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Periaqueductal Gray Glia Modulate Morphine Tolerance Development via Soluble Tumor Necrosis Factor Signaling

Lori Eidson

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ABSTRACT

Each year, over 50 million Americans suffer from persistent pain, including debilitating headaches, joint pain, and severe back pain. Although morphine is amongst the most effective analgesics available for the management of severe pain, prolonged morphine treatment results in decreased analgesic efficacy (i.e., tolerance). Despite significant headway in the field, the mechanisms underlying the development of morphine tolerance are not well understood. The midbrain ventrolateral periaqueductal gray (vlPAG) is a primary neural substrate for the analgesic effects of morphine, as well as for the development of morphine tolerance. A growing body of literature indicates that activated glia (i.e., microglia and astrocytes) facilitate pain transmission and oppose morphine analgesia, making these cells important potential targets in
the treatment of chronic pain. Morphine affects glia by binding to the innate immune receptor toll-like receptor 4 (TLR4), leading to the release of proinflammatory cytokines and opposition of morphine analgesia. Despite the established role of the vIPAG as an integral locus for the development of morphine tolerance, to date, no studies have examined the role of glia activation within this region. Additionally, the role of TLR4 in the development of tolerance has not been elucidated. This dissertation seeks to address the lack of knowledge regarding the role of vIPAG glia and TLR4 in the development of morphine tolerance by (1) Characterizing the effects of chronic morphine and peripheral inflammatory pain on vIPAG glial cell activity; (2) Investigating the role of glia activation within the vIPAG in the development of morphine tolerance; (3) Characterizing the role of the glial receptor TLR4 within the vIPAG in the development of morphine tolerance; and (4) Characterizing the glia to neuron signaling mechanisms involved in the development of morphine tolerance. These experiments, together, provide novel information about the mechanism by which central nervous system glia regulate morphine tolerance, and identify a potential therapeutic target for the enhancement of analgesic efficacy in the clinical treatment of chronic pain.

INDEX WORDS: Opioid, Tolerance, Glia, Toll-like receptor 4, Tumor necrosis factor, Periaqueductal gray
PERIAQUEDUCTAL GRAY GLIA MODULATE MORPHINE TOLERANCE
DEVELOPMENT VIA SOLUBLE TUMOR NECROSIS FACTOR SIGNALING

by

LORI N. EIDSON

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy
in the College of Arts and Sciences
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PERIAQUEDUCTAL GRAY GLIA MODULATE MORPHINE TOLERANCE DEVELOPMENT VIA SOLUBLE TUMOR NECROSIS FACTOR SIGNALING

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May 2015
DEDICATION

I dedicate this work to us and to what we will make of our future. I love you, Mac.
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CHAPTER 1: Introduction
Opioids and the Management of Pain

The perception of pain is an important evolutionary phenomenon that allows escape from danger, avoidance of harmful stimuli, and attention to tissue damage. However, chronic pain perception in the absence of an ongoing injury, or during treatment of an illness, is aversive without purpose and detrimental to quality of life. Chronic pain, defined as pain lasting more than three months, impacts approximately 55% of the population over the age of 20, and includes debilitating headaches, joint pain, severe back pain, and cancer-related pain\(^1,2\). Despite their discovery thousands of years ago, opiates remain the most common and effective option for pain management\(^3\). Indeed, over 90% of chronic pain sufferers receive some form of opiate therapy\(^4\), with morphine being amongst the most commonly prescribed drugs.

In addition to modulating pain, opiates have widespread central and peripheral effects, many of which interfere with the beneficial aspects of opiates\(^5\). Indeed, the negative side effects associated with opiate consumption, including respiratory depression, gastrointestinal immotility, and addiction, render these drugs unsatisfactory for long-term pain management\(^1,6\). Although a multitude of opioids (man-made drugs sharing similar structures with the plant-derived opium\(^6\)) were synthesized in hopes of harnessing the natural analgesic potency of opiates while minimizing the negative side effects, few are as potent as morphine, and the pinnacle, analgesia without the side-effect profile, has not been realized\(^5\).

The average duration of opioid consumption for chronic pain management is 105 days\(^7\). Prolonged morphine treatment reduces analgesic efficacy (i.e., tolerance), thereby requiring steadily larger doses of opiates for the maintenance of analgesia\(^3\). Dose escalation increases the risk of developing negative side effects, including anti-analgesia, addiction, withdrawal, and respiratory depression\(^4\), and is not always sufficient to overcome tolerance and reinstate
analgesic efficacy. Indeed, opioid tolerance is a significant impediment for sufficient pain relief in approximately 60% of patients. Remarkably, the mechanisms underlying the development of morphine tolerance are unknown.

**Opioid Action in the CNS: The PAG & the Descending Analgesic Pathway**

The midbrain periaqueductal gray (PAG) and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord comprise a critical neural circuit for both endogenous and exogenous opioid-mediated analgesia. The PAG was first identified as an essential neural substrate for pain modulation in the 1960s, when it was demonstrated that electrical stimulation of the rat PAG produces analgesia so profound as to allow for invasive abdominal surgery to be performed in the absence of anesthesia. In humans, electrical stimulation of the PAG is still used today for the management of intractable pain. PAG stimulation produced analgesia is attenuated by intra-PAG injection of the mu opioid receptor (MOR) antagonist naloxone, suggesting an opioid-dependent mechanism.

The PAG contains a high density of MOR containing neurons, and microinjection of opiate antagonists into the PAG significantly attenuates the analgesic effects of systemic morphine. Similarly, site-specific lesions of PAG MOR-containing neurons (using the cytotoxin saporin (aka ribosome inactivating protein; RIP) conjugated to the MOR ligand Dermorphin) significantly reduce the antinociceptive effects of systemic morphine suggesting that PAG MOR is critical for morphine action. The density of MOR immunoreactivity within the vPAG is positively correlated with the degree of analgesia produced by morphine, such that male rats with normal levels of MOR immunoreactivity in the vPAG have significantly lower ED values (4.07 mg/kg) compared with animals in which MOR levels were reduced 2-fold.
(12.55 mg/kg). Indeed, in animals with low PAG MOR, systemic administration of 10 mg/kg of morphine results in only a 20% maximum possible analgesic effect in comparison to 100% in animals with a complete complement of MOR. Together, these data indicate that the PAG is an essential site for opioid-mediated analgesia.

Morphine and other opioids bind to neuronal MOR\(^6\), a prototypical G-protein coupled receptor (GPCR), and are generally thought to elicit analgesia by hyperpolarizing GABAergic neurons (‘GABA disinhibition hypothesis’\(^9,11,27-33\)). Indeed, it is the release from tonic GABAergic inhibition that allows morphine to inhibit pain. \textit{In vitro}, MOR binding on PAG neurons inhibits mIPSP frequency and decreases the probability of presynaptic GABA release\(^31,34\). \textit{In vivo}, injection of GABA antagonists into the PAG partially mimics the effects of morphine\(^35\). Physiologically, morphine binding to vlPAG MOR hyperpolarizes GABAergic interneurons\(^34,36\), thereby releasing vlPAG-RVM projection neurons from local tonic inhibition\(^36\). Removal of this tonic inhibition results in the net excitation of RVM neurons, which then signal to the spinal cord to inhibit nociceptive dorsal horn neurons and produce antinociception\(^37-39\) (Figure 1.1). Indeed, lesions of the RVM and spinal dorsal horn abolish PAG stimulation-produced antinociception, indicating that the PAG-RVM-spinal cord circuit is necessary for both exogenous and endogenous pain modulation\(^40,41\).

\textbf{Neuronal Mechanisms of Opioid Tolerance}

In addition to being a critical locus for both endogenous and exogenous pain modulation, the vlPAG is also necessary and sufficient for the development of morphine tolerance\(^24,42-51\). Chronic vlPAG opioid administration results in the rapid development of behaviorally and physiologically defined opioid tolerance. In addition, repeated intra-vlPAG microinjections of morphine\(^49\) or the potent MOR agonists fentanyl\(^43\) or DAMGO\(^47,49\) result in tolerance to
systemically administered morphine. Further, chronic administration of morphine into the ventrolateral, but not lateral or dorsal, PAG induces morphine tolerance\textsuperscript{51}; this effect remains when the downstream target (RVM) is inhibited with the GABA agonist muscimol. Interestingly, these behavioral and electrophysiological changes underlying tolerance are prevented by intra-vlPAG injections of the opioid receptor antagonist naltrexone\textsuperscript{24}, indicating that the vlPAG is sufficient for the development of morphine tolerance\textsuperscript{24}.

While the mechanisms by which morphine tolerance develops are not entirely understood, many current hypotheses include a role for increased glutamatergic and/or decreased GABAergic signaling\textsuperscript{31} that ultimately oppose the hyperpolarizing effects of morphine. Cerebrospinal fluid (CSF) from morphine-tolerant humans contains significantly higher levels of both glutamate and aspartate\textsuperscript{52}, and morphine challenge increases glutamate in the CSF of morphine tolerant rats\textsuperscript{53}. Increased expression of AMPA and NMDA receptor subunits\textsuperscript{54} and increased NMDA receptor binding\textsuperscript{55} in the rat spinal cord has been shown to accompany tolerance development. Along these same lines, blockade of spinal cord glutamatergic signaling by intrathecal administration of NMDA\textsuperscript{56-61} and AMPA\textsuperscript{62,63} receptor antagonists attenuates morphine tolerance. Together, these data indicate that, at least at the level of the spinal cord, opioid tolerance is accompanied by an increase in the excitatory neuroenvironment that is mediated by changes in glutamatergic signaling.

Studies examining the cellular responses of PAG neurons indicate that morphine tolerance, induced by repeated systemic or intra-vlPAG morphine, decreases the ability of opioids to initiate signaling through the PAG-RVM descending analgesic circuit\textsuperscript{44,45}. Repeated pharmacological activation of the PAG-RVM circuit via direct microinjection of the excitatory amino acid agonist kainate or the GABAergic antagonist bicuculine is not sufficient to induce
tolerance, indicating that tolerance requires opiate activation of MOR-expressing GABAergic neurons that synapse onto PAG-RVM output neurons. Indeed, Morgan and colleagues recently demonstrated that chronic vlPAG microinjections of morphine results in tolerance that is dependent on alterations in pre- and post-synaptic GABA release. Chronic systemic morphine results in tolerance as evidenced by a decreased ability of MOR agonists to inhibit Ca\(^{2+}\) and activate K\(^+\) channels in dissociated neuronal cultures from the PAG or PAG slices. While NMDA receptor signaling is not important for PAG-mediated opioid tolerance, data from the PAG supports a role for increased neuroexcitability in tolerance development. For example, intra-PAG microinjections of the cholecystokinin (CCK) antagonist proglumide prevent and even reverse tolerance to repeated PAG microinjections of morphine. CCK excites neurons by opening depolarizing currents and inhibiting K\(^+\) conductance, thereby directly opposing the mechanisms by which morphine hyperpolarizes neurons. Together, these data suggest that increased neuroexcitability at the level of the PAG significantly contributes to opioid analgesic tolerance by decreasing the ability of opioids to hyperpolarize neurons.

**Glial Mechanisms of Opioid Tolerance**

Since the 1990’s basic research has shifted focus from exclusive investigation of neuronal mechanisms underlying opiate analgesia and tolerance to investigation of both neuronal and central nervous system (CNS) glial involvement. It is now well established that chronic morphine induces a robust neuroinflammatory response in the CNS that enhances neuronal excitability and contributes to tolerance. While the importance of the vlPAG in tolerance development is well established, the investigation of glial involvement in opiate signaling has been limited to spinal and medullary loci.
Several lines of evidence implicate opioids as activators of CNS astrocytes and microglia\textsuperscript{85}. In the spinal cord, morphine increases protein levels of the microglia and astrocyte activity markers OX-42 and glial fibrillary acidic protein (GFAP), respectively,\textsuperscript{71,72,91} and induces release of glially derived proinflammatory cytokines\textsuperscript{73,74,81,84,86,87,90}. Proinflammatory cytokines have been shown to decrease GABA receptor expression, increase the number and the conductance of AMPA and NMDA receptors, decrease glutamate transporter proteins, and decrease outward potassium currents\textsuperscript{85}, resulting in an overall increase in neuroexcitability. Functionally, administration of the glial metabolic inhibitors propentofylline, fluorocitrate, and minocycline reduce spinal OX-42, GFAP, and cytokines, and attenuate morphine tolerance\textsuperscript{72,76,87,91-97}. Importantly, glial release of cytokines increases exponentially with repeated morphine administration,\textsuperscript{84} making these excitatory substances key players in the development of morphine tolerance.

Both morphine tolerance and opioid-induced sensitivity to pain (hyperalgesia) following repeated exposure to morphine is still observed in neuronal opioid receptor (µ, δ, and κ) knock out mice\textsuperscript{98}, suggesting that the anti-analgesic effects of morphine are not mediated by traditional opioid receptor signaling. Recent \textit{in vivo} and \textit{in vitro} data confirm this, and demonstrate that the proinflammatory effects of morphine are mediated through the innate immune receptor Toll-like receptor 4 (TLR4)\textsuperscript{81}. TLR4 is found on microglia, and to a lesser degree, astrocytes\textsuperscript{99,100}. Opioids, including morphine, bind to the glycoprotein myeloid differentiation factor-2 (MD-2) on TLR4\textsuperscript{78,79}, and initiate an inflammatory response through nuclear factor kappa B (NF-κB) activation and p38 mitogen activated protein kinase (MAPK) phosphorylation\textsuperscript{81}. Activation of the NFκB pathway results in the robust release of proinflammatory cytokines including tumor necrosis factor alpha, (TNF), Interleukin 1 beta (IL-1β), and Interleukin 6 (IL-6)\textsuperscript{78,79,81}. Spinal
TLR4 activity opposes the acute effects of morphine, including antinociception, and contributes to opioid-induced hyperalgesia. While the brain loci through which TLR4 mediates morphine tolerance have not been investigated, systemic TLR4 antagonists prevent tolerance to systemic morphine.

A nearly ubiquitous characteristic of opioid-induced neuroinflammation is TNF production, likely mediated via TLR4. Chronic systemic or intrathecal morphine administration increases TNF mRNA and protein in the rodent spinal cord, and TNF levels increase with the chronicity of morphine treatment. Inhibition of spinal TNF signaling decreases morphine-induced release of proinflammatory cytokines (e.g., TNF, IL-1β, and IL-6) and activation of p38 MAPK, indicating that TNF induces a positive feedback loop of neuroinflammation that contributes to decreased morphine efficacy.

Functionally, immunomodulatory drugs that attenuate, abolish, or even reverse morphine tolerance (e.g., ibudilast (AV411), minocycline, fluorocitrate, propentofylline) decrease the expression of TNF. However, these immunomodulatory drugs have widespread and non-specific effects, altering expression levels of several cytokines implicated in opioid tolerance, including IL-1β and IL-6. While these data indicate that spinal TNF plays a significant role in morphine-induced inflammation and the development of morphine tolerance, remarkably, very few studies have directly tested the role of TNF in isolation.

Dissertation Aims

Despite the established role of the vlPAG as an integral CNS locus for morphine action, including analgesia and tolerance, studies investigating the modulatory role of glia and cytokine
release have been limited to medullary and spinal loci. The role of PAG TLR4 in the development of tolerance has not been elucidated. This dissertation addressed the dearth of knowledge regarding the role of vIPAG glia and TLR4 in the development of morphine tolerance by (1) characterizing vIPAG glial cell activity in tolerant and non-tolerant male rats in the absence and presence of a persistent pain state using behavioral, immunohistochemical, and western blot analyses; (2) investigating the necessity of vIPAG astrocytes and microglia in the development of morphine tolerance using behavioral analysis coupled with site-specific pharmacological injections of glial metabolic inhibitors; (3) investigating the necessity and sufficiency of vIPAG TLR4 in the development of morphine tolerance using behavioral analysis coupled with site specific injections of TLR4 agonists and antagonists; and (4) investigating the necessity of vIPAG TNF in the development of morphine tolerance using behavioral analysis coupled with lenti-viral or pharmacological administration of an anti-TNF biologic. In these final studies, expression levels of TNF, IL-1β, IL-6, IL-10, and TLR4 were quantified in the vIPAG using quantitative RT-PCR while in situ hybridization was used to quantify mRNA levels of the glutamate transporters GLT-1, GLAST, and EAAC1. Together, these experiments provide novel information about the mechanisms by which central nervous system glia modulate morphine action and the development of tolerance, and will identify potential therapeutic targets for the enhancement of analgesic efficacy in the clinical treatment of chronic pain (Figure 1.2).
Chapter 1 Figures

**Figure 1.1 A schematic of the descending inhibitory pathway for pain modulation.** Adapted from Guo et al. 2006\textsuperscript{13}. Primary afferents relay information regarding noxious stimuli through the dorsal root ganglia (DRG) to terminate within the dorsal horn (DH) of the spinal cord. This noxious input is subject to tonic descending inhibition via projections from the midbrain periaqueductal gray (PAG) to the brainstem rostral ventromedial medulla (RVM) and the spinal cord DH.
We hypothesize that chronic morphine binds to glial TLR4 to induce the release of soluble TNF. Soluble TNF increases the neuronal excitability of PAG neurons by increasing glutamatergic signaling, thereby decreasing morphine’s ability to hyperpolarize GABAergic interneurons. GABAergic signaling tonically inhibits PAG-RVM projection neurons, thereby preventing signaling through the descending analgesic circuit.

**Figure 1.2 A schematic diagram illustrating core hypotheses to be tested.**

We hypothesize that chronic morphine binds to glial TLR4 to induce the release of soluble TNF. Soluble TNF increases the neuronal excitability of PAG neurons by increasing glutamatergic signaling, thereby decreasing morphine’s ability to hyperpolarize GABAergic interneurons. GABAergic signaling tonically inhibits PAG-RVM projection neurons, thereby preventing signaling through the descending analgesic circuit.
CHAPTER 2: Persistent Peripheral Inflammation Attenuates Morphine-induced Periaqueductal Gray Glial Cell Activation and Analgesic Tolerance in the Male Rat

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Abstract

Morphine is among the most prevalent analgesics prescribed for chronic pain. However, prolonged morphine treatment results in the development of analgesic tolerance. An abundance of evidence has accumulated indicating that CNS glial cell activity facilitates pain transmission and opposes morphine analgesia. While the midbrain ventrolateral periaqueductal gray (vlPAG) is an important neural substrate mediating pain modulation and the development of morphine tolerance, no studies have directly assessed the role of PAG-glia. Here we test the hypothesis that morphine-induced increases in vlPAG glial cell activity contribute to the development of morphine tolerance. As morphine is primarily consumed for the alleviation of severe pain, the influence of persistent inflammatory pain was also assessed. Administration of morphine, in the absence of persistent inflammatory pain, resulted in the rapid development of morphine tolerance and was accompanied by a significant increase in vlPAG glial activation. In contrast, persistent inflammatory hyperalgesia, induced by intraplantar administration of Complete Freund’s Adjuvant (CFA), significantly attenuated the development of morphine tolerance. No significant differences were noted in vlPAG glial cell activation for CFA-treated animals versus controls. These results indicate that vlPAG glia are modulated by a persistent pain state, and implicate vlPAG glial cells as possible regulators of morphine tolerance. The development of morphine tolerance represents a significant impediment to its use in the management of chronic pain. We report that morphine tolerance is accompanied by increased glial cell activation within the vlPAG, and that the presence of a persistent pain state prevented vlPAG glial activation and attenuated morphine tolerance.
Introduction

Chronic pain, defined as pain lasting more than 3-6 months, will affect more than one in three Americans at some point in their life\textsuperscript{1,2}. Although morphine is one of the most commonly prescribed analgesics\textsuperscript{4}, secondary side effects (e.g., tolerance) limit its efficacy for long-term chronic pain treatment\textsuperscript{45,114-116}. In the absence of pain, morphine tolerance, defined as the requirement for steadily larger doses of opioids to achieve the same analgesic effect, develops quite rapidly\textsuperscript{45,117,118}. In contrast, clinical studies have consistently reported that the latency to develop morphine tolerance is increased in chronic pain sufferers, although dose escalation is eventually required for the maintenance of analgesic efficacy\textsuperscript{3}. Dose escalation leads to increased risk of developing additional negative side effects, including anti-analgesia, addiction, withdrawal, and respiratory depression\textsuperscript{4}, and is not always sufficient to overcome tolerance and reinstate analgesic efficacy\textsuperscript{3}. As over 90\% of chronic pain sufferers are treated with opioids\textsuperscript{4}, including morphine, elucidation of the mechanisms by which morphine tolerance develops warrants investigation.

The midbrain ventrolateral periaqueductal gray (vlPAG) and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord, comprises an important neural circuit for both endogenous and exogenous opioid-mediated analgesia\textsuperscript{9-13}. In rats, chronic subcutaneous injections of morphine result in tolerance to subsequent doses of morphine, an effect that is eliminated by intra-vlPAG injections of the opioid receptor antagonist naltrexone\textsuperscript{24}. In addition, chronic intra-vlPAG injections of morphine induce tolerance, and this effect remains when the RVM, the primary downstream target of the PAG, is inhibited with the GABA agonist muscimol\textsuperscript{24}. 
An abundance of evidence has accumulated indicating that systemic morphine
administration activates glial cells, including microglia and astrocytes\textsuperscript{51,72,85,86,91,92,119}. Song and
Zhou (2001) reported that chronic morphine administration results in the activation of astrocytes
within the cingulate cortex, hippocampus and spinal cord, and that blockade of glial activation
within the spinal cord attenuates the development of morphine tolerance. Since then, a myriad of
studies have been published implicating glia activation in the development of morphine
tolerance\textsuperscript{85,88} and pain facilitation\textsuperscript{93,120,121}. While it is clear that the activation of microglia and
astrocytes contribute to the development of morphine tolerance, no studies have examined the
role of activated glia within the PAG, a primary site of morphine action. Similarly, the influence
of a persistent inflammatory pain state on PAG glial cell activation has not been assessed. The
present study tested the hypothesis that morphine-induced increases in vlPAG glial cell activity
contribute to the development of morphine tolerance, and that persistent inflammatory pain alters
this activation, resulting in the attenuation of morphine tolerance.

\textbf{Materials and Methods}

\textit{Subjects}

Weight-matched (250-350g) male Sprague Dawley rats (Charles River, Wilmington, MA) were pair-housed on a 12:12 hour light: dark cycle. Access to food and water was available
\textit{ad libitum} throughout the experiments except during behavioral testing. All studies were
approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia State
University, and performed in strict compliance with Ethical Issues of the International
Association for the Study of Pain (IASP) and National Institute of Health (NIH). All efforts were
made to reduce the number of animals used in these experiments and to minimize any possible
suffering by the animal.
Persistent Inflammatory Hyperalgesia

In a subset of animals, persistent inflammatory hyperalgesia was induced by injection of complete Freund’s adjuvant (CFA; Mycobacterium tuberculosis; Sigma; 200 µl), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hindpaw as previously described\textsuperscript{45,122,123}. As intraplantar saline administration results in a short-term inflammatory response, control animals were restrained in a similar manner but did not receive an intraplantar injection.

Experiment 1: Influence of Persistent Inflammatory Pain on Morphine Tolerance

Twenty-four hours following intraplantar CFA injection or handling, animals were administered morphine (5 mg/kg, sc; NIDA) or saline (1 ml/kg, sc) once a day for three consecutive days (CFA+Morphine; CFA+Saline; Handled+Morphine; Handled+Saline). The 5 mg/kg dose was chosen based on our previous studies demonstrating this to be the 50% effective dose (ED\textsubscript{50}) for systemic morphine in male rats\textsuperscript{122,124,125}. Baseline nociceptive thresholds were measured before morphine or saline injections, and 15 minutes following the first and last injection (Injection 1 and Injection 3, respectively). Tolerance was assessed on Day 5 (Day 1 being CFA administration), by injecting cumulative doses of morphine every 20 min, resulting in doses of 3.2, 5.6, 8.0, 10.0 and 18.0 mg/kg as previously described\textsuperscript{45}. Nociception was assessed using the paw thermal stimulator\textsuperscript{126} 15 min after each injection\textsuperscript{122}. Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. A radiant beam of light is positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL). The intensity of the beam was set to produce basal withdrawal rates of 7-9 seconds. A maximal PWL of 20.48 seconds was used to prevent excess tissue damage due to repeated application of the
noxious thermal stimulus. Animals were acclimated to the testing apparatus (30 minutes a day for 3 consecutive days) at the start of the experiment. All behavioral testing took place between 12:00pm and 5:00pm (lights on at 7:00am). All testing was conducted blind with respect to group assignment (i.e., morphine or saline treatment).

Behavioral data analysis and presentation

Behavioral data are expressed in raw seconds. Paw withdrawal latency data were analyzed using repeated measures ANOVA for significant main effect of pain (CFA or handled) and treatment (morphine or saline) across dose. Pre-planned t-tests were used to determine specific group and dose differences when a significant main effect was observed. All values are reported as Mean ± S.E.M.; p< 0.05 was considered statistically significant.

Experiment 2: Anatomical Assessment of Morphine Tolerance

Twenty-four hours following intraplantar CFA or handling, animals were administered morphine (5 mg/kg; sc) or saline (1 ml/kg; sc) once a day for three consecutive days as described above (CFA+Morphine, CFA+Saline, Handled+Morphine, Handled+Saline). One hour following the last injection of morphine or saline, animals were given a lethal dose of Nembutal (160 mg/kg; i.p.) and transcardially perfused with 250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately following blood removal, 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer containing 2.5% acrolein (Polysciences Inc.; Warrington, PA) was perfused through the brain as a fixative. A final rinse with 250 ml of sodium chloride/sodium nitrite solution was perfused through the brain to remove any residual acrolein. Brains were removed and placed in a 30% sucrose solution and stored at 4°C until sectioning. To examine the acute effects of morphine on vIPAG glia activation, a separate group of animals received one sc injection of morphine (or saline) and were sacrificed 1
or 24 hours later. A separate group of animals (CFA+Morphine, CFA+Saline, Handled+Morphine, Handled+Saline) were decapitated immediately following treatment for western blot analysis. Brains were removed, flash frozen in 2-methyl-butane on dry ice, and stored at -80°C.

