was blocked by coadministered LPS-RS (40 μg, i.t.). Finally, EtOH (0.07 μg, i.t.) failed to cause allodynia within 3 hours of injection. All doses were equimolar. There were no baseline differences between any of the groups ($F=0.133$, $p>0.05$).

DISCUSSION

The present series of studies extend our prior observation of TLR4-dependent pain enhancement by the morphine metabolite, morphine-3-glucuronide (Lewis et al., 2010a), by investigating whether such TLR4-dependent effects generalize to glucuronic acid (GA) itself or to other glucuronidated metabolites. These studies present the first evidence that GA and the long-lived ethanol metabolite, Ethyl-β-D-glucuronide (EtG) each activate TLR4 signaling *in vitro* and produce a TLR4-activity dependent allodynia *in vivo*. In addition, our *in silico* modeling based on the recently published crystal structure of TLR4 bound to its co-receptor MD-2 (Park et al., 2009) predicted that EtG would bind to MD-2 at a site in the same region as the recently documented TLR4 antagonist, (+)-naloxone (Hutchinson et al., 2010b). Further, the *in silico* docking of EtG to MD-2 was in a manner that was displaced by the prior docking of (+)-naloxone. These *in silico* results were substantiated *in vitro* using a HEK-TLR4 cell line that was stably transfected to produce a reporter protein in response to TLR4 activation. Studies of these cells revealed that GA, EtG and EtOH induced increases in HEK-TLR4 reporter protein expression were blocked by both (+)-naloxone and the classical TLR4 antagonist, LPS-RS. Lastly, while EtOH was able to increase HEK-TLR4 SEAP expression, it did not enhance pain following an intrathecal injection at a dose equimolar to EtG and GA.

The seemingly contradictory finding that EtOH increased HEK-TLR4 cell reporter protein expression *in vitro*, but failed to cause allodynia when administered intrathecally, may arise from differences in *in vitro* versus *in vivo* conditions. First, EtOH was injected intrathecally at a dose equimolar to allodynia-inducing doses of GA and EtG. Thus EtOH was delivered at a dose of 0.074 μg. It is possible that with such a small dose, there was not sufficient diffusion into spinal tissues from cerebrospinal fluid

(CSF) and/or that EtOH so rapidly diffuses out of the central nervous system that there was insufficient exposure of lumbar dorsal horn to create a detectable change in pain threshold. While a higher dose may potentially cause significant allodynia, based on the HEK-TLR4 results, a higher intrathecal dose of EtOH was not tested here both because the strategy was to compare equimolar doses and because allodynia by higher doses had the potential to result from confounding toxicity of alcohol on central nervous system tissues. A second possible explanation for the apparent contradictory findings for EtOH is that EtOH also enhances the inhibitory function of $GABA_A$ receptors (Lobo and Harris, 2008) which could counteract a TLR4-induced increase in neuronal excitability.

One important question is whether the TLR4-dependent effects of EtG might impact the known symptoms of EtOH use. In a model based on 75 g of EtOH ingestion, serum EtOH peaks 1-2 hours following the end of drinking, while serum EtG peaks approximately 6 hours after the end of drinking (Droenner et al., 2002). Little data are available on CSF levels of EtG, although EtG was detected at 0.31 μg/ml in a human who died due to acute alcohol intoxication (Wurst et al., 1999). Notably, several pain-related effects of EtOH in humans, such as alcohol withdrawal hyperalgesia (Jochum et al., 2010) and headache after both chronic (Martinotti et al., 2010) and acute (e.g. hangover, (Howland et al., 2008) EtOH ingestion, are associated not with high levels of EtOH, but with declining levels of EtOH. Additionally, withdrawal from chronic alcohol exposure in rats has been linked to an increase in microglial activation markers and allodynia (Narita et al., 2007). EtG concentrations peak while EtOH is declining, meaning that EtG levels are highest when these negative side effects are observed. Many alternative hypotheses for these pain-producing side effects have been postulated, however, the exact

cause is unknown. While further study is needed to determine if EtG contributes to the negative side effects of EtOH, the potential exists and warrants additional study.

While EtOH is largely an exogenous drug, with only very small amounts made endogenously, GA is principally found endogenously throughout the body. However, it is usually either conjugated to form uridine 5'-diphosphoglucuronic acid, the substrate for the glucuronyltransferases that form glucuronidated metabolites, or polymerized with N-acetyl-D-glucosamine to form the extracellular matrix component hyaluronan. Hyaluronan polymers have been shown to have anti-inflammatory properties (Huang et al., 2011). However, smaller, low molecular weight polymers have been shown to activate TLR4 (Li et al., 2010; Leu et al., 2011). This finding is of particular significance in several pathological conditions, including pulmonary arterial hypertension (Ormiston et al., 2010), atherosclerosis (Cuff et al., 2001), and rheumatoid arthritis (Naor and Nedvetzki, 2003), where low molecular weight hyaluronan and its adhesion receptor CD44 have been associated with increased inflammation and disease progression. The studies here suggest that the glucuronide component of hyaluronan may contribute to the inflammatory effects of hyaluronan, and also suggest that TLR4 be considered as an additional candidate receptor for these inflammatory effects.

EtG and GA are structurally very similar. A logical question is whether the modification of GA by adding an ethyl group alters the *in silico*, *in vitro* and *in vivo* effects observed. *In silico*, EtG and GA appear to interact with different residues on MD-2, suggesting that there may be differences. However, *in vitro* and *in vivo* the results for the two drugs were not significantly different and in fact quite similar. It appears the

addition of the ethyl group to glucuronic acid did not dramatically alter the effects observed, although it may have altered the most likely predicted docking site *in silico*.

These studies present evidence that the glucuronide molecules GA and EtG may produce pain through TLR4-dependent mechanisms. EtOH also had potential TLR4 activity, but did not cause pain this particualar *in vivo* model. The finding that GA and EtG can cause TLR4-dependent pain in and of themselves has potentially wide-ranging implications for conditions such as hangover, chronic alcohol withdrawal headache and several disorders, such as rheumatoid arthritis and atherosclerosis, linked to low molecular weight hyaluronan. Additionally, the TLR4-dependent pain effect morphine-3-glucuronide has now generalized to additional classes of molecules, including glucuronic acid alone and ethyl glucuronide. This finding suggests that other classes of molecules that are glucuronidated, such as steroid hormones and non-steroidal anti-inflammatories, could also have TLR4-dependent pain producing effects.

CHAPTER 4

CERTAIN STEROID HORMONE GLUCURONIDE METABOLITES CAN CAUSE TOLL-LIKE RECEPTOR 4 ACTIVATION AND ENHANCED PAIN

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ABSTRACT

We have recently shown that several classes of glucuronide metabolites, including the morphine metabolite morphine-3-glucuronide and the ethanol metabolite ethyl glucuronide, cause toll like receptor 4 (TLR4)-dependent signalling *in vitro* and enhanced pain *in vivo*. Another major class of glucuronidated compounds is the steroid hormones, including estrogens and corticosterone. Here we demonstrate that an *in silico* docking study predicts that corticosterone, corticosterone-21-glucuronide, estradiol, estradiol-3-glucuronide and estradiol-17-glucuronide would all interact with the MD-2 component of the TLR4 receptor complex. In addition to each docking with MD-2, the docking of each was altered by pre-docking with (+)-naloxone, a TLR4 signaling inhibitor. As, agonist/antagonist activity cannot be determined from these *in silico* interactions, an *in vitro* study was undertaken to clarify which of these compounds can act in an agonist

fashion. Studies using a cell line transfected with TLR4, necessary co-signaling molecules, and a reporter gene revealed that only estradiol-3-glucuronide and estradiol-17-glucuronide increased reporter gene product, indicative of TLR4 agonism. Finally, in *in vivo* studies, each of the 5 drugs was injected intrathecally at equimolar doses. In keeping with the *in vitro* results, only estradiol-3-glucuronide and estradiol-17-glucuronide caused enhanced pain. For both compounds, pain enhancement was blocked by the TLR4 antagonist lipopolysaccharide from *Rhodobacter sphaeroides*, evidence for the involvement in TLR4 in the resultant pain enhancement. These findings have implications for several chronic pain conditions, including migraine and tempromandibular joint disorder, in which pain episodes are more likely in cycling females when estradiol is decreasing and estradiol metabolites are at their highest.

Recent evidence suggests that certain glucuronide metabolites have neuroinflammatory actions. Specifically, morphine-3-glucuronide, ethyl-β-D-glucuronide and glucuronic acid have been shown to activate the innate immune toll-like receptor 4 (TLR4) and cause TLR4-dependent enhanced pain when administered intrathecally (Lewis et al., 2010a; Lewis et al., 2011a). Another major class of glucuronidated molecules is the steroid hormones. Androgens (Belanger et al., 1994), estrogens (Guillemette et al., 2004), and corticosteroids (Ikegawa et al., 2009) are all glucuronidated as part of metabolism. However, the potential for TLR4 activation and/or enhanced nociception by these glucuronidated metabolites has not been studied.

Several of the steroid hormones also have associations with enhanced pain, including corticosterone (Cort) and estradiol (E_2). Acute stress and acute stress-associated corticosterone release in rats has been shown to inhibit nociception, while chronic stress has been linked to hyperalgesia, although analgesic effects have also been reported (Imbe et al., 2006). Clinically, chronic stress has also been associated with chronic pain conditions such as fibromyalgia, chronic headache, inflammatory bowel disease and tempromandibular joint pain (McEwen and Kalia, 2010). Cort, intriguingly, has both anti-inflammatory and pro-inflammatory effects on inflammation caused by the classic TLR4 ligand lipopolysaccharide (LPS). Cort inhibits proinflammation by LPS when LPS precedes Cort, whereas Cort potentiates proinflammation by LPS when LPS is given 2 or 24 hours following Cort (Frank et al., 2010). One potential explanation for this finding is that the anti-inflammatory effect of Cort was modulated by a proinflammatory effect of its glucuronidated metabolite. Cort is metabolized by extensive degradation or conjugation with a glucuronide group to form corticosterone-21-

glucuronide (CortG). CortG has been found in mouse brain homogenates, indicating that it has the potential to directly affect the central nervous system (Kallonen et al., 2009).

Another steroid hormone that has been linked to increased pain is E_2. Estrogens, including E_2, are the primary female sex hormones and, clinically, women are almost twice as likely to have chronic pain conditions (Craft, 2007). Additionally, the drop from peak E_2 levels during pre-menses in women is associated with an increased risk of migraine and increased tempromandibular joint pain (Craft, 2007). E_2 may also affect the LPS response. Chronic E_2 administration in ovariectomized females and male rats increased the LPS –stimulated inflammatory response in hippocampal microglia *ex vivo* and increased proinflammatory cytokine transcription *in vivo* (Loram et al., 2011). E_2 is metabolized into estradiol-3-glucuronide (E_2-3-G) and estradiol-17-glucuronide (E_2-17-G), and both of these metabolites have also been found in brain tissue homogenates, indicating that they have access to the central nervous system (Kallonen et al., 2009). Neither metabolite is believed to have activity at the estrogen receptors.

The first step to determine if glucuronidated Cort and E_2 metabolites could contribute to the pain-enhancing effects of the parent hormones is to determine if the metabolites have the ability to potentiate pain. Given the TLR4-dependent allodynia caused by other glucuronidated metabolites, we hypothesize that CortG, E_2-3-G and E_2-17-G will cause an increase in TLR4 signaling as well as TLR4-dependent enhanced pain.

MATERIALS AND METHODS

Drugs Cort, E_2, E_2-3-G, and E_2-17-G were purchased from Sigma (St. Louis, MO). CortG was synthesized by the authors (MMF, TS) from D-(+)-glucurono-6,3-lactone.

The lactone was converted to the protected trichloroacetimidate by the procedure of Nakajima et al. (2005), coupled with corticosterone, then deprotected according to the procedure of Ciuffredaa et al. (2009). The identity of the product was confirmed by comparison of ^{1}H and ^{13}C NMR data with that reported by Ciuffredaa et al. (2009). Lipoplysaccharide from *Rhodobacter sphaeroides* (LPS-RS) was purchased from Invivogen (Thousand Oaks, CA) and (+)-naloxone was obtained from the National Institute on Drug Abuse. CortG, E_2-3-G, LPS-RS and (+)-naloxone were dissolved in endotoxin-free sterile water (Hospira, Lake Forest, IL) for *in vitro* studies (Experiment 2) and endotoxin-free, sterile 0.9% saline (Hospira, Lake Forest, IL) for *in vivo* studies (Experiment 3). E_2-17-G was dissolved in 10% DMSO (Sigma, St. Louis, MO) and sterile water for *in vitro* studies (Experiment 2) and 1% DMSO and sterile saline for *in vivo* studies (Experiment 3). Cort and E_2 were dissolved in 100% DMSO for both *in vitro* and *in vivo* studies. Cort, CortG, E_2, E_2-3-G, and E_2-17-G, (+)-naloxone, saline and water were all confirmed to be endotoxin-free by the limulus amebocyte lysate assay (Lonza, Walkersville, MD). Where appropriate, doses are reported as a free base concentration.

