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The "Toll" of Opioid-Induced Glial Activation: Improving the Clinical Efficacy of Opioids by Targeting Glia

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Abstract

Glial activation participates in the mediation of pain including neuropathic pain, due to release of neuroexcitatory, proinflammatory products. Glial activation is now known to occur in response to opioids as well. Opioid-induced glial activation opposes opioid analgesia and enhances opioid tolerance, dependence, reward and respiratory depression. Such effects can occur, not via classical opioid receptors, but rather via non-stereoselective activation of toll-like receptor 4 (TLR4), a recently recognized key glial receptor participating in neuropathic pain as well. This discovery identifies a means for separating the beneficial actions of opioids (opioid receptor mediated) from the unwanted side-effects (TLR4/glial mediated) by pharmacologically targeting TLR4. Such a drug should be a stand-alone therapeutic for treating neuropathic pain as well. Excitingly, with newly-established clinical trials of two glial modulators for treating neuropathic pain and improving the utility of opioids, translation from rats-to-humans now begins with the promise of improved clinical pain control.

Introduction

Normally, a painful stimulus is perceived via a chain of events beginning with the activation of "pain"-receptive sensory nerve fibers. The resultant action potentials relay information of potential or actual tissue injury to pain transmission neurons in the spinal cord dorsal horn. These, in turn, send the information to multiple sites within the brain where various aspects of the pain experience (sensation, analysis of meaning, emotional reactions, etc) are analyzed and responded to. However, pain processing is not a passive process but rather is under powerful modulatory control. Pain messages can be suppressed by drugs like morphine, relayed unaltered, or amplified under conditions such as chronic pain. When chronic pain develops as a result of peripheral nerve injury, for example, these conditions have typically been attributed to a variety of neuronal changes, including altered excitability of sensory neurons, alterations in which neurotransmitters are synthesized and released by various sensory neurons, alterations

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in pain transmission neuron excitability via multiple changes in receptor and ion channel functions, and so on[1].

Intriguingly, powerful modulatory control exists not only for pain, but also for an organism's responses to opioids, such as morphine. Opioids not only suppress pain, they also activate endogenous counter-regulatory mechanisms that, for example, actively oppose opioid-induced pain suppression, enhance analgesic tolerance wherein repeated opioids lose their ability to suppress pain, and enhance dependence wherein organisms require continued opioid exposure to stave off drug withdrawal. These modulatory controls have again been attributed to a variety if neuronal mechanisms, including release of endogenous anti-opioid peptides such as cholecystokinin, internalization and/or desensitization of opioid receptors, alterations in opioid receptor signaling cascades, and so on[2,3].

While modulatory control systems regulating pain and opioid actions have been thought to involve separate mechanisms, the present paper will review recent evidence that suggests that these two phenomena are closely related and involve overlapping mechanisms. This development has been predicted from prior studies focused on the neuronal bases of chronic pain and opioid tolerance, where striking commonalities such as upregulation of NMDA function transcend what initially appeared to be quite different phenomena[4]. The present review will extend the commonalities between chronic pain and various opioid effects to include a non-neuronal component (glial cells, especially microglia and astrocytes) and a distinctly non-traditional mechanism, that being activation of the innate immune receptor expressed by glia, called toll like receptor 4 (TLR4).

Concepts of chronic pain and opioid actions have evolved in recent years with the realization that alterations in neuronal functions fail to capture all of the critical mechanisms involved. Recognition of a role for microglia and astrocytes in these processes first occurred for pain in the early 1990s, but the involvement of glia in modulating opioid actions was not discovered until a decade later. Indeed, the growing recognition of striking similarities in mechanisms underlying chronic pain and opioid tolerance directly led to the discovery of glial involvement in modulating opioid actions. Since glia were so convincingly important in chronic pain, it became a natural question whether they regulated the actions of opioids as well.

The goals of this article are to explore how glial activation impacts both pain and opioid actions. Pain will be considered first, including how glial activation increases neuronal excitability and how glial activation occurs under conditions leading to chronic pain. Included in this latter topic will be a discussion of TLR4 as a key glial activation receptor for the initiation and maintenance of chronic pain via TLR4-induced release of neuroexcitatory products such as proinflammatory cytokines. TLR4 will also be discussed as having a unique role in regulating the actions of opioids, as opioids have now been found to activate TLR4 on glia, in addition to their classical actions occurring through neuronal opioid receptors. Glial activation by opioids is an important phenomenon to understand, as glial activation opposes opioid analgesia and enhances opioid tolerance, dependence, reward and other negative side effects such as respiratory depression. Lastly, the implications of such glial activation will be considered for drug development aimed at improving both clinical pain control and clinical efficacy of opioids.

Role of glial activation in pain enhancement

The conclusion that the activation of microglia and astrocytes are critical to pain enhancement arose from 3 independent lines of study[5]: (a) cell culture studies showing that spinal cord is one of the rare CNS sites where astrocytes are activated in response to substance P, providing the first evidence that spinal cord glia are responsive to "pain" neurotransmitters; (b) anatomy studies that recognized that microglia and astrocytes each upregulate their expression of so-called activation markers in response to peripheral nerve injury that causes neuropathic pain,

and that drugs known to block neuropathic pain block glial activation as well; and (c) *in vivo* studies of sickness-induced changes in physiology and behavior (increased sleep, fever, suppressed food and water intake, enhanced pain, etc.) that recognized that sickness responses, including enhanced pain, are all created as a result of glial activation and proinflammatory cytokine release within the brain and/or spinal cord. These three diverse lines of evidence predicted that glial activation would prove to be intimately important in the creation and maintenance of chronic pain.

The idea that glia contribute to pain has gained widespread support in the past decade[6]. Spinal microglia and astrocytes are activated in every clinically relevant animal model of pain enhancement, including pain arising from trauma-, inflammation- or chemotherapy-induced peripheral nerve damage, bone cancer, spinal cord injury, spinal nerve injury, multiple sclerosis, migraine, radiculopathy, and among others[7] (Textbox 1). Comparable results have now been reported for trigeminal pain models as well[8,9]. Drugs that inhibit proinflammatory glial activation or their proinflammatory products block and/or reverse such pain states (Table 1)[7]. Although microglia and astrocytes release diverse neuroexcitatory substances, key among these for pain sensitivity are proinflammatory cytokines: interleukin-1β (IL-1), tumor necrosis factor-α (TNF), and IL-6, and much of the pain literature has focused on these classically glially derived modulators of neuronal excitability.

