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# Inflammatory Mediators of Opioid Tolerance: Implications for Dependency and Addiction

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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 vlPAG as an integral locus for the development of morphine tolerance, most studies have examined the role of glia activation within the spinal cord. Additionally, the role of TLR4 in the development of tolerance has not been elucidated. This review attempts to summarize what is known regarding the role of vlPAG glia and TLR4 in the development of morphine tolerance. These data, together, provide 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.

### Keywords

Opioid; Tolerance; Glia; Toll-like receptor 4; Tumor necrosis factor; Periaqueductal gray

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## 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 opioid therapy [4], with morphine being amongst the most commonly prescribed drugs.

In addition to modulating pain, opioids have widespread central and peripheral effects, many of which interfere with the beneficial aspects of opioids[5]. Indeed, the negative side effects associated with opioid 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 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[3]. Indeed, opioid tolerance is a significant impediment for sufficient pain relief in approximately 60% of patients[8]. However, the mechanisms underlying the development of morphine tolerance are not completely understood, and the role of inflammation has been largely ignored until relatively recently.

## 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[9–13]. 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[14,15]. In humans, electrical stimulation of the PAG is still used today for the management of intractable pain[16–18]. PAG stimulation produced analgesia is attenuated by intra-PAG injection of the mu opioid receptor (MOR) antagonist naloxone,[19] suggesting an opioid-dependent mechanism.

The PAG contains a high density of MOR containing neurons,[20–22] and microinjection of opioid antagonists into the PAG significantly attenuates the analgesic effects of systemic

morphine[23–25]. Similarly, site-specific lesions of PAG MOR-containing neurons (using the cytotoxin saporin (a ribosome inactivating protein) conjugated to the MOR ligand Dermorphin) significantly reduce the antinociceptive effects of systemic morphine suggesting that PAG MOR is critical for morphine action[26]. The density of MOR immunoreactivity within the vIPAG is positively correlated with the degree of analgesia produced by morphine, such that male rats with normal levels of MOR immunoreactivity in the vIPAG have significantly lower $ED_{50}$ 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]. *In vitro*, MOR binding on PAG neurons inhibits miniature inhibitory postsynaptic potential frequency and decreases the probability of presynaptic GABA release[31,34]. *In vivo*, injection of GABA antagonists into the PAG partially mimics the effects of morphine[35]. In part, morphine binding to vIPAG MOR hyperpolarizes GABAergic neurons[34,36], thereby releasing vIPAG-RVM projection neurons from local tonic inhibition[36]. The PAG signals to RVM neurons, which then signal to the spinal cord to inhibit nociceptive dorsal horn neurons and produce antinociception[37–39]. 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].

## Neuronal Mechanisms of Opioid Tolerance

In addition to being a critical locus for both endogenous and exogenous pain modulation, the vIPAG is critical for the development of morphine tolerance[24,42–51]. Chronic vIPAG opioid administration results in the rapid development of behaviorally and physiologically defined opioid tolerance. In addition, repeated intra-vIPAG 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[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-vIPAG injections of the opioid receptor antagonist naltrexone[24], indicating that the vIPAG is sufficient for the development of morphine tolerance.

Although 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[31]. Cerebrospinal fluid (CSF) from morphine-tolerant humans contains significantly higher levels of both glutamate and aspartate[52], and morphine challenge increases glutamate in the CSF of morphine tolerant rats[53]. Increased expression of AMPA and NMDA receptor subunits[54] and increased NMDA receptor binding[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[56–61] and AMPA[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[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[48]. 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[42]. 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[64]. Although NMDA receptor signaling is not important for PAG-mediated opioid tolerance[65,66], 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[67]. CCK excites neurons by opening depolarizing currents and inhibiting K+ conductance[68–70], 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[71–87]. Although the importance of the vlPAG in tolerance development is well established, the majority of investigation of glial involvement in opioid signaling has been limited to spinal and medullary loci[81,85,88–90].

Several lines of evidence implicate opioids as activators of CNS astrocytes and microglia[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,[71,72,91] and induces release of glially-derived proinflammatory cytokines[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[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[72,76,87,91–97]. Importantly, glial release of cytokines increases exponentially with repeated morphine administration,[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 (mu, delta and kappa) knock out mice[98], suggesting that the anti-analgesic effects of morphine are not mediated by traditional opioid receptor signaling. Recent *in vivo* and *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)[81]. TLR4 is found on microglia, and to a lesser degree, astrocytes[99,100]. Opioids, including morphine, bind to the glycoprotein myeloid differentiation factor-2 (MD-2) on TLR4[78,79], and initiate an inflammatory response through nuclear factor kappa B (NFκB) activation and p38 mitogen activated protein kinase (MAPK) phosphorylation[81]. Activation of the NFκB pathway results in the robust release of proinflammatory cytokines including tumor necrosis factor, (TNF), Interleukin 1 βeta (IL-1β), and Interleukin 6 (IL-6)[78,79,81]. Spinal TLR4 activity opposes the acute effects of morphine, including antinociception, and contributes to opioid-induced hyperalgesia[78,79], and systemic TLR4 antagonists prevent tolerance to systemic morphine[78].