In silico docking simulations

In silico docking simulation methods were identical to those previously described in detail (Hutchinson et al., 2010b). These were employed to examine the docking of Cort, CortG, E_2, E_2-3-G, and E_2-17-G to the TLR4/MD-2 complex. The *in silico* docking analyses were conducted in Experiment 1 using the recently published high-resolution crystalline structure of the dimer of human TLR4 and MD-2 (Park et al., 2009) and the *in*

silico docking software suite AutoDock 4. Briefly, the complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). Modified pdb files were inputted into AutoDock 4.0 (http://autodock.scripps.edu), hydrogens added, and resaved in pdbqt format. Structures were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator (http://cactus.nci.nih.gov/services/translate/). All dockings were executed with Lamarkian genetic algorithms.

In vitro assay for TLR4 signaling

A human embryonic kidney-293 (HEK 293) cell line was used in Experiment 2. This cell line was stably transfected by Invivogen (San Deigo, CA) to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (293-htlr4a-md2cd14; referred to here as HEK-TLR4). In addition, these cells stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors, such as NFκB and AP-1, activated as part of the TLR4 signaling cascade. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

HEK-TLR4 cells were grown at 37^{o}C (5% CO_2; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selection [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h

in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5 x 10^3 cells/well) with the same media. After 48 h, supernatants were removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 μl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 μl test samples were diluted in 45 μl of 1x dilution buffer, transferred to 96-well plates (Thermo, Walthma, MA, USA), and heated at 65^{o}C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 μl/well) was added and, 5 min later, reaction buffer (50 μl/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

In vivo **behavioral assessments**

Animals. Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used for all *in vivo* experiments. Rats (325-375 g at time of experiments) were housed in temperature (23±3 ^{o}C) and light (12 h:12 h light:dark) controlled rooms with water and food given *ad libitum*. All habituation and behavioral testing procedures were performed during the light phase of the daily cycle. All procedures were approved by the University of Colorado-Boulder Institutional Animal Care and Use Committee. Each experimental group contains 6-8 rats.

Intrathecal catheter implantation and injections. Acute intrathecal injections were used to administer drugs in Experiment 3. Catheter placements via the L5/L6 intervertebral approach and drug microinjections were performed based on Milligan et al. (1999). Rats were briefly anaesthetized under isoflurane anesthesia. An 18-gauge needle was placed between L5 and L6 into the intrathecal space and served as a guide. Polyethylene-10 tubing was threaded rostrally through the guide so to terminate over the lumbosacral enlargement. The catheters were pre-loaded with drugs at the intrathecal end and the remainder filled with sterile saline. Upon insertion, the drug was injected with an 8.5 μl flush to ensure complete drug delivery, and the needle and catheter then removed. The rats were anaesthetized for, on average, 5 minutes and had no skin incision or implantations upon awakening.

All drugs administered *in vivo* in Experiment 3 were given intrathecally over lumbosacral spinal cord. All doses were equimolar with the doses of glucuronide metabolites (morphine-3-glucuronide, ethyl glucuronide and glucuronic acid) that were sufficient to cause allodynia in prior studies (Lewis et al., 2010a; Lewis et al., 2011a). Cort was given at a dose of 0.56 μg and CortG was given at a dose of 0.85 μg. E_2 was given at a dose of 0.44 μg, E_2-3-G and E_2-17-G were both given at doses of 0.76 μg. LPS-RS was given at an intrathecal dose of 40 μg, consistent with previous literature showing this dose was capable of reversing neuropathic pain (Hutchinson et al., 2008b).

von Frey test for tactile allodynia. All testing was conducted by an experimenter blind to group assignment. Rats received at least four 1 h habituations to the appropriate

testing apparatus and environment prior to baseline testing, as in our previous studies (Lewis et al., 2010a).

The response threshold to light touch on both plantar hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as described in Milligan et al. (2001). Briefly, a logarithmic range of hairs from 0.406-15.136 g force were used, allowing both analgesia and allodynia to be measured, following the standard up-down procedures previously described (Chaplan et al., 1994). Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described in Milligan et al. (2001).

Statistics

GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all statistical analyses. One-way ANOVAs with appropriate Bonferroni post hocs were used to compare experimental groups on *in vitro* experiments and to confirm that there were no baseline differences on behavioral measures. Two-way repeated-measures ANOVAs with Bonferroni post-hoc tests when appropriate were used to determine statistical significance between behavioral measures. For all analyses, $p<0.05$ was considered significant.

Experimental Procedures

<u>Experiment 1A:</u> Does *in silico* docking predict that Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G will bind to TLR4/MD2?

Initially, the *in silico* docking of Cort, CortG, E_2, E_2-3-G, and E_2-17-G to the entire TLR4 MD-2 dimer complex was conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). Additionally, all the ligands were docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized and the residues on MD-2 that interacted with that conformation determined for each of the experimental drugs.

<u>Experiment 1B:</u> For drugs predicted to dock to the TLR4/MD-2 complex in Experiment 1A, would previously docked (+)-naloxone reduce the likelihood of their *in silico* docking?

The (+)-naloxone conformation from a previous study (Lewis et al., 2010a) was saved as a combined MD-2/(+)-naloxone pdb file and the Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G *in silico* docking was repeated on the combined MD-2/(+)-naloxone complex to determine the change in docking due to the presence of already docked (+)-naloxone.

<u>Experiment 2A:</u> Does *in vitro* Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G cause an increase in TLR4-dependent SEAP expression in HEK-TLR4 cells?

The experimental drugs, Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G were each applied to HEK-TLR4 cells at concentrations of 0, 1, 10 and 100 μM as described above.

Cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4-induced NFκB activity.

<u>Experiment 2B:</u> Is the *in vitro* increase in TLR4-dependent SEAP expression seen in Experiment 2A blocked by the TLR4 inhibitors LPS-RS and/or (+)-naloxone?

For drugs which increase HEK-TLR4 SEAP production in Experiment 2A, the most effective dose (100 μM for each) was applied to HEK-TLR4 cells as described above. Additionally, one of two TLR4 antagonists was coadministered. One, lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS, Invivogen, San Diego, CA), lacks functional TLR4 activity and acts as a competitive TLR4 antagonist. LPS-RS was applied to the HEK-TLR4 cells at the same time as the E_2, E_2-3-G, or E_2-17-G at concentrations of 0, 0.1, 1 or 10 ng/ml. The other, (+)-naloxone, is the opioid-inactive isomer of naloxone and has been shown to antagonize LPS-induced increases in HEK-TLR4 signaling (Hutchinson et al., 2010b). (+)-Naloxone was applied to the HEK-TLR4 cells at the same time as the E_2, E_2-3-G, and/or E_2-17-G at concentrations of 0, 1, 10 or 100 μM. For all assays, cells were incubated for 24 h, then supernatants removed and assessed for SEAP activity, indicative of TLR4 activity.

<u>Experiment 3:</u> Does intrathecal Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G cause tactile allodynia? Is that allodynia TLR4 dependent?

Rats received either Cort (0.56 μg), CortG (0.85 μg), E_2 (0.44 μg), E_2-3-G (0.76 μg), and/or E_2-17-G (0.76 μg) or equivolume (1 μl) vehicle via an acute intrathecal injection, as described above. Both injections had a saline flush for a total injection

volume of 9.5 µl. Rats were tested for tactile allodynia 1 d prior to injection (baseline; BL) and again 1 and 3 h post-injection.

The drugs that produced significant allodynia (in this case, E_2-3-G and E_2-17-G) were coadministered with the TLR4 antagonist LPS-RS. Rats received 0.76 µg of either E_2-3-G, E_2-17-G or equivolume (1 µl) saline with 40 µg LPS-RS via an acute intrathecal injection.

RESULTS

Experiment 1A: *In silico* modeling predicts that Cort, CortG, E_2, E_2-3-G, and E_2-17-G will bind to MD-2.

An *in silico* docking study was done to determine if Cort, CortG, E_2, E_2-3-G, and/or E_2-17-G would be predicted to dock to the TLR4/MD-2 complex. This *in silico* technique has been successfully used previously to model the docking of morphine-3-glucuronide, other opioid drugs, tricyclic antidepressants, ethanol glucuronide, glucuronic acid and other compounds (Hutchinson et al., 2010a; Hutchinson et al., 2010b; Lewis et al., 2010a; Lewis et al., 2011a).

The *in silico* docking of Cort, CortG, E_2, E_2-3-G, and E_2-17-G to the entire TLR4 MD-2 dimer complex was initially conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; genetic algorithm running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). These data demonstrated that the ligands each docked with human MD-2 independent of human TLR4 interactions. Therefore, the ligands were then docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; genetic algorithm

running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). The lowest energy and highest interaction docking conformation was visualized for each drug and the residues on MD-2 that each drug interacted with are listed in Table 4.1. Additionally, Cort, CortG, E_2, E_2-3-G, and E_2-17-G all had a preferred conformation with a negative predicted docking energy, suggesting that they would be likely to dock to the MD2 molecule.

<u>Experiment 1B</u>: (+)-naloxone alters the predicted MD-2 docking conformations of Cort, CortG, E_2, E_2-3-G, and E_2-17-G *in silico.*

Given prior evidence that (+)-naloxone altered the predicted *in silico* docking of other glucuronide molecules and successfully blocked HEK-TLR4 signaling of these same molecules *in vitro* (Hutchinson et al., 2010b; Lewis et al., 2010a; Lewis et al., 2011a) we hypothesized that CortG, E_2-3-G, and E_2-17-G would dock on MD-2 such that when (+)-naloxone was docked to MD2, the drugs of interest would be less likely to dock. Similarly, in Experiment 1A, Cort and E_2 were also predicted to dock to MD-2 at similar residues to the glucuronide metabolites, and we hypothesize that this docking would also be altered by the presence of (+)-naloxone.

The *in silico* docking analysis performed in Experiment 1A was repeated utilizing a saved combined MD-2/(+)-naloxone pdb file to determine the change in docking due to the presence of (+)-naloxone docked to the MD2 molecule. Additionally, for all the molecules, the residues at which the lowest binding energy conformation in Experiment 1A interacted were altered by the presence of (+)-naloxone (Table 4.1). However, the

functional effects of any of these compounds docking to MD2 are beyond the capability of this *in silico* study.

residue	lipid A	(+)-Nal	Cort	CortG	E_2	E_2-3-G	E_2-17-G
24	X	X		***X***			
32	X	X	***X***	***X***		***X***	
44			***X***		**G**		X
46	X	X	***X***	***X***	X	***X***	X
48	X	X		***X***			***X***
52	X	X	***X***	***X***			***X***
54	X	X					
61	X		X	X	X	***X***	X
63	X		X	X	X	X	X
71	X				***X***	***X***	
74	X				***X***	X	***X***
76	X		**G**	X	X	X	X
78	X		**G**	**G**		**G**	
92			**G**				
94	X		**G**	**G**	**G**		X
102					**G**		
104	X		**G**		X	X	X
117	X		**G**	**G**		**G**	X
119	X						**G**
121	X						**G**
133			**G**	***X***		**G**	
135	X		X	X			
147	X		X	**G**	X	X	X
149					**G**		X
151	X	X	X	X	X	X	X
153	X	X		X			

Table 4.1: ***In silico*** **docking studies predict that Cort, CortG, E_2, E_2-3-G, and E_2-17-G will dock to the MD2 portion of the TLR4-MD2 complex.** The residues on MD-2 at which these compounds interact with MD-2 are denoted with an X. Residues that interacted with lipid A chains in the crystal structure determined by Park et al., (2009) are included for reference. When the docking simulations were repeated with (+)-naloxone ((+)-Nal) already bound to the MD2 molecule, the residues at which each drug interacted were altered. The residues that lost interaction with EtG when (+)-naloxone was already docked are denoted with a **X.** Residues that gained interaction when (+)-naloxone was already docked are denoted with a **G**.