Impact of glial activation on neuronal excitability

Under conditions of normal pain responsivity, microglia actively survey the extracellular space in search of potential danger, but are quiescent in terms of releasing neuroexcitatory substances[10]. By contrast, astrocytes are active players in synaptic signaling even under basal conditions because they both receive signals from and signal to synapses[11]. Astrocytes also maintain “housekeeping” functions such as providing energy sources and neurotransmitter precursors to neurons, clearing debris, maintaining homeostasis of extracellular ions, and taking up released neurotransmitters to terminate their actions. On appropriate stimulation (see below), microglia and astrocytes can each shift from their basal-but-active state to an activated state, characterized by a reactive, proinflammatory response profile. In this state, glia release substances that increase neuronal excitability, leading to pain enhancement. These include proinflammatory cytokines (IL-1, TNF, IL-6), chemokines, arachidonic acid and prostaglandins, ATP, excitatory amino acids and D-serine, nerve growth factors, reactive oxygen species, nitric oxide, and enkephalinases. These glial products can directly enhance neuronal excitability[12,13], increase pain-associated neurotransmitter release from sensory afferents[7], upregulate the number and conductance of calcium-permeable AMPA and NMDA receptors[14], potentiate inward currents in tetrodotoxin-resistant sodium channels[15], downregulate GABA receptors, downregulate outward potassium currents[16], downregulate expression of glial glutamate transporters, and downregulate GPCR kinase 2, thereby removing a “brake” on excitability[17]. Thus, both microglia and astrocytes can be triggered by special circumstances to enter a state in which they release a variety of neuroexcitatory substances. How that can occur in response to cues for pathological pain states, such as neuropathic pain, and by exposure to opioids, are explored below.

Triggering glia to enter an activated, proinflammatory state

Several glial activating factors have been identified (Figure 1), including many neuron-to-glia signals. The basic concept is that the peripheral tissue or nerve injury must generate a signal that in turn causes glia in the spinal cord to become activated and to release neuroexcitatory, proinflammatory signals. Neurons can release fractalkine (CX3CL1), monocyte chemotactic protein-1 (CCL2), nitric oxide, substance P, calcitonin gene related peptide, ATP, glutamate and prostaglandins, each of which can activate glia to release pain-enhancing products[7].

Intriguingly, conditions such as peripheral nerve injury can also elicit the release of what have recently come to be called "endogenous danger signals" or "alarmins"[18]. These are signals that something is wrong; that is, there is cellular stress/damage, independent of the release of classical neuronal neurotransmitters or neuromodulators. Such signals include degradation products of the extracellular matrix, components of circulating blood not normally having access to the extracellular space such as fibrinogen, and substances released by stressed, damaged and dying cells, such as nuclear protein HMBG1, heat shock proteins, DNA, and related "self" substances normally hidden from immunological surveillance. On release of these signals, the innate immune pattern recognition receptors TLR4 and TLR2 detect the presence of the danger signal resulting in the activation of TLR-expressing cells.

TLR4 as a glial activation receptor: role in neuropathic pain

Within the CNS, TLR4 is predominantly expressed by microglia, but its expression may be upregulated by astrocytes under neuroinflammatory conditions[19]. Given that TLR4 signaling is activated by messengers of cellular stress, damage and death, TLR4 and hence microglia are well positioned to be key for enhancing pain resulting from injury/inflammation of peripheral or central tissues that release endogenous danger signals (Textbox 2). Indeed, TLR4 has been shown to be a key glial activation receptor for the initiation and maintenance of neuropathic pain[19,20]. Although TLR2 and TLR3 have recently been implicated in neuropathic pain[21,22], most studies have been done on TLR4, and so this receptor is our focus here.

TLR4 signaling occurs via a cascade of events (Figure 2)[23]. Using Gram-negative bacterial lipopolysaccharide (LPS) as the prototypic TLR4 agonist, LPS is transported to the cell by LPS-binding protein, which transfers LPS to the co-receptor cluster determinent-14 (CD14) on the cell membrane. This leads to intracellular activation of acid sphingomyelinase that generates ceramide, which in turn induces generation of a lipid raft containing the co-receptor myeloid differentiation factor 2 (MD2), TLR4, heat shock proteins 70 and 90, among others. CD14 transfers LPS to MD2, leading to first MD2-TLR4 heterodimerization and then homodimerization of MD2-TLR4 pairs. Ensuing intracellular signaling occurs through at least 3 parallel pathways: cell motility and cell survival/apoptosis occurs through the PI3K/Akt pathway; and proinflammatory products such as cytokines result from activation of the NFκB and MAPK pathways. The details of how various endogenous danger signals interact with, bind to, and subsequently activate the TLR4 signaling pathway, and the specific identity of the endogenous danger signals that are involved in neuropathic pain are unknown. What evidence does exist suggests that a similar cascade is involved because neuropathic pain is suppressed in CD14 knockout mice[24], by TLR4 competitive antagonists, by novel TLR4 signaling inhibitors such as (+)-naloxone[19] (see below), by inhibitors of MD2-TLR4 docking[25], and by inhibitors of heat shock protein 90[26].

In summary, using peripheral nerve injury-induced neuropathic pain as the exemplar, nerve damage leads to the activation of microglia and astrocytes within the spinal cord. This occurs as a consequence of signals released by stressed and damaged neurons, including factors that activate the "endogenous danger signal" receptor, TLR4. Upon activation, glia release a variety of neuroexcitatory, pain-enhancing substances, key amongst these being proinflammatory cytokines.

Beyond pathological pain: glial modulation of opioid actions

In the past decade, a series of discoveries have revised our views of the pharmacological actions of opioids. Since 2001, several laboratories have reported that glia become activated in response to opioids and this glial activation leads to the release of proinflammatory products, including proinflammatory cytokines[27-29]. *In vivo*, opioid-induced glial activation has been inferred from: (a) morphine-induced upregulation of microglial and astrocytic activation

markers[30,31], (b) morphine-induced upregulation and/or release of proinflammatory cytokines and chemokines[28,31-33], (c) enhanced morphine analgesia produced by the glial activation inhibitors fluorocitrate, minocycline or ibudilast (AV411)[31,34,35], (d) enhanced morphine analgesia produced by blocking proinflammatory cytokine actions[28,36], and (e) opioid-induced selective activation of microglial p38 MAPK and enhanced morphine analgesia by p38 MAPK inhibitors[30]. *In vitro* studies also document direct actions of opioids on glia[34,37-39].