A nearly ubiquitous characteristic of opioid-induced neuroinflammation is TNF production, likely mediated via TLR4. Chronic systemic[101] 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 TNF levels increase with the chronicity of morphine treatment[84]. 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[54,102,106], 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)[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 implicated in opioid tolerance, including IL-1β[86,101,110] and IL-6[86,87,101]. Although these data indicate that spinal TNF plays a significant role in morphine-induced inflammation and the development of morphine tolerance[72–74,76,84–87,93,111,112], remarkably, very few studies have directly tested the role of TNF in isolation[54,102,106].

## Inflammation significantly contributes to morphine tolerance

Many mechanisms have been proposed to account for opioid tolerance, including MOR decoupling, internalization, and/or down-regulation of MORs[113,114]. However, in comparison to other opioids (e.g., DAMGO, fentanyl[6]), morphine does not result in MOR internalization, and is remarkably weak in terms of decreasing G-protein signaling and receptor desensitization[115,116]. Indeed, chronic morphine increases G-protein efficiency in the vIPAG[42]. Together with our recently published work (reviewed below)[82,83,117], these

data suggest mechanisms other than MOR signaling also contribute to morphine tolerance at the level of the midbrain PAG.

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 OX-42 and Ibal (microglia) and glial fibrillary acidic protein (GFAP; astrocytes)[118]. Glial activation also results in the increased production and release of pro-inflammatory cytokines (including IL-1β, IL-6, and TNF), chemokines, ATP, excitatory amino acids, and NO, all of which increase the excitability of nearby neurons[86,93]. Indeed, glially-derived cytokine release results in increases in the number and conductance of AMPA[119,120] and NMDA[121] receptors, decreases in astrocytic glutamate transporter proteins[85], and down-regulation of GABA receptors[120]. Microglia derived IL-1β and TNF bind to receptors on astrocytes, resulting in the further release of IL-1β, IL-6, and TNF[86]. These cytokines actively oppose the analgesic actions of morphine[71].

Acute and chronic morphine administration activates microglia and astrocytes[28, 48, 49, 72,86,91–93,122], with the degree of glial activation increasing with duration of opioid treatment[81]. 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[71,73,84,86,87]. 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[88].

Complementing literature from the spinal cord and RVM, we have demonstrated that glial activation within the PAG is critical for the development of morphine tolerance. First, we found that administration of a single $ED_{50}$ dose of morphine once a day for 3 days (but *not* administration of a single $ED_{50}$ dose of morphine once a day for *only* 1 day) significantly activated both microglia and astrocytes in the PAG, as indicated by a 2-fold increase in 0X42 and GFAP protein levels (when measured 24 hours later)[83]. 0X42 and GFAP 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 0X42 and GFAP immunoreactivity[82,83]. Persistent peripheral 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 vIPAG glial cell activation for CFA + Saline and CFA + Morphine treated animals versus controls (Handled + Saline)[83]. These data mirror clinical data indicating that peripheral pain delays the development of tolerance[123–126], and suggest that there is something unique about persistent pain that blocks morphine from activating glia. Morgan and colleagues reported that intraplantar CFA prevents tolerance to chronic intra-vIPAG microinjections of morphine[127], and Tonsfeldt et al. recently demonstrated that morphine elicits increased antinociception in female rats following CFA treatment, and that this is dependent on modulation of PAG $GABA_A$ receptor activity[128]. These data corroborate our results, and suggest that pain-induced changes in the vIPAG are responsible for the preservation of

opioid analgesia during a persistent pain state. 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[129–133], peripheral neuropathy[71,90,94,118,132,134,135], formalin[136], and spinal nerve ligation[137] 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.

