OPIOIDS AND GLIA: INVESTIGATING THE MECHANISMS THROUGH WHICH ULTRA-LOW DOSE OPIOID ANTAGONISTS MODULATE OPIOID TOLERANCE AND HYPERALGESIA.

by

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A thesis submitted to the Graduate Program in Pharmacology & Toxicology in the Department of Biomedical and Molecular Sciences In conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Ultra-low doses (ULD) of the opioid receptor antagonists, naloxone and naltrexone, augment the analgesic actions of morphine, block the induction of tolerance, and reverse established tolerance by an unknown mechanism. Preclinical studies demonstrate that chronic morphine administration induces spinal gliosis and that inhibition of gliosis prevents the development of analgesic tolerance to opioids. Thus, this thesis investigated the inhibition of spinal gliosis as a mechanism by which ULD antagonists attenuate analgesic tolerance and opioid-induced hyperalgesia.

Immune cell activation is implicated in the etiology of morphine tolerance and intrathecal catheterization, a technique commonly used to study the spinal effects of drugs, causes profound gliosis. Thus, the first study investigated the effects of catheter-induced gliosis on acute and chronic morphine analgesic tolerance. Catheterization-induced gliosis did not alter antinociceptive responses to acute intrathecal morphine; however, tolerance to chronic morphine was exacerbated in catheterized rats compared to sham and surgery-naïve controls.

The potentiation of analgesic tolerance to chronic morphine by spinal gliosis provided evidence that glia modulate opioid analgesia; therefore, inhibition of opioid-induced activation of glia was explored as a potential mechanism by which ULD antagonists prevent tolerance. The second series of experiments reported morphine-induced activation of spinal microglia and astrocytes was blocked by co-administering ULD naltrexone with morphine. These findings prompted us to elucidate the specific molecular target through which ULD antagonists attenuate opioid analgesia.

Activation of glial Toll-like receptor 4 (TLR4) induces gliosis and may contribute to analgesic tolerance and/or morphine-induced hyperalgesia (MIH). Antagonism of TLR4 by the opioid receptor-inactive (+) stereoisomer of naloxone was identified as a potential mechanism by which ULD antagonists modulate opioid analgesia. Tolerance and MIH developed in mice...
expressing non-functional TLR4 and in wildtype controls. Analgesic tolerance was stereoselectively blocked by ULD (-)naloxone, whereas MIH was blocked by both naloxone enantiomers.

Collectively, these studies demonstrate analgesic tolerance and MIH occur through distinct mechanisms. ULD naloxone attenuates analgesic tolerance likely via an opioid receptor-mediated mechanism that is TLR4-independent. ULD antagonists do not attenuate tolerance via inhibition of spinal gliosis as hypothesized. In contrast, ULD antagonists prevent MIH by inhibiting opioid-induced gliosis in an opioid receptor- and TLR4-independent manner.
Co-Authorship

The research upon which this thesis is based was conducted by Theresa Alexandra Mattioli under the supervision of Dr. Catherine M. Cahill and Dr. Brian Milne. Dr. Catherine M. Cahill performed the implantation of intrathecal catheters used in Chapter 2 and Ms. Maaja Sutak provided spinal tissue used to in the immunohistochemical assay in Chapter 2. Theresa Alexandra Mattioli contributed immunohistochemical (Fig. 8c) and behavioural data (Fig. 8k) and analyzed this data in Chapter 4. Dr. Francesco Ferrini and Dr. Tuan Trang were responsible for the conduct and analysis of all other experiments in Chapter 4 and preparation of the final manuscript based on data in this chapter. Graham Skelhorne-Gross isolated RNA and performed qRT-PCR analysis of spinal tissue in Chapter 5. Ms. Lihua Xue provided technical support throughout the thesis project.
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To my siblings, Justin and Jackie, you have always been a source of balance in my life. Justin, you never fail to put a smile on my face even on the toughest days. I am inspired by your spontaneous and carefree spirit, and have learned that the only limitations are those we impose on ourselves. Jax, you’re my best friend, my confidant, and my role model. You’ve always been there for me, whether the voice of reason, getting me out of trouble, or joining me in mischief, I could not ask for a better sister. Thank you for always seeing the best in me and helping me to see obstacles as challenges to be embraced rather than impossibilities.