Until recently, it was assumed that opioids affect glia through opioid receptors. However, opioids can exert nonstereoselective effects. In contrast to classical opioid receptors, where only (-)-opioid isomers possess function, (+)-opioid agonists suppress (-)-opioid analgesia[40], an effect attributed to glial activation based on propentofylline blockade[41] and independent of classical μ-opioid receptors in knockout mice studies[42]. Opioid hyperalgesia is still observed in mu, delta, and kappa opioid receptor triple knockout mice[43], which again is suggestive of the existence of a non-classical opioid receptor whose nonstereoselective activation opposes analgesia.

Role of TLR4 in counteracting the beneficial actions of opioids

The non-classical, non-stereoselective effects of opioids remained a puzzle until a link was made in 2007 to TLR4[44]. *In vivo*, *in vitro*, and *in silico* approaches provided converging lines of evidence that members of each structural class of opioids activate TLR4 (some nonstereoselectively) and that opioid antagonists such as naloxone and naltrexone nonstereoselectively block TLR4 signaling[45,46]. The consequences of opioid-induced TLR4 signaling are extensive (Textbox 2). Acute blockade of TLR4, genetic knockout of TLR4, or blockade of TLR4 downstream signaling each lead to a marked potentiation of the magnitude and duration of opioid analgesia, with TLR4 modulation of opioid actions in wildtype animals occurring within minutes. Both spinal and supraspinal sites of opioid-TLR4 interactions are implicated[45]. Given the breadth of opioids now documented to interact with TLR4, many off-target opioid effects previously attributed to unilateral opioid action at classical neuronal opioid receptors might in fact result, at least in part, from the duality of opioid actions at TLR4. This dual opioid action hypothesis (glial TLR4 and neuronal opioid receptor) raises the benchmark for establishing unilateral neuronal opioid receptor involvement in a response, over simple naloxone blockade or the use of “opioid-selective” agonists or antagonists, unless they are proven not to be TLR4 signaling activators.

Mechanism of TLR4 activation by opioids

Intriguingly, TLR4 is activated by morphine-3-glucoronide (M3G), a morphine metabolite that is inactive at classical opioid receptors, but not by morphine-6-glucoronide (M6G), the opioid receptor active metabolite[45,46]. Along with nonstereoselectivity, the TLR4 signaling activation by M3G, but not M6G, points to a major difference from the structure activity relationship of the opioid receptor with the reliance of 4,5-epoxymorphinans on the 3’OH for classical opioid receptor activity but not for TLR4 activity. M3G action at TLR4 predicts that intrathecal M3G would induce pain enhancement mediated by TLR4, microglia (given their predominance in TLR4 expression), and proinflammatory cytokines (a downstream product of TLR4 activation). Indeed, this is the case[46]. TLR4 activation by opioids is now implicated in opposing acute and chronic opioid analgesia, and contributing to opioid-induced hyperalgesia, dependence and reward[45,46].

Nonstereoselectivity sets TLR4 apart from classical opioid receptors that bind (-)-opioid isomers but not (+)-isomers. Additionally, this nonstereoselectivity provides a means of specifically blocking opioid-induced glial activation by using drugs such as (+)-naloxone. This would allow (-)-opioids to act neuronally to suppress pain via their actions on classical opioid receptors, while preventing (-)-opioids from simultaneously activating glial TLR4, which

causes the release of pain-enhancing proinflammatory cytokines that oppose or counter-regulate the pain suppressive effects of opioids. The conclusion that (+)- and (-)-naloxone and naltrexone are TLR4 signaling inhibitors derives from their dose-dependent blockade of TLR4 activation by LPS, a classical TLR4 agonist, as indicated by suppression of LPS-induced reporter protein in a TLR4 expressing cell line[19]. Further, using a microglial cell line, (+)- and (-)-naloxone each blocked TLR4 activation-induced gene expression of both proinflammatory cytokines and a microglial activation marker[19].

Although it is clear that TLR4 signaling is activated and inhibited by opioid agonists and opioid antagonists, respectively, where along the signaling cascade this non-stereoselective interaction is occurring remains under investigation (Figure 2). This is because other xenobiotics, for example thalidomide and tricyclic antidepressants, have been shown to modify TLR4 signaling at least in part by exerting intracellular effects downstream of TLR4. Their effects include suppressing MD2 expression and inhibiting acid sphingomyelinse[47,48]. For opioids, the interaction with TLR4 is probably occurring at or very close to MD2/TLR4. *In silico* modeling of opioid interactions with MD2, TLR4, and the MD2-TLR4 complex point to docking of opioids in the LPS binding pocket of MD2, in a manner predictive of altering the MD2-TLR4 complex[45] (Figure 3). Opioid-induced activation of TLR4 signaling is blocked by a competitive inhibitor of LPS, again supportive of an action at the TLR4 complex. Also, morphine analgesia is potentiated in TLR4 knockout mice, supportive of opioid actions at TLR4 rather than downstream sites. Additionally, *in vitro* studies have revealed a requirement for soluble factors in media conditioned by macrophages cultures for allowing opioids to signal effectively through TLR4, suggestive of the requirement of known soluble member(s) of the TLR4 lipid raft complex, such as MD2. From *in vitro* and *in vivo* studies, opioids activate toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) and all three of its downstream signaling pathways (PI3K/Akt, NFkB, MAPK) leading to both membrane ruffling and motility (PI3K/Akt) (Figure 4) and proinflammatory cytokines (NFkB, MAPK). While such data support a role of TLR4, they do not preclude opioid interactions with other TLRs, because opioid effects on TLR2 and TLR9 have been reported[49].

TLR4 and glial activation: implications for drug development

Gaining insights into glial modulation of pain and opioid effects in humans has been constrained by the challenges of assaying glial activation and glial products within the CNS. In rodents, positron emission tomography allows analyses of glial activation through visualizing uptake of labeled fluoroacetate (metabolic inhibitor specific to the Krebs cycle in glia)[50] or labeled ligands of the translocator protein (TSPO; formerly known as the peripheral benzodiazepine receptor), the upregulation of which is a hallmark of activation in microglia[51]. No published studies have used such approaches for studying glial modulation of pain or opioid actions. The only human study of imaging of glial activation in patients reported thalamic microglial activation in amputees with long-duration phantom limb pain[52]. Protein or gene activation analyses of pain- or opioid-related glial activation in humans is in its infancy, with reports supportive of glial activation, enhanced proinflammatory cytokines and suppressed anti-inflammatory cytokines in CSF and/or spinal cord of chronic pain[53-55] and chronic opioid using patients[56]. Genomic analyses of polymorphisms also support alterations in the cytokine system of patients with chronic pain[7] and more recently predisposition to opioid dependence[57]. Given the strength of the animal studies, human research using such approaches is needed.