<u>Experiment 2A:</u> *In vitro* TLR4-dependent SEAP expression in HEK-TLR4 cells is increased by E_2-3-G and E_2-17-G and decreased by E_2 and Cort.

Experiment 1 provided initial evidence suggesting that Cort, CortG, E_2, E_2-3-G and E_2-17-G may have effects on TLR4. In order to determine if these effects were present in a cellular system, HEK-TLR4 cells were utilized. As detailed in the methods, HEK-TLR4 cells have been transfected by Invivogen for overexpression of TLR4 and its co-receptors and a NFκB-dependent SEAP reporter gene. Thus, an increase in TLR4 signaling will lead to an increase in NFκB transcription and subsequent increase in SEAP levels measured. Previous studies have confirmed that HEK cells with the SEAP reporter gene but without TLR4 overexpression did not increase SEAP expression to the classic TLR4 agonist LPS (Hutchinson et al., 2010b). The dose-response functions for Cort, CortG, E_2, E_2, E_2-3-G and E_2-17-G on HEK-TLR4 SEAP expression were calculated.

Cort caused a significant decrease in SEAP production in the HEK-TLR4 cells relative to its 1% DMSO vehicle ($F=9.05$, $p<0.05$). Post-hoc tests showed that Cort decreased SEAP expression relative to vehicle at 0.1, 1 and 10 μM concentrations, but was not significantly different from vehicle at the 100 or 0.01 μM concentrations (Figure 4.1A). CortG, however, caused no significant alterations from its saline vehicle ($F=1.72$, $p>0.05$, Figure 4.1B).

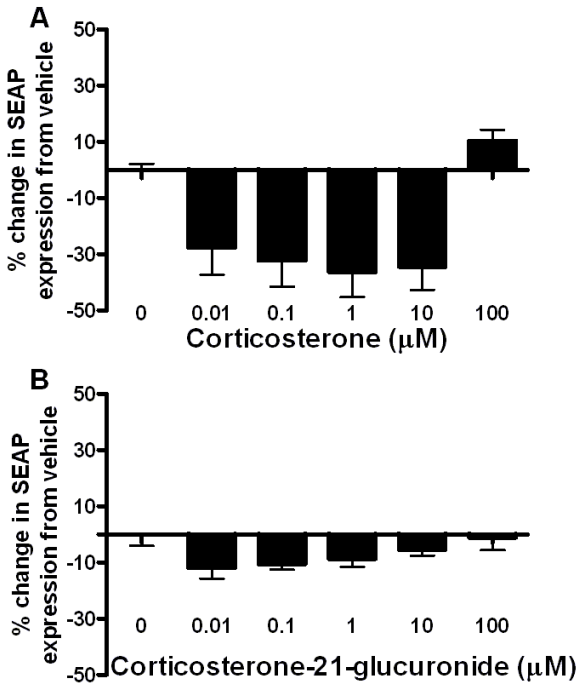

Figure 4.1. Corticosterone caused a significant decrease in HEK-TLR4 cell SEAP reporter expression at 0.1, 1 and 10 μM concentrations ($F=9.05$, $p<0.05$). CortG did not cause any changes in HEK-TLR4 SEAP expression compared to vehicle ($F=1.72$, $p>0.05$, B).

E_2 caused a significant decrease in SEAP expression in the HEK-TLR4 cells ($F=9.60$, $p<0.05$). Post-hoc tests showed that E_2 decreased SEAP expression relative to vehicle at the 0.01, 0.1, 1 and 10 μM concentrations, but caused no change at the 100 μM concentration (Figure 4.2A). E_2-3-G, on the other hand, caused an increase in SEAP production in the HEK-TLR4 cells ($F=19.2$, $p<0.05$). Post-hoc tests confirmed that all five concentrations of E_2-3-G tested (0.01. 0.1, 1, 10 and 100 μM) were significantly increased relative to vehicle (Figure 4.2B). Finally, E_2-17-G also caused a significant increase in HEK-TLR4 SEAP expression ($F=72.81$, $p<0.05$), but post-hoc tests showed this difference only at the 100 μM concentration (Figure 4.2C).

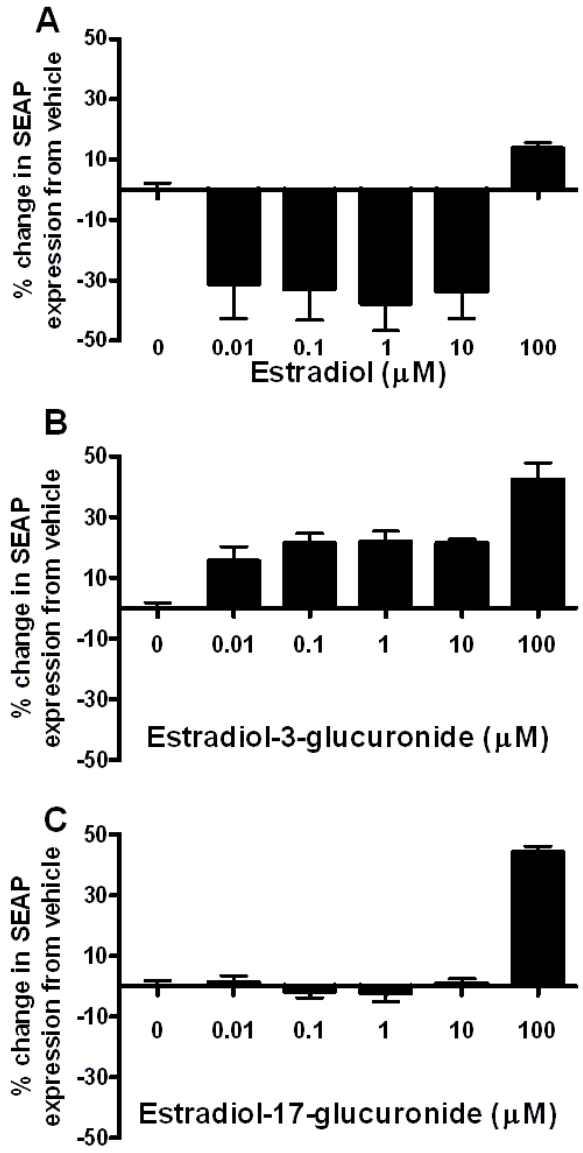

Figure 4.2. E_2 caused a significant decrease in HEK-TLR4 cell SEAP reporter gene expression relative to 1% DMSO vehicle at 0.01, 0.1, 1 and 10 μM concentrations ($F=9.60$, $p<0.05$, A). E_2-3-G caused a significant increase in HEK-TLR4 cell NFκB-dependent SEAP expression relative to water vehicle at 0.01, 0.1, 1, 10 and 100 μM concentrations ($F=19.2$, $p<0.05$, B). E_2-17-G also caused a significant increase in HEK-TLR4 cell SEAP expression relative to 0.1% DMSO vehicle at the 100 μM concentration ($F=72.81$, $p<0.05$).

<u>Experiment 2B:</u> ***In vitro*** **E_2-3-G and E_2-17-G induced increases in SEAP expression are blocked by the TLR4 inhibitors LPS-RS and (+)-naloxone.**

Both E_2-3-G and E_2-17-G caused a robust increase in SEAP expression in the HEK-TLR4 cells in Experiment 2A. To test whether this was a TLR4-specific effect, two TLR4 inhibitors, LPS-RS and (+)-naloxone, were coincubated with E_2-3-G and E_2-17-G to determine if the TLR4 inhibitors blocked the SEAP increases seen with the drugs alone. LPS-RS acts as a competitive antagonist of the LPS effects on TLR4 complex as is structurally similar to LPS but fails to produce a TLR4-dependent proinflammatory response. The second inhibitor, (+)-naloxone, has also been shown to antagonize the LPS response *in vitro* (Hutchinson et al., 2008b) and has been documented by a broad receptor, enzyme, and second messenger screen to have no identified off-target effects (Northcutt et al., 2010).

LPS-RS effectively blocked the increase in HEK-TLR4 cell SEAP expression caused by E_2-3-G ($F=5.22$, $p<0.05$, Figure 4.3A) and E_2-17-G ($F=7.81$, $p<0.05$, Figure 4.3C). Posthoc tests showed that when 100 μM E_2-3-G was incubated with 0.1, 1 or 10 ng/ml LPS-RS, SEAP expression was significantly decreased compared to 100 μM E_2-3-G alone. Similarly, when100 μM E_2-17-G was incubated with 0.1, 1 or 10 ng/ml LPS-RS, SEAP expression was also significantly decreased compared to 100 μM E_2-17-G alone.

(+)-Naloxone also blocked the increase in HEK-TLR4 cell SEAP expression caused by E_2-3-G ($F=6.89$, $p<0.05$, Figure 4.3B) and E_2-17-G ($F=9.17$, $p<0.05$, Figure 4.3D). Posthoc tests showed that when 100 μM E_2-3-G was incubated with 1 or 10 μM (+)-naloxone, SEAP expression was significantly decreased compared to 100 μM E_2-3-G alone. Similarly, when 100 μM E_2-17-G was incubated with 10 or 1 μM (+)-naloxone, SEAP expression was also significantly decreased compared to 100 μM E_2-17-G alone.

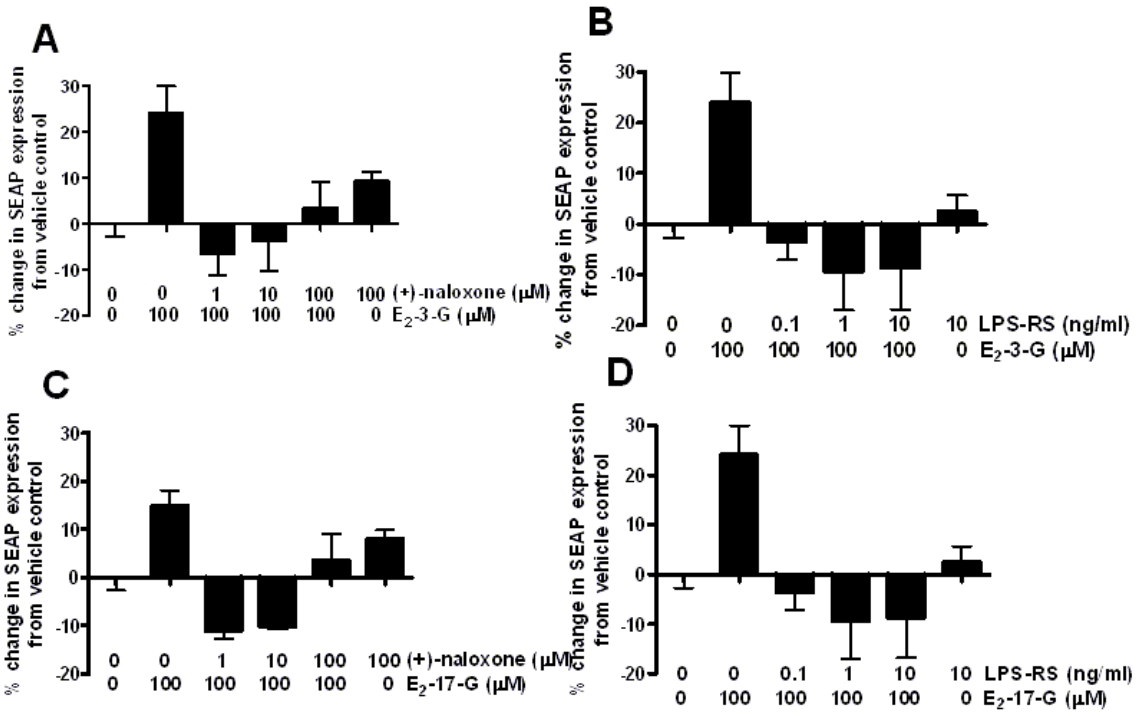

Figure 4.3. The TLR4 antagonist LPS-RS significantly attenuated the HEK-TLR4 cell SEAP increases caused by 100 μM E_2-3-G ($F=5.22$, $p<0.05$, A) or 100 μM E_2-17-G ($F=7.81$, $p<0.05$, C) at all three concentrations tested (0.1, 1 and 10 ng/ml). Similarly, the TLR4 inhibitor (+)-naloxone significantly attenuated the HEK-TLR4 cell SEAP increases caused by 100 μM E_2-3-G ($F=6.89$, $p<0.05$, B), or 100 μM E_2-17-G ($F= 9.17$, $p<0.05$, D) at the 1 and 10 μM concentrations.