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<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACTZ</td>
<td>acetazolamide</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>Ca²⁺</td>
<td>calcium ion</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
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<td>Cl⁻</td>
<td>chloride ion</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CTAP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂</td>
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<td>CTX</td>
<td>cholera toxin</td>
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<tr>
<td>δ</td>
<td>delta</td>
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<tr>
<td>DAMGO</td>
<td>Tyr-D-Ala-Gly-MePhe-Gly-ol</td>
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<td>Deltorphin I</td>
<td>Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂</td>
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<td>Deltorphin II</td>
<td>Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DPDPE</td>
<td>Tyr-D-Pen-Gly-Phe-D-Pen</td>
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<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
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<tr>
<td>eIPSC</td>
<td>evoked inhibitory postsynaptic current</td>
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<td>FLNA</td>
<td>filamin A</td>
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<td>GDP</td>
<td>guanine diphosphate</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>G-protein</td>
<td>trimeric guanine nucleotide binding protein</td>
</tr>
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<td>GIRK</td>
<td>G-protein activated inwardly-rectified potassium channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
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<td>guanine triphosphate</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.t.</td>
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<td>interleukin-1β</td>
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<td>IRAK</td>
<td>interleukin-1 receptor associated kinase</td>
</tr>
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<td>kappa</td>
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<td>KCC2</td>
<td>Potassium chloride co-transporter-2</td>
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<tr>
<td>LP</td>
<td>lumbar puncture</td>
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<td>LPS</td>
<td>lipopolysaccharide from <em>Escherichia coli</em></td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>LPS-RS</td>
<td>lipopolysaccharide from <em>Rhodobacter sphaeroides</em></td>
</tr>
<tr>
<td>μ</td>
<td>percentage of maximum possible effect</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MD-2</td>
<td>myeloid differentiation factor-2</td>
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<td>MIH</td>
<td>morphine-induced hyperalgesia</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>morphine sulfate</td>
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<td>MQAE</td>
<td>N-6-methoxyquinolinium acetoylester</td>
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<td>MyD88</td>
<td>myeloid differentiation factor-88</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>17,17’-bis(cyclo-propylmethyl)-6,6’,7,7’-tetrahydro-4,5,4’,5’-diepoxy-6,6’-(imino)[7,7’-bimorphinan]-3,3’, 14,14’-tetrol</td>
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<tr>
<td>OR</td>
<td>opioid receptor</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDE</td>
<td>phosphodiesterase</td>
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<td>polyethylene</td>
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<td>propentofylline</td>
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<td>PKA/C</td>
<td>protein kinase A or C</td>
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<td>PPADS</td>
<td>pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonate</td>
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<td>PTX</td>
<td>pertussis toxin</td>
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<tr>
<td>-R</td>
<td>receptor</td>
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<td>saline</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>spinal dorsal horn</td>
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<td>sodium dodecyl sulfate</td>
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<td>s.e.m.</td>
<td>standard error of the mean</td>
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<td>tris-buffered saline</td>
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<td>TICAM</td>
<td>TRIF/TIR domain-containing adapter molecule</td>
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<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNP-ATP</td>
<td>2’,3’-O-(2,4,6-trinitrophenyl)adenosine 5’-triphosphate</td>
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<td>TRIF-related adapter molecule</td>
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<td>TIR domain-containing adapter inducing IFN-β</td>
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<td>BDNF-sequestering fusion protein</td>
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<tr>
<td>ULD</td>
<td>ultra-low dose</td>
</tr>
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<td>VGCC</td>
<td>voltage-gated calcium channel</td>
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CHAPTER 1:

INTRODUCTION

1.1 Pain

"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [106]. Thus, the primary purpose of pain is to serve as a warning to the body of impending damage. In normal healthy individuals, primary afferents relay noxious sensory information (e.g. thermal, mechanical and chemical stimuli) from peripheral tissues to the dorsal horn of the spinal cord. Here, these noxious stimuli are transduced into electrical signals and processed, and are subsequently relayed to the brain where they are perceived as painful. In some individuals, the pain associated with an infection or injury does not resolve with healing and may persist for a long period of time; the International Association for the Study of Pain (IASP) defines chronic pain as any pain either continuous or intermittent that persists longer than 6 months [106].