Immunohistochemistry

Hallmarks of glial cell activity include increased cytokine release that correlates with increased expression of the protein markers glial fibrillary acidic protein (GFAP; astrocytes), and CD-11b (OX-42; microglia). Further, increased glial cell activity is evidenced by a profound shift in morphology that can be easily visualized using immunohistochemistry for GFAP and OX-42. Perfused brains were sectioned into 25μm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution at -20°C. A 1:6 series through the rostrocaudal axis of each brain was processed for GFAP and OX-42 immunoreactivity using standard immunohistochemical techniques. Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) immediately followed by a 20-minute incubation in 1% sodium borohydride. The tissue was then incubated in primary antibody solution (rabbit anti-GFAP 1:5,000 or rabbit anti-OX42 1:1000; Abcam) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. After rinsing with KPBS, the tissue was incubated for one hour in secondary antibody (biotinylated IgG goat anti-rabbit 1:600), rinsed with KPBS, and then incubated for one hour in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit, Vector Labs). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), GFAP or OX-42 immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3’-diaminobenzidine (DAB) solution (2 mg/10ml) containing 0.08% hydrogen peroxide in sodium acetate buffer. After 15 minutes,
tissue was rinsed in sodium acetate buffer followed by KPBS. In a subset of sections, GFAP (Rabbit anti-GFAP 1:3,000; Abcam) or OX-42 (Mouse anti-CD11b 1:3000; Serotec) was visualized using a fluorescent secondary antibody (goat anti-rabbit Dylight488 1:50 for GFAP and rabbit anti-mouse Cy3 1:50 for CD11b; Jackson Immunoresearch Laboratories). Following secondary incubation, sections were rinsed in KPBS. DAB and fluorescent sections were mounted out of KPBS onto gelatin-subbed slides, air-dried and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in Xylenes and glass cover-slipped using Permount for DAB reactions or Krystalon for fluorescence.

**Western blotting**

Flash frozen brains were sectioned at 300mm on a cryostat (Leica, Buffalo Grove, IL) and mounted onto slides. One-millimeter bilateral micropunches were taken from 6 levels for the vIPAG (Bregma -8.52, -8.28, -7.92, -7.68, -7.20, and -6.96) and 6 levels of the superior colliculus (Bregma -7.68, -7.20, -6.96, -6.60, -6.24, and -5.80), and homogenized in a 10mM HEPES buffer (pH 7.2). Equal amounts of protein (2mg) along with a standard marker (Bio-Rad, Hercules, CA) were run at 100V for 2 hours through 10% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA), and electro-transferred at 4°C on ice at 250mA for 2 hours onto PVDF membranes (0.2mm pore size; Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk in TBS-Tween 20 (1%) at 4°C overnight, and probed with rabbit anti-GFAP primary antibody (1:300,000; Abcam) in 2% milk/TBS-Tween 20 (1%) for 3 hours at room temperature followed by a 30 minute incubation in HRP-conjugated goat anti-rabbit secondary (1:5000; Abcam) in 2% milk/TBS-Tween 20 (1%). Rabbit anti-b-actin primary (1:10,000; Novus Biologicals) was included as a control for protein loading. Membranes were stripped and reprobed with mouse
anti-rat CD11b (OX-42, 1:700; Serotec) followed by HRP-conjugated goat anti-mouse (OX-42; 1:5000; Abcam) and HRP-conjugated goat anti-rabbit (b-actin; 1:5000; Abcam) secondaries.

**Anatomical data analysis and presentation**

Levels of GFAP and OX-42 immunoreactivity in the vIPAG were compared across treatment groups using semi-quantitative densitometry as previously described\(^{45,131}\). To determine if the observed changes in glia activation were limited to the vIPAG, sections through the superior colliculus (SC), a region containing a high density of m opioid receptors (MOR) but not implicated in opioid modulation of pain, were also analyzed. 12-bit grayscale images that included the region of interest (ROI) were captured using a QImaging Retiga EXi CCD camera (Surrey, BC, Canada) and iVision Image analysis software (Biovision Technologies, Exton, PA). Grayscale values for each image were inverted so that higher values were representative of increased staining levels. Images of three slices through each ROI for each animal were analyzed and data sampled unilaterally. Data sampling occurred by using the drawing tools in iVision to outline the ROI and using the “measure” function to determine an average grayscale pixel value for the outlined area. ROI measures were corrected for nonspecific binding by subtraction of background measure taken from gray matter adjacent to the ROI. Data were analyzed across 3 representative levels through the rostral-caudal axis of the vIPAG (Bregma -7.08, -7.68, and -8.30\(^{130}\)) and superior colliculus (Bregma -7.68, -6.24, and -5.80\(^{130}\)) as previously described\(^{45}\). Densitometry values are presented as the mean ± S.E.M. density of immunoreactivity. Data were analyzed using an ANOVA to determine significant main effects of treatment (morphine, saline) and pain (CFA, handled). Fisher’s post hoc tests were used to determine specific group differences when a significant main effect was observed; \(p \leq 0.05\) was considered statistically significant. For Western blots, band intensities for tissue from the vIPAG and superior colliculus
were visualized at 55kD (GFAP) and 160kD (CD11b), and quantified using ImageJ (NIH, USA) analysis software, as a relative intensity of GFAP or CD11b band divided by the intensity of the β-actin band. Data are expressed as the mean ratio ± S.E.M of protein of interest/β-actin. Data were analyzed for significant main effects of treatment (morphine, saline) and pain (CFA, handled) using an ANOVA, and Fisher’s HSD was used for post-hoc analysis; p < 0.05 was considered statistically significant.

**Results**

**Experiment 1: Persistent Peripheral Inflammation Attenuated Morphine Tolerance**

To assess the initial analgesic potency of morphine, and the degree and time course for development of morphine tolerance, paw withdrawal latencies (PWL) were determined for both the injured (ipsilateral; right) and uninjured (contralateral; left) hindpaws at baseline, and after Day 1 and Day 3 of morphine or saline. Contralateral (uninjured) PWL did not differ between CFA+Saline and Handled+Saline groups at any time point (Figure 2.1A). By contrast, intraplantar CFA significantly decreased ipsilateral PWL 24, 48, and 96 hours post injection as compared with handled controls (CFA+Saline versus Handled+Saline; Figure 2.1B) indicating the development of persistent hyperalgesia. Administration of morphine on Day 1 and 3 significantly increased both contralateral and ipsilateral PWLs as compared with saline controls (Figure 2.1A and B, respectively). For the contralateral paw, administration of morphine (Day 1) produced comparable levels of analgesia in the CFA+Morphine and Handled+Morphine groups (Figure 2.1A). However, the degree of analgesia produced by morphine on Day 3 was significantly attenuated in the Handled+Morphine vs. the CFA+Morphine animals, suggesting the development of morphine tolerance (Figure 2.1A). In the ipsilateral hindpaw, administration of morphine on Day 1 produced anti-hyperalgesia in CFA treated animals as indicated by a
return to normal baseline PWL (CFA+Morphine; Handled+Saline, Figure 2.1B). In contrast to the decreased analgesia observed in Handled+Morphine animals on Day 3, morphine produced a significant increase in ipsilateral PWL of injured animals on Day 3 as compared to Day 1 (CFA+Morphine; Injection 1 and Injection 3, Figure 2.1B) indicating lack of tolerance development.

Assessment of morphine tolerance

Morphine tolerance, assessed on Day 5 using a cumulative dosing paradigm, was only observed in non-CFA treated animals. As shown in Figure 2.2, the antinociceptive potency of morphine was significantly decreased in both the ipsilateral and contralateral hindpaw of uninjured animals that received 3 consecutive days of morphine (Handled+Morphine) as compared with uninjured animals that received saline (Handled+Saline; Figures 2.2A and C). Indeed, animals that received 3 days of saline reached 100% maximum possible analgesia (MPE) at the 8 mg/kg dose. In contrast, 100% MPE was not noted until the 18 mg/kg dose in animals that received 3 prior days of morphine. Neither the antinociceptive nor the antihyperalgesic potency of morphine was different in CFA+Morphine treated animals as compared with CFA+Saline treated animals (Figures 2.2B and D) indicating lack of tolerance development. Indeed, no differences in PWLs produced by morphine were noted for all doses tested. Together these data indicate that persistent inflammatory pain attenuates the development of tolerance to morphine.

As glia activation in the spinal cord has been implicated in the development of morphine tolerance, the next series of experiments examined if glia were similarly activated within the vlPAG, and if persistent inflammatory pain altered their activation.

Experiment 2: Morphine Tolerance is Associated with Increased Glial Cell Activation in the vlPAG
Increased activity of astrocytes, as evidenced by an increase in GFAP immunoreactivity, was only observed in non-CFA treated animals that received morphine (Handled+Morphine, Figure 2.3B). A representative example of vlPAG GFAP staining in animals administered CFA+Morphine versus Handled+Morphine is shown in Figure 2.3A. Western blots confirmed increased activity of astrocytes, as evidenced by an increase in relative band intensity of GFAP/b-actin, was only observed in non-injured animals that received morphine (Handled+Morphine, Figure 2.3C).

Similar to what was noted for astrocytes, microglia activity, as evidenced by OX-42 immunoreactivity, was significantly increased in animals that received morphine in the absence of pain (Handled+Morphine, Figure 2.4B). A representative example of vlPAG OX-42 staining in animals administered CFA+Morphine versus Handled+Morphine is shown in Figure 2.4A. A trend towards increased activity of microglia, as evidenced by an increase in relative band intensity of OX-42/b-actin, was only observed in animals that received morphine in the absence of pain (Handled+Morphine, Figure 2.4C); however, it did not reach statistical significance.

Peripheral inflammation induced by intraplantar CFA did not illicit significant increases in vlPAG glial cell activity (Figures 2.3 and 2.4). Importantly, one injection of morphine (5 mg/kg) was not sufficient to alter vlPAG GFAP (Figure 2.5A) or OX-42 levels (Figure 2.5B) at 24 hours post-morphine. Similarly, no increase in GFAP or OX-42 levels were noted 1 hour post-morphine (data not shown). No significant group differences were noted in superior colliculus GFAP or OX-42 immunoreactivity ($F_{3,23}=2.089$, $p=0.1295$ and $F_{3,10}=1.416, p=0.2690$, respectively) or protein level ($F_{3,28}=0.232$, $p=0.8730$ and $F_{3,6}=1.822$, $p=0.2435$, respectively), indicating that changes in vlPAG glial cell activity are region specific (data not shown).


**Discussion**

The present experiments tested the hypothesis that vlPAG glial cell activity contributes to the development of morphine tolerance. Clinical studies indicate that chronic pain attenuates the development of morphine tolerance\(^3,132\); however, animal studies have yielded variable results\(^133-135\). Therefore, the impact of persistent inflammatory hyperalgesia on morphine tolerance development and glial activation were also investigated. Here we report that (1) short-term daily administration of an ED\(_{50}\) dose of morphine was sufficient to induce morphine tolerance; (2) persistent inflammatory pain induced by intraplantar CFA significantly attenuated morphine tolerance; and (3) increased vlPAG microglia and astrocyte activity was only observed in those animals made tolerant to morphine. Together, these data suggest a potential role for vlPAG microglia and astrocytes in the development of morphine tolerance, and suggest that persistent inflammatory pain attenuates morphine tolerance by inhibiting morphine-induced vlPAG glial cell activation.

*Increases in vlPAG microglia and astrocyte activity correlate with the development of morphine tolerance*

Many mechanisms have been proposed to account for opioid tolerance, including decoupling, internalization and/or down-regulation of m opioid receptors\(^136,137\), upregulation of NMDA receptor function\(^138-140\), down-regulation of glutamate transporters\(^141,142\), and production of nitric oxide (a known mediator of NMDAR function)\(^85\). These mechanisms were all thought to implicate some form of ‘neuronal adaptation’\(^85\). However, it is becoming increasingly clear that activated glia mediate many of these ‘neuronal adaptations’ that contribute to morphine tolerance\(^85\). Consistent with previous reports, here we find that tolerance to morphine developed rapidly in the absence of pain\(^24,44,45\). Indeed, one ED\(_{50}\) dose of morphine (5 mg/kg) injected
subcutaneously for three days was sufficient to induce behaviorally defined tolerance. Paralleling the development of tolerance, GFAP and OX-42 protein levels increased significantly within the vlPAG, suggesting the activation of astrocytes and microglia, respectively. A large body of evidence has accumulated implicating opioids as activators of spinal astrocytes and microglia\textsuperscript{86,87,89,119}. In both mice and rats, morphine increases spinal GFAP and OX-42 protein levels\textsuperscript{107,143}, as well as glially derived proinflammatory cytokines\textsuperscript{74,87}. Inhibition of spinal glia or cytokine release increases the analgesic efficacy of morphine\textsuperscript{74,87}, and attenuates morphine tolerance\textsuperscript{72,86,91,92,119}. Our novel findings in the vlPAG parallel the data from studies of spinal cord glia, and indicate that supraspinal glial cell activity may also contribute to the development of morphine tolerance.

Under basal conditions glia survey the environment for pathogens, debris, and regulate ion and neurotransmitter levels in the synapse to modulate neuronal excitability\textsuperscript{89}. The activation of glia results in the release of excitatory substances that oppose morphine analgesia (e.g., proinflammatory cytokines)\textsuperscript{89}. Glial release of cytokines increases with chronicity of morphine administration\textsuperscript{85}, making these excitatory substances key players in the development of morphine tolerance. Glially-derived cytokine release, particularly tumor necrosis factor alpha (TNFa) and interleukin-1 beta (IL-1b), results in increased density and conductance of neuronal AMPA\textsuperscript{144,145} and NMDA\textsuperscript{146} receptors, decreased astrocytic glutamate transporter proteins (GLT-1, GLAST)\textsuperscript{89}, and down-regulation of neuronal GABA receptors\textsuperscript{145}. These cytokine-induced changes, among others\textsuperscript{147,148}, effectively increase neuronal excitability. Morphine binds to neuronal m opioid receptors (MOR) in the vlPAG that are primarily located on GABAergic neurons\textsuperscript{149-151}; MOR binding in the vlPAG disinhibits GABAergic PAG-RVM projection neurons\textsuperscript{152}, resulting in the net excitation of the PAG-RVM-spinal cord descending pain modulatory circuit. Glial-induced
increases in the excitability of vIPAG MOR-containing neurons may act to alter the inhibitory properties of morphine, thereby decreasing analgesic efficacy and contributing to the development of morphine tolerance.

*Persistent inflammatory pain prevented morphine-induced increases in vIPAG glial cell activity and attenuated the development of morphine tolerance*

The results of the present study demonstrate that the presence of persistent pain alters both the development of morphine tolerance and morphine-induced vIPAG glial cell activation. The finding that persistent peripheral inflammation attenuates morphine tolerance is consistent with the clinical literature demonstrating that opioid tolerance is attenuated in chronic pain sufferers. Indeed, clinical studies have repeatedly shown that morphine tolerance develops most robustly in those individuals consuming morphine in the absence of pain. In the present study, male rats given CFA 24 hours before the 3-day morphine administration regimen showed significant increases in analgesia to all challenge doses of morphine, as compared with non-injured animals. Several factors may contribute to the pain-induced attenuation in morphine tolerance. First, morphine, given in conjunction with peripheral inflammation, failed to illicit the increases in vIPAG microglia and astrocyte activity observed in non-CFA treated animals given morphine. Indeed, peripheral inflammatory pain blocked both morphine tolerance and morphine-induced glial cell activation within the vIPAG. As glia are not activated, no cytokine release would be expected, and, therefore, no net change in neuronal excitability. Alternatively, cannabinoids, which are released within the PAG during peripheral pain, have been shown to influence both glial activity and morphine analgesia. Cannabinoid receptors are robustly expressed within the vIPAG, with approximately 32% of cannabinoid receptor 1 (CB1) expressing neurons also expressing μ opioid receptor. Functionally, intra-PAG administration
of a CB1 agonist enhances morphine analgesia\textsuperscript{158}, and systemic administration of cannabinoids, along with morphine, leads to the attenuation of morphine tolerance\textsuperscript{158-160}. Endocannabinoids also possess potent anti-inflammatory properties\textsuperscript{161}, which would likely block the activation of glia. Indeed, systemic administration of the cannabinoid receptor agonist WIN 55,212-2 prevents microglia and astrocyte activation, and decreases the release of the proinflammatory cytokines interleukin (IL)-1\beta, IL-6 and tumor necrosis factor (TNF)-\alpha in the spinal cord\textsuperscript{162}.

In the present study, no glia activation was noted following administration of CFA alone. These results are in contrast with previous reports that peripheral pain, including CFA\textsuperscript{163-166}, peripheral neuropathy\textsuperscript{71,90,94,127,165,167,168}, formalin\textsuperscript{169}, and spinal nerve ligation\textsuperscript{170} induce significant glial activation within the spinal cord. However, given the roles of the spinal cord and PAG in pain facilitation and pain modulation, respectively, it is not entirely surprising that there would be differential pain-induced regulation of glial activation in these two sites. Together, these studies suggest that inflammation elicits differential glial responses in a CNS region-dependent manner, and prevents morphine-induced increases in vPAG glial cell activity.

\textit{How opioids activate glia}

Opioid hyperalgesia is still observed in neuronal opioid receptor (mu, delta, and kappa) knockout mice\textsuperscript{98}, suggesting that the anti-analgesic affects of morphine (e.g., anti-analgesia and tolerance) are mediated by non-neuronal opioid receptors. Indeed, it was recently discovered that morphine analgesia is modulated not only by classical neuronal opioid receptors but also by non-classical glial receptor activity\textsuperscript{78}. Opioids have been shown to bind to Toll-like receptor 4 (TLR4)\textsuperscript{78}, an innate immune receptor located on microglia and astrocytes, and an abundance of evidence has accumulated indicating that TLR4 activity opposes morphine analgesia\textsuperscript{78,89}. Functionally, animals that receive TLR4 antagonism, as well as TLR4 knockout mice, exhibit increased
responsiveness to the analgesic properties of acute morphine administration. Similarly, systemic administration of TLR4 antagonists attenuates morphine tolerance. To date, the specific role of TLR4 in morphine tolerance development has not been elucidated. However, given our findings that the development of morphine tolerance correlates with increased vlPAG glial cell activation, and the evidence showing that TLR4 is expressed on rat PAG glia, future studies investigating the potential role of vlPAG TLR4 in the development of morphine tolerance are warranted.

Conclusions

There is extensive literature supporting a critical role for glial cell activation in the development of morphine tolerance. Our findings that increased vlPAG glial activity is concurrent with the development of morphine tolerance, and that pain inhibits both vlPAG glial reactivity and morphine tolerance development suggests that vlPAG glia play a significant role in the development of morphine tolerance. Taken together, our results may provide a direct neurobiological mechanism whereby chronic inflammatory pain attenuates the development of morphine tolerance, and implicate vlPAG glial cells as key regulators of this phenomenon.

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Figure 2.1 Summary of results from morphine analgesia testing on day 1 and day 3.
Contralateral (A) and ipsilateral (B) PWL (in seconds) following intraplantar CFA or handling (Baseline), and after the first and third injection of morphine or saline in CFA+Morphine (n=6), CFA+Saline (n=7), Handled+Morphine (n=6), and Handled+Saline (n=5) treated male rats. The first and third injection of morphine caused an increase in contralateral and ipsilateral PWL as compared with saline controls (p< 0.05; A & B, respectively). Contralateral PWL did not differ between CFA+Saline and Handled+Saline groups at any time point (p> 0.05; A). CFA treatment caused a significant decrease in ipsilateral PWL at all time points as compared with handled controls (p< 0.05; CFA+Saline; Handled+Saline; B). While uninjured animals treated with morphine showed a decrease in analgesia to the third injection as compared with the first (p< 0.05; A), CFA treated animals showed an increase in antihyperalgesia to the third injection (p< 0.05; B). Asterisks indicate significant differences between CFA+Morphine and Handled+Morphine groups.
Figure 2.2 Summary of results from morphine tolerance testing on day 4.
PWL (in seconds) as a function of cumulative doses of morphine in handled (A & C), and CFA treated (B & D) male rats. Both ipsilateral (A & B) and contralateral (C & D) PWL data are presented. Animals received 3 consecutive days of morphine (5 mg/kg; sc, open circles) or saline (1 ml/kg; sc, filled circles). CFA+Morphine treated animals (n=7) did not differ from CFA+Saline treated animals (n= 4) in response to cumulative morphine on day 5 (ipsilateral; $F_{1,9}=1.128, p=0.3159$ & contralateral; $F_{1,9}=1.470, p=0.2563$). Handled+Morphine treated animals (n= 9) showed a significant decrease in PWL in response to cumulative morphine on day 5 as compared with Handled+Saline animals (n=5; ipsilateral; $F_{1,12}=21.702, p=0.0006$ & contralateral; $F_{1,12}=20.373, p=0.0007$).
Figure 2.3 Summary of vlPAG GFAP protein following morphine tolerance. Representative fluorescent photomicrographs of GFAP immunoreactivity in the vlPAG of animals treated with CFA+Morphine (a) and Handled+Morphine (b) (A). Densitometry of GFAP immunoreactivity in the vlPAG (B). Administration of morphine, in the absence of CFA (Handled+Morphine; n=7), resulted in a significant increase in GFAP immunoreactivity within the vlPAG (F3,22=10.022, p=0.0002). No differences in GFAP levels were noted for the CFA+Morphine (n=11), CFA+Saline (n=4) or Handled+Saline control groups (n=4). Relative band intensity of GFAP/β-actin in the vlPAG (C). Administration of morphine, in the absence of CFA (Handled+Morphine; n=5), resulted in a significant increase in relative band intensity of GFAP/β-actin in the vlPAG (F3,19=10.256, p=0.0003). No differences in GFAP levels were noted for the CFA+Morphine (n=5), CFA+Saline (n=7) or Handled+Saline (n=6) control groups.
Figure 2.4 Summary of vlPAG OX-42 following morphine tolerance.
Representative fluorescent photomicrographs of OX-42 immunoreactivity in the vlPAG of animals treated with CFA+Morphine (a) and Handled+Morphine (b) (A). Densitometry of OX-42 immunoreactivity in the vlPAG (B). Administration of morphine, in the absence of CFA (Handled+Morphine; n=8), significantly increased OX-42 immunoreactivity within the vlPAG (F$_{3,19}$=9.270, p=0.0005). No differences in OX-42 levels were noted for the CFA+Morphine (n=7), CFA+Saline (n=4) or Handled+Saline (n=4) control groups. Relative band intensity of OX-42/b-actin in the vlPAG (C). Administration of morphine, in the absence of CFA (Handled+Morphine; n=5), resulted in an increase in relative band intensity of OX-42/b-actin in the vlPAG (F$_{3,10}$=2.544, p=0.1151); however, it did not reach significance.
Figure 2.5 Summary of GFAP and OX-42 protein in the vlPAG following acute morphine.

Densitometry of GFAP (A) and OX-42 (B) immunoreactivity in the vlPAG in Handled+Morphine (n=3), CFA+Morphine (n=4), Handled+Saline (n=4), and CFA+Saline (n=5) animals 24 hours following one morphine or saline injection. Neither CFA nor morphine (5 mg/kg, sc) increased vlPAG GFAP ($F_{(3,12)}$=0.494; $p=0.693$) or OX-42 ($F_{(3,12)}$=0.162; $p=0.9198$) levels in the vlPAG as compared to handled and saline controls.
CHAPTER 3: Blockade of Toll-like Receptor 4 Attenuates Morphine Tolerance and Facilitates the Pain Relieving Properties of Morphine

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Abstract

The ventrolateral periaqueductal gray (vlPAG) is an integral locus for morphine action. Although it is clear that glia contribute to the development of morphine tolerance, to date, the investigation of their role has been limited to spinal and medullary loci. Opioids induce a neuroinflammatory response that opposes acute and long-term analgesia, thereby limiting their efficacy as therapeutic agents. Recent data suggest that the innate immune receptor Toll-like receptor 4 (TLR4), along with its co-receptor myeloid differentiation factor-2 (MD-2), mediates these effects. To date, the brain loci through which TLR4 modulates morphine tolerance have not been identified. We have previously demonstrated that chronic subcutaneous morphine results in tolerance that is accompanied by increases in vlPAG glial cell activity. Using in vivo pharmacological manipulations of vlPAG glia and TLR4 in the adult male rat, we show that intra-vlPAG administration of the general glial cell metabolic inhibitor propentofylline or the astrocyte activity inhibitor fluorocitrate attenuate tolerance to morphine. Characterization of MD-2 expression within the PAG revealed dense MD-2 expression throughout the vlPAG. Further, antagonizing vlPAG TLR4 dose-dependently prevented the development of morphine tolerance, and vlPAG microinjections of TLR4 agonists dose-dependently produced a ‘naïve’ tolerance to subsequent challenge doses of morphine. Finally, using a model of persistent inflammatory pain and pharmacological manipulation of TLR4 we demonstrate that systemic antagonism of TLR4 potentiated acute morphine antihyperalgesia. These results, together, indicate that vlPAG glia regulate morphine tolerance development via TLR4 signaling, and implicate TLR4 as a potential therapeutic target for the treatment of pain.
Introduction

Opioids remain an integral part of clinical pain management. However, acute and chronic morphine induces a CNS proinflammatory glial response that actively opposes the analgesic effects of morphine, and contributes to the development of tolerance. Morphine-induced neuroinflammation is evidenced by increases in spinal microglial and astrocyte activity markers OX-42 and glial fibrillary acidic protein (GFAP), respectively. Release of glially derived proinflammatory cytokines, hallmarks of neuroinflammation, is also induced by morphine. Intrathecal glia inhibitors (e.g., propentofylline) decrease morphine-induced cytokine release, and attenuate morphine tolerance. Similarly, blockade of spinal cytokine action attenuates tolerance, indicating that CNS glia modulate morphine action.