<u>Experiment 3:</u> E_2-3-G and E_2-17-G cause tactile allodynia that is blocked by the TLR4 antagonist LPS-RS.

The *in silico* and *in vitro* studies above strongly suggest that E_2-3-G and E_2-17-G, but not Cort, CortG or E_2, are capable of activating TLR4. Previous studies have shown that other glucuronide metabolites that can act as TLR4 agonists are able to produce pain *in vivo* (Lewis et al., 2010a). To determine if the drugs investigated here caused increase tactile sensitivity, equimolar Cort, CortG, E_2, E_2-3-G and E_2-17-G were injected via an acute intrathecal injection over the lumbar enlargement and allodynia measured 1 and 3 hours following injection. Experiments 1 and 2 predict that E_2-3-G and E_2-17-G will

enhance TLR4 signaling. Previous studies suggest that TLR4 activity can cause enhanced pain in vivo (Hutchinson et al., 2008b; Lewis et al., 2010a). To determine if TLR4 activity was necessary for drugs which caused allodynia in this study, a TLR4 antagonist, LPS-RS was coadministered and allodynia again assessed 1 and 3 hours post-injection. LPS-RS successfully blocked E_2-3-G and E_2-17-G induced SEAP expression in HEK-TLR4 cell and has been used to block pain following glucuronic acid or ethyl glucuronide administration *in vivo* (Lewis et al., 2010a).

No differences between right and left hind paws were detected. Thus, results are reported as the average of the response thresholds recorded for both paws. There were also no baseline differences between any of the behavior groups ($F=1.86$, $p>0.05$).

Intrathecal Cort (0.56 μg) and its equivolume (1 μl) 1% DMSO vehicle each caused an increase in tactile sensitivity relative to baseline, and did not differ from each other ($F=0.33$, $p>0.05$, Figure 4.4A). Intrathecal CortG (0.85 μg), however, did not cause allodynia to develop relative to its saline vehicle ($F=0.17$, $p>0.05$, Figure 4.4B).

Similar to Cort, intrathecal E_2 (0.44 μg) and its equivolume (1 μl) 1% DMSO vehicle both caused a decrease in sensitivity relative to baseline but did not differ from each other ($F=2.17$, $p>0.05$, Figure 4.4C). Intrathecal E_2-3-G (0.76 μg), however, caused a robust tactile allodynia 1 and 3 hours post injection which was blocked by 40 μg LPS-RS ($F=42.8$, $p<0.05$, Figure 4.4D). E_2-17-G (0.76 μg) also caused significant tactile allodynia to develop 3 hours post injection that was blocked by 40 μg LPS-RS ($F=9.303$, $p<0.05$, Figure 4.4E).

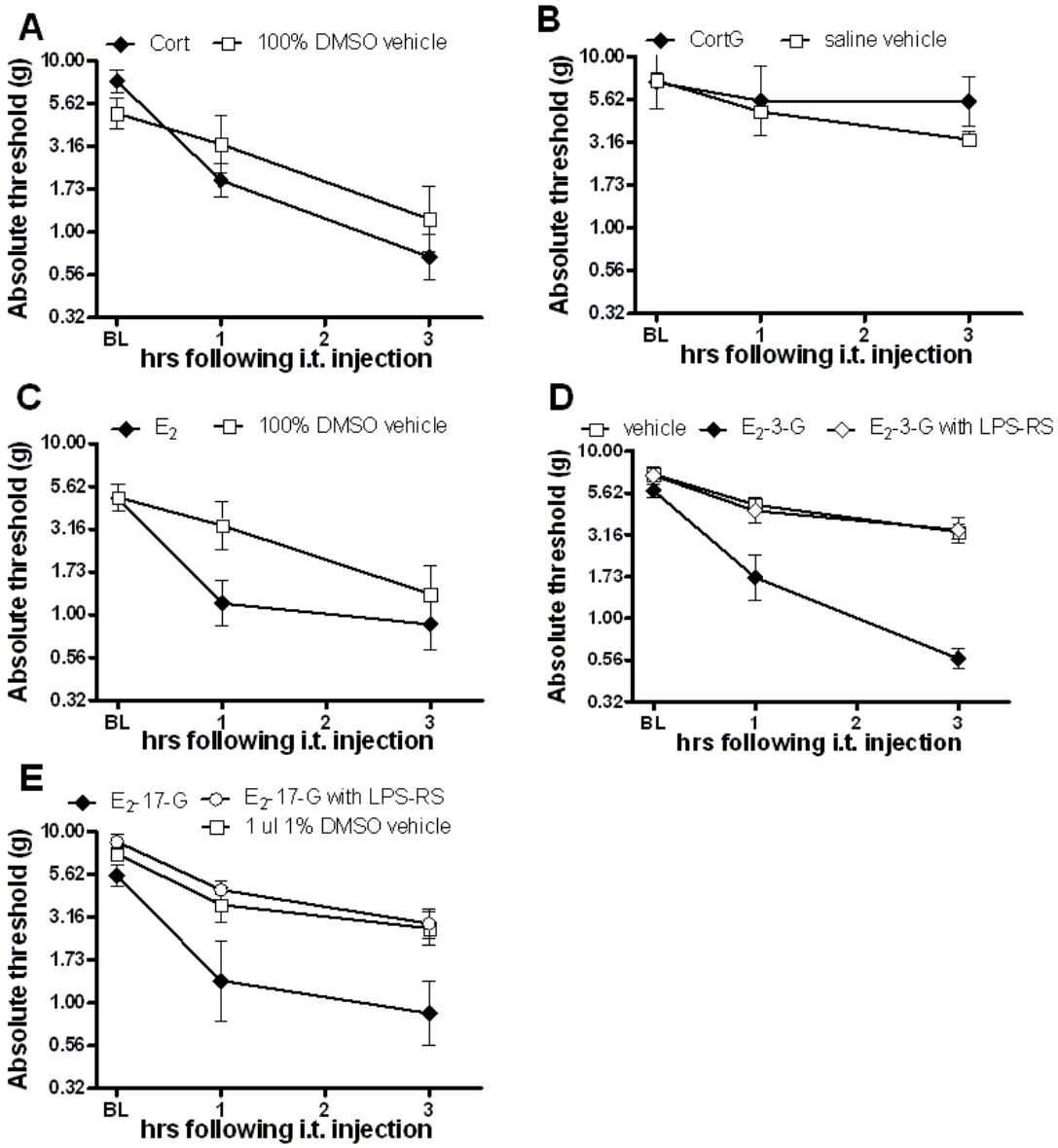

Figure 4.4. Intrathecal injection of Cort (0.56 μg, F=0.33, p>0.05, A), CortG (0.85 μg, F=0.17, p>0.05, B) or E_2 (0.44 μg, F=1.07, p>0.05, C) failed to cause significant allodynia relative to vehicle controls. Intrathecal injection of E_2-3-G (0.76 μg, F=42.8, p<0.05, D) and E_2-17-G (0.76 μg, F=4.63, p<0.05, E) caused significant allodynia to develop. Coadministration of LPS-RS (40 μg, intrathecal) blocked the development of the allodynia caused by E_2-3-G (F=24.36, p<0.05, D) and E_2-17-G (F=6.02, p<0.05, E).

DISCUSSION

These studies sought to determine if the steroid hormone glucuronide metabolites corticosterone-21-glucuronide (CortG), estradiol-3-glucuronide (E_2-3-G) and estradiol-17-glucuronide (E_2-3-G), along with the parent molecules corticosterone (Cort) and estradiol (E_2), caused TLR4 activation and/or potentiated pain responses when administered intrathecally. We present the first evidence that the naturally occurring glucuronide metabolites E_2-3-G and E_2-17-G cause TLR4 activation *in vitro* and a potent TLR4-dependent pain response *in vivo.* Additionally, each molecule tested, including Cort, CortG, and E_2 as well as E_2-3-G and E_2-17-G, was predicted to dock to MD-2 in an *in silico* model utilizing a recently published TLR4-MD2 crystal structure (Park et al., 2009). Further, the docking of each was altered when i*n silico* modeling was repeated using an MD-2 structure with the TLR4 inhibitor (+)-naloxone already docked, indicating a (+)-naloxone sensitive docking. However, whether that interaction is as an antagonist or agonist to TLR4 signaling is unknown from the *in silico* docking alone. An *in vitro* study using HEK cells transfected with TLR4 and necessary co-signaling molecules showed that E_2-3-G and E_2-17-G caused a dose-dependent increase in NFκB-dependent SEAP reporter gene protein expression. Cort and E_2, both in 1% DMSO vehicles, significantly reduced reporter gene protein expression relative to vehicle. CortG produced no significant changes on TLR4 reporter protein. When administered intrathecally, E_2-3-G and E_2-17-G caused a pain response that was blocked by the TLR4 antagonist LPS-RS. Cort, CortG and E_2 did not cause a difference in pain sensation relative to their vehicles.

One inherent issue with Cort and E_2 results is that, in addition to the potential for TLR4 activity, they also have potent effects on the glucocorticoid and estrogen receptors, respectively. It is unknown whether CortG also acts on glucocorticoid receptors, while

E_2-3-G and E_2-17-G are not believed to have effects on estrogen receptors (Guillemette et al., 2004). The purpose of the present studies was to determine the potential of these drugs to cause TLR4 agonism, resulting in increased pain in a healthy *in vivo* model. Future studies might more directly assess the potential for TLR4 agonism independent of glucocorticoid or estrogen receptor function by coadministering glucocorticoid or estrogen receptor antagonists, or to assess the potential of these drugs as TLR4 antagonists. The present studies have not investigated potential dual roles for these compounds, in which the hormone receptor and TLR4 might cause opposing effects.

CortG is the first of six glucuronide metabolites tested in this methodology (Lewis et al., 2010a; Lewis et al., 2011a) that failed to either increase HEK-TLR4 cell reporter protein *in vitro* or cause potentiated pain responses *in vivo*. One potential explanation is that, as discussed above, CortG may bind to glucocorticoid receptors. Glucocorticoids are known to inhibit many inflammatory functions (Barnes, 2010), although in certain circumstances may also enhance LPS-induced inflammation (Frank et al., 2010). As with Cort, this anti-inflammatory action may mask a potential TLR4 agonist effect. Another possible explanation is that the structure of CortG is such that it lacks TLR4 agonism. Elucidating which explanation is correct will aid in the understanding of the nature of the TLR4 agonism in glucuronidated molecules.

The finding that estrogen metabolites can, by themselves, have TLR4-dependent, pain-producing effects has implications for several chronic pain disorders. For instance, among women who suffer from migraines, about 60% are more likely to have a migraine during perimenstrual stage of their menstrual cycle, between ovulation and menses (Kornstein and Parker, 1997). During this stage, there is a precipitous drop in circulating

estrogen levels. Notably, very high levels of estrogens, as in the third trimester of pregnancy, as well as very low levels of estrogens, as in post-menopause, both sharply reduce the risk of migraines (Craft, 2007). The risk conferred during the perimenstrual stage, then, is not due exclusively to the level of estrogens, but rather due to the abrupt decline in circulating estrogens during this stage. A similar link between the perimenstrual stage and pain episodes was found in women suffering from tempromandibular joint disorder (LeResche et al., 2003). The perimenstrual stage of estrogen withdrawal is also the period during which estradiol-glucuoronide concentrations will be maximal (Stanczyk et al., 1980; LeResche et al., 2003). The studies here suggest that the TLR4 activation and enhanced pain caused by E_2-3-G and E_2-17-G could contribute to the increase risk, in women affected by the disorder, of menstrual migraine or tempromandibular joint pain in the perimenstrual period. Were this link substantiated in future studies, it would create a novel treatment target in these challenging disorders.

These studies have shown that naturally produced steroid hormone metabolites, E_2-3-G and E_2-17-G are predicted to bind to MD-2 *in silico,* increase HEK-TLR4 cell reporter gene protein levels and cause TLR4-dependent pain. The finding that endogenously produced, naturally circulating molecules can have TLR4 effects is somewhat counterintuitive. TLR4s are generally thought to detect danger signals, such as lipopolysaccharide from gram negative bacterial cell walls, indicative of bacterial invasion, and degraded membrane components that are not typically found extracellularly, indicative of tissue damage (Osterloh and Breloer, 2008). While glucuronic acid, another endogenous molecule, has also been shown to activate TLR4

(Lewis et al., 2011a), it is typically found conjugated to form uridie diphosphoglucuronic acid or polymerized to form hyaluronan, rather than freely available. We present the first evidence that an endogenously produced molecule can activate TLR4 and produce increased pain sensation.