1.1.1 Social and Economic Impact of Chronic Pain

Estimates indicate that up to 100 million American adults suffer from chronic pain, with an economic impact of $560-$630 billion due to health care costs and loss in productivity [26]. Few studies have been able to accurately estimate the impact of chronic pain on Canadians, although one study reports that 1.5 million Canadians (ages 12-44) are affected [211]. A longitudinal study found a cumulative incidence of 36% of Canadians over the age of 25 suffered from chronic pain [200]. The risk of chronic pain increased with age in women, suggesting that these numbers will increase as the baby boomer population ages. Chronic pain is associated with the worst quality of life compared to other chronic diseases [40], and it is difficult to fully estimate the impact of this disease on the individual or on society as a whole. Presently, chronic
pain is not well managed; many patients have reduced sensitivity to traditional analgesics, such as morphine and other opioids, warranting the need for more efficacious treatments.

1.2 Opioid Analgesics

Opioids have been used as analgesics for centuries. Opium, an extract from the poppy, *Papaver somniferum*, was first used as a postoperative analgesic in the late 1700s [89], until its active component, morphine, was later isolated and purified [214]. In addition to analgesia, opioids have been used for their sedative, anti-diarrheal, antitussive, and euphoric effects. Despite morphine’s inherently high analgesic efficacy, the use of morphine and other opioids to treat chronic pain states is largely limited by the development of analgesic tolerance and opioid-induced hyperalgesia, which is an exaggerated sensitivity to a noxious stimulus. Opioids are also limited in their use and prescription due to their propensity for addiction, although uncommon in chronic pain patients when used appropriately.

Opioid receptors (OR) are expressed widely throughout the central and peripheral nervous systems (see [65] for review). These receptors have been identified in various regions of the brain (e.g. cerebral cortex, thalamus, hypothalamus, amygdala, periaqueductal gray, etc.) and within the spinal cord, on both neurons and glia [72,73,205,233], and are also found on many peripheral tissues. Three ORs have been identified based on cloning and functional studies: mu (µ) [34], delta (δ) [74,128,287] and kappa (κ) [287]. All three of these receptors are G-protein coupled receptors (GPCR), comprised of seven transmembrane spanning domains, with highly conserved sequence homology within the transmembrane helices and greater variation in their termini and extracellular domains [34]. Several receptor subtypes (µ₁, µ₂, µ₃, δ₁, δ₂, κ₁, κ₂, κ₃) have been proposed based on *in vivo* pharmacological responses (reviewed by [65,198]), however, their existence has not been confirmed by molecular evidence; only three genes have been identified to date. Alternative gene splicing [180], post-translational modifications to receptor mRNA, or differential binding of receptors to intracellular mediators at the membrane
may account for the varied responses. An alternate theory suggests receptor dimerization may be responsible for the heterogeneity of opioid receptor responses; physical interactions between ORs [84,85] and with other GPCRs (e.g. beta-adrenergic receptors [121]) have been reported. The agonist itself may dictate the intracellular signaling cascade induced, with each agonist inducing a distinct conformational change in the OR; the unique receptor-effector complexes that result consequently activating signaling cascades in a distinct manner[126,199,210]. This concept is commonly referred to as biased agonism or ligand-directed signaling (see [188] for detailed review).

Most clinically available opioids induce analgesia primarily via activation of the μOR, but also exhibit activity, albeit with lower affinity, at δ- and κORs (Table 1.1). As a GPCR, ORs interact with trimeric guanine nucleotide binding proteins (G-proteins), showing preference for the pertussis toxin (PTX)-sensitive inhibitory Gαᵯᵢ/o proteins [31,32,55,138,219], although they also interact with the cholera toxin (CTX)-sensitive stimulatory Gₛ protein [83,218,270]. At least twenty Gα subunits have been identified, which may account in part for the differing responses resulting from opioid agonist receptor activation [87]. Evidence suggests OR interactions with Gα subunits are influenced by subunit availability or stoichiometric expression and subunit localization [45]. Following agonist binding, GPCRs undergo a conformational change, resulting in the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), activating the G-protein. The Gα subunit dissociates from the Gβγ subunits, which remain bound as a dimer, each initiating its own signaling cascade via interaction with other effectors (see [45] for detailed review). Opioid agonists induce analgesia primarily by three mechanisms (Fig. 1.1). In brief, activation of presynaptic ORs located at the nerve terminals of primary afferents inhibit the opening of voltage-gated calcium channels [257] thereby preventing the influx of calcium ions into the presynaptic terminal and the subsequent release of excitatory neurotransmitters (e.g. glutamate, substance P) by exocytosis into the synapse. Additionally, post-synaptic OR activation
Table 1.1 Opioid Receptor Ligands