Opioids, including morphine, bind to myeloid differentiation factor-2 (MD-2) of the innate immune receptor toll-like receptor 4 (TLR4), leading to initiation of the TLR4 signaling cascade that results in a proinflammatory response. TLR4 is found primarily on microglia, and to a lesser degree on astrocytes. Spinal TLR4 activity opposes the acute effects of morphine, including antinociception, and contributes to opioid-induced hyperalgesia. Unlike classical opioid receptors, which only bind the (-)-stereoisomer of opioids, TLR4 binds opioids in a non-stereoselective fashion with both (-)- and (+)-ligands affecting the signaling cascade and modulating opioid analgesia. Indeed, (+)-morphine decreases acute intrathecal (-)-morphine analgesia. Similarly, in vitro studies show that both (-) and (+)-naloxone block (-)-morphine-induced TLR4 activation. Although systemic antagonism of TLR4 prevents the development of tolerance to systemic morphine, the brain loci through which TLR4 mediates morphine tolerance have not been investigated.
The PAG, and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord, comprises an essential neural circuit for opioid-mediated analgesia. Tolerance is quick to develop following repeated administration of morphine into the ventrolateral PAG (vlPAG). Blocking opioid binding in the vlPAG with the antagonist naltrexone significantly attenuates the development of tolerance to systemically administered morphine, indicating that key mechanisms underlying morphine tolerance are localized in the vlPAG.

Although it is clear that CNS activation of glia contribute to the development of morphine tolerance, no studies to date have examined the role of activated glia within the PAG, despite extensive evidence indicating its importance for morphine action.

Here we tested the hypothesis that vlPAG glia activation contributes to morphine tolerance development through action at TLR4. As morphine is primarily consumed for the alleviation of severe pain, in our final experiments we used an animal model of persistent inflammatory pain to test the hypothesis that TLR4 blockade would enhance the antihyperalgesia effects of acute morphine.

**Materials and Methods**

**Subjects**

Weight-matched (250-350g) male Sprague Dawley rats (Charles River; Wilmington, MA) were pair-housed on a 12:12 hour light:dark cycle (lights on a 7:00 am). Access to food and water was available *ad libitum* throughout the experiments except during behavioral testing. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia State University, and performed in strict compliance with Ethical Issues of the International Association for the Study of Pain (IASP) and National Institute of Health (NIH). All efforts were
made to reduce the number of animals used in these experiments and to minimize any possible suffering by the animal.

*Intra-vlPAG Cannulae Implantation*

Animals in experiments 1, 2.2, & 2.3 were anesthetized to a deep surgical plane with 5% Isoflurane (Baxter Healthcare Corporation; Deerfield, IL) and maintained at 2-5% Isoflurane throughout surgery. Animals were implanted with bilateral guide cannulae (22 gauge; Plastics One; Roanoke, VA) aimed at the ventrolateral PAG (AP: 1.7 mm, ML: ± 0.6 mm, DV: -5.0 mm from lambda) using stereotaxic techniques as previously described\textsuperscript{114}. Skull screws and dental acrylic were applied to hold cannulae securely in place. Skin staples were used to close the site, and animals were given 0.05 ml Baytril (sc) to prevent infection. Injection cannulae were inserted into guide cannulae once every 24 hours to acclimate the animals to the injection procedure and maintain cannulae patency. As repeated insertion of the injection cannulae has the potential to cause tissue damage-induced glial activation, we compared OX-42 and GFAP staining levels in the vlPAG for animals treated with vlPAG saline and animals that were treated with sc saline\textsuperscript{173} and found no differences in expression (data not shown).

*Experiment 1.1: Influence of vlPAG glial cell activation on morphine tolerance development*

Following recovery from cannulae implantation (1 week) animals were given morphine (5 mg/kg; sc, NIDA; Bethesda, MD) once a day for three days to induce behavioral tolerance to challenge doses of morphine. The 5 mg/kg dose was chosen based on our previous studies demonstrating this to be the 50% effective dose (ED\textsubscript{50}) for systemic morphine in male rats\textsuperscript{122,124,125}. The time course of morphine injection was chosen based on our previous data demonstrating this to be sufficient for the induction of morphine tolerance\textsuperscript{45,173}. Control animals received sterile saline (1 ml/kg; sc) once a day for three days.
**Glia Inhibition**

Separate groups of animals were treated with one of 3 glia inhibitors: the general glial cell inhibitor propentofylline (10 fmol or 100 fmol, SIGMA; St. Louis, MO), the microglia inhibitor minocycline (10 fmol, 1 pmol or 10 pmol; SIGMA), or the astrocyte inhibitor fluorocitrate (1 fmol or 10 pmol; SIGMA). Glia inhibitors were administered 16 hrs. and 1 hr. prior to the first morphine injection and 30 min. prior to the last two morphine injections based on the injection protocols of Cui, Wei, Tawfik and colleagues^95,96,176^ Previous studies suggest that preemptive doses (i.e., the 16 and 1 hour doses) of these inhibitors are necessary to counteract morphine-induced glial cell activation^95,176^. The 30-minute time point was chosen based on previous work using minocycline^96^. All doses were chosen based on intracranial doses known to affect glia without affecting neurons, and known to have no effect on the microglia and astrocyte activity of naïve animals^176^. All drugs were administered bilaterally (0.25 ml/ side/ 2min). Control animals received intra-vlPAG microinjections of vehicle (sterile saline; 0.25 µl/side). Microinjections were slowly infused through a 5 ml Hamilton Syringe at a rate of 0.125 ml/minute using a Harvard Apparatus 11 Plus Syringe Pump (Holliston, MA) to allow for diffusion of the drug and to minimize tissue damage. Injection cannulae were left in place for 60 seconds following the microinjections to prevent backflow upon removal.

**Morphine challenge**

Tolerance was assessed on day 5 (day 1 being the first day of vlPAG microinfusions) by injecting cumulative doses of morphine every 20 min, resulting in doses of 3.2, 5.6, 8.0, and 10.0 mg/kg (sc) as previously described^45,173^. Nociception was assessed using the paw thermal stimulator 15 min after each injection^122,126^. Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. A radiant beam of light is
positioned under the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL). A maximal PWL of 20.48 seconds was used to prevent excess tissue damage due to repeated application of the noxious thermal stimulus. Animals were acclimated to the testing apparatus (30 minutes a day for 3 consecutive days) at the start of the experiment. All behavioral testing took place between 12:00 pm and 5:00 pm (lights on at 7:00 am). All testing was conducted blind with respect to group assignment.

Data Analysis and Presentation

PWLs are measured in seconds and the half-maximal antinociceptive effect (D$_{50}$) and 95% confidence intervals (CI) were calculated from dose-response curves generated using GraphPad software$^{43,45}$. The lower limit for calculating D$_{50}$ values was the mean baseline score, and the upper limit was the mean paw withdrawal latency (PWL) following administration of the highest morphine dose. Changes in D$_{50}$ between groups were assessed using ANOVA, and Fisher’s post-hoc tests were used to determine specific group differences when a significant main effect was observed. All values are reported as Mean D$_{50}$ ± 95% CI; p<0.050 was considered significant.

Experiment 1.2: Anatomical Assessment of vlPAG Glial Cell Inhibition

One hour following tolerance assessment, animals were given a lethal dose of Nembutal (160 mg/kg; i.p.) and transcardially perfused with 250 ml of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator to remove blood from the brain. Immediately following blood removal, 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5% acrolein (Polysciences Inc.; Warrington, PA) was perfused through the brain as a fixative. A final rinse with 250 ml of sodium chloride/sodium nitrite solution was perfused through the brain to remove
any residual acrolein. Brains were removed and placed in a 30% sucrose solution and stored at 4°C until sectioning.

**Cannulae Placement Verification**

Perfusion fixed brains were sectioned into 25 µm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution at -20°C. A 1:6 series through the rostrocaudal axis of each brain was Nissl stained, coverslipped, and cannulae placement was verified visually using a Nikon microscope (10X magnification). Animals with bilateral cannulae located outside of the vlPAG (e.g., deep mesencephalic nucleus (DpMe)) were considered “cannulae misses” and were included for analysis for site specificity.

**Immunohistochemistry**

As a positive control that propentofylline, minocycline, and fluorocitrate inhibited microglial and astrocyte activity, immunoreactivity of vlPAG glial cell activity markers were analyzed. Hallmarks of glial cell activity include increased cytokine release that correlates with increased expression of the protein markers CD-11b (OX-42; microglia), and glial fibrillary acidic protein (GFAP; astrocytes)\(^{86}\). Further, increased glial cell activity is evidenced by a profound shift in morphology that can be easily visualized using immunohistochemistry for OX-42 and GFAP\(^{127}\). Perfused brains were sectioned into 25 µm coronal sections with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution\(^{128}\) at -20°C. A 1:6 series through the rostrocaudal axis of each brain was processed for OX-42 and GFAP immunoreactivity using standard immunohistochemical techniques\(^{129}\). Briefly, sections were rinsed extensively in potassium phosphate-buffered saline (KPBS) immediately followed by a 20-minute incubation in 1% sodium borohydride. The tissue was then incubated in primary antibody solution (mouse anti-CD11b (OX-42) 1:3000, Serotec; Raleigh, NC or rabbit anti-
GFAP 1:5,000; Abcam; Cambridge, MA) in KPBS containing 1.0% Triton-X for one hour at room temperature followed by 48 hours at 4°C. After rinsing with KPBS, the tissue was incubated for one hour in secondary antibody (biotinylated IgG goat anti-mouse or anti-rabbit; 1:600, Jackson ImmunoResearch; West Grove, PA), rinsed with KPBS, and then incubated for one hour in an avidin-biotin peroxidase complex (1:10; ABC Elite Kit; Vector Labs; Burlingame, CA). After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), OX-42 or GFAP immunoreactivity was visualized as a black reaction product using nickel sulfate intensified 3,3’-diaminobenzidine (DAB) solution (2 mg/10 ml) containing 0.08% hydrogen peroxide in sodium acetate buffer. After 15 minutes, tissue was rinsed in sodium acetate buffer followed by KPBS. Following secondary incubation, sections were rinsed in KPBS. Sections were mounted out of KPBS onto gelatin-subbed slides, air-dried and dehydrated in a series of graded alcohols. Tissue-mounted slides were then cleared in Xylenes and glass cover-slipped using Permunt.

Anatomical data analysis and presentation

Levels of OX-42 and GFAP immunoreactivity in the vlPAG were compared across treatment groups using semi-quantitative densitometry as previously described. 45,131,173 12-bit grayscale images that included the region of interest (ROI) were captured using QImaging Retiga EXi CCD camera (Surrey, BC, Canada) attached to a Nikon microscope and iVision Image analysis software (Biovision Technologies; Exton, PA). Grayscale values for each image were inverted so that higher values were representative of increased staining levels. Images of three slices through each ROI for each animal were analyzed and data sampled unilaterally. Data sampling occurred by using the drawing tools in iVision to outline the ROI and using the “measure” function to determine an average grayscale pixel value for the outlined area. ROI measures were corrected for nonspecific binding by subtraction of background measure taken from gray matter adjacent to
the ROI. Data were analyzed across 3 representative levels through the rostral-caudal axis of the vlPAG (Bregma -7.08, -7.68, and -8.30) as previously described\textsuperscript{45,130}. Densitometry values are presented as the mean ± S.E.M. density of immunoreactivity. Data were analyzed using an ANOVA to determine significant main effects of treatment. Fisher’s post hoc tests were used to determine specific group differences when a significant main effect was observed; \( p \leq 0.050 \) was considered statistically significant.

**Experiment 2: Modulation of Morphine Tolerance by vlPAG TLR4**

The TLR4/MD-2 complex binds opioids including morphine and mediates glial cell activation\textsuperscript{73,78,79,89}. While TLR4 signaling has been implicated in glially-mediated morphine opposition, the specific role of TLR4 activation in the development of morphine tolerance is largely unknown. Our overarching hypothesis is that morphine activation of TLR4 within the vlPAG is a primary mechanism underlying the development of tolerance.

**Experiment 2.1: Myeloid Differentiation Factor-2 expression within the PAG**

**Immunohistochemistry**

Normal male Sprague Dawley rats were perfused as described above. A 1:6 series through the rostral caudal axis (Bregma -6.72, -7.08, -7.68, -8.04, -8.28, and -8.76) of the PAG was processed for immunohistochemical labeling of MD-2 as a positive control for the presence of the MD-2/TLR4 complex. Both the dorsolateral (dlPAG) and vlPAG were examined. The dlPAG (Bregma -6.72, -7.08, -7.68, and -8.04) was included as this region is virtually devoid of the mu opioid receptor (MOR), expresses very little morphine-induced Fos, and has not been implicated in morphine tolerance development\textsuperscript{51,125}. Immunohistochemistry is as described above with the exception of the primary (rabbit anti-MD-2 primary antibody; 1:200; Abcam) and secondary (biotinylated IgG goat anti-rabbit secondary; 1:600, Jackson ImmunoResearch) antibodies.
Twelve-bit grayscale images that included the region of interest (ROI) were captured using a QImaging Retiga EXi CCD camera and iVision Image analysis software to visualize MD-2 expression within the PAG. Anatomical data analysis and presentation are as described above, except here we sampled 2 sections per animal per Bregma level.

Experiment 2.2: Necessity of vlPAG TLR4 in morphine tolerance development

Following recovery from cannulae implantation (1 week) animals were given morphine (5 mg/kg; sc; NIDA) once a day for three consecutive days to induce behavioral tolerance to a challenge dose of morphine. Control animals received saline (1 ml/kg; sc) once a day for three consecutive days. Immediately following sc injections, animals received bilateral intra-vlPAG microinjections of the competitive TLR4 antagonist LPS-RS (1.7 µg or 2.4 µg /0.5 µl/side; Invivogen; San Diego, CA), the stereoselective TLR4 antagonist (+)-naloxone (5 µg/0.5 µl/side; NIDA) or saline (0.5 µl/side; as a vehicle control). LPS-RS (produced by *R. sphaeroides*) was chosen as a TLR4 antagonist as it has been shown to competitively bind to TLR4, enhance morphine analgesia, prevent release of cytokines, and block the morphine-induced TLR4 signaling cascade. The LPS-RS dose was chosen based on intracranial LPS doses, and the fact that LPS and LPS-RS are typically administered at the same dose. (+)-naloxone was chosen as this TLR4 antagonist readily crosses the blood brain barrier, and has been shown to enhance morphine analgesia and block the morphine-induced TLR4 signaling cascade. The (+)-naloxone dose was chosen based on intra-PAG doses of (-)-naloxone and the fact that (-)- and (+)-naloxone block (-)-morphine-induced TLR4 signaling to a similar degree, and on a similar time course. Tolerance assessment took place 24 hours following 3 days of drug administration as described above. Briefly, animals were given cumulative doses of sc morphine in the absence of intra-vlPAG microinjections and PWLs were measured in response to a
noxious thermal stimulus. Following tolerance assessment animals were perfused and brains removed and sectioned for visualization of cannulae location. To determine site specificity, animals with bilateral cannulae located outside of the vlPAG (e.g., DpMe) were included in the analysis. Data analysis and presentation are as described above.

Experiment 2.3: Sufficiency of vlPAG TLR4 activation to induce ‘naïve’ tolerance to morphine
Experimental methods are identical to Experiment 2.2. To test for the sufficiency of vlPAG TLR4 to induce tolerance, a separate group of animals received bilateral intra-vlPAG injections of the prototypical TLR4 agonist LPS (1.7 µg or 5 µg /0.5 µl/side; Sigma, St. Louis, MO), the TLR4 agonist KDO2 (0.5 µg, 1 µg, or 5µg/0.5µl/side; Avanti Polar Lipids), the stereoselective TLR4 agonist (+)-morphine (5 µg/0.5 µl/side; NIDA), or vehicle control (saline; 0.5 µl/side) once a day for three consecutive days. Animals in this experiment did not receive sc (-)-morphine until the final testing day (morphine challenge). LPS is a prototypical TLR4 agonist and has been shown to competitively bind to TLR4, resulting in the production of cytokines, and attenuation of acute morphine analgesia. The chosen LPS dose was based on intracranial LPS doses that are sufficient to induce glial activation and cytokine expression. Similar doses of KDO2-Lipid A were chosen because KDO2 is a substructure of LPS with endotoxin activity that is equal to that of native LPS. Finally, the (+)-morphine dose was chosen based on vlPAG doses of (−)-morphine, and the fact that (+)- and (−)-morphine activate the TLR4 signaling cascade to a similar degree in vitro. Twenty-four hours following intra-vlPAG injections of TLR4 agonists, animals were given cumulative challenge doses of sc morphine and PWLs were tested to assess tolerance development as described above. Data analysis and presentation are as described above.

Experiment 3: Influence of TLR4 on acute morphine antihyperalgesia
Morphine is primarily consumed for the alleviation of severe pain, and chronic morphine use is associated with tolerance development. Therefore, our final series of experiments were conducted to determine if blockade of vIPAG TLR4 with (+)-naloxone would potentiate the antihyperalgesic effect of morphine in a model of persistent inflammatory pain.

**Persistent Inflammatory Hyperalgesia**

In a separate group of animals, persistent inflammatory hyperalgesia was induced by injection of complete Freund’s adjuvant (CFA; 200 µl; Sigma; St. Louis, MO), suspended in an oil/saline (1:1) emulsion, into the plantar surface of the right hindpaw as previously described. Twenty-four hours following intraplantar CFA injection, acute antihyperalgesia was measured by injecting cumulative doses of morphine every 20 min, resulting in doses of 3.2, 5.6, 8.0, and 10.0 and 18.0 mg/kg (sc; NIDA). Hyperalgesia was assessed using the paw thermal stimulator at baseline, and 15 min after each injection, as described above. Animals received one sc injection of (+)-naloxone (8 mg/kg, sc; NIDA) or saline (1 ml/kg, sc) with the first dose of morphine to produce 2 treatment groups ((+)-Naloxone/Morphine; Vehicle/Morphine). A separate group of animals received repeated injections of saline on the same time-course as the cumulative morphine doses to determine if (+)-naloxone induces antihyperalgesia in the absence of morphine ((+)-Naloxone/Vehicle). The 8 mg/kg dose of (+)-naloxone was chosen based on previous studies demonstrating it to be sufficient to potentiate acute morphine analgesia in the absence of pain. Animals were acclimated to the testing apparatus (30 minutes a day for 3 consecutive days) at the start of the experiment. All behavioral testing took place between 12:00pm and 5:00pm (lights on at 7:00am). All testing was conducted blind with respect to group assignment. Data analysis and presentation are as described above.
Results

Experiment 1: Inhibition of vlPAG Microglia and Astrocyte Activity Attenuated Morphine Tolerance: vlPAG infusions of propentofylline attenuated morphine tolerance.

Administration of cumulative doses of morphine produced an increase in paw withdrawal latency (PWL) in all animals tested (Figure 3.1A). An analysis of variance revealed a significant main effect of treatment ($F_{(4,27)} = 7.84; p = 0.0002$), with the antinociceptive potency of morphine being greatest in rats pretreated with vlPAG Vehicle + sc Saline for 3 days ($D_{50} = 2.499 \text{ mg/kg, CI } = 1.917 - 3.081$). In contrast, pretreatment with vlPAG Saline + sc Morphine resulted in a significant 4-fold rightward shift in the dose-response curve ($D_{50} = 10.64 \text{ mg/kg, CI } = 7.636 - 13.65; p = 0.0004$), indicating tolerance to morphine. Intra vlPAG pretreatment with 100 fmol of propentofylline significantly increased the antinociceptive potency of morphine ($D_{50} = 5.48 \text{ mg/kg, CI } = 3.503 - 7.464$) as compared with animals made tolerant to morphine (vlPAG Vehicle + sc Morphine; $p=0.0054$). However, the $D_{50}$ for these animals was significantly increased as compared with saline controls (vlPAG Vehicle + sc Saline; $p = 0.030$), indicating an attenuation, but not elimination, of tolerance. 10 fmol of propentofylline significantly increased the antinociceptive potency of morphine ($D_{50} = 4.02 \text{ mg/kg, CI } = 2.203 - 5.787$) as compared with animals made tolerant to morphine (Vehicle + sc Morphine; $p=0.0046$). Indeed, animals treated with 10fmol propentofylline did not differ from saline controls (vlPAG Vehicle + sc Saline; $p=0.054$) indicating a complete abolishment of tolerance. Animals pretreated with 10fmol propentofylline in the absence of morphine (vlPAG PPF + sc Saline; $D_{50} = 4.318 \text{ mg/kg, CI } = 1.730 - 6.905, n = 3$) did not differ from vehicle controls (vlPAG Vehicle + sc Saline; $p = 0.0796$, data not shown) indicating that chronic pretreatment with propentofylline alone does not potentiate morphine analgesia. Additionally, these effects were site specific as animals treated
with 10fmol propentofylline outside of the PAG (cannulae misses; n=5) and sc morphine (D_{50} = 11.53, CI = 6.633 - 16.43) significantly differed from animals treated with vlPAG Vehicle + sc Saline (p = 0.0012), and did not differ from vlPAG Vehicle + sc Morphine treated animals (p = 0.735, data not shown). Together, these results indicate that propentofylline is sufficient to attenuate the development of tolerance to sc morphine, and suggest that glial cell activity in the vlPAG contributes to the development of morphine tolerance.

*Inhibition of vlPAG microglia activity with minocycline was not sufficient to attenuate morphine tolerance.*

Morphine tolerance, assessed using a cumulative dosing paradigm, was observed in all groups that received sc morphine once a day for 3 days (Figure 3.1B). An analysis of variance of the data indicated a significant main effect of treatment (F_{(4,34)} = 8.82; p < 0.0001). Post-hoc analysis revealed that the antinociceptive potency of morphine was significantly decreased in animals that received 3 days of vlPAG Vehicle + sc Morphine (D_{50} = 10.13 mg/kg, CI = 6.991 – 13.27) as compared with animals that received 3 days of vlPAG Vehicle + sc Saline (D_{50} = 2.37 mg/kg, CI = 1.793 – 2.938), indicating tolerance to morphine (p = 0.0006). Animals that received intra-PAG injections of the microglial cell activity inhibitor minocycline had D_{50}s that did not differ from animals made tolerant to morphine (10fmol: D_{50} = 10.35 mg/kg, CI = 9.185 – 11.51, p = 0.445; 1pmol: D_{50} = 8.67 mg/kg, CI = 6.882 – 10.46, p = 0.196; 10pmol: D_{50} = 10.03 mg/kg, CI = 8.031 – 12.02, p = 0.483). Additionally, all animals treated with minocycline had D_{50}s that were significantly greater than animals treated with vlPAG Vehicle + sc Saline (10fmol: p < 0.0001; 1pmol: p < 0.0001; 10pmol: p < 0.0001) indicating tolerance to morphine. These results indicate that blockade of vlPAG microglial cell activity is not sufficient to attenuate the development of morphine tolerance.
Inhibition of vlPAG astrocyte activation with fluorocitrate attenuated morphine tolerance.

Administration of cumulative doses of morphine produced an increase in paw withdrawal latency (PWL) in all animals tested (Figure 3.1C). An analysis of variance indicated a significant main effect of treatment ($F_{(3,22)} = 10.50; p = 0.0002$). Morphine antinociception was significantly decreased in animals that received vlPAG Vehicle + sc Morphine ($D_{50} = 10.44 \text{mg/kg}, \text{CI} = 7.488 – 13.39$) as compared with animals that received vlPAG Vehicle + sc Saline ($D_{50} = 2.39 \text{mg/kg}, \text{CI} = 1.817 – 2.966$), indicating tolerance to morphine ($p = 0.0004$). 1 fmol and 10 pmol of the astrocyte activity inhibitor fluorocitrate attenuated tolerance to morphine as compared with animals treated with vlPAG Vehicle + sc Morphine (1fmol: $D_{50} = 5.61 \text{mg/kg}, \text{CI} = 4.406 – 6.819, p = 0.018$; 10pmol: $D_{50} = 6.36 \text{mg/kg}, \text{CI} = 5.337 – 7.381, p = 0.023$). However, animals treated with fluorocitrate showed a significant rightward shift in the dose-response curve as compared with animals treated with vlPAG Vehicle + sc Saline (1fmol: $p = 0.0003$; 10pmol: $p <0.0001$). These results indicate that fluorocitrate is sufficient to attenuate, but not eliminate, the development of morphine tolerance, and suggests that vlPAG astrocyte activity contributes to the development of morphine tolerance.

Experiment 1.2: Anatomical Assessment of vlPAG Glial Cell Inhibition

To assess the efficacy of the metabolic glial inhibitors administered, immunohistochemistry, in combination with densitometry, was used to measure microglia and astrocyte activity within the PAG. In agreement with our previous findings, subcutaneous injections of morphine once daily for three days significantly increased vlPAG microglia activity as evidenced by an increase in OX-42 immunoreactivity (Figure 3.2A) in comparison to saline controls ($F_{(8,19)} = 6.54; p = 0.002$). vlPAG microinfusions of the microglial cell metabolic inhibitor minocycline at 10 fmol, 1 pmol, and 10 pmol concentrations significantly decreased microglia ($p = 0.0008$, 0.0002,
and p < 0.0001, respectively; Figure 3.2A), but not astrocyte activity (p=0.15, 0.36, and 0.25, respectively; data not shown), compared with animals treated with vlPAG Vehicle + sc Morphine. The effect was dose-dependent with the highest concentration of minocycline being most effective at decreasing morphine-induced microglial cell activation. These results confirm that minocycline specifically inhibited microglia, and not astrocytes, and suggest that minocycline’s inability to attenuate morphine tolerance was not due to insufficient microglia inhibition by our drug infusions.

Similar to what was noted for microglia, and in agreement with our previous findings, chronic subcutaneous morphine significantly increased vlPAG astrocyte activity, as evidenced by an increase in GFAP immunoreactivity (Figure 3.2B) as compared with saline controls (F(8,17) = 7.14; p = 0.038). vlPAG microinfusions of the astrocyte metabolic inhibitor fluorocitrate (1 fmol & 10 pmol) dose dependently decreased astrocyte (p= 0.059 and 0.0001, respectively; Figure 3.2B) but not microglia activity (p= 0.11 and 0.56, respectively; data not shown), in the vlPAG as compared with animals treated with vlPAG Vehicle + sc Morphine. This effect was dose-dependent such that only the 10 pmol dose significantly decreased morphine-induced astrocyte activation. These results confirm that fluorocitrate was specifically targeting astrocytes, and not microglia.