There are several possible explanations for why TLR4 detects numerous compounds with a glucuronide component. One, several classes of bacteria, such as streptococci, have a hyaluronan capsule surrounding their cell walls (Maclennan, 1956). Several studies have shown that TLR4 detects low molecular weight hyaluronan as the polymers degrade (Li et al., 2010; Leu et al., 2011). It is possible that the destruction of bacterial capsules and presence of low molecular weight hyaluronan became a danger signal of bacterial invasion, and one which was detected by TLR4. Another potential explanation is that, while the chemical structure of the exogenously made estradiol glucuronide metabolites is identical to that produced endogenously, that other differences between endogenously and exogenously produced metabolites exist that result in the effects observed. All drugs tested free of endotoxin contamination on the LAL assay, one obvious concern, but there could be more subtle differences in the metabolism of these steroid hormones. Finally, TLR4 is a pattern recognition receptor that responds to a range of molecular structures. While it evolved to detect danger signals, it is possible that it happens to detect a small number of molecules just by random chance. That is, molecules that are structurally similar to the danger signals detected by TLR4 also act as agonists without being danger signals themselves.

The studies here present evidence that the estradiol metabolites, E_2-3-G and E_2-17-G, are predicted to bind to MD-2 *in silico,* increase the production of a HEK-TLR4

reporter protein *in vitro* and cause potent allodynia when administered intrathecally. Additionally, we determined that the parent molecules, Cort and E_2 as well as the glucuronide metabolite CortG, are not TLR4 agonists in sum with other potential physiological effects. These findings could have broad implications for migraine and other disorders, such as fibromyalgia and tempromandibular join disorder (Craft, 2007), in which female hormone levels have been linked to painful episodes.

CHAPTER 5

THE OPIOID-INACTIVE TOLL-LIKE RECEPTOR 4 INHIBITOR (+)-NALOXONE REVERSES MULTIPLE MODEL OF CHRONIC NEUROPATHIC PAIN IN RATS.

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ABSTRACT

We have previously demonstrated that both the opioid receptor antagonist (-)-naloxone and the non-opioid active (+)-naloxone isomer antagonize toll-like receptor 4 (TLR4) and can reverse neuropathic pain following chronic constriction injury in rats. The present studies expand on this finding to determine that (+)-naloxone was able to reverse a second model of neuropathic pain, spinal nerve ligation. Further, (+)-naloxone was similarly effective 2 weeks after nerve injury and up to 4 months after nerve injury. Utilizing cell lines transfected with either TLR4 or TLR2, necessary co-signaling molecules and a reporter gene, the specificity of (+)-naloxone antagonism was tested. (+)-Naloxone successfully antagonized the increase in TLR4 reporter gene product caused by the classic ligand, lipopolysaccharide, but failed to antagonize the increase in TLR2 reporter gene product caused by the TLR2 agonist Pam3CSK4. These studies provide additional evidence that supports the TLR4 antagonist (+)-naloxone as a novel candidate for the treatment of neuropathic pain.

Chronic neuropathic pain is extremely difficult to treat, with many patients gaining insufficient relief from existing treatments. It is increasingly recognized that the proinflammatory molecules secreted by glial cells following nerve damage contribute to the establishment and maintenance of chronic neuropathic pain (Milligan and Watkins, 2009). However, there are currently no approved treatments that target this cellular response. A potential target for developing therapeutics would be to reverse the ongoing activation of cells upstream of the production of proinflammatory cytokines, rather than the more common approach of blocking the effects of the proinflammaory cytokines.

One mechanism proposed for the proinflammatory activation of glial cells in response to nerve injury is activation through toll-like receptor 4 (TLR4). TLR4 is a pattern recognition receptor that produces robust proinflammatory responses in cells when stimulated by its classic ligand, lipopolysaccharide (Lehnardt et al., 2003). TLR4 is found on, at least, microglia in the brain (Bsibsi et al., 2002) and has a complex signaling process involving a receptor complex, including CD14 and MD-2, and the activation of multiple downstream transcription factors (Dauphinee and Karsan, 2006).

TLR4 signaling can be temporarily inhibited in a number of ways. Two forms of inactive LPS, mutant LPS and LPS from *Rhodobacter sphaeroides* (LPS-RS), can competitively antagonize the LPS response. siRNA against TLR4 has also been show block the LPS response (Wu et al., 2010). Finally, the small molecule antagonist naloxone can block TLR4 signaling (Hutchinson et al., 2008b; Hutchinson et al., 2010b). Notably, inhibition of TLR4 by all four methods has been shown to reduce the pain following sciatic nerve injury (Hutchinson et al., 2008b; Wu et al., 2010). However, the

ability of TLR4 inhibition to reverse the pain caused by other models of neuropathic pain has not been tested.

The antagonism of TLR4 signaling by naloxone is especially intriguing from a clinical perspective. While siRNA and the large lipopolysaccharide molecules are unable to cross the blood-brain barrier, naloxone has well known CNS effects following systemic administration, making it a better candidate for pharmaceutical treatment of pain. The best studied effects of naloxone are its ability to block opioid receptors. However, only the (-)-naloxone isomer antagonizes opioid receptors, while both the (-) and (+)-naloxone isomers have been shown to antagonize TLR4 and reverse neuropathic pain (Hutchinson et al., 2008b). (+)-Naloxone, then, is a blood-brain barrier permeable small molecule that has the ability to antagonize the TLR4 receptor without antagonizing the endogenous opioid system. (+)-Naloxone has also been screened on a wide range of CNS targets with no known off-target effects (Northcutt et al., 2010).

This series of studies seeks to further our understanding of the reversal of neuropathic pain by naloxone. Specifically, we hypothesize that the ability of (+)-naloxone to relieve neuropathic pain will generalize from the chronic constriction injury sciatic model (Hutchinson et al., 2008b) to a spinal nerve ligation model. Additionally, we hypothesize that (+)-naloxone will be similarly effective at relieving neuropathic pain of long duration in both the spinal nerve ligation and sciatic nerve injury models. Finally, we predict that (+)-naloxone will antagonize the LPS response on TLR4 signaling, but not the effect of a TLR2 agonist on TLR2 signaling, which would further indicate its specificity.

MATERIALS AND METHODS

Subjects

Adult, male, pathogen-free Sprague-Dawley rats (Harlan Labs, Madison, WI) were used Experiments 1, 2 and 5. Adult, male pathogen-free Sprague-Dawley rats (supplied by Laboratory & Animal Services, Waite Campus, University of Adelaide) were used in Experiment 3. Rats were 130-170 g at time of surgery in Experiments 1, 2, and 5; and 325-375 g at time of surgery in Experiment 3. Rats in both locations were housed in temperature (23±3 ^{o}C) and light (12 h:12 h light:dark) controlled rooms with water and food available *ad libitum*. All habituation and behavioral testing procedures were performed during the light phase of the daily cycle. Experimental procedures in Experiments 1, 2 and 5 were approved by the University of Colorado-Boulder Institutional Animal Care and Use Committee. The experimental procedure in Experiment 3 was approved by the University of Adelaide Animal Ethics Committee. Each experimental group contains 6-9 rats.

Drugs

(+)-Naloxone was obtained from the National Institute on Drug Abuse. For *in vivo* studies, the toll like receptor 4 antagonist lipopolysaccharide (LPS) and the toll like receptor 2 antagonist Pam3CSK4 (Pam; Invivogen, San Diego, CA) were used. The vehicle for all drugs was sterile endotoxin free saline (Hospira, Lake Forest, IL). (+)-Naloxone, Pam and saline were confirmed to be endotoxin-free by the limulus amebocyte lysate assay (Lonza, Walkersville, MD), performed according to manufacturers instructions.

Spinal Nerve Ligation (SNL)

In Experiments 1, 2 and 5, neuropathic pain was induced through ligation of the 5th and 6th lumbar spinal nerves, as described previously (Kim and Chung, 1992; Kim et al., 1997; Hutchinson et al., 2008b). Briefly, under isoflurane anesthesia, the left L5 and L6 spinal nerves were isolated and each nerve root tightly ligated with 6-0 silk suture. Sham controls had an identical surgery procedure except without the ligation of the spinal nerves.

Chronic Constriction Injury (CCI) of the Sciatic Nerve

A second model of neuropathic pain used in Experiment 3 was the CCI model of partial sciatic nerve injury (Bennett and Xie, 1988). CCI was performed at the mid-thigh level of the left hindleg as previously described (Milligan et al., 2004a). In brief, under isoflurane anesthesia, the sciatic nerve was isolated and loosely tied with four sterile chromic gut sutures (4-0 chromic gut, Ethicon, Somerville, NJ, USA).

Von Frey test for tactile allodynia

All behavioral testing was conducted by an experimenter blind to group assignment. Rats received at least four 1 h habituations to the testing apparatus and environment prior to baseline testing, as in our previous studies (Lewis et al., 2010b). Baseline response thresholds were determined prior to nerve injury and rats tested periodically from 3 days post-injury until time of experiment.

The response threshold to light touch on both plantar hind paws was measured using calibrated microfilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA), as

described previously (Milligan et al., 2001). Briefly, a logarithmic range of hairs from 0.406-15.136 g force were used, allowing both analgesia and allodynia to be measured, following the standard up-down procedures previously described (Chaplan et al., 1994). Responses were fitted to a Gaussian integral psychometric function using a maximum-likelihood fitting method as described previously (Milligan et al., 2001).

***In vitro* assay for TLR4 signaling**

Two human embryonic kidney-293 (HEK 293) cell lines were used in Experiment 5. One cell line was stably transfected by Invivogen (San Deigo, CA) to over-express human TLR4 and co-receptor molecules (MD-2, CD14) (referred to here as HEK-TLR4). The second cell line was stably transfected to overexpress human TLR2 (referred to here as HEK-TLR2; Invivogen, San Diego, CA). In addition, both cell lines stably express an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by transcription factors activated as part of the TLR2 and 4 signaling cascades. Secreted alkaline phosphatase (SEAP) protein is produced as a consequence of TLR4 activation.

Both HEK-TLR4 and HEK-TLR2 cell lines were grown at 37^{o}C (5% CO_2; VWR incubator model 2300) in 10 cm dishes (Greiner Bio-One, CellStar 632171; Monroe, NC, USA) in normal supplement selection media (DMEM media [Invitrogen, Carlsbad, CA, USA] supplemented with 10% fetal bovine serum [Hyclone; Logan, UT, USA], HEK-TLR4 selecton [Invivogen]; Penicillin 10,000 U/ml [Invitrogen]; streptomycin 10 mg/ml [Invitrogen], Normocine [Invivogen], and 200 nM L-Glutamine [Invitrogen]). The cells were then plated for 48 h in 96 well plates (Microtest 96 well plate, flat bottom, Becton Dickinson; 5 x 10^3 cells/well) with the same media. After 48 h, supernatants were

removed and replaced with fresh media. Drugs tested were added in concentrations indicated and incubated for 24 h. Supernatants (15 μl) were then collected from each well for immediate assay.

SEAP levels in the supernatants were assayed using the Phospha-Light System (Applied Biosystems) according to the manufacturer's instructions. This chemiluminescent assay incorporates Tropix CSPD chemiluminescent substrate. The 15 μl test samples were diluted in 45 μl of 1x dilution buffer, transferred to 96-well plates (Thermo, Walthma, MA, USA), and heated at 65^{o}C in a water bath (Model 210, Fisher Scientific, Pittsburgh, PA, USA) for 30 min, then cooled on ice to room temperature. Assay buffer (50 μl/well) was added and, 5 min later, reaction buffer (50 μl/well) is added and allowed to incubate for 20 min at room temperature. The light output is then measured in a microplate luminometer (Dynex Technologies, #IL213.1191, Chantilly, VA, USA).

Statistics

GraphPad Prism (version 5 for Windows, San Diego, CA) software was used for all statistical analyses. Two-way repeated-measures ANOVAs with Bonferroni post-hoc tests when appropriate were used to determine statistical significance between behavioral measures. One-way ANOVAs with appropriate Bonferroni post hocs were used to compare experimental groups on *in vitro* experiments and to confirm that there were no baseline differences on behavioral measures. For all analyses, $p<0.05$ was considered significant.

Experimental Procedures

Experiment 1: Does (+)-naloxone reverse neuropathic pain 2 weeks following SNL?