<table>
<thead>
<tr>
<th></th>
<th>µOR</th>
<th>δOR</th>
<th>κOR</th>
</tr>
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<tbody>
<tr>
<td>Agonists</td>
<td>Morphine, Fentanyl, Methadone, Meperidine, DAMGO</td>
<td>Deltorphin I, II DPDPE, SNC80, TAN-67</td>
<td>Salvinorin A, Ketocyclazocine U-50,488, U-69593</td>
</tr>
<tr>
<td>Antagonists</td>
<td>Naltrexone, Naloxone (non-selective)</td>
<td>Naltrindole Naltriben</td>
<td>NorBNI</td>
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<tr>
<td></td>
<td>CTOP</td>
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<td>CTAP</td>
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**Abbreviations:** CTAP, D-Phe-Cys-Tyr,D-Trp-Arg-Thr-Pen-Thr-NH2; CTOP, D-Phe-Cys-Tyr,D-Trp-Orn-Thr-Pen-Thr-NH2; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; **Deltorphin I**, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH2; **Deltorphin II**, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH2; DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen; **NorBNI**, 17,17'-bis(cycloproplmethyl)-6,6',7,7'-tetrahydro-4,5,4',5'-diepox-6,6'-imino[7,7'-bimorphinan]-3,3',14,14'-tetro; **SNC80**, (±)-4-[(α-R)-α-[2S,5R]-4-allyl-2,5-dimethyl-1-piperazinyl]-2-methoxybenzyl]-N,N-diethylbenzamide; **TAN-67**, 2-methyl-4a(alpha)-(3-hydroxyphenyl)-1,2,3,4,5,12a(alpha)-octahydro-quinolino[2,3,3,g]isoquinoline; **U-50,488**, trans-2,3-dichloro-N-methyl-N[2]-1-pyrrolidinyl]-cyclohexyl]benzeneacetamide; **U-69,593**, (5a,7a,8β)-(−)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspirol(5)dec-8-yl]phenyl-benzene acetamide.
Stimulation of a primary afferent neuron by a noxious stimulus generates an electrical impulse, or action potential, that is transmitted along the axon to the synaptic terminal within the dorsal horn of the spinal cord. Here, depolarization of the pre-synaptic membrane initiates opening of voltage gated calcium (Ca\(^{2+}\)) channels (VGCCs), resulting in Ca\(^{2+}\) influx, and triggering the release of excitatory neurotransmitters (e.g. glutamate, ATP, substance P) from synaptic vesicles into the synaptic cleft. The neurotransmitters diffuse across the cleft and bind to their specific receptors (e.g. glutamate binds to ionotropic NMDA and AMPA receptors) post-synaptically resulting in an influx of cations such as sodium (Na\(^{+}\)) and Ca\(^{2+}\) which in turn causes depolarization and initiation of a secondary action potential that is transmitted to the brain (e.g. thalamus, cerebral cortex etc.).

Opioid agonists inhibit neurotransmission via: (1) activation of presynaptic opioid receptors (ORs) that inhibit the opening of VGCCs to prevent Ca\(^{2+}\) influx and the release of excitatory neurotransmitters; (2) post-synaptic OR activation stimulates G-protein-activated inwardly rectifying potassium channels (GIRKs), thus hyperpolarizing the neuron and preventing generation of a secondary action potential and nociceptive transmission to the brain; (3) OR activation inhibits adenylyl cyclase, decreasing cyclic-adenosine monophosphate (cAMP) signaling, and decreases protein kinases A/C (PKA/PKC) and mitogen-activated protein kinase (MAPKs) activity (not shown).
stimulates G protein-activated inwardly rectifying potassium channels (GIRKs; [63,107,174]), thus hyperpolarizing the neuron to prevent excitation and subsequent transmission of the noxious stimulus (see [44], for review). OR activation also inhibits adenylyl cyclase activity, decreasing cyclic-adenosine monophosphate (cAMP) signaling [117], and modulates activity of protein kinases A/C (PKA/PKC) and mitogen-activated protein kinases (MAPKs). Collectively, these G-protein and MAPK-mediated effects reduce neuronal excitation and inhibit nociceptive transmission to the brain resulting in analgesia.