Finally, vlPAG microinfusions of the general glial cell metabolic inhibitor propentofylline (10 fmol and 100 fmol) significantly decreased morphine-induced activation of microglia and astrocytes, as evidence by reduced vlPAG expression of OX-42 (Figure 3.2A; p = 0.002 and 0.0002, respectively) and GFAP (Figure 3.2B; p = 0.0003 and 0.0005, respectively) compared with animals treated with vlPAG Vehicle + sc Morphine. These results indicate that administration of propentofylline inhibits morphine-induced activation of both microglia and
astrocytes, and together with our behavioral data, indicate that concurrent blockade of both microglia and astrocytes was sufficient to attenuate the development of tolerance to morphine.

Experiment 2: vlPAG TLR4 was Both Necessary and Sufficient for the Development of Morphine Tolerance

Results from Experiment 1 indicate that both microglia and astrocyte activity within the vlPAG increase in response to chronic morphine administration, and that blockade of microglia and astrocyte, or just astrocyte activation, attenuates the development of morphine tolerance. To investigate the mechanism by which morphine affects glia and contributes to tolerance, we characterized MD-2 expression in the dlPAG and vlPAG, and tested the hypothesis that morphine-induced activation of TLR4 within the vlPAG mediates morphine tolerance development.

Experiment 2.1: dlPAG and vlPAG Localization of MD-2

In these studies, immunohistochemistry was used to characterize MD-2 expression within the vlPAG. The dlPAG, a region not implicated in morphine tolerance development \(^{51}\), was also examined. As indicated in the photomicrograph (Figure 3.3A; inset), MD-2 is present and densely expressed within the vlPAG. This region of the PAG contains a high density of mu opioid receptors (MOR) \(^{22,114,150,183-186}\). A comparison of vlPAG (Bregma -6.72 through -8.76) and dlPAG (Bregma -6.72 through -8.04; Figure 3A) revealed significantly greater MD-2 immunoreactivity in the vlPAG as compared with the dlPAG (F\(_{(4,1)}\) = 11.275; p = 0.0284). MD-2 expression was homogeneous across levels of the vlPAG (F\(_{(3,5)}\) = 2.702; p = 0.062) and dlPAG (F\(_{(4,3)}\) = 1.413; p = 0.2872; Figure 3.3B). Post-hoc analysis revealed significantly greater MD-2 immunoreactivity in the vlPAG as compared with the dlPAG at both rostral (Bregma -6.72; p = 0.0458) and caudal (Bregma -8.04; p = 0.0380) levels (Figure 3.3B).
Experiment 2.2: Antagonism of vlPAG TLR4 with LPS-RS or (+)-naloxone attenuated the development of morphine tolerance.

An analysis of variance of the data indicated a significant main effect of treatment ($F_{(3, 22)} = 5.35; p=0.0064; $ Figure 3.4A). The antinociceptive potency of morphine was significantly decreased in animals that received 3 days of morphine (s.c.; 5 mg/kg) and intra-vlPAG saline ($D_{50} = 10.86$ mg/kg, CI = 7.463 – 14.26) as compared with animals that received 3 days of saline (1ml/kg) and intra-vlPAG vehicle ($D_{50} = 6.03$ mg/kg, CI = 5.216 – 6.835; $p = 0.0051$), indicating tolerance to morphine (Figure 3.4A). vlPAG microinjections of the TLR4 antagonist LPS-RS dose-dependently eliminated the development of morphine tolerance. The antinociceptive potency of morphine was significantly increased in animals treated with 2.4 $\mu$g of LPS-RS ($D_{50} = 4.501$ mg/kg, CI = 3.398 – 5.605) as compared with animals made tolerant to morphine ($p = 0.0031$) indicating blockade of tolerance. These results were limited to the vlPAG as the $D_{50}$ for animals treated with 2.4 mg LPS-RS into misplaced cannulae (cannulae misses, $D_{50} = 8.237$, CI = 7.320 – 11.15) did not differ from vehicle controls (vlPAG Vehicle + sc Morphine, $p = 0.4555$; data not shown). No significant difference in $D_{50}$ was noted in animals treated with 1.7 mg of LPS-RS ($D_{50} = 11.54$ mg/kg, CI = 5.447 – 17.62) as compared with the vlPAG Vehicle + sc Morphine group ($p = 0.4168$). These results indicate that vlPAG TLR4 is necessary for the development of morphine tolerance.

The mu opioid receptor (MOR) is stereoselective such that only the (-)-stereoisomers of opioids bind. Conversely, opioid agonists and antagonists (e.g., naloxone) affect TLR4 in a non-stereoselective fashion, with both (-)- and (+)-stereoisomers affecting the signaling cascade in a manner that maintains their agonistic and antagonistic properties.\textsuperscript{77,78}
The antinociceptive potency of morphine was significantly decreased ($F_{(3,21)} = 8.968; p = 0.0005$; Figure 3.4B) in animals that received 3 days of morphine (s.c.; 5 mg/kg) and intra-vlPAG saline ($D_{50} = 10.75 \text{ mg/kg}, CI = 7.274 – 14.23$) as compared with animals that received 3 days of saline (s.c; 1ml/kg) and intra-vlPAG vehicle ($D_{50} = 5.801 \text{ mg/kg}, CI = 5.002 – 6.600; p = 0.0050$) indicating tolerance to morphine (1.8-fold rightward shift in the dose-response curve). Similar to what was observed for LPS-RS, antagonism of TLR4 with (+)-naloxone (5 µg/0.5 µl) abolished the development of morphine tolerance. The antinociceptive potency of morphine was significantly increased in animals treated with (+)-naloxone ($D_{50} = 3.529 \text{ mg/kg}, CI = 2.344 – 4.715$), as compared with animals treated with vlPAG Vehicle + sc Morphine ($p=0.0101$; Figure 3.4B). (+)-naloxone also resulted in a significant leftward shift in the dose-response curve as compared with the vlPAG Vehicle+sc Saline group ($p = 0.0056$). However, (+)-naloxone pretreatment in the absence of morphine was without effect. Indeed, the $D_{50}$ for the vlPAG (+)-naloxone+ sc Saline group ($D_{50} = 6.315\text{ mg/kg}, CI = 5.440 – 6.830$) did not differ from the vlPAG Vehicle+sc Saline group ($p = 0.5979$), indicating that vlPAG TLR4 inhibition does not affect antinociception in the absence of morphine, and suggesting that TLR4 is not constitutively activated. These results further support our hypothesis that vlPAG TLR4 is necessary for the development of morphine tolerance.

_Experiment 2.3: Activation of vlPAG TLR4 with LPS, KDO2, or (+)-morphine induced ‘naïve’ tolerance._

Intra-vlPAG microinjections of the TLR4 agonist LPS dose-dependently induced tolerance to morphine ($F_{(3,24)} = 6.190; p = 0.0029$; Figure 3.5A). Indeed rats pretreated with vlPAG LPS (5 µg) alone ($D_{50} = 9.113 \text{ mg/kg}, CI = 8.052 – 10.170$) did not differ from rats given morphine once a day for three days ($D_{50} = 10.81 \text{ mg/kg}, CI = 7.354 – 14.260; p = 0.1958$), indicating ‘naïve’
tolerance to morphine. Additionally, rats pretreated with vlPAG LPS (5 µg) or rats treated with vlPAG Vehicle + sc Morphine showed significant decreases in the antinociceptive potency of morphine (p = 0.0001 and p = 0.0050, respectively) as compared with rats treated with vlPAG Vehicle (D_{50} = 5.896 mg/kg, CI = 5.093 – 6.700). The lowest dose of LPS (1.7 µg; D_{50} = 6.462 mg/kg, CI = 5.439 – 7.485) was without effect (i.e. these animals did not show signs of tolerance as compared with vehicle controls (p = 0.1936)). These results indicate that vlPAG TLR4 agonism is sufficient to induce the development of morphine tolerance.

Similar to what was noted for LPS, intra-vlPAG microinjections of the TLR4 agonist KDO2 dose-dependently reduced the antinociceptive potency of morphine (F(4,26)=4.659; p=0.0057; Figure 3.5B) with the 5 µg dose having the most robust effect. The antinociceptive potency of morphine did not differ in animals pretreated with 5 µg of KDO2 (D_{50}=7.922 mg/kg, CI = 6.391 – 9.453) as compared with morphine-pretreated animals (D_{50}=10.84 mg/kg, CI = 7.415 – 14.260; p = 0.0816) indicating the development of ‘naïve’ tolerance. Indeed, both KDO2 (5mg) and morphine pretreatment caused a significant rightward shift in the morphine dose-response curve (p = 0.0044 and p = 0.0015, respectively) as compared with saline controls (D_{50} = 5.720 mg/kg, CI = 5.024 – 6.416).

Intra-vlPAG administration of the TLR4 stereoselective agonist (+)-morphine also induced “naïve” morphine tolerance (F(2,21)=5.949; p= 0.0090; Figure 3.5C). Indeed, the D_{50} for animals pretreated with 5 µg of (+)-morphine (D_{50} = 8.218 mg/kg, CI = 7.144 – 9.293) did not differ from animals that received 3 days of (-)-morphine (sc) (D_{50} = 10.86 mg/kg, CI = 7.461 – 14.26; p=0.0513). Additionally, both (-)- and (+)-morphine groups had a significant increase in D_{50} (p=0.0072 and p=0.0037, respectively) as compared with saline controls (D_{50} = 5.921 mg/kg, CI = 5.014 – 6.828). Together, these results are the first to indicate that morphine activation of
vlPAG glia contributes to the development of morphine tolerance, and that vlPAG TLR4 is both necessary and sufficient for the development of morphine tolerance.

Experiment 3: Acute Systemic Antagonism of TLR4 Potentiated Acute Morphine Antihyperalgesia in a Model of Persistent Inflammatory Pain

Experiment 3.1: (+)-naloxone significantly increased acute morphine antihyperalgesia

The results from the studies above indicate that antagonism of TLR4 attenuates the development of tolerance to morphine, providing additional evidence that glia actively oppose the antinociceptive actions of morphine. Therefore, in our final series of experiments we tested if blockade of TLR4 potentiated the analgesic effects of acute morphine. As morphine is primarily consumed for the alleviation of severe pain, these studies were conducted in animals experiencing persistent inflammatory hyperalgesia. Systemic antagonism of TLR4 with (+)-naloxone (8 mg/kg) significantly potentiated acute morphine antihyperalgesia as compared with sc vehicle/sc morphine treated animals ($t_{(11)} = 3.811; p = 0.0014$; Figure 3.6). Indeed, the potency of morphine was significantly increased in animals treated with (+)-naloxone ($D_{50} = 3.415 \text{ mg/kg, CI = 3.093 – 3.737}$) as compared with vehicle controls ($D_{50} = 5.609 \text{ mg/kg, CI = 4.727 – 6.490}$). Consistent with previous results, (+)-naloxone, in the absence of morphine, exhibited acute antihyperalgesic effects resulting in a slight increase in PWL across the testing period $^{181}$. These results, together with our previous studies, indicate that TLR4 opposes both the acute and chronic effects of morphine.

Discussion

The present set of experiments tested the hypothesis that vlPAG glia contribute to the development of morphine tolerance through the innate immune receptor toll-like receptor 4 (TLR4). Here we report that (1) vlPAG microinjections of the general glial metabolic inhibitor...
propentofylline or the astrocyte inhibitor fluorocitrate significantly decreased morphine-induced glia activation in the vIPAG, and attenuated the development of tolerance to systemically-administered morphine; (2) vIPAG TLR4 antagonism prevented the development of tolerance to systemic morphine; and (3) vIPAG TLR4 agonism induced a ‘naïve’ tolerance to subsequent challenge doses of morphine. Based on these findings we hypothesized that blockade of TLR4 would potentiate the antinociceptive effects of morphine in a model of persistent inflammatory pain. Indeed, systemic antagonism of TLR4 significantly enhanced the acute antihyperalgesic effects of systemic morphine. Together, these data demonstrate a role for vIPAG glia in the development of morphine tolerance, establish the vIPAG as a CNS locus through which TLR4 mediates morphine tolerance development. The results of our acute studies implicate TLR4 as a potential therapeutic target for the treatment of pain.

**vIPAG glia contribute to morphine tolerance**

It is now well established that opioids, including morphine, activate spinal glia to produce a potent proinflammatory response that opposes both acute and chronic morphine analgesia. Morphine administration increases the expression of microglia and astrocyte activity markers OX-42 and GFAP and induces the release of glially derived proinflammatory cytokines. In agreement with our previous findings, here we show that in the absence of pain, tolerance to morphine developed rapidly. Indeed, administration of one ED$_{50}$ dose of morphine (5 mg/kg) for three days was sufficient to induce behaviorally defined tolerance. Paralleling the development of tolerance, OX-42 and GFAP protein levels increased significantly within the vIPAG, suggesting the activation of microglia and astrocytes, respectively. These results demonstrate that the vIPAG proinflammatory response contributes to the development of morphine tolerance. Indeed, intra-PAG administration of propentofylline, which inhibits both
microglia and astrocyte activity by reducing proinflammatory cytokine release, dose-dependently decreased vlPAG microglia and astrocyte activity, and attenuated tolerance to morphine \cite{93,94}. Decreased astrocyte activity appears to drive this effect in part, as inhibition of astrocytes alone with fluorocitrate led to an attenuation of tolerance. Proinflammatory cytokines, particularly tumor necrosis factor alpha (TNFa), increase the density and conductance of neuronal GluR2-lacking AMPA receptors (via neuronal PI3K and TNFR1 but not TNFR2), and decrease GABA\textsubscript{A} receptors via endocytosis. These changes increase mEPSCs and decrease mIPSCs in hippocampal slices and cultured hippocampal neurons \cite{145}. The proinflammatory cytokine IL-1\textbeta significantly increases neuronal GluR2-lacking AMPA receptors, albeit to a lesser extent \cite{144,145}. TNFa also decreases astrocytic glutamate transporter proteins (GLT-1, GLAST) in the spinal cord presumably leaving more glutamate available in the synapse \cite{97}. Together, these cytokine-induced changes, among others, effectively increase neuronal excitability \cite{147,148}. This increased excitation may contribute to morphine tolerance by opposing the inhibitory properties of morphine.

Our findings within the PAG complement a vast literature indicating that spinal cord glia contribute to morphine tolerance development, and indicate a role for supraspinal glial cell activity as well \cite{72,74,84,92}. Consistent with our results, intrathecal administration of propentofylline increases acute opioid analgesia and attenuates morphine tolerance \cite{87,93}. However, in contrast to the spinal cord, where either inhibition of microglia or astrocytes alone is sufficient to attenuate the development of morphine tolerance, here inhibition of astrocytes alone was effective in attenuating tolerance \cite{72,96}. While microglia are classically viewed as \textit{initiators} of neuroinflammation, chronic morphine can cause neurons to release factors that activate both microglia and astrocytes (e.g., fractalkine, dynorphin, and nitric oxide), and astrocytes can
express TLR4\textsuperscript{84,85,99,187}. Therefore, it is conceivable that while morphine activates both microglia and astrocytes, blocking astrocyte activation alone reduces opiate-induced changes in neuronal excitability that contribute to morphine tolerance development and maintenance.

\textit{vlPAG TLR4 modulates morphine tolerance development}

There is extensive literature supporting a critical role for glial cell activation in the development of morphine tolerance. Until relatively recently, however, the mechanism by which opioids induce the activation of glia was unknown. Demonstration that opioid hyperalgesia is still observed in neuronal opioid receptor (m, d, and k) knockout mice led to the consideration that the anti-analgesic effects of morphine (e.g., tolerance) may be mediated by non-neuronal opioid receptors\textsuperscript{98}. Interestingly, glial activation induced by other stimuli such as neuropathic pain or lipopolysaccharide (LPS) administration, also reduces the analgesic efficacy of intrathecal morphine, an effect attenuated by intrathecal administration of the neuronal opioid receptor inactive antagonist (+)-naloxone\textsuperscript{188}.

It is now clear that TLR4 activity opposes morphine analgesia. Hutchinson et al. provided convincing \textit{in vitro}, \textit{in vivo}, and \textit{in silico} data indicating that TLR4, along with its co-receptor MD-2, binds opioid agonists and antagonists (e.g., morphine and naloxone) in a nonstereoselective fashion with both the (-) and (+)-stereoisomers affecting the TLR4 signaling cascade in a manner that maintains their agonistic and antagonistic properties\textsuperscript{78}.

Functionally, animals that receive TLR4 antagonism, as well as TLR4 knockout mice, exhibit increased responsiveness to the analgesic properties of acute morphine administration\textsuperscript{89}. In addition, systemic (+)-naloxone prevents the development of tolerance to systemic (-)-morphine\textsuperscript{78}. Here we establish the vlPAG as a key CNS locus through which TLR4 signaling mediates morphine tolerance. Importantly we show dense, and homogeneous expression of MD-
2 across the rostro-caudal extent of the vIPAG, and report that MD-2 immunoreactivity is significantly greater in the vIPAG as compared to the dIPAG, a subregion of the PAG not implicated in morphine tolerance development. Further, vIPAG microinfusions of the TLR4 competitive antagonist LPS-RS dose-dependently eliminated the development of morphine tolerance to sc morphine. Here, we further demonstrate that intra-vIPAG administration of the opioid receptor inactive form of naloxone, (+)-naloxone, which blocks TLR4 signaling in vitro, completely abolished the development of tolerance to systemic morphine. These results suggest that vIPAG TLR4 is necessary for the development of morphine tolerance. Lastly, we show that vIPAG microinfusions of several TLR4 agonists induced a ‘naïve’ tolerance to subsequent challenge doses of morphine. vIPAG infusions of the prototypical TLR4 agonist LPS, the synthetic TLR4 agonist KDO2, and the opioid receptor inactive form of morphine, (+)-morphine, in the absence of systemic (-)-morphine, all resulted in a tolerance to subsequent doses of (-)-morphine, indicating that activation of vIPAG TLR4 is sufficient to induce tolerance.

**TLR4 opposes acute antihyperalgesia**

Our results demonstrating that acute inhibition of TLR4 potentiated morphine analgesia in a model of persistent pain have exciting implications for the clinical treatment of chronic pain. This work complements previous work demonstrating that TLR4 agonism enhances pain, and that TLR4 antagonism potentiates the analgesic efficacy of morphine in the absence of pain. Further, our results support the finding that the opioid receptor inactive, (+)-stereoisomer of naloxone attenuates pain in the absence of morphine. (+)-naloxone has been demonstrated to be blood-brain barrier permeable with pharmacokinetic properties similar to its widely prescribed stereoisomer (-)-naloxone, indicating that (+)-naloxone could readily translate to clinical applications. Although morphine is amongst the most effective analgesics available,
chronic morphine treatment leads to a myriad of negative side effects including tolerance. Morphine tolerance is particularly problematic as it is often addressed by increasing the dose of morphine prescribed. Dose escalation leads to increased risk of developing additional negative side effects, including anti-analgesia, addiction, withdrawal, and respiratory depression, and is not always sufficient to overcome tolerance and reinstate analgesic efficacy. Interestingly, the proinflammatory glial response to opioids has been implicated in many of the negative side effects associated with opioid treatment (e.g., withdrawal, reward, dependence, respiratory depression, and tolerance). As over 90% of chronic pain sufferers are treated with opioids, including morphine, these data have exciting implications for pain management. Indeed, decreasing the dose of morphine prescribed by co-administration of (+)-naloxone could enhance morphine analgesia and prevent negative consequences of opioid therapy including tolerance development.

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Figure 3.1 Summary of morphine tolerance following vlPAG glial metabolic inhibition.

PWL represented as mean D$_{50}$ ± 95% confidence intervals (CI) as a function of cumulative injections of morphine in rats treated with (A) vlPAG vehicle + sc Saline (●, n=6), vlPAG vehicle + sc Morphine (○, n=9), and vlPAG propentofylline (10fmol ▼, n=5, 100fmol ▲, n=9) and sc Morphine, (B) vlPAG vehicle + sc Saline (●, n=6), vlPAG vehicle + sc Morphine (○, n=8), and vlPAG minocycline (10fmol ▼, n=9; 1pmol ◆, n=12; or 10pmol ▲, n=4) and sc Morphine, and (C) vlPAG vehicle + sc Saline (●, n=6), vlPAG vehicle + sc Morphine (○, n=9), and vlPAG fluorocitrate (1fmol ▼, n=5; 10pmol ▲, n=6) and sc Morphine.
Figure 3.2 Summary of glial inhibition following vlPAG metabolic glial inhibitors. Densitometry of OX-42 (A) and GFAP (B) immunoreactivity in the vlPAG in vlPAG Saline + sc Saline (n=6), vlPAG Saline + sc Morphine (n=8), vlPAG propentofylline (10 fmol, n=5 and 100 fmol, n= 9) + sc Morphine, vlPAG minocycline (10 fmol, n=9; 1 pmol, n= 12; and 10 pmol, n=4) + sc Morphine, and vlPAG fluorocitrate (1 fmol, n=5 and 10 pmol, n=6) + sc Morphine groups. *p < 0.05, †p < 0.01, and ‡p < 0.001 indicate that groups are significantly different from the vlPAG Saline + sc Morphine group. Insets are representative photomicrographs (10X) of OX-42 (A) and GFAP (B) immunoreactivity in the vlPAG (Bregma -7.08) of animals treated with vlPAG Saline + sc Saline, vlPAG Saline + sc Morphine, vlPAG 10fmol PPF + sc Morphine, and vlPAG 10pmol Mino + sc Morphine (from left to right; A) and vlPAG Saline + sc Saline, vlPAG Saline + sc Morphine, vlPAG 10fmol PPF + sc Morphine, and vlPAG 10pmol FC + sc Morphine (from left to right; B).
Figure 3.3 Summary of MD-2 in the dorsal and ventrolateral PAG.
Myeloid differentiation factor-2 (MD-2) immunoreactivity in the vlPAG and dlPAG of normal adult male rats (n=5) collapsed across Bregma levels (A) and separated by Bregma level (B) represented as mean ± SEM. Note that dlPAG is not present at Bregma -8.28 and -8.76. Representative photomicrographs of dlPAG and vlPAG MD-2 immunoreactivity shown at 4X (middle) and 10X (right) magnifications (A, inset). Aq denotes the location of the cerebral aqueduct.
Figure 3.4 Summary of morphine tolerance following TLR4 antagonism.
PWL represented as mean D\textsubscript{50} ± 95% confidence intervals (CI) as a function of cumulative injections of morphine in rats treated with (A) vlPAG Vehicle + sc Saline (○, n=8), vlPAG Vehicle + sc Morphine (○, n=7), and vlPAG LPS-RS (1.7 mg ▼, n=5; 2.4 mg ▲, n=6) + sc Morphine, and (B) vlPAG Vehicle + sc Saline (●, n=8), vlPAG Vehicle + sc Morphine (●, n=7), and vlPAG (+)-Naloxone (5 mg/0.5 ml) + sc Morphine (◇, n=6), and vlPAG (+)-Naloxone + sc Vehicle (◆, n=4).
Figure 3.5 Summary of morphine tolerance following TLR4 agonism. PWL represented as mean $D_{50} \pm 95\%$ confidence intervals (CI) as a function of cumulative injections of morphine in rats treated with (A) vlPAG Vehicle (●, n=8), vlPAG Vehicle + sc Morphine (○, n=7), and vlPAG LPS (1.7 mg ▼, n=7 or 5 mg ▲, n=6) (B) vlPAG Vehicle (●, n=10), vlPAG Vehicle + sc Morphine (○, n=7), and vlPAG KDO2 (0.5 mg ▼, n=5; 1 mg ◆, n=3; or 5 mg ▲, n=6) and (C) vlPAG Vehicle (●, n=7), vlPAG Vehicle + sc Morphine (○, n=7), and vlPAG (+)-morphine (5 mg ◆, n=10).
Figure 3.6 Summary of acute morphine antihyperalgesia following acute (+)-naloxone.

PWL represented as mean $D_{50} \pm 95\%$ confidence intervals (CI) across time (minutes) in rats treated with one injection of sc (+)-naloxone (8 mg/kg) and cumulative injections of sc morphine (●, n=5), one injection of sc vehicle (1ml/kg) and cumulative injections of sc morphine (○, n=8), and one injection of sc (+)-naloxone (8 mg/kg) and repeated injections of sc vehicle (□, n=3). All injections were subcutaneous. (+)-naloxone (or vehicle control) was injected immediately after baseline testing (Time 0). Twenty-four hours before PWL testing all animals received an injection of CFA into the hindpaw (200 µl).
CHAPTER 4: Periaqueductal Gray Toll-like Receptor 4 Modulates Morphine Tolerance via Soluble Tumor Necrosis Factor Signaling

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Abstract

Opioid-based narcotics remain an integral part of clinical pain management with morphine being one of the most effective drugs. However, the acute and long-term analgesic efficacy of morphine is limited by the ensuing neuroinflammatory response. The midbrain periaqueductal gray (PAG) is an essential neural substrate for opioid-mediated analgesia, and we have previously reported that chronic morphine administration activates ventrolateral PAG (vlPAG) glia via the innate immune receptor toll-like receptor 4 (TLR4), leading to the development of behaviorally-defined tolerance. TLR4 activation results in a robust release of cytokines, including tumor necrosis factor a (TNF), and TNF signaling can increase the excitability of neurons by modulating glutamate homeostasis and signaling via neuronal TNF receptor 1 (TNFR1). Our overarching hypothesis is that TNF-induced increases in neural excitability oppose the hyperpolarizing effects of morphine, resulting in opioid tolerance. Using a lentiviral vector encoding dominant negative TNF (DN-TNF), here we demonstrate that intra-PAG sequestration of soluble TNF (solTNF) abolished tolerance to systemic morphine as well as naïve tolerance to morphine induced by intra-vlPAG injections of the TLR4 agonist LPS. Additionally we demonstrate the efficacy of the novel, BBB permeable DN-TNF peptide XPro®1595 in preserving morphine efficacy following chronic morphine exposure. Tolerance was accompanied by a significant increase in vlPAG TLR4 and proinflammatory cytokine (IL-1β) mRNA and decrease in vlPAG astrocytic glutamate transporter protein mRNA (GLT-1 and GLAST) that was attenuated or eliminated by sequestration of solTNF. These results support our working hypothesis and indicate that morphine binds to TLR4 within the vlPAG, leading to the release of solTNF. Our results further suggest that solTNF mediates morphine tolerance in the PAG via neuronal TNFR1 signaling and augmentation of glutamate homeostasis.
Introduction

Opioid therapy remains the most common and effective strategy for severe and chronic pain management\(^3\). However, decreased analgesic efficacy over time (i.e., tolerance) significantly impedes treatment for approximately 60% of the patient population\(^8\). It is now clear that opioids, including morphine\(^72-81\), elicit a robust neuroinflammatory response that enhances nociceptive transmission\(^71\) and contributes to opioid tolerance\(^72-75,82-87\). The proinflammatory effects of morphine are not mediated via signaling at classical opioid receptors, but rather, through the innate immune receptor TLR4\(^81\). Opioids bind to myeloid differentiation factor-2 (MD-2), a glycoprotein that binds to both LPS and the extracellular domain of TLR4\(^78,79\). Opioid binding to the TLR4/MD-2 complex results in the activation of MAP kinases and NF-κB, leading to the robust release of proinflammatory cytokines, including TNF, IL-1β, and IL-6\(^78,79,81\).