All rats underwent baseline (BL) von Frey testing followed by SNL surgery. Two weeks following surgery, after robust and stable tactile allodynia had developed, rats were given 100 mg/kg (+)-naloxone subcutaneously (s.c.) or equivolume saline vehicle. Tactile thresholds were tested prior to and 1 and 3 hours following s.c. injection.

Experiment 2: Does (+)-naloxone reverse neuropathic pain at 8 wk after SNL?

Two months post-SNL surgery, the efficacy of s.c. (+)-naloxone in increasing tactile thresholds was again tested. Six rats used in Experiment 1 were used in this experiment, along with 6 sham rats from another, similar experiment. All rats underwent BL von Frey testing and then SNL or sham surgery. Beginning 8 weeks following surgery, rats were again assessed for their von Frey response and were then given (+)-naloxone (100 mg/kg, s.c.) or equivolume saline vehicle in a counterbalanced within-subjects design with a one-week washout period between injections. Tactile thresholds were assessed prior to and 1 and 3 hours following each s.c. injection.

Experiment 3: Does (+)-naloxone reverse neuropathic pain 16 wk after CCI?

All rats underwent BL von Frey testing and then CCI surgery. Four months later, (+)-naloxone (60 mg/kg, s.c.) or equivolume saline vehicle was given. Tactile thresholds were assessed prior to and 30, 60, 90, 120 and 180 minutes following s.c. injection.

Experiment 4: What is the dose-response function of (-)-naloxone on neuropathic SNL pain?

Rats underwent SNL surgery following BL von Frey testing. Two weeks following surgery, as in Experiment 1, rats were given 1, 10 and 100 mg/kg doses of s.c. (-)-naloxone or equivolume saline vehicle in a within subjects design, starting with the lowest dose and with a one-week washout between doses. Tactile sensitivity was assessed prior to and 1 and 3 h following each injection.

Experiment 5: Does (+)-naloxone antagonize HEK-TLR4 or HEK-TLR2 cell SEAP expression?

HEK-TLR4 cells were stimulated with 0.1 ng/ml LPS to produce a robust increase in SEAP signal. The efficacy of 1, 10 and 100 μM (+)-naloxone to inhibit the LPS signal was tested by coincubating each (+)-naloxone dose with 0.1 ng/ml LPS. Following a 24 h coincubation, supernatants were then assayed for SEAP expression.

Similarly, HEK-TLR2 cells were stimulated with 1 ng/ml Pam to produce a robust increase in SEAP signal of a similar magnitude to the LPS signal investigated in the HEK-TLR4 cells. The efficacy of 1, 10 and 100 μM (+)-naloxone to inhibit the Pam signal was tested by coincubating each (+)-naloxone dose with 1 ng/ml Pam. Following a 24 h coincubation, supernatants were then assayed for SEAP expression.

RESULTS

For all *in vivo* behavioral studies, no differences in tactile thresholds were detected between the ipsilateral and contralateral paws prior to or following drug administration; thus, results are reported as the average of both paws.

Experiment 1: (+)-Naloxone reverses SNL-induced neuropathic pain.

Prior research has shown that (+)-naloxone (100 mg/kg) was sufficient to reverse allodynia induced by chronic constriction injury (CCI) (Hutchinson et al., 2008b). In order to test whether the reversal of neuropathic pain generalized to another neuropathic pain condition, we utilized a spinal nerve ligation (SNL) model. All rats developed significant allodynia following SNL. Fourteen days post SNL surgery, rats were given (+)-naloxone (100 mg/kg, s.c.) or equivolume saline. There were no significant differences between groups in baseline tactile sensitivity ($t=0.878$, $p>0.05$) or the established allodynia prior to injection ($t=0.774$, $p>0.05$). Baselines are lower than typically reported due to the particularly young and small rats used (~150 g at time of surgery). The (+)-naloxone dose significantly reversed the allodynia compared to saline controls ($F=7.27$, $p<0.05$, Figure 5.1), with post-hocs showing significant reversal at 1 hour after injection.

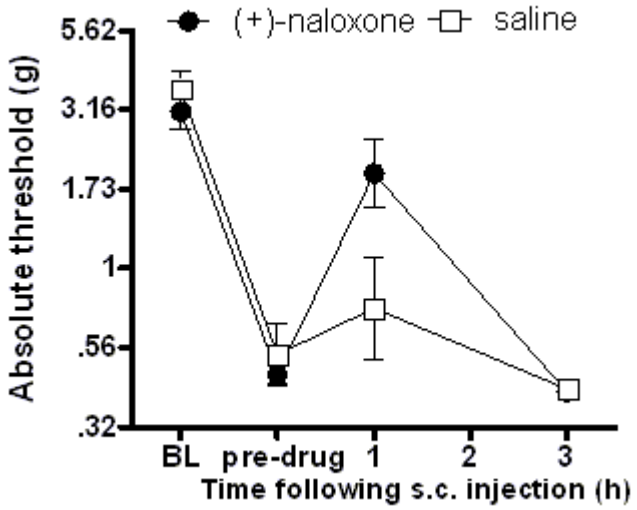

Figure 5.1. (+)-Naloxone reverses SNL pain 2 weeks following injury. There were no baseline differences between groups. All rats developed significant allodynia following SNL surgery which was significantly reversed 1 h following 100 mg/kg s.c. dose of (+)-naloxone.

<u>Experiment 2:</u> (+)-Naloxone reverses long established SNL-induced neuropathic pain.

Long-established neuropathic pain can be more difficult to reverse than recently established neuropathic pain. To test whether (+)-naloxone would be effective against long-established neuropathic pain, rats were given (+)-naloxone (100 mg/kg, s.c.) and equivolume saline vehicle 2 months following SNL or sham surgery in a within-subjects design. There were no significant differences in baseline thresholds ($t=1.80$, $p>0.05$). (+)-Naloxone significantly reversed established allodynia in rats with SNL, but did not alter the tactile sensitivity of rats that underwent the sham surgery ($F=19.2$ $p>0.05$, Figure 5.2).

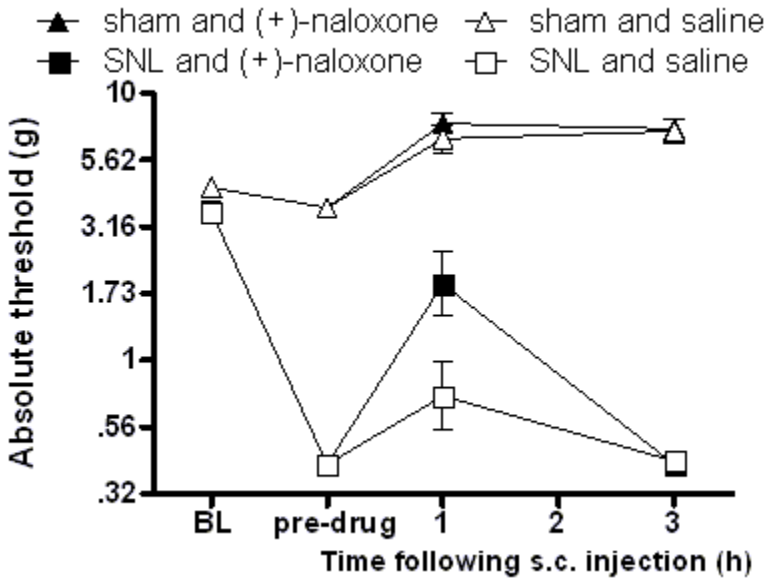

Figure 5.2. (+)-Naloxone reverses SNL pain 8 weeks following injury. Rats with SNL surgery developed significant allodynia(Kim and Chung, 1992; Kim et al., 1997; Hutchinson et al., 2008b) while rats who underwent a sham surgery did not. SNL-induced allodynia was significantly reversed 1 h following 100 mg/kg s.c. dose of (+)-naloxone, but saline vehicle did not significantly change SNL allodynia in this within-subjects design. Neither (+)-naloxone nor saline altered the response thresholds of the rats who received sham surgery.

<u>Experiment 3:</u> (+)-Naloxone reverses long established CCI-induced neuropathic pain.

Experiment 2 showed that (+)-naloxone was capable of reversing long-established SNL pain. To determine if this ability generalized to other neuropathic pain models, and in neuropathic pain of an even longer duration, rats 4 months post-CCI surgery were given (+)-naloxone (60 mg/kg) or equivolume saline vehicle. In addition to determine if (+)-naloxone reverses long-established neuropathic pain in multiple models, the CCI model is better characterized long after initial injury (Milligan et al., 2006a; Milligan et al., 2006b). A dose of 60 mg/kg was utilized to determine if a lower dose would also be effective. There were no baseline difference between groups ($t=0.05$ $p>0.05$) and no

difference prior to injection. (+)-Naloxone caused a significant reversal of CCI-induced allodynia ($F=75.3$, $p<0.05$. Figure 5.3). Post-hoc tests showed a significant reversal at 60, 90 and 120 minutes following injection.

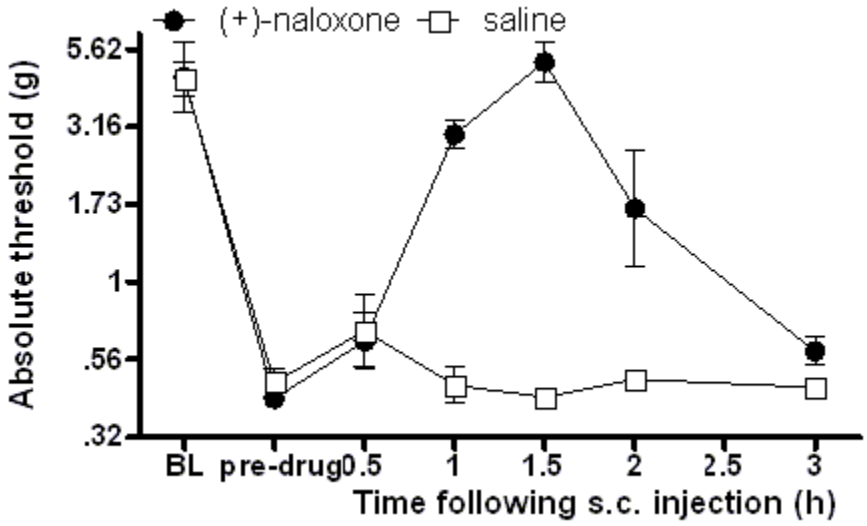

Figure 5.3. (+)-Naloxone reversed CCI pain of 4 months duration. There were no significant differences at baseline testing between groups. All rats developed chronic allodynia following CCI surgery. (+)-Naloxone (60 mg/kg, s.c.) significantly reversed allodynia 60, 90 and 120 minutes following injection.

<u>Experiment 4:</u> (-)-Naloxone reverses neuropathic pain at 100 mg/kg but not at 10 mg/kg.

Hutchinson et al. (2008b) demonstrated that a single 100 mg/kg (s.c.) dose of either (+)- or (-)-naloxone reversed neuropathic pain in a CCI model. Experiment 1 showed that the (+)-naloxone effect generalized to a second neuropathic pain model, SNL while Experiment 3 suggested that a lower dose of (+)-naloxone (60 mg/kg s.c.) was also effective. In Experiment 4, the dose-response function for the (-)-naloxone reversal of SNL pain was determined using a within-subjects design. (-)-Naloxone, rather than (+)-naloxone was chosen for study here for simplicity of being commercially available. This

choice was based on the fact that *in vitro*, *in vivo* (Hutchinson et al., 2008b), and *in silico* (Hutchinson et al., 2010b) tests indicate comparable effects of (+)- and (-)-naloxone on blockade of TLR4.

All rats developed allodynia following SNL. There was a significant effect of drug ($F=9.373$, $p<0.05$), with post hoc tests showed (-)-naloxone significantly reversed allodynia at the 100 mg/kg dose, but not at the 10 or 1 mg/kg doses. The magnitude of (+)- and (-)-naloxone reversal of SNL-induced allodynia was comparable, further evidence for the non-stereoselective nature of naloxone reversal of neuropathic pain.