1.3 Opioid Tolerance

With repeated opioid administration, the analgesic effects often diminish and larger doses are required in order to achieve an equal response. Pharmacologically, this is characterized by a rightward shift in the dose-response curve for opioid-induced effects. The concept of antinociceptive tolerance is widely accepted in animal models, but there is some debate regarding the phenomenon in humans. Observed dose escalations in patients are often attributed to disease progression (e.g. cancer pain) rather than a loss in analgesic efficacy [37,230]. The molecular mechanisms underlying opioid tolerance and hyperalgesia are not well understood, however, it is hypothesized that the mechanisms underlying tolerance and hyperalgesia overlap considerably with those involved in chronic pain signaling (for review, see [157]).

A number of potential mechanisms which regulate opioid receptor signaling have been implicated [42]. OR regulation occurs in a manner similar to β2-adrenoceptors, another GPCR whose regulation is well characterized [79]. Upon agonist binding, it is thought that the μOR is phosphorylated by G-protein receptor kinases (GRKs; [265]), promoting the binding of β-arrestins [36]; arrestins initiate receptor uncoupling from the activated G-protein and termination of continued receptor signaling ([46] for review). β-arrestin binding also triggers endocytosis of the receptor [79,203] which is then sorted for either lysosomal degradation [252] or resensitization by dephosphorylation and subsequent recycling to the cell surface [291].
Figure 1.2 Opioid Receptor Signaling and Tolerance. Adapted from [291]. (A) Upon agonist binding, the μOR undergoes a conformational change initiating substitution of GTP for GDP activating the G-protein heterotrimer (α, β, and γ subunits). G-protein receptor kinases (GRKs) phosphorylate the μOR, promoting the binding of β-arrestins to initiate receptor uncoupling from the activated G-protein terminating receptor signaling. β-arrestin may also initiate receptor endocytosis where it is sorted for lysosomal degradation or resensitization (dephosphorylation) and recycled to the cell surface. (B) Representation of a μOR signaling complex composed of multiple proteins that interact and participate in receptor signaling.
1.3.1 Mechanisms of Opioid Tolerance

The cause of opioid tolerance, or the loss of opioid receptor sensitivity, has been widely investigated with many studies providing contradictory evidence. Differential agonist efficacies for G-protein signaling (as discussed above) may be one such factor. An agonist’s ability to induce endocytosis, and thus receptor resensitization, may also be important to the development of tolerance. In theory, agonists with low internalization efficacies would produce less tolerance than those with high efficacies. This hypothesis has not been confirmed as studies provide conflicting results (see [62,165,290], for review). It has also been reported that inhibition of μOR coupling to effectors, such as β-arrestin-2 (presumably inhibiting receptor internalization) may enhance analgesia and attenuate tolerance development [18–20]; see [61], for contradictory evidence). In β-arrestin-2 knockout mice, acute morphine-induced antinociception, measured by response latencies in the hot-plate test (a thermal nociceptive test primarily mediated supraspinally), was significantly potentiated and prolonged compared to wildtype littermates [20]. Additionally, antinociceptive tolerance to chronic morphine, measured by hot-place responses and the hot water tail-immersion paradigm (an assessment of spinal reflex to thermal nociceptive stimuli) was attenuated in β-arrestin-2 knockout mice [18,19]. GRKs also appear to play a critical role in opioid tolerance; as previously described, these enzymes phosphorylate ORs after agonist binding, promoting the recruitment of β-arrestins. In GRK-3 knockout mice, chronic treatment with a selective κOR agonist (U50,488) produced significantly less antinociceptive tolerance, measured by the hot water tail-immersion test, compared to wild-type littermates [163]. As the evidence indicates, there is great discord in the literature regarding the molecular basis of opioid tolerance. Taken together, these studies suggest the development of tolerance is the consequence of a number of cellular adaptations, rather than the result of a single alteration in receptor signaling.
1.3.2 Bimodal Theory of Tolerance