A nearly ubiquitous characteristic of opioid-induced neuroinflammation is TNF production. Chronic systemic or intrathecal morphine administration increases TNF mRNA\(^84,86,102,103\) and protein\(^53,86,101,102,104,105\) in the rodent spinal cord, and proinflammatory cytokine levels increase with the chronicity of morphine treatment\(^84\). Inhibition of spinal TNF signaling decreases morphine-induced release of proinflammatory cytokines and attenuates p38 MAPK activation\(^54,102,106\). Functionally, immunomodulatory drugs that attenuate, abolish, or even reverse morphine tolerance (e.g., ibudilast (AV411)\(^97,107\), minocycline\(^76,91,96,97,108\), fluorocitrate\(^72,82\), propentofylline\(^82,87,93,94,109\)) decrease the expression of TNF. However, these immunomodulatory drugs have widespread and non-specific effects, altering expression levels of several cytokines also implicated in opioid tolerance, including IL-1β\(^86,101,110\) and IL-6\(^86,87,101\). While these data indicate that spinal TNF plays a significant role in morphine-induced
inflammation and the development of morphine tolerance\textsuperscript{72-74,76,84-87,93,111,112}, remarkably few studies have directly tested the role of TNF in isolation\textsuperscript{54,102,106}.

TNF naturally exists in two forms; the more common form, transmembrane TNF (tmTNF), is cleaved by the TNF converting enzyme TACE to release the less abundant form, soluble TNF (solTNF)\textsuperscript{190}. solTNF binds exclusively to TNF receptor I (TNFRI), which signals via the proinflammatory pathways p38 MAPK and NFkB\textsuperscript{112,191}. In contrast, tmTNF binds to either TNFRI or TNFRII, and is a critical factor for innate immunity to infection\textsuperscript{192}.

To date, anti-TNF biologics used to test the role of TNF in opioid tolerance attenuate the actions of both forms of TNF. For example, viral expression\textsuperscript{106} or chronic infusion\textsuperscript{54,102} of decoy TNF receptors in the spinal cord, which alter the action of both solTNF and tmTNF, attenuates morphine tolerance. Currently there are FDA-approved non-selective biological inhibitors that sequester both forms of TNF, but these drugs have been associated with neurological deficits and demyelinating disease in patients who were previously neurologically normal\textsuperscript{193,194}.

TNF significantly increases neuronal excitability, in part, through TNFRI signaling. For example, morphine-induced TNF signaling decreases expression of the astrocytic glutamate transporter GLT-1 and glutamate/aspartate transporter GLAST\textsuperscript{54}, and increases the expression of AMPA and NMDA receptor subunits in the spinal cord\textsuperscript{54}. In hippocampal slices, TNF signaling through TNFRI (solTNF and tmTNF) increases neuronal GluR2-lacking AMPA receptor expression and conductance, and decreases neuronal GABA\textsubscript{A} receptor expression\textsuperscript{145}. As morphine exerts its analgesic effects by hyperpolarizing neurons\textsuperscript{28,31,42,64}, TNF-mediated alterations in neuroexcitability may provide an underlying mechanism driving the development of opioid tolerance\textsuperscript{62,63,195-204}.
Previous studies examining the impact of glia signaling on morphine tolerance were focused on the spinal cord dorsal horn. However, the midbrain ventrolateral periaqueductal gray (vlPAG) has been shown repeatedly to be a more critical site for morphine action\textsuperscript{24,45,175,205,206}. Repeated administration of morphine into the vlPAG results in the rapid development of tolerance\textsuperscript{49,50}, while intra-vlPAG administration of the opioid antagonist naltrexone significantly attenuates tolerance to \textit{systemic} morphine\textsuperscript{24}. Opioid tolerance is mediated, in part, via vlPAG TLR4 signaling\textsuperscript{82,83}. Administration of the TLR4 agonist LPS directly into the PAG induces a ‘naïve’ tolerance to morphine, while intra-PAG administration of the TLR4 antagonists LPS-RS or (+)-naloxone abolishes morphine tolerance\textsuperscript{82}. As morphine-induced TLR4 signaling results in robust TNF release\textsuperscript{81}, the present studies tested the hypothesis that morphine tolerance is due to altered vlPAG astrocytic glutamate homeostasis and increased neuronal excitability that is mediated by TLR4 solTNF signaling.

\textbf{Materials and Methods}

\textit{Subjects}

Pathogen free male Sprague Dawley rats (250-350g; Charles River; Wilmington, MA) were pair-housed on a 12:12 hour light:dark cycle (lights on at 7:00 am). Access to food and water was available \textit{ad libitum} except during behavioral testing. The Institutional Animal Care and Use Committee (IACUC) at Georgia State University approved all studies. All efforts were made to reduce the number of animals used and to minimize any possible suffering by the animal.

\textit{Cloning and preparation of lentiviral stocks}

The inhibitor protein dominant-negative TNF (DN-TNF) is a well-characterized variant of native human TNF that has been engineered to rapidly form heterotrimers with native soluble
TNF (solTNF) in rat and mouse brain. DN-TNF heterotrimerization with native solTNF effectively sequesters native solTNF and precludes it from initiating signaling through TNFRI by preventing receptor binding\textsuperscript{207}, while sparing the beneficial effects mediated by the transmembrane TNF (tmTNF) signal\textsuperscript{192,207}. The human full-length DN-TNF DNA sequence (TNF variant with two point mutations; A145R/I97T\textsuperscript{207}) and the enhanced green fluorescent protein DNA sequence (GFP; reporter) were subcloned into a constitutive self-inactivating lentiviral vector under the chicken b-actin cytomegalovirus (CAG) promoter. Both DN-TNF-GFP and GFP (control virus) lentiviral stocks were produced and purified at Emory University’s Viral Vector Core according to previously published protocols\textsuperscript{208}. The final titers were 1 x 7\textsuperscript{7} IU/ml for lenti-DN-TNF-GFP and 2 x 10\textsuperscript{8} IU/ml for lenti-GFP control. The feasibility and efficacy of the virus have been well demonstrated \textit{in vitro} and \textit{in vivo}\textsuperscript{208-210}

\textit{Lentivirus infection and morphine treatment}

Animals in Experiment 1.1 received bilateral intra-vlPAG (AP: 1.7 mm, ML: ± 0.6 mm, DV: -5.5 mm from lambda) microinfusions (2.5 µl/ 4 minutes/ side)\textsuperscript{207} of a lentiviral vector encoding DN-TNF and GFP (lenti-DN-TNF) or encoding GFP only (lenti-GFP) using a Hamilton syringe (5 µl) attached to a stereotaxic arm. The syringe was left in place for 60 seconds following each infusion to prevent backflow upon removal. All animals were anesthetized to a deep surgical plane with 5% Isoflurane (Baxter Healthcare Corporation; Deerfield, IL) and maintained at 2-5% Isoflurane throughout microinfusions. Skin staples were used to close the skin, and animals were given topical triple antibiotic ointment (Bacitracin, Neomycin, Polymyxin) to prevent infection and topical lidocaine hydrochloride (2%; Akorn, Inc.) to ameliorate pain. One week following virus infusions, animals were administered morphine (5 mg/kg, sc; National Institute on Drug Abuse (NIDA), Bethesda, MD) or vehicle
(sterile saline; 1 ml/kg; sc) once a day for 3 consecutive days, resulting in four treatment groups: lenti-DN-TNF + Morphine (n = 10), lenti-DN-TNF + Saline (n = 5), lenti-GFP + Morphine (n = 10), and lenti-GFP + Saline (n = 4). Animals were removed from the lenti-DN-TNF + Saline group (n=5) and from the lenti-GFP + Saline group (n = 6) because cannulae were located outside of the PAG, or they did not show robust GFP expression in the vlPAG. The 5 mg/kg dose was chosen based on our previous studies demonstrating this to be the 50% effective dose (ED50) for systemic morphine in male rats122,125.

_Ventrolateral periaqueductal gray cannulation, lentivirus infection, and LPS microinfusions_

In Experiment 1.2 animals were bilaterally cannulated and infused with lenti-DN-TNF or lenti-GFP with a Hamilton syringe (5 µl) inserted through guide cannulae. Animals were implanted with bilateral guide cannulae (22 gauge; Plastics One; Roanoke, VA) aimed at the ventrolateral PAG (AP: 1.7 mm, ML: ± 0.6 mm, DV: -5.0 mm from lambda) using stereotaxic techniques as previously described82,114. Animals recovered for 1 week before lentivirus infection. Using a Hamilton syringe (5 µl) inserted through implanted guide cannulae (5.5 mm DV), animals received bilateral intra-vlPAG microinfusions (2.5 µl/ 4 minutes/ side)207 of lenti-DN-TNF-GFP or lenti-GFP as described above. To test a direct role for TLR4-mediated TNF release in morphine tolerance development, animals in Experiment 1.2 received bilateral intra-vlPAG infusions of the TLR4 agonist lipopolysaccharide (LPS; 5 µg/ 0.5µl/ side/ 2 minutes; Sigma) once a day for three consecutive days to induce a naïve tolerance to morphine as previously described82. LPS is the prototypical TLR4 agonist and has been shown to competitively bind to TLR4, resulting in the production of TNF. LPS doses and injection time-course were chosen based on our previous studies demonstrating that this dosing is sufficient to induce naïve tolerance to morphine82. Control animals received vehicle (sterile saline; 0.5µl/
side/ 2 minutes) once a day for three consecutive days resulting in the following experimental groups: lenti-DN-TNF + PAG LPS (n = 9), lenti-DN-TNF + PAG Saline (n = 6), lenti-GFP + PAG LPS (n = 4), and lenti-GFP + PAG Saline (n = 6). Animals were removed from the lenti-DN-TNF + PAG LPS (n=1), lenti-DN-TNF + PAG Saline group (n=4), lenti-GFP + PAG LPS (n = 6), and from the lenti-GFP + PAG Saline group (n = 4) because cannulae were located outside of the PAG, or they did not show robust GFP expression in the vlPAG.

*XPro®1595 and morphine treatment*

XPro®1595 is a novel, brain-permeant, dominant negative TNF variant with FDA IND status that selectively diminishes the inflammatory signal of soluble TNF (> 2500-fold)\(^{192,207}\) in the same manner as the virally expressed DN-TNF in Experiment 1\(^{211}\). A preemptive dose of XPro®1595 (10 mg/kg; sc) was administered 1 day before the first morphine (5mg/kg; sc) injection to allow DN-TNF adequate time to reach the brain. A booster injection of XPro®1595 was administered with the 3\(^{rd}\) morphine injection to maintain DN-TNF levels in the brain (half life = 18 hours; personal communication from Dr. Malu Tansey). Control animals received equivolume saline, resulting in the following experimental groups: XPro + Morphine (n = 11), XPro + Saline (n = 8), Vehicle + Morphine (n = 11), and Vehicle + Saline (n = 8).

*Nociceptive testing*

Nociception was assessed using the paw thermal stimulator\(^{126}\). Briefly, for this test, the rat is placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30\(^{\circ}\)C. A radiant beam of light is positioned under the plantar surface of the hindpaw and the time for the rat to remove the paw from the thermal stimulus is electronically recorded as the paw withdrawal latency (PWL). A maximal PWL of 20.48 seconds was used to prevent excess tissue damage due to repeated application of the noxious thermal stimulus. Animals were acclimated to
experimenter handling (once a day for one week) prior to testing, and acclimated to the testing apparatus (30 minutes a day for 3 consecutive days) 3 days before the first sc injection. All behavioral testing took place between 12:00 pm and 5:00 pm (lights on at 7:00 am). Groups were coded and testing was conducted blind with respect to group assignment. All groups were represented in each round of testing and testing order was counterbalanced across testing days. In Experiments 1.1 and 2, paw withdrawal latencies (PWL) were determined prior to and 15 minutes post-injection (morphine or saline; day 1 and day 3) to assess the time course of tolerance development.

Tolerance Assessment

Morphine tolerance was assessed on day 4 by injecting cumulative doses of morphine every 20 min, resulting in doses of 1.8, 3.2, 5.6, 8.0, 10.0, and 18.0 mg/kg (sc) as previously described\(^4\),\(^5\),\(^17\). Day 1 was defined as the first day of morphine pretreatment (Experiments 1.1 & 2), LPS pretreatment (Experiment 1.2), or vehicle pretreatment. Nociception was assessed using the paw thermal stimulator 15 min after each injection\(^12\),\(^2\) as described above.

Behavioral data analysis and presentation

Paw withdrawal latencies (PWL; seconds) were measured in triplicate and averaged for each animal. Half-maximal effective dose (ED\(_{50}\)) and 95% confidence intervals (CI) were calculated from dose-response curves generated using GraphPad/Prism software\(^8\). Changes in ED\(_{50}\) between groups were assessed using ANOVA, and Fisher’s post hoc tests were used to determine specific group differences when a significant main effect was observed (GraphPad/Prism). All values are reported as Mean ED\(_{50}\) ± 95% CI; p < 0.050 was considered significant.

Determination of lentivirus expression
Immediately following tolerance assessment, all animals from Experiment 1 were decapitated, and brains removed, flash frozen in 2-methylbutane on dry ice, and stored at -80°C until processing. All tissue processing was conducted using RNase free technique, and working surfaces and slide boxes were treated with RNase Away (Thermo Fisher Scientific). Brains were sectioned into 25 µm coronal sections, immediately slide mounted, and stored in slide boxes with desiccant at -80°C. A 1:6 series through the rostrocaudal axis of the PAG (Bregma -7.04 to -8.30) was used to verify virus infection by visualizing GFP using a Nikon microscope (10 X magnification) with a fluorescent (FITC) filter. Animals with GFP spread located outside of the PAG were excluded from all analyses.

*GLT-1, GLAST, and EAAC1 in situ hybridization*

In a subset of animals (n= 4-6/group), a separate 1:6 series through the caudal axis of the PAG (Bregma -7.04 to -8.30) was used for in situ hybridization. DNA fragments of GLAST, GLT-1 and, to determine which cell type (glia or neuron) is responsible for alterations in glutamate homeostasis, we examined the neuronal excitatory amino acid carrier 1 (EAAC1). DNA fragments were amplified from rat brain cDNA using polymerase chain reaction (PCR). PCR products (1371-bp, 1363-bp, and 1411-bp for GLAST, GLT-1, and EAAC1, respectively) were inserted into pCRII vector (Invitrogen, CA) and were sequenced to verify identification of each gene (Genbank accession number, NM_019225, NM_017215, and NM_013032, respectively). Sense and antisense $^{35}$S-UTP-labelled RNA probes for GLAST, GLT-1, and EAAC1 mRNA were generated using in vitro transcription with the linearized plasmids and T7 or SP6 RNA polymerases (Promega, WI). Brain sections were hybridized with the probes and washed as previously described$^{212}$. The sections were then exposed to Kodak BioMax MR films for 4 weeks.
In situ hybridization densitometry

\(^{14}\)C microscales (GE Healthcare Life Sciences, USA) were used to create standard curves (R\(^2\) > 0.97) for each assay. Scion Image software (NIH and Scion Corp., USA) and a MTI CCD 72 camera and Northern Light box (Imaging Research, Inc., CN) were used to capture 12-bit grayscale images of each section. Optical densities in an area of 0.8 X 1.0 mm were measured in the vIPAG and inferior colliculus (IC). The mean pixel value from 3 sections of each brain per region was recorded and measures corrected for nonspecific binding by subtracting background adjacent to the ROI that lacked hybridization. Changes in mRNA between groups were assessed using ANOVA, and Fisher’s post hoc tests were used to determine specific group differences when a significant main effect was observed (SPSS). Mean specific hybridization is reported as the disintegrations per minute per milligram of tissue (dpm/mg) \(\pm\) SEM; p < 0.050 was considered significant.

Immunoassay determination of XPro®1595 levels

Cerebrospinal fluid (CSF), plasma, and brains were collected from animals pretreated with XPro + Morphine (n=6), XPro + Saline (n=3), Vehicle + Morphine (n = 6), and Vehicle + Saline (n = 3; Experiment 2) for measurement of XPro®1595 concentrations. Animals were removed from the XPro + Saline group (n=3) and from the Vehicle + Saline group (n = 3) because of damage to the tissue. Immediately prior to decapitation, animals were anesthetized with BeuthanasiaD (160 mg/kg; i.p.) and cerebrospinal fluid (CSF) was collected via the cisterna magna puncture method\(^{213}\) with minor modifications. Rats were positioned with the nose pointed downward at a \(~45^\circ\) angle and the cisterna magna was exposed by dissecting through the musculature overlying the posterior atlantooccipital membrane. The membrane was punctured with a glass pipette tip, and was collected via capillary action, and CSF was stored at -80°C. Rats
were then decapitated as described above, and trunk blood was collected in EDTA coated tubes, gently mixed, and put on ice for 1-2 hours. Tubes were centrifuged at 2000 rpm for 18 minutes at 4°C and the plasma was isolated and stored at -80°C. Immediately after trunk blood collection, brains were removed and flash frozen as described above. All tissue collection was complete within 2 minutes. Midbrain, CSF and plasma (diluted 1:4) levels of XPro®1595 were measured using the Meso Scale Discovery anti-human TNF ultrasensitive electrochemiluminescent detection immunoassay (cat # K151BHC-1) and quantified using the Meso Scale Discovery SECTOR Imager 2400-A (Meso Scale Diagnostics, LLC, Rockville, MD). To increase assay sensitivity for determination of levels211, XPro®1595 protein was used instead of the supplied human TNF protein for generation of standards. Data were analyzed using the Meso Scale Discovery integrated data analysis software, which converts signals to pg/ml values. The remaining tissue lysates were assayed for total protein using a Pierce BCA protein assay kit (Thermo Scientific) to ensure equivalent protein loading across samples, and measured on a Spectramax plate reader at 562 nm using SoftMax v5.2 software (Molecular Devices, CA). Data are presented as ng/ml. All samples were assayed in duplicate by an experimenter blinded to treatment history.

vtPAG cytokine and TLR4 qPCR

Brains were collected from animals (n = 5 per group) treated with XPro + Morphine, XPro + Saline, Vehicle + Morphine, and Vehicle + Saline, blocked (Bregma -6.96 to -8.52)130,173, and flash frozen as described above. One-millimeter bilateral micropunches were taken through the vtPAG, and stored in RNAse, DNase, and pyrogen free tubes (1.5ml Fisher Scientific) until RNA isolation for quantitative RT-PCR of proinflammatory cytokines and TLR4 mRNA. Assays were conducted using RNase free technique. Total RNA was isolated from
vLPAG tissue punches using a standard TRIzol Reagent method (Invitrogen Life Technologies, Carlsbad, CA); concentration and purity was assayed on a nanospec, and all RNA was diluted to 60 ng/ml with nanopure water, and converted to cDNA using the Cloned AMV First-Strand cDNA Synthesis Kit (Life Technologies). cDNA sequences were found using GenBank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Measurement of gene expression included the following: Tlr4, Tnf, Il6, Il1b, Il10, and the housekeeping gene Gapdh. Primer sequences were taken from previously published sources (TLR4214, IL-10215, and IL-6165) or designed (TNF, IL-1β, GAPDH) using PrimerQuest (Integrated DNA Technologies, Inc. (IDT), Coralville, IA), and tested for sequence specificity using the Basic Local Alignment Search Tool (BLAST) at NCBI (Tlr4; NM_019178, Tnf; NM_012675, Il6; NM_012589, Il1b; NM_031512, Il10; NM_012854, and Gapdh; NM_017008). All primers were obtained from IDT. Amplification products from step down PCR (60 °C – 50°C) were run on a 2% agarose gel with 0.002% ethidium bromide at 5V per cm for 60 minutes with a DNA ladder (GeneRuler, Thermo Scientific) to verify gel position and lack of doublets as a first pass analysis of primer specificity (Figure 6f). Bands were isolated under UV light, and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen; Venlow, Limburg). Amplification products of forward and reverse primers were sequenced by the GSU Biology Department Core Facility and analyzed with 4Peaks software (nucleobytes.com; Netherlands, Amsterdam) and BLAST to verify identification of each gene (Tlr4; 96/79% (forward/reverse) query coverage with NM_019178, Tnf; 80/43% with NM_012675, Il6; 75/82% with NM_012589, Il1b; 100/91% with NM_031512, and Il10; 89/88% with NM_012854). Finally, primer specificity was further tested via melt curve analysis to verify one single peak (one amplification product), and serial dilution curve analysis to test efficiency, sensitivity, and working range. An acceptable slope (CT value for each dilution
against the log of the quantity of cDNA used for each reaction) was between ± 0.1 units from the control (CYCLOB, slope -3.2 to -3.4). Following primer validation, a temperature gradient PCR was run to determine the optimal annealing temperature for each primer set (57°C was best for all), and primers (forward and reverse) were titrated for optimal concentration pairings (listed in Table 4.2). All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA. Primer sequences are presented in Table 4.2. PCR amplification of cDNA was performed using SYBR Select Master Mix (Invitrogen Life Technologies).

Formation of PCR product was monitored and quantified in real time using the 7500 Fast Real-Time PCR System (Life Technologies). Relative gene expression was determined by taking the expression ratio of the gene of interest to Gapdh. Data are normalized to the Vehicle + Saline condition. Changes in mRNA between groups were assessed using an ANOVA on ranks (Kruskal-Wallis), and Mann Whitney U post-hoc comparisons were used to determine specific group differences when a significant main effect was observed (SPSS). We used nonparametric statistics as our data did not reach the assumptions of normality; Vehicle + Saline controls had very high variability which lead to a lack of homogeneity of variance in the data. Data is expressed as median; p < 0.050 was considered significant.

Results

Sequestration of vlPAG soluble TNF eliminated tolerance to systemic morphine

The inhibitor protein dominant-negative TNF (DN-TNF) is a well-characterized variant of native human TNF that has been engineered to rapidly form heterotrimers with native soluble TNF (solTNF) in rat and mouse brain. Human DN-TNF and GFP, or GFP alone were expressed in the vlPAG via lenti-viral infection (lenti-DN-TNF or lenti-GFP, respectively). DN-TNF heterotrimerization with native solTNF effectively sequesters native solTNF and precludes it
from initiating signaling through neuronal TNFRI by preventing receptor binding, while sparing the beneficial effects mediated by the transmembrane TNF (tmTNF) signal. All animals included in behavior analysis demonstrated robust virus-induced GFP expression (Figure 1b) in the vIPAG as indicated by FITC microscopy (Figure 4.1b). Baseline and post-injection PWLs did not differ between lenti-DN-TNF and lenti-GFP animals treated with systemic saline, indicating that virus expression did not alter basal nociceptive thresholds (Figure 4.1c, black symbols). Administration of morphine on Day 1 and Day 3 produced an increase in PWL in both lenti-DN-TNF treated animals and lenti-GFP controls (red symbols). There was no difference in the analgesic potency of morphine between lenti-GFP and lenti-DN-TNF treated animals on Day 1 (p = 0.837), indicating that virus expression did not alter acute morphine analgesia. ANOVA revealed a significant main effect of treatment on Day 3 (F(3,21) = 29.49; p < 0.000), with the antinociceptive potency of morphine being significantly lower in animals pretreated with lenti-GFP as compared with lenti-DN-TNF (p < 0.000). Morphine efficacy did not differ between days in animals pretreated with lenti-DN-TNF, indicating maintenance of analgesia (i.e., no tolerance). Administration of cumulative doses of morphine on Day 4 produced an increase in PWL in all animals tested (Figure 1d). ANOVA revealed a significant main effect of treatment (F(3, 195) = 49.64; p < 0.0001). Post hoc analysis revealed that pretreatment with lenti-GFP + Morphine resulted in a significant threelfold rightward shift in the dose-response curve (ED50 = 7.47 mg/kg) as compared with lenti-GFP + Saline controls (ED50 = 2.50 mg/kg; p = 0.010; Table 1), indicating tolerance to morphine. Intra-vIPAG pretreatment with lenti-DN-TNF preserved the antinociceptive potency of morphine (ED50 = 2.66 mg/kg) compared with animals made tolerant to morphine (lenti-DN-TNF + Morphine vs. lenti-GFP + Morphine; p = 0.013). Indeed, these animals did not differ from saline controls (lenti-DN-TNF + Morphine vs. lenti-GFP + Saline; p
Together, these results indicate that vlPAG solTNF is necessary for the development of tolerance to subcutaneous morphine.