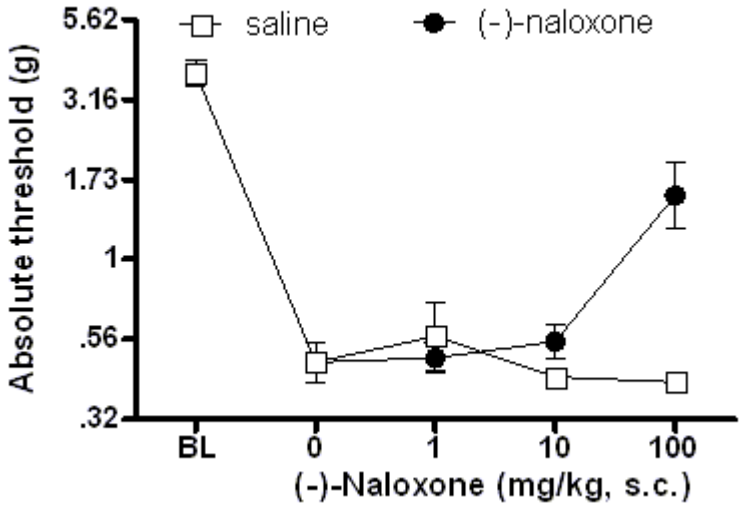

Figure 5.4. (-)-Naloxone significantly reversed SNL pain at a dose of 100 mg/kg, but not at lower doses. All rats developed significant allodynia in this within-subjects design.

<u>Experiment 5:</u> (+)-Naloxone antagonizes stimulated HEK-TLR4 cell SEAP expression, but not stimulated HEK-TLR2 cell SEAP expression.

Previous evidence suggests that (+)-naloxone can inhibit LPS-induced TLR4 signaling (Hutchinson et al., 2008b). Another TLR, TLR2, has been linked to similar proinflammatory responses to exogenous drugs.(Hutchinson et al., 2010a) TLR2, in fact,

shares cosignaling molecules with TLR4, such as MD2 (Dziarski and Gupta, 2000) and TIRAP (Yamamoto et al., 2002) as well as a similar downstream intracellular cascade (O'Neill, 2008). Because the exact site of (+)-naloxone action is not yet known, it is conceivable that (+)-naloxone could inhibit TLR2 function given shared cell signaling steps. To investigate whether (+)-naloxone is also a TLR2 inhibitor as well as a TLR4 inhibitor, HEK-TLR4 and HEK-TLR2 cells were stimulated with a LPS or Pam, respectively, and the ability of (+)-naloxone to inhibit the agonist response measured.

In HEK-TLR4 cells (Fig. 5A), (+)-naloxone significantly inhibited the stimulation of LPS ($F=112.3$, $p<0.05$). Post-hoc tests showed that when 1, 10 and 100 μM (+)-naloxone was coincubated with LPS, the SEAP expression was significantly reduced compared to LPS alone (Figure 5.5A). In HEK-TLR2 cells (Fig. 5B), there was also a significant difference between groups ($F=24.08$, $p<0.05$). However, post hoc tests determined that neither 1, 10 nor 100 μM (+)-naloxone coincubated with Pam was significantly different from Pam alone (Figure 5.5B). The statistical significant differences were between Pam stimulated SEAP expression and vehicle or (+)-naloxone alone. In summary, (+)-naloxone was able to block the HEK-TLR4 effects of LPS, but not the HEK-TLR2 effects of Pam.

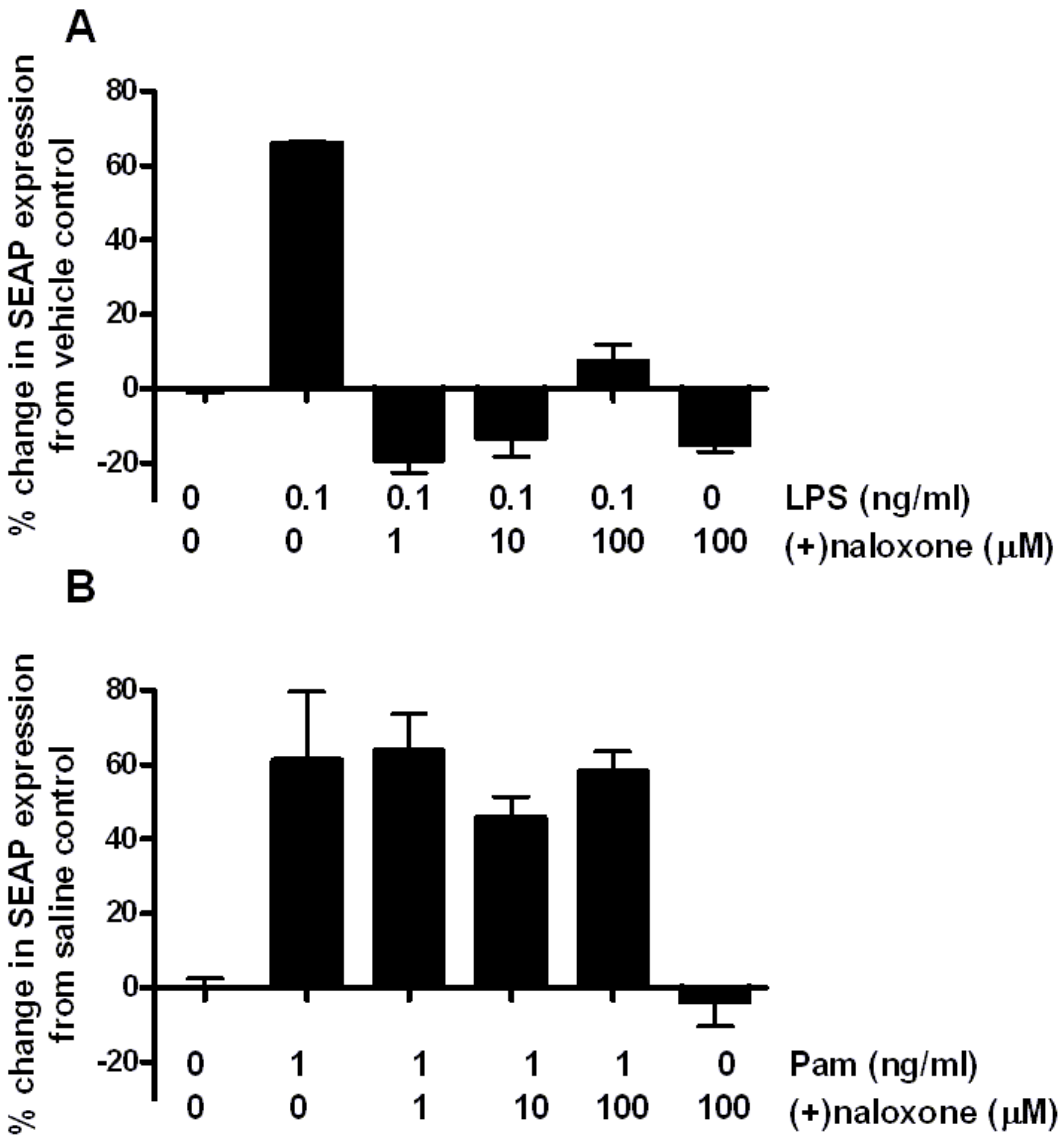

Figure 5.5. (+)-Naloxone antagonizes stimulated HEK-TLR4 cell SEAP expression, but not stimulated HEK-TLR2 cell SEAP expression. The increase in SEAP expression seen in LPS stimulated HEK-TLR4 cells was significantly blocked by coincubation with 1, 10 or 100 μM (+)-naloxone. The increase in SEAP expression caued by Pam in HEK-TLR2 cells was unaffected by coincubation with 1, 10 or 100 μM (+)-naloxone.

DISCUSSION

The studies presented here expand our knowledge of the TLR4 antagonist (+)-naloxone and its ability to reverse neuropathic pain behaviors. The ability of (+)-

naloxone to reverse neuropathic pain in models beyond chronic constriction injury (CCI) was tested using a spinal nerve ligation (SNL) model of neuropathic pain. (+)-Naloxone successfully reduced SNL-induced allodynia 1 hour following subcutaneous injection, indicating that (+)-naloxone was effective on multiple models of neuropathic pain. We also present the first evidence that (+)-naloxone can reverse longstanding neuropathic pain. (+)-Naloxone reversed the allodynia expressed either 2 months following SNL or 4 months following CCI. Given the success of (+)-naloxone in reversing neuropathic pain, and prior observation that (+)-naloxone and (-)- naloxone produce comparable reversal of CCI-induced neuropathic pain (Hutchinson et al., 2008b), we conducted a dose-response study on the commercially available (-)-isomer of naloxone for its ability to reverse SNL-induced allodynia. The effective dose of (-)-naloxone was 100 mg/kg. Hence, as with CCI-induced pain (Hutchinson et al., 2008b), (-)-naloxone was similarly effective to (+)-naloxone in reversing SNL pain at the 100 mg/kg dose confirming the nonstereoselective nature of naloxone's effect. Finally, utilizing HEK cells transfected with either TLR4 or TLR2 and necessary co-signaling molecules, the ability of (+)-naloxone to inhibit TLR4 and TLR2 signaling was tested. (+)-Naloxone inhibits LPS-induced increases in TLR4 reporter protein, but did not affect Pam-induced increase in TLR2 reporter protein, further evidence of its specificity as a TLR4 inhibitor.

The effectiveness of (+)-naloxone in reversing neuropathic pain up to 4 months after nerve injury provides additional support for a role of TLR4 in perpetuating neuropathic pain. Previous work has shown that both TLR4 knockout and co-receptor CD14 knockout mice developed reduced analgesia and hyperalgesia following L5 spinal nerve transaction (Tanga et al., 2005; Cao et al., 2009). Additionally, siRNA blockade of

TLR4 given at time of surgery was also able to reduce the development of CCI neuropathic pain (Wu et al., 2010). However, the ability of (+)-naloxone to reverse neuropathic pain, as shown here and in Hutchinson et al.(2008b), as well as the ability of mutant LPS and LPS-RS to reverse neuropathic pain (Hutchinson et al., 2008b) suggest that TLR4 is being continually stimulated during these neuropathic pain conditions.

Given the role of TLR4 in ongoing neuropathic pain, it is a potential target for novel pharmaceutical treatment. TLR4 is principally found on microglia in the central nervous system (Bsibsi et al., 2002), but has been shown to be upregulated on neurons following ischemic injury (Tang et al., 2007). When activated, it leads to the upregulation of several MAPKs and activation of the transcription factor NFκB (Dauphinee and Karsan, 2006). Ultimately, TLR4 activation can lead to the production and release of proinflammatory cytokines, including interleukin-1β and tumor necrosis factor-α (Dauphinee and Karsan, 2006). (+)-Naloxone has previously been shown to reduce the production of proinflammatory cytokines *in vitro* and reduce the microglial activation marker CD11b *in vivo* (Hutchinson et al., 2008b). This finding suggests that glial activation inhibition and reduced proinflammatory cytokine production are potential mechanisms for (+)-naloxone reversal of neuropathic pain. Notably, other compounds that reduce proinflammatory cytokine production and reduce microglial cell activation have also been shown to be effective against neuropathic pain in rodent models (Ledeboer et al., 2005; Ledeboer et al., 2006).

(+)-Naloxone provides a blood-brain barrier permeable, small molecule, TLR4 antagonist that reverses neuropathic pain in multiple models, months after onset, making it an intriguing potential treatment. While (-)-naloxone is similarly effective on

neuropathic pain, it has the disadvantage of also inhibiting the endogenous opioid system, which reduces its applicability. One limitation of (+)-naloxone is its short time of effect. The effect of s.c. (+)-naloxone lasted less than 3 hours here and in prior studies (Hutchinson et al., 2008b). One potential candidate to improve upon the pain reversal of (+)-naloxone is (+)-naltrexone. (+)-Naltrexone is a small molecule that, similar to (+)-naloxone, is the non-opioid active isomer of the opioid receptor antagonist (-)-naltrexone. In humans, naltrexone has a half life of about 4 hours, significantly longer half-life than the 1-1.5 hour half-life of naloxone, and is also blood-brain barrier permeable (Dawson, 2004). (+)-Naltrexone antagonizes the LPS-induced increase in HEK-TLR4 cell reporter gene expression, reverses neuropathic pain behaviors when given intrathecally (Hutchinson et al., 2008b), and docks to MD-2 *in silico* (Hutchinson et al., 2010b). However, the lack of commercial availability is a serious impediment to investigating the effects of both (+)-naloxone and (+)-naltrexone and several key studies have not yet been performed due to limited availability of the compounds.

Current evidence suggests that (+)-naloxone is relatively specific to the TLR4 receptor. In a screen of central nervous system targets other than TLR4, including neuronal neurotransmitter receptors, hormone receptors, and enzymes, (+)-naloxone did not have a significant binding affinity to any target (Northcutt et al., 2010). Here, we show that (+)-naloxone also did not affect Pam-induced TLR2 activation. Currently, no known off-target effects of (+)-naloxone exist; however, additional screening of immune system targets would be informative. A lack of off-target effects decreases the chances that (+)-naloxone will have negative side effects. Additionally, (-)-naloxone has been

used clinically for years to reverse opioid actions, such as respiratory depression, with a good safety profile.