On the basis of the overwhelming evidence from animal studies supportive of a role of glia in pathological pain states, the U.S. Food and Drug Administration (FDA) has now approved two glia-targeting drugs for Phase 2 clinical trials for the treatment of neuropathic pain: ibudilast (Avigen's AV411) and propentofylline (Solace's SLC022). In addition, ibudilast has FDA

approval for testing its ability to increase the clinical efficacy of opioids. While having distinct mechanisms of action[58,59], both ibudilast and propentofylline are orally available, blood-brain barrier permeable, glial activation inhibitors (based on microglial and astrocyte activation marker suppression) that have strong support treating pain from various rodent models[58,59]. Ibudilast has a long history of safety in humans for the treatment of asthma and post-stroke dizziness. Propentofylline has previously been tested in humans as far as Phase 3 trials for treating Alzheimer's disease, with the trial discontinued for lack of efficacy. In addition to their well-characterized effects *in vitro* and *in vivo*, a recent novel finding is that ibudilast, but not pentoxyfyline (close relative of propentofylline), is a TLR4 signaling inhibitor[45]. Furthermore, given the structural diversity of exogenous pharmacotherapies and endogenous small molecule "alarmins" that have TLR4 activity, a far wider range of existing xenobiotics (as well as those in development for a myriad of TLR4 indications) may have TLR4 activity. This presents the possibility of drug-drug and/or drug/-TLR4 interactions at the previously unrecognized xenobiotics-TLR4-glial interface. Further, both AV411 and SLC022 possess the ability to enhance the production of anti-inflammatory cytokines, in addition to suppressing proinflammatory ones. This is important as it would replace the important glially-derived neuroprotective, negative feedback factors that would be missing if only proinflammatory products were to be suppressed.

This action of ibudilast as a TLR4 signaling inhibitor raises the more general issue of what is desirable in a glial inhibitor. Pervasive glial inhibitors such as fluorocitrate or fluoroacetate are not feasible, as these metabolic poisons shut down glial uptake of extracellular excitatory amino acids, leading to seizures[59] and have mostly been tested in inflammatory models (Table 1). Drugs such as ibudilast and propentofylline have both neuronal and glial effects[58,59], and it is clear that they have multiple effects[45,58,60]. This suggests that (+)-naloxone analogs should be considered that specifically target TLR4 rather than having broad effects as do propentofylline and ibudilast. While one could argue that blocking TLR4, classically known as the endotoxin receptor, could have detrimental effects, the immune system is redundant in its safeguards and has multiple avenues for recognizing and eliminating bacteria, such as opsonization and internalization by pathways independent of TLR4. In addition, the clinical experience of chronic high dose (-)-naltrexone use is well tolerated in other indications, and studies of TLR4 knockout and TLR4 mutant mice by-and-large reveal no health concerns in the absence of a functional TLR4 system. Such evidence, in addition to the data reviewed above, advocates serious consideration of orally available, blood-brain barrier permeable, TLR4 (and probably TLR2) inhibitors for clinical trials as stand alone treatments of chronic pain and as adjunct therapeutics for improving the clinical utility of opioids.

Concluding remarks

As reviewed above, while glia in their basal state play important roles in maintaining the health and normal functioning of the nervous system, their inappropriate proinflammatory activation concurrent with chronic pain pathologies and opioid administration can dramatically amplify pain and detrimentally alter the actions of opioids. While proinflammatory responses by glia can be important for inducing resolution of CNS immune challenges, neuroprotection, and repair, under conditions of chronic pain and opioid exposure, glial activation is not advantageous and instead detrimental to health and CNS functioning. Importantly, the glial actions described here do not stand and act alone to produce the behavioral consequences, since previously established neuronal pathways will unquestionably have to signal, precipitate, and propogate the response. Instead, the data reviewed here suggests that glia activation, in some cases resulting from TLR4 signaling, contributes significantly to the dysregulation of neuronal functioning under these altered physiological states. While TLR4 is by no means the only receptor-mediated activation pathway possessed by glia, TLR4 activation facilitates glial activation and neuroexcitability under conditions of chronic pain where endogenous danger

signals are elevated and in response to opioids. The results from animal studies to date predict that controlling endogenous danger signal- and opioid-induced glial activation and the resultant proinflammation using pharmacotherapies will: be stand-alone treatments for diverse pathological pain states, enhance the ability of opioids to suppress pain, suppress the development of opioid tolerance, suppress the development of opioid dependence, suppress opioid reward linked to drug abuse, and suppress other negative side-effects of opioids such as respiratory depression. The broader implications of glial attenuation by novel pharmacotherapies beyond their intended beneficial actions will need to be examined and carefully monitored, but the opportune clinical experience based on existing previously uncharacterized glial modulators designed and prescribed for other purposes, such as naltrexone and minocycline, is promising as no negative consequences of glial attenuation have been identified to date with these agents. With two glial modulatory drugs now entering clinical trials, the exciting translation to humans is finally beginning.