**Sequestration of vlPAG solute TNF eliminated morphine tolerance induced by vlPAG LPS**

To test our hypothesis that the effects of solTNF are mediated by TLR4 signaling, a separate group of animals received pretreatment with lenti-DN-TNF or lenti-GFP and intra-vlPAG infusions of the TLR4 agonist LPS to induce a naïve tolerance to morphine. All animals included in behavior analysis demonstrated robust virus-induced GFP expression in the vlPAG as indicated by FITC microscopy (Figure 4.2a). Administration of cumulative doses of morphine on Day 4 (first morphine exposure) produced an increase in PWL in all animals tested (Figure 4.2b). ANOVA indicated a significant main effect of treatment ($F_{(3,167)} = 37.31; p < 0.000$).

Replicating our previously published findings, 3 vlPAG infusions of LPS was sufficient to induce ‘naïve’ morphine tolerance, as indicated by a significant rightward shift in the dose response curve (lenti-GFP + PAG LPS; ED$_{50}$ = 6.00 mg/kg) as compared with lenti-GFP + PAG Saline controls (ED$_{50}$ = 2.37 mg/kg; $p = 0.015$; Table 4.1). Intra-vlPAG pretreatment with lenti-DN-TNF preserved the antinociceptive potency of morphine (lenti-DN-TNF + PAG LPS; ED$_{50}$ = 3.26 mg/kg) compared with animals made tolerant to morphine by vlPAG LPS (lenti-GFP + PAG LPS; $p = 0.012$). Indeed, lenti-DN-TNF + PAG LPS animals did not differ from lenti-DN-TNF + PAG Saline controls ($p = 0.067$). These results support our hypothesis that morphine acts through TLR4 to increase solTNF signaling, and decrease morphine efficacy.

**Chronic systemic morphine decreased vlPAG GLT-1 and GLAST mRNA in a solTNF-dependent manner**

We next tested the hypothesis that solTNF contributes to tolerance development by decreasing GLT-1 and GLAST, the primary source of glutamate reuptake in the brain, and
investigated changes in the neuronal glutamate transporter EAAC1. *In situ* hybridization (ISH), in combination with densitometry, was used to measure GLT-1, GLAST, and EAAC1 mRNA in the caudal vlPAG (Figure 4.3). ANOVA indicated a significant main effect of treatment on vlPAG GLT-1 mRNA ($F_{(3,23)} = 3.58; p = 0.029$, Figure 4.3a). *Post hoc* analysis revealed that morphine administration for 3 days significantly decreased vlPAG GLT-1 mRNA, as compared with saline controls (lenti-GFP + Morphine vs. lenti-GFP + Saline; $p = 0.004$). Lentiviral expression of DN-TNF in the vlPAG rescued the expression of GLT-1 mRNA. Indeed lenti-DN-TNF + Morphine animals did not differ from lenti-GFP + Saline controls ($p = 0.128$). Similar to what was noted for GLT-1 mRNA, chronic morphine significantly decreased vlPAG GLAST mRNA ($F_{(3,26)} = 6.681; p = 0.002$, Figure 4.3b) as compared with saline controls (lenti-GFP + Morphine vs. lenti-GFP + Saline; $p < 0.000$). vlPAG DN-TNF expression was sufficient to prevent the morphine-induced decrease in vlPAG GLAST mRNA, as lenti-DN-TNF + Morphine animals did not differ from lenti-GFP + Saline controls ($p = 0.494$). There were no differences noted in vlPAG EAAC1 mRNA expression ($F_{(3,26)} = 0.876; p = 0.466$), suggesting that morphine preferentially alters astrocytic glutamate transport in the vlPAG. Morphine decreased astrocytic glutamate transporter mRNA in a site specific manner, as the adjacent inferior colliculus (IC) did not differ in GLT-1 or GLAST mRNA ($F_{(3,26)} = 1.489; p = 0.241$ and $F_{(3,23)} = 1.471; p = 0.249$, respectively). The inferior colliculus was chosen because there is a high expression of MOR in this region, but the inferior colliculus is not involved in morphine analgesia. Interestingly, ANOVA indicated a significant effect of treatment on IC EAAC1 mRNA ($F_{(3,26)} = 3.720; p = 0.024$, Figure 4.3c inset), with morphine significantly increasing expression as compared with controls (lenti-GFP + Morphine vs. lenti-GFP + Saline; $p < 0.000$). These results support our
hypothesis, and suggest that solTNF contributes to morphine tolerance development by altering astrocyte-mediated glutamate homeostasis in the vlPAG.

*Chronic vlPAG TLR4 agonism decreased vlPAG GLT-1 and GLAST mRNA in a solTNF-dependent manner*

Given that LPS binding to TLR4 is a potent inducer of TNF expression, and that chronic vlPAG LPS administration leads to morphine tolerance\(^8^2\), we also hypothesized that GLT-1 and GLAST would be decreased in animals made tolerant to morphine by LPS. Similar to what was noted for chronic systemic morphine exposure, chronic vlPAG microinfusions of LPS decreased vlPAG GLT-1 and GLAST mRNA (\(F_{(3,12)} = 7.268; p = 0.005\), and \(F_{(3,10)} = 7.321; p = 0.007\), respectively), and did not alter vlPAG EAAC1 mRNA (\(F_{(3,10)} = 1.982; p = 0.166\); Figure 4.4). Lenti-GFP + PAG LPS animals had significant decreases in GLT-1 and GLAST mRNA as compared with lenti-GFP + PAG Saline animals (\(p = 0.005\), Figure 4.4a and \(p = 0.002\), Figure 4.4b, respectively). vlPAG DN-TNF expression rescued GLT-1 mRNA (lenti-DN-TNF + PAG LPS vs. lenti-GFP + PAG Saline; \(p = 0.723\)), and increased, but did not rescue GLAST mRNA (lenti-DN-TNF + PAG LPS vs. lenti-GFP + PAG Saline; \(p = 0.044\)). There were no differences in IC GLAST or EAAC1 mRNA (\(F_{(3,10)} = 0.347; p = 0.792\), Figure 4.4b inset and \(F_{(3,13)} = 0.659; p = 0.592\), Figure 4.4c inset, respectively), however LPS significantly decreased GLT-1 mRNA in the IC (\(F_{(3,12)} = 3.169; p = 0.0064\), lenti-GFP + PAG LPS vs. lenti-GFP + PAG Saline, \(p = 0.015\), Figure 4.4a inset). These results support our hypothesis, and indicate that LPS alters vlPAG astrocytic glutamate uptake in a similar manner to morphine.

*Systemic administration of XPro®1595 prevented morphine tolerance*

Finally, we tested the ability of a novel, brain permeant human DN-TNF peptide (XPro®1595) to preserve morphine analgesia. XPro®1595 is a dominant negative TNF variant
with FDA IND status that selectively diminishes the inflammatory signal of soluble TNF (> 2500-fold)\textsuperscript{192,207} in the same manner as the virally expressed DN-TNF in Experiment 1. An ELISA for human TNF (hTNF) revealed robust presence of hTNF in plasma, CSF, and midbrain tissue from animals treated with XPro\textsuperscript{®}1595 (Figure 4.5c, d, and e). Indeed, midbrain levels strongly correlated with CSF levels of hTNF ($R^2 = 0.867; y = 0.76x + 0.350$), indicating efficient transport into the brain. hTNF was not detected in Vehicle + Morphine or Vehicle + Saline treated animals (data not shown). No differences in baseline nociceptive thresholds or acute (Day 1) morphine analgesia were noted between groups (Figure 4.5a). Animals pretreated with Vehicle + Morphine showed signs of tolerance following the third sc morphine injection, while animals pretreated with XPro\textsuperscript{®}1595 + Morphine did not ($F_{(3,17)} = 17.046; p < 0.000$).

Administration of cumulative doses of morphine on Day 4 produced an increase in nociceptive thresholds in all animals tested (Figure 5b). ANOVA revealed a significant main effect of treatment ($F_{(3,258)} = 87.31, p < 0.0001$). Pretreatment with Vehicle + Morphine resulted in a significant threefold rightward shift in the dose-response curve ($ED_{50} = 8.03$ mg/kg), as compared with Vehicle + Saline animals ($ED_{50} = 2.31$ mg/kg; $p < 0.001$; Figure 4.5), indicating tolerance to morphine. Systemic pretreatment with DN-TNF (XPro\textsuperscript{®}1595) preserved the antinociceptive potency of morphine (XPro + Morphine; $ED_{50} = 3.08$ mg/kg) compared with animals made tolerant to morphine (Vehicle + Morphine; $p < 0.001$). These data indicate that animals given systemic XPro\textsuperscript{®}1595 + Morphine did not develop tolerance to morphine as compared with animals given Vehicle + Morphine. Together, these results support our hypothesis that XPro\textsuperscript{®}1595 could feasibly be used in conjunction with opioid therapy to maintain analgesic efficacy in the clinic.
Systemic administration of XPro®1595 abolished morphine-induced increases in vlPAG TLR4 and IL-1β mRNA

TNF and TLR4 are major regulators of cytokine expression in the CNS (e.g., IL-6, IL-1β, and TNF), and cytokines including IL-1β and IL-6 have been implicated in morphine tolerance development. Our final experiment tested the hypothesis that morphine increases proinflammatory cytokine expression in the vlPAG via solTNF signaling. Changes in vlPAG TLR4 mRNA were also assayed. Quantitative reverse transcriptase PCR (qPCR) revealed that chronic morphine resulted in a median 49% increase in vlPAG TNF mRNA as compared with Vehicle + Saline treated animals (Figure 4.6a). Similar results were noted for IL-6 gene expression, with morphine increasing vlPAG IL-6 mRNA by 8% as compared with Vehicle + Saline controls. However, the increase in TNF and IL-6 gene expression was not statistically significant (Kruskal-Wallis, H = 4.00, 2 df; p = 0.136 and H = 1.56, 2 df; p = 0.457, respectively). There were no differences in IL-10 mRNA (H = 2.42, 2 df; p = 0.298). A one-way ANOVA on ranks indicated a significant main effect of treatment on IL-1β (Figure 4.6b) and TLR4 (Figure 4.6c) mRNA (H = 8.05, 2 df; p = 0.018 and H = 7.49, 2 df; p = 0.024, respectively). Morphine significantly increased vlPAG IL-1β mRNA by 3,500% as compared with Vehicle + Saline controls, a nearly 30-fold increase as compared with the 120% increase noted for XPro + Saline treated controls (W = 15; p = 0.016, Figure 4.6b). Co-administration of systemic XPro®1595 and morphine eliminated the increase in IL-1β gene expression as these animals did not differ from XPro + Saline controls (W = 26; p = 0.841). Finally, post hoc analysis revealed a significant 53% increase in vlPAG TLR4 gene expression in Vehicle + Morphine treated animals as compared with XPro + Saline controls (25% decrease, W = 15; p = 0.016). Co-administration of XPro®1595 with morphine normalized TLR4 mRNA as XPro +
Morphine and XPro + Saline animals did not differ (W = 17.5; p = 0.063). We used nonparametric statistics as our data did not reach the assumptions of normality; Vehicle + Saline controls had very high variability which lead to a lack of homogeneity of variance in the data. In combination with our previous results demonstrating a role for vIPAG TLR4\(^{82}\), these data indicate that solTNF regulates gene expression of the proinflammatory cytokine IL-1\(\beta\) and the innate immune receptor TLR4, and implicate solTNF as an important mediator of vIPAG TLR4-mediated tolerance development.

**Discussion**

The present set of experiments tested the hypothesis that vIPAG TLR4 contributes to morphine tolerance development via soluble TNF-dependent modulation of glutamate homeostasis. Here we report that (1) vIPAG microinjections of lenti-DN-TNF rescued the morphine-induced decrease in astrocytic glutamate transporter mRNA (GLT-1 and GLAST) in the vIPAG, and prevented the development of tolerance to systemically administered morphine; (2) vIPAG microinfusions of the TLR4 agonist LPS induced a naïve tolerance to systemic morphine, as previously reported\(^{82}\), and significantly decreased GLT-1 and GLAST mRNA in the vIPAG in a manner that paralleled the effects of morphine; (3) vIPAG microinjections of lenti-DN-TNF prevented LPS-induced tolerance to morphine, and rescued or increased vIPAG GLT-1 and GLAST gene expression, respectively; and (4) sequestration of solTNF with systemic administration of XPro\(^{®}\)1595 prevented morphine tolerance development, and decreased (TLR4) and eliminated (IL-1\(\beta\)) morphine-induced increases in proinflammatory factors, and attenuated the trending increases in other cytokines (TNF, and IL-6 mRNA) in the vIPAG. Together, these data indicate that the development of morphine tolerance is correlated with increased proinflammatory gene expression and decreased astrocytic glutamate transporter
mRNA in the vlPAG that is TLR4 dependent. Sequestration of vlPAG soluble TNF normalizes these changes and prevents morphine tolerance. Together, these data are the first to isolate soluble TNF signaling as necessary for opioid tolerance, and the first to implicate PAG cytokines and PAG astrocytic glutamate transporters in morphine tolerance.

Morphine and other opioids bind to neuronal MOR\(^6\) and hyperpolarize GABAergic neurons to elicit analgesia\(^9,27-29\). Indeed, opioids have a direct inhibitory effect on most MOR expressing neurons\(^30\) including those in the descending analgesic circuit (i.e., PAG, RVM, and spinal cord)\(^9,11,30-33\). MOR binding in the vlPAG hyperpolarizes GABAergic interneurons\(^34,36\), thereby releasing vlPAG-RVM projection neurons from local tonic inhibition\(^36\). In PAG slices, morphine inhibits mIPSP frequency and decreases the probability of presynaptic GABA release\(^31,34\). This effect is enhanced in the presence of anti-inflammatory compounds including cyclooxygenase inhibitors and 5-lipoxygenase inhibitors\(^31\). Indeed, microinfusion of cyclooxygenase inhibitors into the PAG produces robust analgesia\(^216\). Together with the present results, these data suggest that morphine hyperpolarization of PAG neurons is critical for opioid induced analgesia, and that proinflammatory signaling in the PAG opposes morphine analgesia by decreasing morphine hyperpolarization of GABAergic PAG interneurons.

In agreement with our previous findings\(^45,82,173\), here we show that tolerance to morphine developed rapidly. Indeed, systemic administration of one ED\(_{50}\) dose of morphine (5 mg/kg) for 3 days was sufficient to induce tolerance as indicated by a significant 3-fold rightward shift in the morphine dose response curve. A significant decrease in vlPAG GLT-1 and GLAST mRNA paralleled the development of tolerance, demonstrating that chronic morphine decreases astrocyte-mediated glutamate uptake in the vlPAG. No change was noted in vlPAG neuronal EAAC1 mRNA, suggesting that chronic opioids preferentially alter astrocyte-mediated
glutamate homeostasis in the vlPAG. In controls, GLT-1 mRNA levels were approximately 3 times higher than GLAST and EAAC1, suggesting that GLT-1 is the main source of glutamate uptake in the PAG. Chronic morphine did increase EAAC1 in the inferior colliculus, pointing to a potential role for opioid mediated glutamate plasticity in auditory midbrain regions\textsuperscript{217}. vlPAG soluble TNF was necessary for the molecular and behavioral effects of chronic morphine on glutamate transporter expression, as DN-TNF prevented morphine-induced decreases in GLT-1 and GLAST mRNA in the vlPAG and preserved morphine analgesia.

The present results confirm our previous data indicating that vlPAG astrocytes contribute to the development of morphine tolerance\textsuperscript{82}, and complement a vast literature demonstrating a role for glia\textsuperscript{85} and excitatory neurotransmission\textsuperscript{62,63}. Astrocytes are responsible for the majority of glutamate uptake in the CNS via GLT-1 and GLAST\textsuperscript{218}, thereby terminating glutamatergic signaling\textsuperscript{219-221}. A significant increase in CSF glutamate and aspartate has been reported in morphine-tolerant humans\textsuperscript{52}, and morphine challenge increases glutamate in the CSF of morphine-tolerant rats\textsuperscript{53}. Increased glutamate uptake by GLT-1 attenuates morphine tolerance in mice\textsuperscript{222}, further indicating a role for astrocytes. Chronic morphine also increases AMPA and NMDA receptor subunits\textsuperscript{54} and NMDA receptor binding\textsuperscript{55} in the rat spinal cord, while blocking NMDA\textsuperscript{56-61} and AMPA\textsuperscript{62,63} glutamatergic signaling attenuates tolerance. Together with our results, these data suggest that a breakdown in astrocyte-mediated glutamate homeostasis significantly contributes to opioid tolerance.

The present studies also establish that vlPAG soluble TNF signaling is necessary for the decreased expression levels of vlPAG GLT-1 and GLAST mRNA and morphine tolerance. These findings corroborate previous studies demonstrating a role for spinal cord TNF in morphine tolerance\textsuperscript{54,102,106} and opioid-induced modulation of GLT-1 and GLAST\textsuperscript{102}. Acute or
chronic intrathecal Etanercept, a decoy TNF receptor that sequesters both solTNF and tmTNF, attenuates tolerance to systemic morphine\textsuperscript{106} and the ensuing changes in the glutamatergic system\textsuperscript{54}. Our data from the vPAG complement these findings in the spinal cord, and demonstrate for the first time that soluble TNF, and not tmTNF signaling, mediates morphine tolerance development and alterations in astrocytic glutamate homeostasis.

Our results are in contrast to previous data indicating that modulation of glutamate signaling in the PAG does not prevent tolerance to morphine\textsuperscript{65}. Morgan and colleagues demonstrated that the NMDA receptor antagonist MK-801 microinjected into the ventral PAG (vPAG) does not prevent tolerance to intra-vPAG morphine\textsuperscript{65}. However, chronic morphine administration does not alter NMDA receptor expression in the PAG\textsuperscript{66}, indicating a need to investigate the involvement of alternative mechanisms of glutamate signaling in the PAG. It is important to note that in addition to regulating GLT-1 and GLAST expression, TNF is a major driver of inflammation, increasing the expression of TLR4 and other proinflammatory factors, including glutamate, nitric oxide and inducible nitric oxide synthase, cyclooxygenase 2, TNF, and IL-1\textbeta; these factors all increase neuronal excitability and contribute to opioid tolerance\textsuperscript{71}. Indeed, TNF alters excitation of neurons in several systems. For example, TNF increases the density and conductance of neuronal AMPA receptors and decreases GABA\textsubscript{A} receptors in hippocampal slices\textsuperscript{145}. Functionally, these changes increase miniature EPSCs and decrease miniature IPSCs\textsuperscript{145}. The effects of TNF on hippocampal AMPA and GABA receptor expression occur via neuronal TNFRI but not TNFRII\textsuperscript{145}, implicating soluble TNF. Additionally, TNF, and to a lesser extent IL-1\textbeta, significantly increases neuronal GluR2-lacking AMPARs \textit{in vitro}\textsuperscript{144}. While alterations in vPAG AMPA and GABA\textsubscript{A} receptors were not investigated in the present study, Bobeck and colleagues recently demonstrated that chronic intra-vPAG morphine results
in tolerance that is dependent on alterations in both pre- and post-synaptic GABA release. As TNF can modulate many of the excitatory changes observed in the development of opioid tolerance, preventing solTNF signaling in the vlPAG likely decreases excitation more robustly than any single modulation of glutamate signaling in isolation.

It is now clear that the innate immune receptor TLR4, but not MOR, mediates morphine-induced cytokine release (including TNF) and the induction of tolerance. Here we show that behaviorally defined tolerance to morphine, and the concurrent decrease in GLT-1 and GLAST mRNA, is mediated by vlPAG TLR4 signaling. Consistent with our previous findings, vlPAG infusions of the prototypical TLR4 agonist LPS, in the absence of chronic morphine, resulted in behaviorally-defined tolerance, indicating that activation of vlPAG TLR4 is sufficient to induce tolerance. Intra-vlPAG LPS also significantly decreased GLT-1 and GLAST mRNA in the vlPAG that was on par with the reduction noted in animals made tolerant by chronic systemic morphine. vlPAG LPS administration did not alter neuronal EAAC1 mRNA, although there was a trend towards a decrease, suggesting that chronic TLR4 activation and systemic morphine preferentially alter astrocyte-mediated glutamate homeostasis in a similar manner. Together, these results suggest that vlPAG TLR4 signaling mediates the alterations in glutamatergic signaling induced by chronic morphine. vlPAG soluble TNF was necessary for the molecular and behavioral effects of chronic vlPAG LPS administration, as vlPAG DN-TNF attenuated LPS-induced decreases in vlPAG GLT-1 and GLAST mRNA, and preserved morphine analgesia. These data complement our previous results demonstrating the necessity and sufficiency of vlPAG TLR4 signaling in the development of morphine tolerance and further demonstrate that TLR4-mediated increases in soluble TNF signaling result in enhanced neuroexcitability in the vlPAG and induction of morphine tolerance.
XPro®1595 is a novel, brain-permeant, dominant negative TNF variant with FDA IND status that selectively diminishes the inflammatory signal of soluble TNF (> 2500-fold)\(^{192,207}\) in the same manner as the virally expressed DN-TNF. Here we demonstrate that systemically administered XPro®1595 (DN-TNF) decreased opioid-induced inflammation in the vlPAG and prevented morphine tolerance. XPro®1595 administration also prevented the morphine-induced increase in IL-1β and attenuated the significant increase in TLR4 mRNA in the vlPAG, and eliminated the trending increase in TNF and IL-6 mRNA. Together, these data corroborate previous results indicating that TNF is a major driver of inflammation, and support XPro®1595 as a potential addition to opioid therapy in clinical pain management. XPro®1595 may be preferable over current FDA approved anti-TNF biologics (Etanercept, Infliiximab, Adalimumab) as these drugs block both forms of TNF and are associated with encephalic lesions, neuritis, multiple sclerosis, and other demyelinating diseases\(^{194}\).

Our results are consistent with previous studies implicating IL-1β in glutamatergic plasticity and opioid tolerance\(^7^1\). For example, opioids increase IL-1β mRNA and protein \textit{in vitro} and \textit{in vivo}\(^{223,224}\), and modulation of spinal IL-1β attenuates opioid tolerance development\(^225\). IL-1β also increases neuronal GluR2-lacking AMPAR expression\(^{144}\), contributing to an increased excitatory environment. In the present study, opioid tolerance was accompanied by a significant increase in vlPAG TLR4 mRNA, indicating that chronic morphine primes vlPAG glia to overrespond to subsequent morphine challenges by increasing gene expression of the receptor substrate for opioid-mediated inflammation (TLR4\(^{78,81}\)). The simultaneous increase in IL-1β and TLR4 mRNA is of note as these factors share a similar signaling domain (Toll/IL-1 receptor (TIR) domain) that associates with MyD88 to induce robust inflammation\(^{226}\).
Contrary to what has been noted in the spinal cord, chronic morphine did not increase vlPAG TNF mRNA, however, there was a trend towards an increase. TNF is found in very low concentrations in the CNS (femtomolar to picomolar range), and binds with high affinity to a relatively small number of receptors\textsuperscript{227}. In \textit{vitro}, TNF protein is significantly increased by the TLR4 agonist LPS, in a TACE-dependent manner, 30 minutes following LPS application\textsuperscript{228}. In \textit{vivo}, acute intrathecal morphine analgesia is reduced by TNF within 5 minutes of morphine administration\textsuperscript{74}, suggesting that morphine leads to the quick release of \textit{soluble} TNF protein via TACE-mediated cleavage of tmTNF. As TNF is primarily responsible for initiating the production of other proinflammatory cytokines\textsuperscript{71}, morphine signaling through TLR4 may induce rapid cleavage of tmTNF to solTNF protein and stimulate the production of IL-1β and TLR4 mRNA to modulate glutamate homeostasis. TACE inhibition reduces TNF, IL-6, and IL-1α in equine species \textit{in vivo} and in human cells \textit{in vitro} to reduce pain\textsuperscript{229,230}. Together with these dissertation results (Chapter 1-4), these findings suggest that TACE inhibition may preserve morphine analgesia and prevent tolerance development in the vlPAG by preventing TLR4-mediated \textit{soluble} TNF signaling. A trend towards an increase in vlPAG IL-6 mRNA was also noted, and there was no morphine-induced alteration in the anti-inflammatory cytokine IL-10. Together these data indicate that chronic morphine induces alterations in vlPAG inflammation that parallel and diverge from what has been noted in other CNS regions.

In summary, the development of morphine tolerance was paralleled by increased proinflammatory gene expression and decreased astrocytic glutamate transporter mRNA in the vlPAG. Our results further show that exclusive sequestration of solTNF prevents opioid induced neuroinflammation, alterations in glutamate homeostasis, and morphine tolerance, indicating that tmTNF signaling (TNFRI and TNFRII) does not contribute to opioid tolerance development. As
TNFRII is protective against glutamate excitotoxicity, TNFRII signaling may be a critical countermeasure to opioid-induced neuroexcitability that is important to preserve. These data are also the first to demonstrate a direct role for PAG cytokines in tolerance development. Chronic vlPAG LPS resulted in behavioral and molecular phenotypes that paralleled morphine-treated animals, suggesting that vlPAG innate immune receptor signaling mediates the proinflammatory effects of opioids. Finally, our results support XPro®1595 as a potential anti-inflammatory gene therapy to complement opioid therapy in clinical pain management, as XPro®1595 injected systemically was efficiently transported to midbrain tissue, normalized vlPAG neuroinflammation, and effectively preserved behaviorally defined morphine analgesia. Together, these data suggest that the ability of morphine to hyperpolarize neurons in the PAG is critical for opioid induced analgesia, and that proinflammatory signaling in the PAG can oppose morphine analgesia by decreasing the ability of morphine to hyperpolarize GABAergic PAG interneurons.