Chronic neuropathic pain is a widespread condition that often remains resistant to current treatments. Reduced TLR4 signaling has been shown to reduce neuropathic pain behaviors in rats in these studies and others (Tanga et al., 2005; Hutchinson et al., 2008b; Cao et al., 2009; Wu et al., 2010). (+)-Naloxone, an orally available, blood brain barrier permeable small molecule, has been shown here to reverse neuropathic pain two weeks to four months after the onset of pain. (+)-Naloxone antagonizes TLR4 signaling and has no known off-target effects, having demonstrated no TLR2 inhibitory effects here, no opioid receptor activity (Iijima et al., 1978), and no binding affinity in a screen of CNS targets (Northcutt et al., 2010). The effectiveness of (+)-naloxone on neuropathic pain, ease of administration and existing safety record in clinical settings make it an intriguing compound for future study.

CHAPTER 6

DISCUSSION

The studies here have advanced our knowledge of the role of TLR4 in acute and chronic pain states. We have demonstrated that a group of TLR4 agonists, glucuronide metabolites, transiently enhance pain behaviors when given intrathecally, an effect blocked by the TLR4 antagonist LPS-RS. The finding that many glucuronide metabolites can act on TLR4 suggests that they be considered more carefully in drug development than is currently the standard. Additionally, it suggests that glucuronide metabolites be considered as contributors to well known side effects of the parent drugs. The opioid-inactive (+)-naloxone, shown here to reverse multiple models of neuropathic pain, is a blood-brain barrier permeable TLR4 signaling inhibitor. (+)-Naloxone also blocked morphine-3-glucuronide-induced pain behaviors, suggesting it may be a candidate compound to mitigate the TLR4-dependent pain enhancement of these glucuronide metabolites. Finally, these glucuronide studies combined to develop an approach for testing TLR4 agonism *in vitro* and predicting pain behaviors *in vivo*.

I. TLR4 in Pain States

Traditionally, researchers and regulatory agencies alike have considered the majority of glucuronide metabolites as biologically inert (Davis-Bruno and Atrakchi, 2006). In the survey here of 6 different glucuronide compounds, including opioid, steroid hormone and alcohol metabolites, 5 were found to produce TLR4-dependent pain

following intrathecal injection. These data suggest that glucuronidation should be considered as a potential bioactivation step, with TLR4 agonism as a possible result. A thorough study of morphine-3-glucuronide (M3G) showed that it caused this enhanced pain through increased proinflammatory cytokine production, a common end product of TLR4 activation and a likely mechanism for pain enhancement.

Prior studies of exogenous drugs that activate TLR4 have been necessarily confounded by dual actions of TLR4 signaling and the target drug effects (Hutchinson et al., 2010a; Hutchinson et al., 2010b). However, several of the glucuronide metabolites tested, including glucuronic acid (GA), ethyl glucuronide (EtG) and the estradiol glucuronides (E_2-3-G, E_2-17-G), lose their activity at the site of parent drug action, leaving TLR4 as the only known receptor on which they can act. The finding of a potential mechanism for drug side effects that is separate from the parent drug site of action presents the intriguing possibility that one could block the side effects without reducing the drug's efficacy.

Notably, for all of the parent drugs whose metabolites were found to produce TLR4-dependent pain, including morphine, ethanol and estradiol, the withdrawal of the parent drug can trigger various states of enhanced pain. Withdrawal from chronic morphine has long been associated with a hyperalgesia, which has recently been linked to an increase in proinflammatory signaling (Raghavendra et al., 2004b). Withdrawal from both acute (Howland et al., 2008) and chronic (Jochum et al., 2010; Martinotti et al., 2010) alcohol can cause headaches and other hypersensitivities. And the period of declining estradiol in menstruating females is the period of greatest risk for migraine (Kornstein and Parker, 1997), temporomandibular joint pain (LeResche et al., 2003) and

other pain disorders. For each of these diverse drugs, the period of drug withdrawal will is the time when glucuronoide metabolites are in highest concentration relative to the parent drug (Stanczyk et al., 1980; Droenner et al., 2002; LeResche et al., 2003; Dale et al., 2007). While numerous neuronally-based mechanisms have been postulated for these withdrawal symptoms, the findings of these studies suggest metabolites and TLR4 signaling should be considered as an additional hypothesis. Were such a hypothesis substantiated, it would provide a novel avenue for treating these hyperalgesic effects, that of TLR4 signaling inhibition and/or glial inhibition.

The opioid-inactive (+)-naloxone isomer, in addition to its ability to reverse multiple models of chronic neuropathic pain (Lewis et al., 2011c), was also able to reduce the hyperalgesia and allodynia produced by M3G. This suggests that (+)-naloxone, shown to be a TLR4 signaling inhibitor in numerous different situations (Hutchinson et al., 2008b; Hutchinson et al., 2010b; Lewis et al., 2011b), might be an effective co-treatment with molecules known to produce glucuronide sensitivities in addition to its potential as an effective treatment for neuropathic pain.

II. Method for screening TLR4 agonists

The studies presented here provide further evidence that TLR4 activation can contribute to enhanced pain. Numerous exogenous drugs were shown to activate TLR4 and cause enhanced pain. One useful application of these studies is the development of a screening procedure for potential TLR4 agonists that may have the potential to enhance pain.

HEK-TLR4 cells are transfected with TLR4 and necessary co-signaling molecules, along with an NFκB-dependent SEAP reporter gene. An increase in NFκB activation following TLR4 signaling leads to an increase in SEAP gene expression. HEK-TLR4 cells SEAP expression in these studies can predict the amount of pain that develops following intrathecal injection (Figure 6.1). All intrathecal injections were equimolar. There was a significant correlation between HEK-TLR4 cell NFκB-dependent SEAP expression and the degree of pain induced (r^2=0.72, $p<0.05$). HEK-TLR4 cell SEAP expression induced by 100 μM concentration of drug predicted the amount of pain that developed 3 hours following intrathecal injection. All measures are reported as percent vehicle in order to have a meaningful comparison between compounds with different solubility requirements.

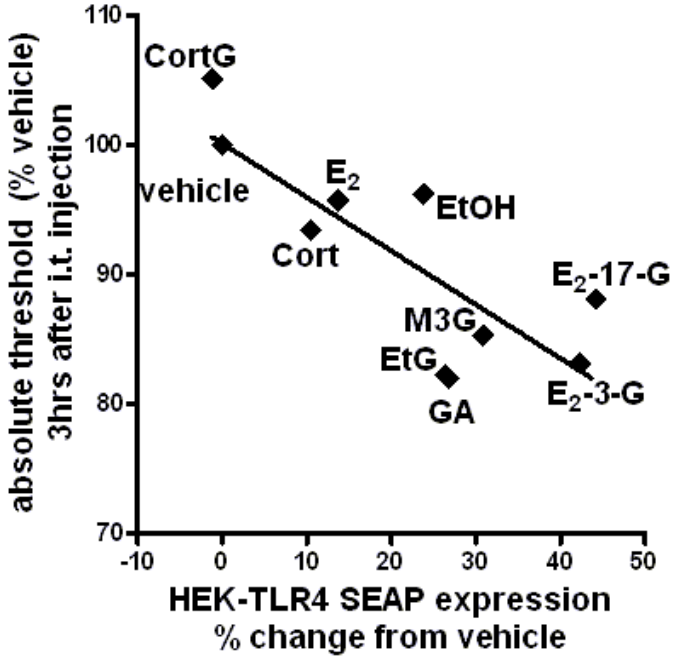

Figure 6.1 The degree of HEK-TLR4 cell NFκB-dependent SEAP expression predicts the degree of pain induced by the compound.

Previous papers have been able to predict agonist activity in the HEK-TLR4 cell assay based on an *in silico* docking model similar to that undertaken here (Hutchinson et al., 2010a; Hutchinson et al., 2010b). The present studies were not able to predict HEK-TLR4 SEAP expression or pain behaviors based on a simple correlation to the *in silico* predicted docking energy. However, the present studies investigated glucuronide metabolites from a range of different drug classes, unlike the previous papers which investigated either opioid or tricyclic compounds, respectively. This limited the complexity of the model that could be developed. Additionally, the molecules in the present studies were tested strictly for agonism at TLR4, not antagonism, so certain molecules that were predicted to dock to MD-2 *in silico*, but failed to enhance HEK-TLR4 SEAP expression or pain, may in fact be TLR4 signaling antagonists. It is likely that a more detailed *in silico* analysis utilizing molecules structurally related and a more complex model would successfully predict *in vitro* TLR4 agonism.

Another question is whether the effects of the glucuronide metabolites are due exclusively to the glucuronic acid (GA) component, or whether they are enhanced by the presence of the parent drug group. GA and the structurally similar ethyl glucuronide had nearly identical performance on both the intrathecal injection pain behavior test and the HEK-TLR4 cell assay. M3G and E_2-3-G both had somewhat greater HEK-TLR4 cell SEAP expression, but pain behavior was near the floor of our testing abilities such that, were there a real decrease in pain thresholds relative to GA, our test may not have been able to detect it. Notably, CortG didn't induce any pain behavior at all. A glucuronide group alone does not seem to confer TLR4 activity, and the results are inconclusive as to

whether the parent drug conformation contributes to the degree of HEK-TLR4 SEAP expression and pain behavior.

III. Potential implications and future directions

Although glucuronidation is largely considered a deactivating step, one class of glucuronide metabolites, the acyl glucuronides, has previously been linked to negative side effects, including immunogenicity (Bailey and Dickinson, 2003). Acyl glucuronides are derived from compounds with a carboxylic group, including the common nonsteroidal anti-inflammatory drugs naproxen and ibuprofen. Both naproxen and ibuprofen have been linked to hypersensitivity reactions, including rash, fever and immunocytopenia, in a small subset of users (Bailey and Dickinson, 2003). The findings here suggest that the glucuronide metabolites of these drugs be considered as potential contributors to these rare, immogenic hypersensitivity reactions. Additionally, other classes of glucuronide metabolites, including the acyl glucuronides and drug classes with negative side effects associated with withdrawal of drugs, should be tested for potential TLR4 signaling and/or proinflammatory responses in the CNS.

The survey of glucuronide metabolites was not designed to determine which cell types were involved, only to determine if TLR4 was a component. As TLR4 can be expressed on most of the major cell types in the CNS, the exact mechanism is not known. The finding of TLR4 involvement in many of these metabolites suggests that a more in-depth study of mechanisms and cell types, similar to that performed for morphine-3-glucuronide, would be fruitful. Additionally the finding of increased proinflammatory response in cerebrospinal fluid and lumbar dorsal horn in M3G suggest that a more

detailed analysis of the proinflammatory response to other glucuronide metabolites is warranted.

IV. Conclusions

This dissertation expanded on the knowledge of TLR4 signaling in pain states. We present the first evidence that a group of molecules, conjugated glucuronide metabolites, can induce TLR4 activity and TLR4-dependent acute pain sensitization. Specifically, glucuronic acid, morphine-3-glucuronide, ethyl glucuronide, estradiol-3-glucuronide and estradiol-17-glucuronide were all found to enhance TLR4 signaling in an *in vitro* model and to cause pain sensitization *in vivo* that was blocked by the TLR4 antagonist LPS-RS. This finding has broad potential implications for these metabolites to contribute to parent drug effects. In particular, withdrawal from morphine, alcohol and estradiol is associated with an increase in pain sensation, and also corresponds to the time of highest glucuronide metabolite concentrations. These findings further suggest that glucuronidation be considered a bioactivation step with the potential to produce active metabolites with TLR4 activity.

This work also increased our understanding the role of (+)-naloxone, a TLR4 signaling inhibitor with no known off-target effects. (+)-Naloxone is a orally available blood-brain barrier permeable small molecule, making it of greater pharmaceutical potential than other larger, non blood-brain barrier permeable options. Building on the work of Hutchinson et al. (2008b), (+)-naloxone was found to reverse multiple models of neuropathic pain, up to 4 months following initial nerve injury. This finding also suggests an ongoing role for TLR4 long after the establishment of neuropathic pain. (+)-

Naloxone was also found to block the enhanced pain following intrathecal M3G injection, suggesting it may also have applications for reducing the effects of glucuronide metabolites.

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