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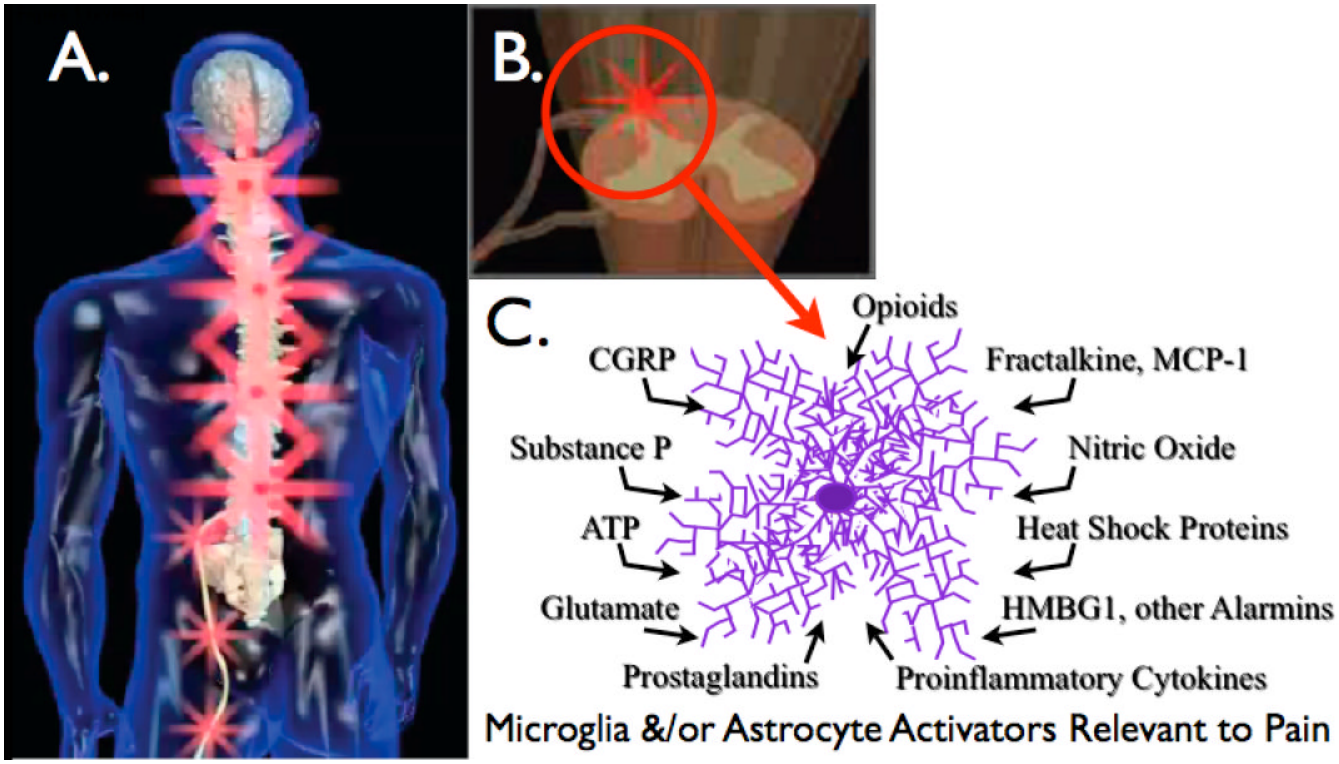

Figure 1. Glial activation by peripheral neuropathy

(A) Peripheral nerve injury leads to spinal amplification of incoming pain messages in addition to spontaneous pain signaling. This is indicated by the magnification of red "stars" symbolizing pain signals moving from the periphery into spinal cord, with amplification of pain messages being relayed to higher brain centers. **(B)** Spinal amplification of pain occurs within the spinal cord dorsal horn, a key site for the dynamic regulation of pain processing. This is the site where incoming sensory fibers synapse with neurons that relay pain messages up to brain via the spinothalamic and other pathways. This is also the site where glia and other immunocompetent cells can amplify pain via the release of neuroexcitatory substances such as proinflammatory cytokines. **(C)** The release of neuroexcitory, proinflammatory products by glia occurs in response to microglial and astrocyte activation. This activated state can occur in response to a variety of neuron-to-glia signals including neuronal chemokines (fractalkine, MCP-1), neurotransmitters (glutamate, ATP, substance P, CGRP), neuromodulators (nitric oxide, prostaglandins), endogenous danger signals (also called "alarmins"; e.g., heat shock proteins, the nuclear protein HMGB1), in addition to xenobiotics including opioids.