**Acknowledgements**

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Table 4.1 Summary of morphine ED50s following chronic morphine administration.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>ED50 (mg/kg)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>lenti GFP + Saline</td>
<td>4</td>
<td>2.50</td>
<td>1.86 – 3.06</td>
</tr>
<tr>
<td>lenti GFP + Morphine</td>
<td>10</td>
<td>7.47</td>
<td>6.90 - 8.03</td>
</tr>
<tr>
<td>lenti DN-TNF + Saline</td>
<td>5</td>
<td>3.93</td>
<td>2.97 – 4.88</td>
</tr>
<tr>
<td>lenti DN-TNF + Morphine</td>
<td>10</td>
<td>2.66</td>
<td>2.14 – 3.18</td>
</tr>
<tr>
<td>$F_{(3,195)} = 49.64; p &lt; 0.0001$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lenti GFP + PAG Saline</td>
<td>6</td>
<td>2.37</td>
<td>2.05 – 2.70</td>
</tr>
<tr>
<td>lenti GFP + PAG LPS</td>
<td>4</td>
<td>6.00</td>
<td>5.42 – 6.58</td>
</tr>
<tr>
<td>lenti DN-TNF + PAG Saline</td>
<td>6</td>
<td>3.00</td>
<td>2.61 – 3.40</td>
</tr>
<tr>
<td>lenti DN-TNF + PAG LPS</td>
<td>9</td>
<td>3.26</td>
<td>2.90 – 3.62</td>
</tr>
<tr>
<td>$F_{(3,167)} = 37.31; p &lt; 0.0001$</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle + Saline</td>
<td>8</td>
<td>2.30</td>
<td>1.99 – 2.60</td>
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<tr>
<td>Vehicle + Morphine</td>
<td>11</td>
<td>7.35</td>
<td>6.82 – 7.88</td>
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<tr>
<td>XPro + Saline</td>
<td>8</td>
<td>3.18</td>
<td>2.69 – 3.67</td>
</tr>
<tr>
<td>XPro + Morphine</td>
<td>11</td>
<td>2.25</td>
<td>1.86 – 2.65</td>
</tr>
<tr>
<td>$F_{(3,258)} = 87.31; p &lt; 0.0001$</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>
Table 4.2 Summary of qPCR primer specifications.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Target Sequence (Fwd/Rev)</th>
<th>Primer Concentration (nM; Fwd/Rev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnf</td>
<td>TGTACCTTATCTACTCCCAGGTTCCTCT/ GTGTGGGTGAGGAGACGTGA</td>
<td>200/400</td>
</tr>
<tr>
<td>Il1b</td>
<td>AAGACCCAAGCACCTTCTTT/ AGACAGCACAGGCTTTT</td>
<td>400/400</td>
</tr>
<tr>
<td>Tlr4</td>
<td>TCCCTGCATAGAGGTACCTTC/ CACACCTGGATAAAATCCAGC</td>
<td>400/400</td>
</tr>
<tr>
<td>Il6</td>
<td>GGGACTGATGTTGGTGACAGCC/ CATATGTAATTAAGCTCCGACTTGTG</td>
<td>400/400</td>
</tr>
<tr>
<td>Il10</td>
<td>TAAGGGTTACTTGGGTTGACC/ TATCCAGAGGTCTTCAGC</td>
<td>200/400</td>
</tr>
</tbody>
</table>
Chapter 4 Figures

**Figure 4.1 Summary of acute morphine analgesia and morphine tolerance following vlPAG soluble TNF sequestration.**

Viral vector-mediated sequestration of soluble TNF in the vlPAG prevents the development of tolerance to morphine. (a) Schematic of lenti-GFP and lenti-DN-TNF lentiviral vectors. Both lenti-GFP and lenti-DN-TNF vectors contain a chicken b-actin cytomegalovirus enhancer/promoter (CAG), b-globin intron, a central polypurine tract of HIV-1 (ppt), a woodchuck hepatitis virus posttranscriptional response element (WPRE), and a self-inactivating deletion in the 3’ LTR. The lenti-DN-TNF vector contains an internal ribosome entry site (IRES) for GFP expression following the sequence for pro-human DN-TNF (A145R/I97T). (b) Representative locations of lenti-GFP (circles) and lenti-DN-TNF (squares) microinfusions in the caudal vlPAG (Bregma – 8.03), and representative light and FITC images of injection location and GFP expression from the same section. Dotted lines indicate aqueduct location. (c) Paw withdrawal latency (s) during nociceptive testing on day 1 (D1) and day 3 (D3) at baseline and 15 minutes post saline or morphine injection in groups pre-treated with lenti-GFP or lenti-DN-TNF. Baseline nociceptive thresholds did not differ on D1 or D3. Lenti-GFP + Saline (n = 4; black circles) and lenti-DN-TNF + Saline (n= 5; black squares) groups do not differ at any time point. There was no difference in the analgesic potency of morphine between lenti GFP (n = 10) and lenti-DN-TNF (n = 10; red symbols) treated animals on D1 (p = 0.84). The antinociceptive potency of morphine was significantly lower in animals pretreated with lenti-GFP as compared with lenti-DN-TNF (p < 0.000) on D3. Data are represented as mean PWL ± SEM. (d) Paw withdrawal latency (s) during cumulative morphine injections on D4 in animals pretreated with lenti-GFP + Saline (black circles), lenti-GFP + Morphine (red circles), lenti-DN-TNF + Saline (black squares), and lenti-DN-TNF + Morphine (red squares). Pretreatment with lenti-GFP + Morphine resulted in a three fold rightward shift in the dose-response curve compared with lenti-GFP + Saline controls (p = 0.01), indicating tolerance to morphine. Sequestration of vlPAG solTNF prevented tolerance as lenti-DN-TNF + Morphine animals did not differ from lenti-GFP + Saline controls (p = 0.15). Data are represented as mean PWL ± SEM.
Figure 4.2 Summary of morphine tolerance following vlPAG LPS and solTNF sequestration.

Aq stands for aqueduct. Viral vector-mediated sequestration of soluble TNF in the vlPAG prevents the development of morphine tolerance induced by vlPAG LPS microinfusions. (a) Representative locations of lenti-GFP (circles) and lenti-DN-TNF (squares) microinfusions in the vlPAG (Bregma -8.04), and representative FITC photomicrograph of GFP expression in the vlPAG. Aq indicates the location of the aqueduct. (b) Paw withdrawal latency (s) during cumulative morphine injections in animals pretreated with lenti-GFP + PAG Saline (n = 4; black circles), lenti-GFP + PAG LPS (n = 4; red circles), lenti-DN-TNF + PAG Saline (n = 6; black squares), and lenti-DN-TNF + PAG LPS (n = 9; red squares). Pretreatment with lenti-GFP + LPS resulted in a significant rightward shift in the dose-response curve compared with lenti-GFP + PAG Saline controls (p = 0.015), indicating tolerance to morphine. Sequestration of vlPAG solTNF prevented tolerance as lenti-DN-TNF + PAG LPS animals did not differ from lenti-DN-TNF + PAG Saline controls (p = 0.067). Data are represented as mean PWL ± SEM.
Figure 4.3 Summary of vlPAG glutamate transporter mRNA following chronic morphine and vlPAG sequestration of solTNF.

Viral vector-mediated sequestration of soluble TNF in the vlPAG prevents the morphine-induced decrease in astrocytic glutamate transporter mRNA. (a) Representative photomicrographs (left inset) of GLT-1 specific hybridization in the caudal PAG (Bregma -8.04) of animals treated with lenti-GFP + Saline (left) and lenti-GFP + Morphine (right). Aq denotes the aqueduct and the red box represents the sampling region. Mean GLT-1 specific hybridization in the inferior colliculus
(right inset) did not differ between groups (p = 0.241). Mean GLT-1 specific hybridization in the caudal vPAG (main graph). Morphine (once a day for 3 days) significantly decreased vPAG GLT-1 mRNA as compared with lenti-GFP + Saline controls (p = 0.004). Sequestration of soluble TNF in the vPAG prevented the morphine-induced decrease in GLT-1 mRNA as lenti-DN-TNF + Morphine animals did not differ from lenti-GFP + Saline controls (p = 0.128). (b) Representative photomicrographs (left inset) of GLAST specific hybridization in the caudal PAG (Bregma -8.04) of animals treated with lenti-GFP + Saline (left) and lenti-GFP + Morphine (right). Mean GLAST specific hybridization in the inferior colliculus (right inset) did not differ between groups (p = 0.249). Mean GLAST specific hybridization in the caudal vPAG (main graph). Morphine (once a day for 3 days) significantly decreased vPAG GLAST mRNA as compared with lenti-GFP + Saline controls (p < 0.000). Sequestration of soluble TNF in the vPAG prevented the morphine-induced decrease in GLAST mRNA as lenti-DN-TNF + Morphine animals did not differ from lenti-GFP + Saline controls (p = 0.494). (c) Representative photomicrographs (left inset) of neuronal EAAC1 specific hybridization in the caudal PAG (Bregma -8.04) of animals treated with lenti-GFP + Saline (left) and lenti-GFP + Morphine (right). Mean EAAC1 specific hybridization was increased in the inferior colliculus (right inset) in animals treated with lenti-GFP + Morphine as compared with lenti-GFP + Saline controls (p < 0.000). Mean EAAC1 specific hybridization in the caudal vPAG (main graph) did not differ between groups (p = 0.466). Specific hybridization is reported as the mean disintegrations per minute per milligram of tissue (dpm/mg) ± SEM; n = 4/6 per group).
Figure 4.4 Summary of vlPAG glutamate transporter mRNA following chronic vlPAG LPS and sequestration of solTNF.
Viral vector-mediated sequestration of soluble TNF in the vlPAG prevents or attenuates the LPS-induced decrease in astrocytic glutamate transporter mRNA. (a) Representative photomicrographs (left inset) of GLT-1 specific hybridization in the caudal PAG (Bregma -8.04)
of animals treated with lenti-GFP + PAG Saline (left) and lenti-GFP + PAG Morphine (right). Aq denotes the aqueduct and the red box represents the sampling region. Mean GLT-1 specific hybridization in the inferior colliculus (right inset) was decreased in animals treated with lenti-GFP + PAG LPS as compared with lenti-GFP + PAG Saline controls (p = 0.015). Mean GLT-1 specific hybridization in the caudal vIPAG (main graph). LPS (once a day for 3 days) significantly decreased vIPAG GLT-1 mRNA as compared with lenti-GFP + PAG Saline controls (p = 0.005). Sequestration of soluble TNF in the vIPAG prevented the morphine-induced decrease in GLT-1 mRNA as compared with lenti-DN-TNF + PAG LPS animals did not differ from lenti-GFP + PAG Saline controls (p = 0.723). (b) Representative photomicrographs (left inset) of GLAST specific hybridization in the caudal PAG (Bregma -8.04) of animals treated with lenti-GFP + PAG Saline (left) and lenti-GFP + PAG LPS (right). Mean GLAST specific hybridization in the inferior colliculus (right inset) did not differ between groups (p = 0.347). Mean GLAST specific hybridization in the caudal vIPAG (main graph). LPS (once a day for 3 days) significantly decreased vIPAG GLAST mRNA as compared with lenti-GFP + Saline controls (p = 0.002). Sequestration of soluble TNF in the vIPAG (lenti-DN-TNF + LPS) attenuated, but did not eliminate, the LPS-induced decrease in GLAST mRNA as compared with lenti-GFP + PAG Saline controls (p = 0.044). (c) Representative photomicrographs (left inset) of neuronal EAAC1 specific hybridization in the caudal PAG (Bregma -8.04) of animals treated with lenti-GFP + PAG Saline (left) and lenti-GFP + PAG LPS (right). Mean EAAC1 specific hybridization in the inferior colliculus (right inset) did not differ (p = 0.592). Mean EAAC1 specific hybridization in the caudal vIPAG (main graph) did not differ between groups (p = 0.166). Specific hybridization is reported as the mean disintegrations per minute per milligram of tissue (dpm/mg) ± SEM; n = 4/6 per group.
Figure 4.5 Summary of acute morphine analgesia and morphine tolerance following systemic XPro1595 and morphine.

Systemic administration of XPro®1595 prevents the development of tolerance to morphine. (a) Paw withdrawal latency (s) during nociceptive testing on day 1 (D1) and day 3 (D3) at baseline and 15 minutes post saline or morphine injection in groups pre-treated with XPro®1595 or Vehicle (saline). Baseline nociceptive thresholds did not differ on D1 or D3. Vehicle + Saline (n = 8; black circles) and XPro + Saline (n= 8; black squares) groups do not differ at any time point. There was no difference in the analgesic potency of morphine between Vehicle (n = 11) and XPro®1595 (n = 11; red symbols) treated animals on D1 (p = 0.84). The antinociceptive potency of morphine was significantly lower in animals pretreated with Vehicle as compared with XPro®1595 (p < 0.000) on D3. Data are represented as mean PWL ± SEM. (b) Paw withdrawal latency (s) during cumulative morphine injections on D4 in animals pretreated with Vehicle + Saline (black circles), Vehicle + Morphine (red circles), XPro + Saline (black squares), and XPro + Morphine (red squares). Pretreatment with Vehicle + Morphine resulted in a significant rightward shift in the dose-response curve compared with Vehicle + Saline controls (p < 0.000), indicating tolerance to morphine. Sequestration of solTNF (XPro + Morphine) prevented tolerance as compared with Vehicle + Morphine controls (p < 0.001). Data are represented as mean PWL ± SEM. (c, d, e) Midbrain, CSF, and plasma expression of DN-TNF in animals treated with XPro®1595 as measured with ELISA for human TNF (n= 3/6 per group).
Figure 4.6 Summary of cytokine and TLR4 mRNA in the vlPAG following chronic systemic XPro1595 and morphine.

Systemic administration of XPro®1595 prevents morphine induced increases in vlPAG IL-1β and TLR4 mRNA. (a) Tnf, (b) Il1β, (c) Tlr4, (d) Il6, and (e) Il10 mRNA levels relative to the housekeeping gene Gapdh, normalized to the Vehicle + Saline group in the vlPAG of animals treated with Saline + Morphine, XPro + Saline, and XPro + Morphine. (b) Morphine significantly increased vlPAG IL-1β (p = 0.018) and (c) TLR4 (p = 0.024) mRNA in the vlPAG. Systemic XPro®1595 prevented the morphine induced increase in IL-1β and TLR4 mRNA as these animals did not differ from XPro + Saline treated controls (p = 0.841 and p = 0.063, respectively). Data are represented as median % change from Vehicle + Saline controls; n = 5 per group. (f) All primer pairs produced amplicons of appropriate size as compared to the control DNA ladder. Representative schematic of vlPAG tissue punch location (top left). PCR amplicons from each gene of interest were run on a 2% agarose gel (main photomicrograph) to demonstrate primer specificity. All genes localized to regions appropriate to their predicted size.
CHAPTER 5: General Discussion
vlPAG astrocyte activity significantly contributes to morphine tolerance.

Chronic pain, defined as pain lasting more than three months, impacts approximately 55% of the population over the age of 20. Opioid-based narcotics are the most prevalent therapeutic treatment for the management of pain. Although morphine is amongst the most effective analgesics available, prolonged morphine treatment results in decreased analgesic efficacy (i.e., tolerance), resulting in the requirement for steadily larger doses of opiates for the maintenance of analgesic efficacy. Many mechanisms have been proposed to account for opioid tolerance, including MOR decoupling, internalization, and/or down-regulation of MORs. However, in comparison to other opioids (e.g., DAMGO, fentanyl), morphine does not result in MOR internalization, and is remarkably weak in terms of decreasing G-protein signaling and receptor desensitization. Indeed, chronic morphine increases G-protein efficiency in the vlPAG. Together with our data (Chapter 2 – 4), these data suggest other mechanisms contribute to morphine tolerance at the level of the midbrain PAG.

Non-opioid receptor-related hypotheses, including alterations in neuronal excitability and glutamatergic signaling have also been proposed. For example, upregulation of NMDA and AMPA receptor function, down-regulation of glutamate transporters, and production of nitric oxide (NO; a known mediator of NMDAR function) have all been shown to contribute to tolerance. However, it is now becoming increasingly clear that ‘activated’ glia (microglia and astrocytes; the ‘non’-neuronal cells of the CNS) mediate many of these effects, as well as other adaptations that contribute to morphine tolerance.

Under basal conditions, microglia and astrocytes survey the environment for pathogens, including viruses and bacteria. Disturbances in homeostasis results in the rapid activation of glia, evidenced by a profound shift in morphology that can be easily visualized using
immunohistochemistry for OX42 (CD11b; microglia) and glial fibrillary acidic protein (GFAP; astrocytes). Glial activation also results in the increased production and release of pro-inflammatory cytokines (including IL-1β, IL-6, and TNFα), chemokines, ATP, excitatory amino acids, and NO, all of which increase the excitability of nearby neurons. Indeed, glially-derived cytokine release results in increases in the number and conductance of AMPA and NMDA receptors, decreases in astrocytic glutamate transporter proteins, and down-regulation of GABA receptors. Microglia derived IL-1β and TNF bind to receptors on astrocytes, resulting in the further release of IL-1β, IL-6, and TNF. These cytokines actively oppose the analgesic actions of morphine reducing pain relief.

Acute and chronic morphine administration activates microglia and astrocytes, with the degree of glial activation increasing with duration of opioid treatment. Increased opioid consumption and the ensuing glial activation ultimately results in the opposition of morphine analgesia and the development of morphine tolerance. Studies by Song and Zhou (2001), as well as others, have shown that inhibition of spinal glia activation with general glial metabolic inhibitors results in a partial attenuation in morphine tolerance. Interestingly, spinal glia activation induced by other stimuli, such as neuropathic pain or lipopolysaccharide (LPS; a potent TLR4 agonist) administration, also reduces the analgesic efficacy of morphine.

Complementing literature from the spinal cord and RVM, here we demonstrate that glial activation within the PAG is critical for the development of morphine tolerance. First, we found that administration of a single ED50 dose of morphine once a day for 3 days (but not administration of a single ED50 dose of morphine once a day for only 1 day; Chapter 2) significantly activated both microglia and astrocytes in the PAG, as indicated by a 2-fold increase in OX42 and GFAP protein levels (when measured 24 hours later).
expression within the PAG paralleled the development of morphine tolerance such that animals that were tolerant to the antinociceptive effects of morphine had the highest OX42 and GFAP immunoreactivity (Chapter 2 and 3). Persistent inflammatory hyperalgesia induced by intraplantar administration of complete Freund’s adjuvant (CFA), significantly attenuated the development of morphine tolerance, and no significant differences were noted in vlPAG glial cell activation for CFA + Saline and CFA + Morphine treated animals versus controls (Handled + Saline; Chapter 2). These data mirror clinical data indicating that peripheral inflammatory pain delays the development of tolerance\textsuperscript{122,153-155}, and suggest that there is something unique about persistent inflammatory pain that blocks morphine from activating glia.

Shortly after publication of Chapter 2, Morgan and colleagues reported that intraplantar CFA prevents tolerance to chronic intra-vlPAG microinjections of morphine\textsuperscript{235}. These data corroborate our results, and suggest that pain-induced changes in the vlPAG are responsible for the preservation of opioid analgesia during a persistent inflammatory pain state. No PAG glia activation was noted following administration of CFA alone (Chapter 2). These results suggest that peripheral pain site-specifically prevents glial cell activation in the PAG, and are in contrast to several studies at the level of the spinal cord and medulla (i.e., RVM) demonstrating that peripheral pain, including CFA\textsuperscript{163-166,176}, peripheral neuropathy\textsuperscript{71,90,94,127,165,167,168}, formalin\textsuperscript{169}, and spinal nerve ligation\textsuperscript{170} induce significant glia activation. However, given the unique roles of the PAG and spinal cord in pain modulation and pain facilitation, respectively, it is not entirely surprising that there would be differential pain-induced regulation of glial activation in these two sites. Chronic constriction of nerves (chronic constriction injury; CCI) induces robust hyperalgesia and also induces robust increases in RVM and spinal cord microglia and astrocyte activity\textsuperscript{176}. CCI-induced increases in pain sensitivity are attenuated by glial cell inhibitors
(propentofylline, minocycline, and fluorocitrate) injected intrathecally (at doses identical to Chapter 3 studies), indicating that pain induces glial cell activity at the level of the spinal cord and RVM, but not at the level of the PAG.

Interestingly, neuropathic pain is thought to result from nerve damage and the slow death of neurons, and burn pain induces robust inflammation. Cell death increases following neuropathic pain and burn pain, and cell death is regulated, in part, by TLR4 and TNF signaling. Cell death results in the release of cytokines (TNF, IL-1β, IL-6), alarmins (heat shock proteins and fragments of self DNA that indicate nuclear cell damage), reactive oxygen species (nitric oxide synthase; NOS, inducible nitric oxide synthase iNOS, and nitric oxide; NO), and other proinflammatory factors (e.g., heat shock proteins) that activate glia, excite neurons, and prevent morphine analgesia. Glia regulate all of these effects via TLR4 signaling, and blocking glia (particularly TNF) decreases excitotoxicity and increases morphine efficacy in the clinic and in the laboratory (Chapter 2 – 4). Crucially, neuropathic pain and burn pain induce more inflammation than other painful conditions, and morphine is not an effective acute or long-term analgesic for the treatment of neuropathic pain or burn pain in the clinic. Together with our data, these data suggest that certain types of pain facilitate neuroinflammation (neuropathic and burn pain) more robustly than others (CFA and formalin) to rapidly increase neuronal excitability and prevent the inhibitory effects of morphine. This positive feedback loop of neuroinflammation is partially regulated by TLR4 and TNF. Further investigation of neuronal death markers in the PAG would contribute to our understanding of how neurons fit in to this puzzle.

Together, these studies suggest that persistent inflammatory pain elicits differential glial responses in a CNS region-dependent manner, and prevents morphine-induced increases in
vlPAG glial cell activity. While we did not investigate the mechanisms whereby pain inhibits vlPAG glia to preserve analgesia, evidence suggests a role for endogenous cannabinoids. Cannabinoids are released within the PAG during peripheral pain\textsuperscript{156}, and cannabinoid receptors are robustly expressed within the vlPAG, with approximately 32\% of cannabinoid receptor 1 (CB1)-expressing neurons also expressing MOR\textsuperscript{157}. Functionally, intra-PAG administration of a CB1 agonist enhances morphine analgesia\textsuperscript{158}, and systemic administration of cannabinoids, along with morphine, leads to the attenuation of morphine tolerance\textsuperscript{158-160}.

Endocannabinoids possess potent anti-inflammatory properties\textsuperscript{161}, which would likely block the activation of glia. Indeed, systemic administration of the cannabinoid receptor agonist WIN 55,212-2 prevents morphine- or pain-induced glia activation and decreases the release of the proinflammatory cytokines IL-1β, IL-6, and TNF in the spinal cord\textsuperscript{159,162}. Recent data also indicate that cannabinoids and opioids synergize in the vlPAG to increase opioid-mediated analgesia\textsuperscript{158,244} suggesting that cannabinoid signaling in the PAG inhibits glia to enhance opioid analgesia. Together, our results complement data from the spinal cord and RVM demonstrating increased OX-42 and GFAP following chronic morphine. Additionally, we hypothesize that pain leads to the release of endogenous cannabinoids (anandamide) in the vlPAG\textsuperscript{156,245}, and that cannabinoids suppress glia activity to prolong the efficacy of opioids (Chapter 2). This hypothesis has not been tested. However, preliminary data from an incomplete collaboration with Dr. Heather Bradshaw\textsuperscript{246-251}, demonstrated that CFA induced the robust release of anandamide and 2-arachidonoylglycerol (2-AG) in the vlPAG (personal communication/side project). Additionally, Dr. Bradshaw’s analysis revealed that this effect was greater in females, suggesting that cannabinoids may mediate analgesia in females, while morphine is not effective\textsuperscript{45,114,123,252,253}. These hypotheses remain to be tested.
vlpag microinfusions of the general glial cell inhibitor propentofylline or the astrocyte inhibitor fluorocitrate significantly attenuated both morphine-induced vlPAG glial cell activation and tolerance (Chapter 3). While minocycline significantly attenuated microglial cell activation in the vlPAG, morphine tolerance was not affected, suggesting that vlPAG astrocytes may be more critical for the maintenance, and not initiation, of tolerance. These data are in contrast to results from the spinal cord demonstrating that inhibition of either astrocytes or microglia in isolation attenuates morphine tolerance to morphine. Chronic intrathecal morphine increases microglial p38 MAPK to induce tolerance to morphine, and pretreatment with intrathecal minocycline partially restores analgesia. These data, in combination with data from Chapter 2 demonstrating that CFA does not increase vlPAG glia activity as it does in the spinal cord, indicate that overlapping and distinct mechanisms underlie glia-mediated tolerance in the CNS. In combination with data from the spinal cord and RVM, our data indicate that neuroinflammation contributes to morphine tolerance at every level of the descending pain modulatory circuit.

**vlPAG TLR4 is necessary and sufficient for morphine tolerance**

It is well established that morphine activates glia and that glia contribute to morphine tolerance. However, it was not until 2010 that the mechanism by which morphine activates glia was discovered. At that time, the innate immune receptor, TLR4, was shown to bind opioids like morphine and mediate glial cell activation. TLR4 is found primarily on microglia, and to a lesser degree on astrocytes, but not neurons. TLR4 recognizes the endotoxin lipopolysaccharide (LPS; the prototypical TLR4 agonist), endogenous danger signals including “alarmins” (e.g., fragments of self DNA in the extracellular space that indicate cell nucleus damage), and certain xenobiotics including both synthetic (e.g., morphine, naloxone, oxycodone,
buprenorphine, fentanyl\textsuperscript{78} and endogenous (e.g., M3G\textsuperscript{79}) opioids. Interestingly, MOR binds M6G, but not M3G\textsuperscript{6}. Conversely, TLR4 binds M3G but not M6G\textsuperscript{79,89,254}. Unlike MOR, which only binds the (−)-stereoisomer of opioids, TLR4 binds opioid agonists and antagonists in a non-stereoselective fashion that maintains their agonistic and antagonistic properties at TLR4\textsuperscript{78}. TLR4 agonists, including opioids and LPS, bind to the MD2 region of TLR4 resulting in activation of three separate signaling cascades; the PI3K/Akt, NFκB, and the MAPK pathway\textsuperscript{81}. The former results in cell motility and apoptosis and the latter two pathways are responsible for the production of proinflammatory substances such as cytokines.