## vlPAG TLR4 and 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[78]. TLR4 is found primarily on microglia, and to a lesser degree on astrocytes, but not neurons[99,100]. 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[78]) and endogenous (e.g., M3G[79]) opioids. Interestingly, MOR binds M6G, but not M3G [6]. Conversely, TLR4 binds M3G but not M6G[79,89,148]. 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[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[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[78,117]. Recent *in vivo* and *in vitro* data demonstrate that the innate immune receptor TLR4, but not MOR[81], mediates morphine-induced cytokine release (including TNF)[78,79,81]. Chronic morphine increases TLR4 mRNA expression [79,117,149] and downstream products of the TLR4 signaling cascade (e.g., IL-1β); similarly, systemic TLR4 antagonists attenuate tolerance to systemic morphine[78,82]. These data further elucidate the mechanisms by which morphine alters glia activity, and suggest that vIPAG glia contribute to the development of morphine tolerance via TLR4 signaling.

We recently demonstrated, for the first time, the expression of the TLR4 co-receptor myeloid differentiation factor 2 (MD2) in the PAG, indicating the presence of TLR4[82]. 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 a mechanism whereby morphine may preferentially activate glia in the vIPAG. Indeed morphine-induced OX-42, Ibal, and GFAP expression is most robust in ventral PAG regions[83]. Our recent data also indicate that vIPAG microinfusions of TLR4 antagonists, including (+)-naloxone, dose-dependently prevented tolerance to systemic morphine. In

parallel, we showed that vIPAG microinfusions of TLR4 agonists (in the absence of morphine) dose-dependently produced a naive tolerance to subsequent challenge doses of morphine (indicated by a significant 3-fold rightward shift in the morphine dose-response curve)[82,117]. Together these data are the first to identify a CNS locus through which TLR4 signaling modulates opioid tolerance. It is important to note that studies demonstrating the necessity of MOR signaling in tolerance development using 'opioid-specific' ligands may need to be re-evaluated, as TLR4 binds several of these ligands, including naloxone, in a manner that maintains their agonistic and antagonistic properties[78].

## vIPAG TLR4 modulates morphine tolerance via soluble TNF signaling

Although TNF contributes to morphine-induced inflammation and the development of morphine tolerance at the level of the spinal cord[72–74,76,84–87,93,111,112], remarkably, few studies have investigated the role of TNF signaling within the vIPAG, and very few studies have directly tested the role of TNF in isolation[54,102,106]. 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 neuroexcitotoxic free radicals (NO, NOS, iNOS); these signaling factors have all been implicated in opioid tolerance[71]. IL-1β and TNF bind 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 neurotrophic factor; BDNF); this effectively induces a positive feedback loop of neuroinflammation[71,85,88,89]. 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[150]. BDNF also increases AMPA subunits and the NMDAR subunit NR2A[151,152]. These subunits have been referred to as 'anti-opioid subunit' due to the fact that NR2A knock-out mice do not develop morphine tolerance[153]. Together, these data indicate that morphine induced glial activation results in the release of an overwhelming number of factors that result in a 'hyper-excitatory' environment that contributes to morphine tolerance.

Corroborating a vast body of work in the spinal cord, we recently demonstrated that chronic morphine-induced tolerance significantly increased vIPAG TLR4 mRNA[117] and increased proinflammatory cytokine expression (IL-1β, TNF, and IL-6) in the vIPAG. These results confirm previous studies 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[149]. We also demonstrated that morphine tolerance was accompanied by a significant decrease in astrocytic glutamate transporter mRNA (GLT-1 and GLAST) in the vIPAG[117]. Neuronal glutamate transporter mRNA (EAAC1) was not affected by chronic morphine administration, suggesting that opioids preferentially alter vIPAG astrocytic glutamate uptake to oppose the hyperpolarizing effects of morphine, and lead to tolerance. Chronic intra-vIPAG microinjections of the TLR4 agonist LPS (in the absence of morphine) mimicked the effects of morphine on GLT-1 and GLAST in the vIPAG[117], 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*[54,119–121] studies have reported that

cytokines increase the number and conductance of AMPA and NMDA receptors, decrease astrocytic glutamate transporter proteins (GLT-1 and GLAST) [53,54,159], decrease GABA receptors and GABA currents [120], and increase presynaptic release of neurotransmitters[71].

TNF is a major inflammatory signal released upon TLR4 activation. 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[160]. 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[160,161]. Using a lentiviral vector encoding dominant negative TNF (DN-TNF) or a brain-permeable DN-TNF peptide (XPro®1595), we have demonstrate that vIPAG sequestration of soluble TNF (solTNF) abolishes tolerance to systemic morphine as well as naive tolerance to morphine induced by intra-vIPAG injections of the TLR4 agonist LPS[117]. vIPAG injections of lenti-DN-TNF also prevented the morphine-induced decreases in GLT-1 and GLAST, and systemically injected XPro®1595 prevented the morphine-induced increase in IL-1β and TLR4 mRNA in the vIPAG, 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 (that blocks solTNF and tmTNF signaling) was sufficient to rescue morphine analgesia in morphine tolerant mice, and prevent morphine-induced alterations in glutamatergic signaling[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 important for morphine tolerance.