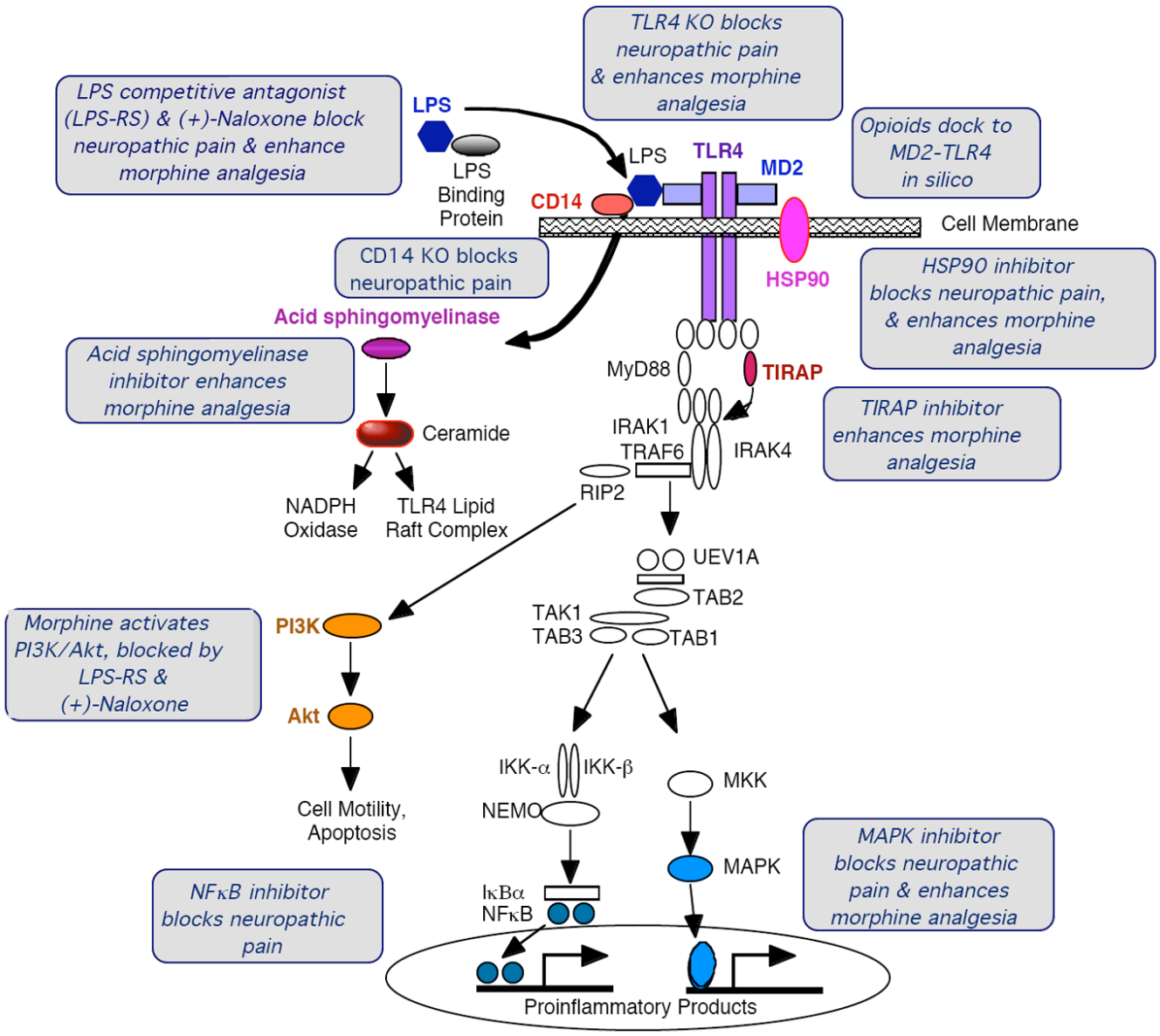

Figure 2. TLR4 signaling cascade and evidence for modulation by opioids

TLR4 signaling occurs via a cascade of events. Gram-negative bacterial lipopolysaccharide (LPS; dark blue hexagon) is the prototypic TLR4 agonist that is transported to the cell via LPS-binding protein (gray oval), which transfers LPS to CD14 on the cell membrane (red oval). This leads to intracellular activation of acid sphingomyelinase (purple oval), which generates ceramide (red oval). Ceramide induces the generation of a lipid raft containing the co-receptor myeloid differentiation factor 2 (MD2) (light blue rectangle), TLR4 (purple rectangles), and heat shock protein (HSP) 70 and HSP90 (pink oval), among other elements. Ceramide also activates NADPH oxidase, that leads to peroxynitrite formation. CD14 transfers LPS to MD2 (light blue rectangle), leading to both MD2-TLR4 heterodimerization and then homodimerization of MD2-TLR4 pairs. Ensuing intracellular signaling occurs through toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) to at least 3 parallel pathways: cell motility and cell survival/apoptosis occur through the IP3K/Akt pathway (yellow ovals), and proinflammatory products such as cytokines result from activation of the

NFκB (dark blue circles) and MAPK (medium blue oval) pathways. The gray boxes provide summaries of the converging evidence that opioids interact with the TLR4 signaling cascade. The TLR4 signaling pathway schema is a modified from illustrative figure of Dauphinee and Karsan http://www.nature.com/labinvest/journal/v86/n1/fig_tab/3700366f1.html#figure-title

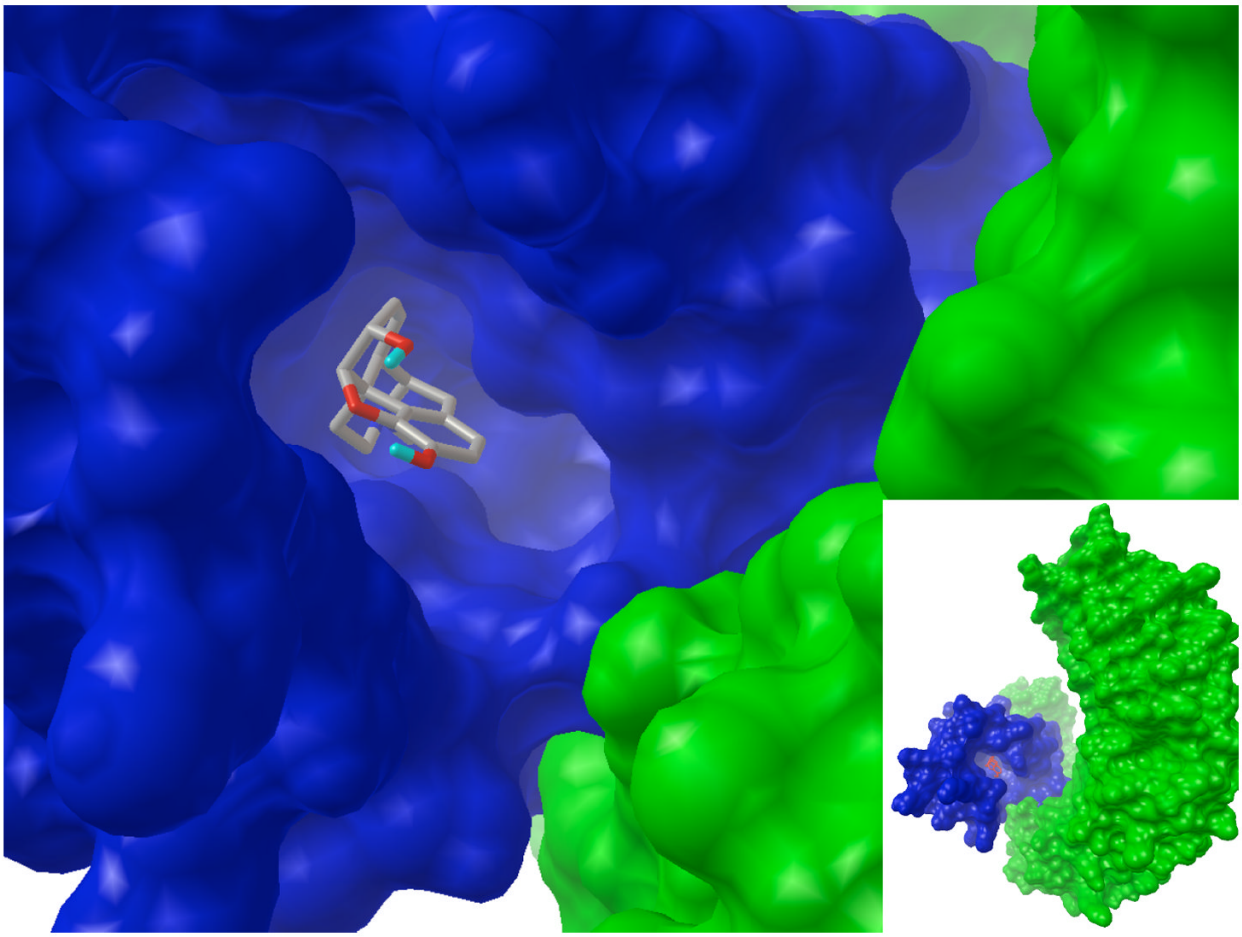

Figure 3. Computer modeling of opioid interaction with the TLR4-MD2 complex

In silico docking of (-)-morphine to the 3D crystalline structure of the human MD2 (blue) and TLR4 (green) complex demonstrates that the preferred binding conformation of (-)-morphine is to the LPS binding domain of MD2.