It is now clear that TLR4 signaling contributes to the development of morphine tolerance\textsuperscript{78}. Recent \textit{in vivo} and \textit{in vitro} data demonstrate that the innate immune receptor TLR4, but not MOR\textsuperscript{81}, mediates morphine-induced cytokine release (including TNF)\textsuperscript{78,79,81}. Chronic morphine increases TLR4 mRNA expression (Chapter 4)\textsuperscript{79,255} and downstream products of the TLR4 signaling cascade (e.g., IL-1β; Chapter 4), and systemic TLR4 antagonists attenuate tolerance to systemic morphine\textsuperscript{78}. Results from Chapter 3 further elucidate the mechanisms by which morphine alters glia activity, and indicate that vlPAG glia regulate morphine tolerance development via TLR4 signaling. For the first time, we demonstrate expression of the TLR4 co-receptor myeloid differentiation factor 2 (MD2) in the PAG, indicating the presence of TLR4. MD-2 immunoreactivity was significantly denser in PAG regions important for morphine analgesia (lateral and ventrolateral PAG) as compared with other subnuclei (dorsal PAG), indicating that morphine may preferentially activate glia in the vlPAG. Indeed morphine-induced OX-42, Iba1, and GFAP expression is most robust in ventral PAG regions (unpublished observations). vlPAG microinfusions of TLR4 antagonists, including (+)-naloxone, dose-dependently prevented tolerance to systemic morphine, and vlPAG microinfusions of TLR4...
agonists (in the absence of morphine) dose-dependently produced a naïve tolerance to subsequent challenge doses of morphine, as indicated by a significant 3-fold rightward shift in the morphine dose-response curve (Chapter 3). Together these data are the first to identify a CNS locus through which TLR4 modulates opioid tolerance, and indicate that vlPAG TLR4 signaling is necessary and sufficient for the development of morphine tolerance. It is important to note that studies demonstrating the necessity of MOR signaling in tolerance development using ‘opioid-specific’ ligands need to be re-examined, as TLR4 binds several of these ligands in a manner that maintains their agonistic and antagonistic properties\(^{78}\).

Using a model of persistent inflammatory pain (CFA) we also demonstrated that systemic antagonism of TLR4 with (+)-naloxone potentiated the antihyperalgesic effects of acute morphine. These data suggest that while one 24 hour pretreatment with morphine is not sufficient to increase vlPAG glial cell activity in the absence of pain (Chapter 2), acute morphine induces TLR4 signaling that counteracts the acute antihyperalgesic potency of opioids (Chapter 3). These results are the first to demonstrate that pharmacological manipulations that preferentially target TLR4 improve the analgesic potency of acute morphine, and complement previous studies demonstrating potentiation of acute opioid antinociception by TLR4 antagonism in the absence of pain\(^ {78,79}\). Together with our results from Chapter 2, these data indicate that in the absence of pain, morphine is viewed as a pathogen in the CNS, resulting in the activation of the innate immune system via vlPAG TLR4 to contribute to opioid tolerance. Indeed, recent data indicate that morphine induces neuroinflammation via the same mechanisms as the endotoxin LPS\(^ {81}\). As morphine activates vlPAG glia more rapidly in the absence of pain (Chapter 2), the remainder of the experiments in this dissertation focused on chronic morphine administration in
the absence of pain in order to elucidate the mechanisms whereby vIPAG TLR4 contributes to morphine tolerance.

**vIPAG TLR4 modulates morphine tolerance via soluble TNF signaling**

While TNF contributes to morphine-induced inflammation and the development of morphine tolerance at the level of the spinal cord, remarkably, no studies have investigated the role of TNF signaling within the vIPAG, and very few studies have directly tested the role of TNF in isolation. Additionally, the specific roles of the two natural forms of TNF (tmTNF and solTNF) have not been dissected. Glia activation by opioids induces the production of the cytokines IL-1β, IL-6, and TNF, as well as neuroecitotoxic free radicals (NO, NOS, iNOS); these signaling factors have all been implicated in opioid tolerance. IL-1β and TNF binds to their target receptors on astrocytes and microglia resulting in the further release of proinflammatory factors (e.g., IL-1β, IL-6, TNF, ATP, nitric oxide synthase; NOS, and brain derived neurotrphic factor; BDNF); this effectively induces a positive feedback loop of neuroinflammation. BDNF binds to its neuronal receptor TrkB, which further contributes to neuronal excitability by initiating a depolarizing shift in anion reversal potential and increasing intracellular Cl⁻ such that GABA binding becomes depolarizing. BDNF also upregulates AMPA subunits and the NMDAR subunit NR2A. These subunits have been referred to as ‘anti-opioid subunit’ due to the fact that NR2A knock-out mice do not develop morphine tolerance. Together, these data indicate that morphine induced glial activation results in the release of an overwhelming number of factors that contribute to morphine tolerance.

Products released from neurons following chronic morphine administration can also act on glia to result in the release of cytokines, suggesting substantial proinflammatory wind-up with
chronic morphine exposure. Indeed, repeated morphine administration causes neurons to release fractalkine and NO\textsuperscript{84}. Fractalkine is a chemokine (like a cytokine that controls immune cell motility) that is only produced by neurons. The fractalkine receptor (CX3CR1) is only found on glia. Binding results in robust inflammation suggesting neuron-driven inflammatory wind-up\textsuperscript{84,166,259-261}. NO can cause activation of microglial p38 MAPK, and the release of IL-1β, IL-6, TNF, ATP, glutamate, prostaglandins, cholecystokinin (CCK), cyclooxygenase (COX), and further excitation of astrocytes\textsuperscript{88,261}. COX is a precursor for prostaglandin production, and both prostaglandins\textsuperscript{104} and spinal and vlPAG CCK\textsuperscript{67,262,263} have all been shown to contribute to morphine tolerance. These results suggest that opioid actions at TLR4 and MOR synergize and result in a robust positive-feedback loop of neuroinflammation, and a concurrent increase in the excitability of neurons that opposes the hyperpolarizing effects of morphine, and contributes to tolerance.

Corroborating a vast body of work in the spinal cord, here we demonstrate that tolerance to systemic morphine was accompanied by increased proinflammatory cytokine expression (IL-1β, TNF, and IL-6) in the vlPAG (Chapter 4). Additionally, chronic morphine significantly increased vlPAG TLR4 mRNA confirming previous results suggesting that chronic morphine induces an increase in the immune receptor substrate to which it binds, thereby priming glia to over-respond to subsequent opioid exposures\textsuperscript{255}. Morphine tolerance was also accompanied by a significant decrease in astrocytic glutamate transporter mRNA (GLT-1 and GLAST) in the vlPAG. Neuronal glutamate transporter mRNA (EAAC1) was not affected by chronic morphine administration, suggesting that opioids preferentially alter vlPAG astrocytic glutamate uptake to oppose the hyperpolarizing effects of morphine, and lead to tolerance. Chronic intra-vlPAG microinjections of the TLR4 agonist LPS (in the absence of morphine) mimicked the effects of
morphine on GLT-1 and GLAST in the vlPAG (Chapter 3), suggesting that TLR4 mediates the inflammatory effects of chronic morphine. These results are consistent with previous studies demonstrating that cytokines increase neuronal excitability. Indeed, in vitro and in vivo\textsuperscript{54,144-146} studies have reported that cytokines increase the number and conductance of AMPA and NMDA receptors, decrease astrocytic glutamate transporter proteins (GLT-1 and GLAST)\textsuperscript{53,54,200}, decrease GABA receptors and GABA currents\textsuperscript{145}, and increase presynaptic release of neurotransmitters\textsuperscript{71}.

The inhibitor protein dominant-negative TNF (DN-TNF) is a well-characterized variant of native human TNF that has been engineered to effectively sequester native solTNF and preclude it from initiating signaling through TNFRI by preventing receptor binding\textsuperscript{207}. The use of DN-TNF to manipulate solTNF signaling is highly advantageous in that it spares the beneficial effects mediated by the transmembrane TNF (tmTNF) signal\textsuperscript{192,207}. Using a lentiviral vector encoding dominant negative TNF (DN-TNF) or a brain-permeable DN-TNF peptide (XPro\textsuperscript{®}1595), we demonstrate that vlPAG sequestration of soluble TNF (solTNF) abolishes tolerance to systemic morphine as well as naïve tolerance to morphine induced by intra-vlPAG injections of the TLR4 agonist LPS. vlPAG injections of lenti-DN-TNF also prevented the morphine-induced decreases in GLT-1 and GLAST, and systemically injected XPro\textsuperscript{®}1595 prevented the morphine-induced increase in IL-1\textbeta and TLR4 mRNA in the vlPAG, and eliminated the trending increase in TNF and IL-6 mRNA. These results complement work from Shen and colleagues demonstrating that chronic intrathecal morphine induces tolerance that is accompanied by decreases in spinal GLT-1 and GLAST, and increases in AMPA and NMDA receptor subunits. These authors further showed that one intrathecal injection of the general TNF decoy receptor Etanercept was sufficient to rescue morphine analgesia in morphine tolerant
mice, and prevent morphine-induced alterations in glutamatergic signaling\textsuperscript{54,102}. Our results are consistent with this work, and indicate for the first time that solTNF (TNFRI), and not tmTNF (TNFRI and TNFRII) signaling, is necessary for morphine tolerance (Chapter 4).

Our data are also novel in that we identify a neural locus through which TLR4 contributes to morphine tolerance, and indicate that TLR4-induced soluble TNF signaling (through TNFRI) is responsible for the anti-analgesic effects of morphine-TLR4 binding. Together, these data support our working hypothesis and indicate that morphine binds to TLR4 within the vlPAG, leading to the release of solTNF. Our results further suggest that solTNF mediates morphine tolerance in the PAG via TNFRI signaling and augmentation of glutamate homeostasis (Figure 1.2). Given that PAG-mediated analgesia depends largely on the ability of opioids to inhibit vlPAG MOR-expressing GABAergic interneurons\textsuperscript{12,19,22,39,43,47,65,114,150,183-186,264-282}, our data suggest that TLR4 signaling contributes to opioid tolerance by decreasing the ability of morphine to hyperpolarize vlPAG GABAergic interneurons, thereby maintaining tonic inhibition of vlPAG-RVM projections neurons, and preventing opioid analgesia.

\textbf{Summary}

The midbrain periaqueductal gray (PAG), and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord, comprises an essential neural circuit for opioid-mediated analgesia\textsuperscript{9,11,12,175,283}. PAG-mediated analgesia depends largely upon the ability of opioids to inhibit vlPAG MOR-expressing GABAergic interneurons\textsuperscript{12,19,22,39,43,47,65,114,150,183-186,264-282}, thereby releasing PAG-RVM projection neurons from tonic inhibition\textsuperscript{34,36}. A multitude of studies have demonstrated that the vlPAG is both necessary and sufficient for the initiation and maintenance of morphine tolerance\textsuperscript{24,49}. Indeed, our lab reported that tolerance to systemic morphine is accompanied by the inability of acute systemic opioids to activate the PAG-RVM
circuit in male rats. Studies outlined within this dissertation tested the overarching hypothesis that morphine-induced vlPAG glia activity contributes to morphine tolerance. Opioids, including morphine, bind to the glial receptor TLR4 resulting in the robust release of proinflammatory cytokines (including tumor necrosis factor a; TNF). TLR4 and TNF signaling increase neuronal excitability in a number of model systems, and are strongly implicated in opioid tolerance development. This dissertation specifically identified the role of TLR4 and TNF in the development of morphine tolerance in a CNS region critical for opioid induced analgesia: the midbrain vlPAG.

We hypothesized that vlPAG glia modulate the analgesic efficacy of morphine via signaling through the innate immune receptor TLR4 and downstream products of the TLR4 signaling cascade, specifically via the cytokine TNF. In particular, we postulated that morphine binds to neuronal MOR as well as glial TLR4 in the PAG, and that concurrent activity at these receptors modulates the analgesic efficacy of morphine. Two opposing mechanisms were proposed: (1) opiate binding at MOR, resulting in hyperpolarization of GABAergic neurons and induction of opiate analgesia; and (2) opiate binding at glial TLR4 leading to increased vlPAG cytokine signaling and increased neuroexcitability. This increase in vlPAG neuroexcitability opposes the hyperpolarizing effects of opiates and limits the inability of morphine to disinhibit the PAG-RVM circuit (Figure 5.1). These hypotheses were tested in four specific aims.

With these data, glia modulation of opioid tolerance has been reported at every major level of the descending analgesic circuit: PAG, RVM, and spinal cord dorsal horn. Our results are the first to identify (1) a role for PAG glia in the development of morphine tolerance; (2) a neural locus through which TLR4 modulates morphine tolerance development; (3) sTNF as the important TNF form mediating opioid tolerance and alterations in glutamate homeostasis;
and (4) release of PAG cytokines in the development of morphine tolerance. Additionally, these studies identify the anti-TNF biologic XPro®1595 as a potential tool to accompany opioid therapy in the clinic. XPro®1595 may be preferable over current FDA approved anti-TNF biologies (Etanercept, Infliximab, Adalimumab) as these drugs block both forms of TNF and are associated with encephalic lesions, neuritis, multiple sclerosis, and other demyelinating diseases. Here we have demonstrated that exclusive sequestration of solTNF prevents opioid induced neuroinflammation and the ensuing changes in glutamate homeostasis and development of morphine tolerance. Importantly, these data indicate that tmTNF signaling (TNFRI and TNFRII) does not contribute to opioid tolerance. As TNFRII is protective against glutamate excitotoxicity, these data indicate that TNFRII signaling may be a critical countermeasure to opioid-induced neuroexcitability.

Our results suggest that XPro®1595 may be an ideal drug candidate to pursue clinically. Interestingly, glia, TLR4, and TNF have all been implicated in the increased nociception that characterizes painful neuropathic diseases including neuropathic pain and rheumatoid arthritis. The mechanisms underlying glia mediated hyperalgesia are strikingly similar to the mechanisms underlying opioid tolerance development, indicating that anti-TNF biologics could complement opioid therapy in the clinic by suppressing nociceptive signals at the site of injury and in the spinal cord, as well as preserving morphine analgesic efficacy by preventing opioid-induced glia activation within the vIPAG, thereby attenuating the development of morphine tolerance.

There are several questions that are left unanswered with the conclusion of this dissertation. Here we identify the following as important next logical steps: (1) Characterize the effects of TACE inhibition on spinal cord, RVM, and vIPAG glia activity and the development of morphine tolerance; (2) Characterize the effects of soluble TNF sequestration on the ability of
chronic and acute morphine to inhibit GABA release in the vlPAG and to maintain tonic inhibition of PAG-RVM projection neurons (via electrophysiological experiments and microdialysis); (3) Characterize the necessity and sufficiency of TLR4, TNF, and TACE to inhibit PAG-RVM signaling (fluorogold (FG) into the RVM with IHC) and neuronal activation within the PAG (Fos IHC)\textsuperscript{253}. We would hypothesize a significant decrease in FG positive and Fos + colocalization in vlPAG cells in tolerant animals, as previously described by Morgan, Loyd, and Murphy (2006). We would predict that intraplantar CFA treatment (Chapter 2), TLR4 antagonism (Chapter 3), soluble TNF sequestration (Chapter 4), or TACE antagonism (Chapter 5) would maintain morphine analgesia by allowing for release of PAG-RVM circuit upon MOR binding. These manipulations may also prevent tolerance and rescue opioid analgesia; (4) Induce tolerance in animals given pain (e.g., CFA) by prolonging chronic morphine exposure until tolerance develops, and then characterize the effects of vlPAG glia, TLR4, soluble TNF, and TACE in tolerance development in a several different pain states (e.g., CFA, CCI, migraine, burn pain, and formalin); (5) Characterize the effects of vlPAG glia, TLR4, soluble TNF, and TACE in tolerance development using multiple behavioral measures of nociception and analgesia including thermal tests (e.g., Hargreaves paw thermal stimulator to stimulate thermal nociceptors; Chapter 2 – 4), mechanical tests (von Frey filament test to stimulate mechanical nociceptors), and visceral tests (visceral distention to stimulate silent nociceptors in the gut). These results will help determine which chronic pain conditions should be treated with morphine and anti-inflammatory therapy in the clinic; (6) Females should be made tolerant to morphine by using an effective dose for the sex and administering morphine until they show behavioral tolerance to multiple pain tests (mentioned in future directions 5). When tolerant, \textit{this dissertation should be repeated in females}. The role of glia and TACE should also be
characterized in a model of migraine, as females have higher incidence of migraine, and migraine has a robust inflammatory component; (7) Finally, XPro®1595 should be characterized in all of the 6 hypotheses/future directions stated above. This drug has been successfully used as a neuroprotector in a Hemiparkinsonian rat model\textsuperscript{211,284}, and soluble TNF has been implicated in increased blood brain barrier permeability in neurodegenerative diseases. Indeed, lenti-DN-TNF attenuates the symptoms associated with inflammation (cytokines), neuronal excitability, cell death, and cognitive impairment in rodent models of Alzheimer’s disease Parkinson’s disease\textsuperscript{207-211,285-290}. It is necessary for cognitive health to maintain tmTNF signaling\textsuperscript{191,231,291-293}. XPro®1595 (BBB DN-TNF peptide) has exciting clinical potential as it sequesters the proinflammatory soluble TNF, while maintaining innate immunity to infection (e.g., tmTNF). Answering the above 7 questions, as well as delving into the pain and endocannabinoid story in males and females, will elucidate the role of neuroinflammation and neuroexcitability in the development of morphine tolerance and could enhance chronic morphine treatment for a variety of clinical pain conditions safely. These hypotheses remain to be rigorously tested.
Figure 5.1 A schematic diagram illustrating major dissertation conclusions.
Chronic morphine binds to vIPAG TLR4 and leads to solTNF signaling that increases proinflammatory gene expression (TLR4, IL-1β) and decreases astrocytic glutamate transporter mRNA (GLT-1 and GLAST) in the vIPAG. These changes effectively increase the availability of glutamate in the synapse, thereby decreasing the ability of morphine to hyperpolarize GABAergic interneurons. These changes associated with morphine tolerance prevent morphine from initiating signaling through the descending analgesic circuit. The next step is to inhibit TACE to test our hypothesis that TACE mediates TLR4 mediated soluble TNF signaling and glutamatergic alterations in the vIPAG to alter tolerance to mu opioid receptor agonists.
REFERENCES


Carstens, E., Stelzer, B. & Zimmermann, M. Microinjections of glutamate or morphine at coincident midbrain sites have different effects on nociceptive dorsal horn neurons in the rat. *Neurosci Lett* 95, 185-191 (1988).


Loyd, D. R., Morgan, M. M. & Murphy, A. Z. Sexually dimorphic activation of the periaqueductal gray-rostral ventromedial medullary circuit during the development


Viviani, B. *et al.* Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. *J Neurosci* 23, 8692-8700 (2003).


Wu, H. E., Sun, H. S., Cheng, C. W., Terashvili, M. & Tseng, L. F. dextro-Naloxone or levo-naloxone reverses the attenuation of morphine antinociception induced by


Jensen, T. S. & Yaksh, T. L. Comparison of the antinociceptive action of mu and delta opioid receptor ligands in the periaqueductal gray matter, medial and paramedial ventral medulla in the rat as studied by the microinjection technique. *Brain Res* 372, 301-312 (1986).


APPENDIX: Curriculum Vitae
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B. Positions and Honors

2014 Invited Speaker: Emory University School of Medicine (Dr. Malu Tansey’s laboratory seminar): Ventrolateral Periaqueductal Gray Toll-like Receptor 4 Modulates Morphine Tolerance via Tumor Necrosis Factor α Signaling

2013- Invited student member: Sigma Xi Scientific Research Society

2013 Invited Speaker: Brains and Behavior Retreat; GSU, Atlanta, GA: Modulation of Opioid Tolerance by Toll-like Receptor 4 in the Midbrain Periaqueductal Gray of Male Rats

2012 Invited Speaker: GSU Neuroscience Institute Breakfast and Lecture (NIBL): Periaqueductal Gray Glia Modulate Morphine Tolerance in the Male Rat via Toll-like Receptor 4 (TLR4)

2012 Teaching assistant: Neuroscience 3000 (Principles of Neuroscience)

2012 Invited instructor: GSU Neuroscience 3000 (Principles of Neuroscience): Introduction to Glia

2012 Invited instructor: GSU Neuroscience 3000 (Principles of Neuroscience): Psychoactive Drugs and the CNS

2008-2010 Instructor: Biology 1104 (Introduction to biology laboratory)

2008- Student member: Society for Neuroscience (SfN)

2008- Student member: International Association for the Study of Pain (IASP)

2005-2006 Undergraduate student member: Society for Behavioral Neuroendocrinology

2014 Georgia State University Center for Neuromics award to support ongoing research

2013 Georgia State University Center for Neuromics award to support ongoing research

2013 Sigma Xi, Scientific Research Society Grant-In-Aid of Research

2013 Georgia State University College of Arts and Sciences Dissertation Grant Award

2013 Kenneth W. and Georganne F. Honeycutt Merit-based Fellowship

2012 Georgia State University Brains & Behavior (B&B) Fellowship

2006 Graduated Magna Cum Laude with Advanced Honors

2001-2006 Dean’s list

Service and Outreach

2008-2014 Laboratory supervisor and research mentor:
Undergraduate honors thesis students: Jean-Marc Sauzier and Laura Butkovitch
Undergraduate honors students: Brittany Grandy, Vincent Laufer, Whitney Hugeulet, Hela Eichenbaum, Sierra Moore (Presidential honors undergraduate), Brynn Travis, Elizabeth Sandy, and Tyler Upchurch
Other undergraduate mentees: LaTrenda Dumes (McNair Fellow), Ronek Shah, Preston Giradot, Ozaer Faroqui, and Lauren Hanus
2012   *Primary research mentor*: Medical Student Summer Research Program (Kehinde Idowu; Meharry Medical College, Nashville, TN)

2011   *Primary research mentor*: Center for Behavioral Neuroscience’s (CBN) Institute on Neuroscience (ION) research program for high school students (Anthony Jordan)

2009-2011   *Primary research mentor*: Center for Behavioral Neuroscience’s (CBN) Behavioral Research Advancement in Neuroscience (BRAIN) Program for undergraduates (Nina Waldron, Todd Jackson (2nd place in poster competition), and Anthony Jordan (1st place in poster competition))

2008-2014   *Organizer (2010-2011) and volunteer (2008-2014)*: GSU Neuroscience Institute graduate student recruitment

2012-2013   *Invited instructor*: GSU Neuroscience 3000 (Principles of Neuroscience): Sheep Brain Dissections

2009-2012   *Science educator*: Brain Awareness Month elementary and middle school visits

2010-2011   *President*: GSU Neuroscience Institute Graduate Student Association (NGSA)

2010-2011   *Manager*: Neuroscience Institute Undergraduate Placement (UP) Program to fit motivated and qualified undergraduates with appropriate research labs

2011   *Organizer*: Brains & Behavior Graduate Student Retreat to facilitate research collaborations (Big Canoe, GA)

2011   *Organizer and host*: Student invited speaker (Dr. Ronald R Hoy, Cornell University, Department of Neurobiology and Behavior)

2006   *Science educator*: Center for Behavioral Neuroscience (CBN) Zoo Atlanta Exposition

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**C. Publications**

**Manuscripts**


**Poster presentations**

**Eidson LN**, Tansey MG, Murphy AZ (2014) Poster presentation, Periaqueductal gray toll-like receptor 4 (TLR4) modulates morphine tolerance development via soluble tumor necrosis factor alpha signaling. Brains and Behavior Retreat, Atlanta, GA.

Eidson LN, Doyle HH, Butkovich LM, Murphy AZ (2013). Sex Differences In Astrocyte Activity Within The Periaqueductal Gray: Role In Pain And Analgesia. Society for Behavioral Neuroendocrinology, Atlanta, Georgia.


Eidson LN, Murphy AZ (2011) Poster presentation, Midbrain Periaqueductal Gray-Mediated Morphine Tolerance and Glial Cell Activation is Altered in the Absence and Presence of Persistent Pain. 10th European Meeting on Glial Cells in Health and Disease (EuroGlia), Prague, Czech Republic.


Eidson LN, Murphy AZ (2009) Poster presentation, Midbrain Periaqueductal Gray-Mediated Morphine Tolerance and Glial Cell Activation is Altered in the Absence and Presence of Persistent Pain. ACSfN Poster Preview, Atlanta, GA.


Eidson LN, Mao Y, Pallas SL (2008) Poster presentation, Calbindin expressing GABAergic interneurons may play a role in the development of a visual map in cross-modal primary auditory cortex (A1). South East Nerve Net, Atlanta, GA.

Eidson LN, Mao Y, Pallas SL (2008) Poster presentation, Calbindin expressing GABAergic interneurons may play a role in the development of a visual map in cross-modal primary auditory cortex (A1). Georgia State Poster Day, Atlanta, GA.

Eidson LN, Mao Y, Pallas SL (2008) Poster presentation, Calbindin expressing GABAergic interneurons may play a role in the development of a visual map in cross-modal primary auditory cortex (A1). Computational Neuroscience Conference, Atlanta, GA.


D. Awards and Grants
Ongoing
2013- Kenneth W. and Georganne F. Honeycutt Merit-based Fellowship ($1,000 per year)
2012- Georgia State University Brains & Behavior (B&B) Fellowship ($500 per year + stipend)
Completed
2014  Georgia State University Center for Neuromics award to support ongoing research
*Ventrolateral Periaqueductal Gray Toll-like Receptor 4 Modulates Morphine Tolerance via Tumor Necrosis Factor α Signaling* ($1,000 total)
2013  Sigma Xi, Scientific Research Society Grant-In-Aid of Research: *The Role of Periaqueductal Gray Glial Cell Activity in Morphine Tolerance Development* ($970 total)
2013  Georgia State University College of Arts and Sciences Dissertation Grant Award
*Modulation of Morphine Tolerance by Glial Receptor Toll-like Receptor 4 in the Midbrain Periaqueductal Gray of Male Rats* ($1,000 total)
2011 Georgia State University Center for Neuromics award to support ongoing research
*Modulation of Morphine Tolerance by Glial Receptor Toll-like Receptor 4 in the Midbrain Periaqueductal Gray of Male Rats* ($1,500 total)
2007 NSF REU Supplement to sponsor Lori Eidson (PI: S.L. Pallas). *Functional and Anatomical Analysis of Cortical Plasticity After Surgically Induced Sensory Manipulations* ($5,997 total)