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 vIPAG, 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. Given that PAG-mediated analgesia depends largely on the ability of opioids to inhibit vIPAG MOR-expressing GABAergic neurons[12,19,22,39,43,47,65,162–186], our data suggest that TLR4 signaling contributes to opioid tolerance by decreasing the ability of morphine to hyperpolarize vIPAG GABAergic neurons, thereby maintaining tonic inhibition of vIPAG-RVM projections neurons, and preventing opioid analgesia.

## Summary

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 recent 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) solTNF 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 (see Figure 1).

Additionally, our 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 biologics (Etanercept, Infliximab, Adalimumab) as these drugs block both forms of TNF and are associated with encephalic lesions, neuritis, multiple sclerosis, and other demyelinating diseases[187]. Our studies have further 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) is not sufficient for opioid tolerance development. As TNFRII is protective against glutamate excitotoxicity[188], these data indicate that TNFRII signaling may be a critical countermeasure to opioid-induced neuroexcitability.

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## Highlights

- Our recent studies establish a role for PAG microglia, and in particular TLR4 signaling, in the development of morphine tolerance.
- We present data indicating that soluble TNF (solTNF) is the important TNF isoform mediating opioid tolerance and alterations in glutamate homeostasis and that exclusive sequestration of solTNF in the vlPAG prevents opioid-induced neuroinflammation and the ensuing changes in glutamate homeostasis and development of morphine tolerance.
  - Our 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).

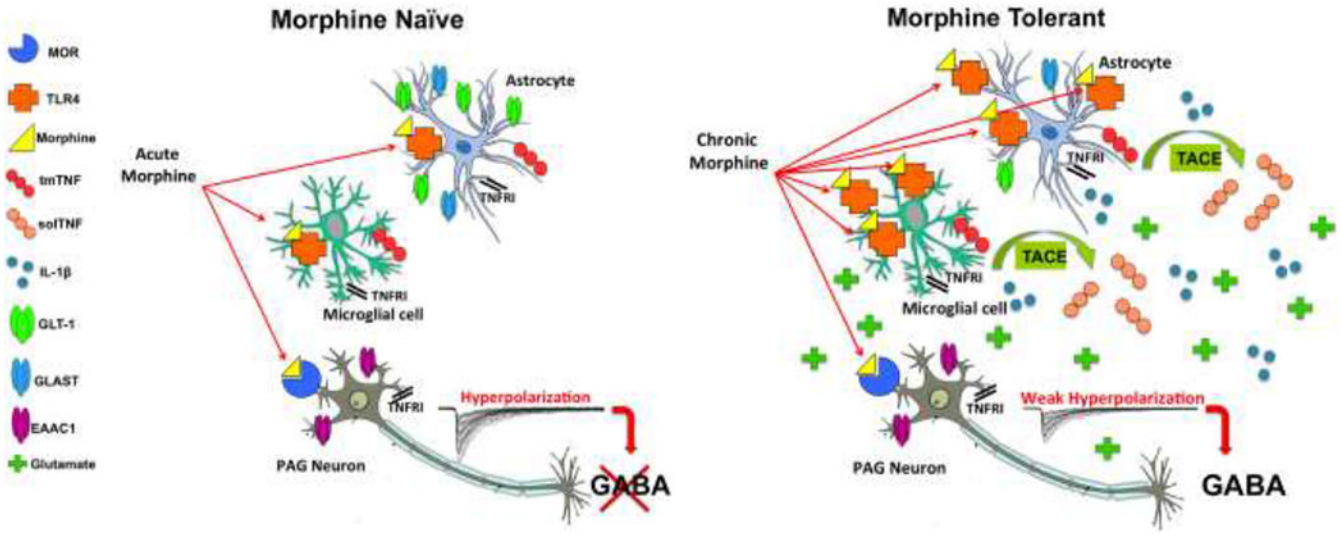


**Figure 1. A schematic diagram illustrating major conclusions and hypotheses.**

Chronic morphine binds to vlPAG 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 vlPAG. These changes effectively increase the availability of glutamate in the synapse, thereby decreasing the ability of morphine to hyperpolarize GABAergic neurons. These changes associated with morphine tolerance prevent morphine from initiating signaling through the descending analgesic circuit.