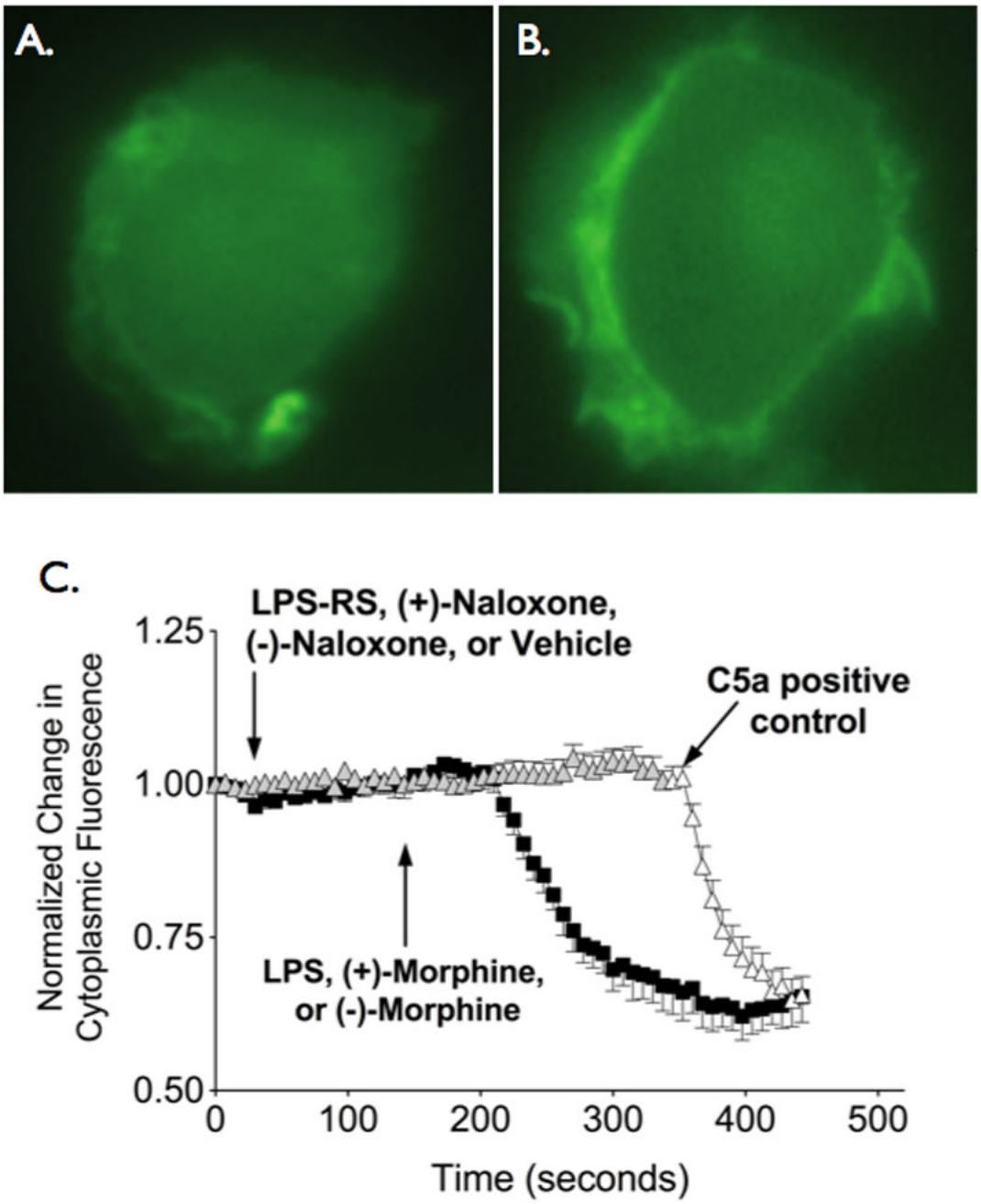

Figure 4. Live cell imaging of opioid-induced TLR4 signaling

Opioid interactions with TLR4 can be imaged in real time using a RAW264.7 mouse macrophage cell line that stably expresses green fluorescent protein labeled Akt1 (GFP-Akt1), as Akt is a component of one of the 3 parallel signaling pathways activated in response to TLR4 agonists. **(A)** Under basal conditions, Akt1 is diffusely distributed in the cytosol. **(B)** On activation, Akt1 rapidly moves to the membrane site where an Akt1 activating event is occurring. Membrane ruffling is also reflective of activation of the PI3K/Akt pathway downstream of TLR4. **(C)** Mobilization of GFP-Akt1 from the cytosol is easily quantified. At the **first (downward) arrow**, a TLR4 antagonist [Gray Triangles; either the competitive TLR4 antagonist LPS-RS (an LPS variant expressed by the bacterium *Rhodobacter sphaeroides*

which helps the pathogen evade the host's immune system by blocking TLR4), (+)-naloxone, or (-)-naloxone] or vehicle [Black Squares] is added to the live cell cultures. Cytoplasmic Akt1 fluorescence remains unchanged until TLR4 agonists (the classic TLR4 agonist LPS, (+)-morphine, or (-)-morphine) are added at the **second (upward) arrow**. Cells that are exposed only to TLR4 agonists (Black Squares) show a rapid loss of fluorescence from the cytosol as GFP-Akt1 mobilizes to the cell membrane. In the presence of TLR4 antagonists (Grey Triangles), TLR4 agonists now fail to induce GFP-Akt1 activation, so cytosolic fluorescence remains stable. As a positive control that these antagonist-blocked cells are in fact responsive, complement 5a (C5a) is then added at the **third arrow** (White Triangles) as a positive control since C5a activates PI3K/Akt1 but via a pathway independent of TLR4. Panel 3 is modified from Hutchinson et al.[45].

TABLE 1

Pain facilitation in animal models is prevented (p) or reversed (r) by inhibition of spinal glial activation or proinflammatory cytokine actions:

Model	Intervention [prevention (p) vs. reversal (r) noted for each]
Bone cancer	IL-1ra (r)[61]
Carrageenan, subcutaneous	minocycline (p), IL-1 knockout (p)
Chemotherapy induced neuropathy	propentofylline (SLC022) (p)[62] IL-10 (r)[63], IL-1ra (r)[63], ibudilast (AV411) (r)[64], CB2 agonist (p)[65], minocycline (p)[66]
Colon irritation	minocycline (r)[67]
Complete Freund's adjuvant	IL-1ra (p,r)[68,69], IL-1 KO (p), fluorocitrate (p)[69], CB2 agonist (r)[70]
Dorsal root compression/inflammation	IL-1ra, sTNFR minocycline (p)[71]
Dynorphin, intrathecal	IL-1ra (p), IL-10 (p)
EAE model of multiple sclerosis pain	IL-10 (r)[72]
Facial nerve chronic constriction injury	IL-1ra (r)[73]
Formalin, subcutaneous	fluorocitrate (p), IL-1ra (p), minocycline (p), IL-1 KO (p)
Fractalkine, intrathecal	minocycline (p), IL-1ra (p), anti-IL-6 (p)
HIV-1 gp120, intrathecal	fluorocitrate (p), IL-1ra (p), sTNFR (p), anti-IL6 (p), minocycline (p)[74], IL-10 (p)[75]
Inferior alveolar and mental nerve transection	minocycline (p)
Infraorbital nerve injury	propentofylline (SLC022) (r)[8], fluorocitrate (r)[8], minocycline (r)[8], sTNFR (r)[8], IL-1ra (r)[8]
Monoarthritis	Fluorocitrate (r)[6], minocycline (p)[6]
Myositis	minocycline (p)[76], anti-TNF (p)[76]
Mustard oil, topical	fluorocitrate (p)
Phospholipase A2, subcutaneous	fluorocitrate (p), IL-1ra (p), sTNFR (p)
Postoperative incisional pain	fluorocitrate (r), IL1ra overexpressing transgenetics & IL1 receptor KO (p)[77], IL-1ra (r)[77], pentoxifylline (p)[78], CB2 agonist (r)[79]
Sciatic nerve injury	IL-1ra (r), IL-10 (r), IL-1 knockout (p), ibudilast (AV411) (r)[58], fluorocitrate (r)[80], minocycline (p)[81,82], pentoxifylline (p)[81], CB2 agonist (r)[70]
Sciatic nerve inflammation	fluorocitrate (p), minocycline (p), IL-1ra (p), sTNFR (p), anti-IL-6 (p), IL-10 (p)[83]
Sciatic nerve tetanic stimulation	fluorocitrate (p)
Spinal cord injury	IL-10 (p), sTNFR (p)[84], minocycline (p,r) [84,85], ibudilast (AV411) (r), propentofylline (p)[86]
Spinal nerve root injury	methotrexate (p,r)
Spinal nerve transection or ligation	propentofylline (SLC022) (p,r), minocycline (p)[87], ibudilast (AV411) (r)[58], pentoxifylline (p)[88], IL-1 KO (p), CB2 agonist (r)[89], IL-1ra + sTNFR (p,r), IL-1 receptor KO (p), IL1ra overexpresssing transgenic (p)
Supradural "inflammatory soup"	ibudilast (AV411) (r)
Tempormandibular joint region inflammation	anti-IL6 (p)[90]
Tibia fracture model of complex regional pain syndrome	pentoxifylline (p)[91]
Zymosan, subcutaneous	fluorocitrate (r)[80]

Abbreviations: anti-IL6: neutralizing antibody against interleukin-6, anti-TNF: neutralizing antibody against tumor necrosis factor-α, CB2: cannabinoid type 2, KO: knockout mice, IL-1ra: interleukin-1 receptor antagonist, sTNFR: soluble TNF receptor, IL-10: interleukin-10.

Modified and updated from Watkins et al.[7]. Citations are found in this 2007 review unless otherwise indicated.

TABLE 2

Clinically relevant efficacy of opioids is improved in animal models by inhibition of glial activation or proinflammatory cytokine actions:

Model	Direction of effect	Intervention
Opioid induced acute analgesia	enhanced	minocycline[28, 34], ibudilast (AV411)[31], IL-10[32], IL-1ra[28, 32, 36], IL-1 signaling KOs[36], IL-ra over-expressing transgenics[36], classic TLR4 antagonists[45], (+)-naloxone[45], (-)- naloxone[45], ultra-low (-)-opioid antagonists [92], sTNFR[28], anti-IL-6[28], p38 MAPK inhibitor[28]
Opioid induced analgesia for neuropathic pain	enhanced	propentofylline (SLC022)[93], pentoxifylline[93] IL-1ra + TNF soluble receptors + anti-IL6[94]
Morphine analgesic tolerance	suppressed	ibudilast (AV411)[64], IL-10[32], IL-1ra[32], fluorocitrate[95], minocycline[35], pentoxifylline[81], (+)-naloxone[45], propentofylline (SLC022)[33], IL-1 signaling KOs[36], IL-ra overexpressing transgenics[36], IL-10 + IL-1ra[28], IL-1 converting enzyme inhibitor + IL1ra[28]
Morphine withdrawal-induced pain enhancement	suppressed	IL-10[32], IL-1ra[32], propentofylline (SLC022)[33], (+)-naloxone[45], IL-10 + IL-1ra[28], IL-1 converting enzyme inhibitor + IL1ra[28]
Morphine withdrawal-induced pain enhancement in neuropathic rats	suppressed	IL-1ra + TNF soluble receptors + anti-IL6[94]
Precipitated opioid withdrawal behaviors	suppressed	ibudilast (AV411)[31], (+)-naloxone[45], minocycline[31]
Spontaneous opioid withdrawal behaviors	suppressed	ibudilast (AV411)[31]
Morphine induced respiratory depression	suppressed	minocycline[34]
Morphine induced dopamine release from brain “reward” area (nuc. Accumbens)	suppressed	(+)-naloxone, ibudilast (AV411)[96]
Morphine induced conditioned place preference	suppressed	minocycline[34], propentofylline (SLC022)[97]
Morphine induced glial activation (IHC)	suppressed	ibudilast (AV411)[31], pentoxifylline[98], propentofylline (SLC022)[99], fluorocitrate[95] minocycline[74]
Morphine induced proinflammatory cytokines & chemokines	suppressed	propentofylline (SLC022)[99], (+)-naloxone[19], ibudilast (AV411)[31]

Abbreviations: anti-IL6: neutralizing antibodies against interleukin-6, IL-1ra: interleukin-1 receptor antagonist, KOs: knock out mice, TLR4: toll like receptor 4, sTNFR: soluble TNF receptor, IL-10: interleukin-10.