There are a number of alternate hypotheses proposed to explain the onset of tolerance to the antinociceptive effects of opioid agonists. One hypothesis that has garnered much attention over the last two decades is the bimodal opioid receptor theory proposed by Crain & Shen [55]. The premise of the bimodal theory is that morphine and other opioid agonists may induce excitatory and inhibitory effects at ORs in a dose-dependent manner. In dorsal root ganglia (DRG) neurons, low (nanomolar) doses of morphine induced excitatory effects, such as increased calcium (Ca\(^{2+}\)) conductance (lengthened action potential duration; APD), decreased potassium (K\(^+\)) conductance (increased post-synaptic excitability)[219], and stimulation of adenylyl cyclases by the G\(\beta\gamma\) dimer [30,266]. In contrast, higher (micromolar) doses decreased neuronal excitation with shortened Ca\(^{2+}\)-mediated APD and increased K\(^+\) conductance [219]. The excitatory effects of opioids are blocked by pretreatment of DRG neurons in vitro with cholera toxin-A, which inhibits the activation of associated receptors by ADP-ribosylation of Gs [82,231], confirming mediation by Gs [218]. On the contrary, inhibitory effects are blocked by pretreatment with pertussis toxin (ADP-ribosylates G\(_i\) and G\(_{io}\))[258]), indicating mediation by G\(_{io}\) [219]. Therefore, the pronounced analgesia induced by activation of inhibitory G\(_{io}\)-coupled ORs by therapeutic doses (micromolar concentrations) of opioid agonists presumably masks the anti-analgesic effects of the G\(_s\)-coupled receptor activation, presenting only as analgesia [54]. Chronic exposure to morphine increased excitatory effects with no change in G\(_\alpha\)-protein expression [111,251]. Increased G\(_s\) and decreased G\(_{io}\)-coupling to μORs following chronic morphine administration were reported in vitro [29] and in vivo in rat brain and spinal cord [29,251,269]. Thus, opioid analgesic tolerance may be the consequence of a switch from the inhibitory G\(_{io}\)-mediated to excitatory G\(_s\)-mediated OR signaling.
Figure 1.3 Bimodal Theory of Opioid Tolerance. Adapted from [56]. Opioid agonists dose-dependently induce inhibitory or excitatory effects via μOR activation. High (μM) doses activate OR coupled to G<sub>i/o</sub> proteins causing decreased neuronal excitation with shortened Ca<sup>2+</sup>-mediated action potential duration (APD) and increased K<sup>+</sup> conductance, resulting in analgesia. Conversely, low (nM) doses of opioid agonists induce excitatory effects via G<sub>s</sub> coupled μORs leading to increased calcium (Ca<sup>2+</sup>) conductance and APD, decreased potassium (K<sup>+</sup>) conductance, and stimulation of adenylyl cyclase (AC), collectively enhancing nociceptive neurotransmission.
1.3.3  Involvement of Spinal Glia in Tolerance

1.3.3.1  Glial Activation

Recently, opioid tolerance has been explained on the basis of augmented glial function, as chronic morphine treatment leads to activation of spinal astrocytes and microglia. Spinal glia were thought to be merely support cells within the CNS, responsible for the maintenance of neurons and CNS homeostasis. It is now accepted that glia actively communicate with neurons and modulate synaptic transmission [115]. Reactive glia are characterized by cell hypertrophy, proliferation [64], and increased expression of cellular markers, such as glial fibrillary acidic protein (GFAP) in astrocytes and CD11b/CR3 or ionized calcium-binding adapter molecule-1 (Iba-1) in microglia [97,175,194,196,228]. Once activated, glia release a variety of pronociceptive and neuroexcitatory substances (e.g. prostaglandins, excitatory amino acids, interleukins, nitrogen oxide species, brain-derived neurotrophic factor (BDNF), ATP, glutamate, etc.), which may enhance pain transmission by nociceptive neurons [49,134,193,194,228,237]. With the crucial role spinal glia play in modulation of nociceptive signaling, the involvement of these cells in opioid tolerance and hyperalgesia is being widely investigated.

1.3.3.2  Opioid-Induced Glial Activation

Glial activation (gliosis) may be induced by a number of endogenous and exogenous factors, including nerve injury or infection. The potential role of gliosis in opioid tolerance is supported by studies that demonstrate reduced opioid analgesic efficacy in rodents with established glial activation due to nerve injury [7,137,194,274]. Johnston and Westbrook [119] also reported reduced morphine-induced analgesia in rats pre-treated with lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria and a potent activator of spinal glia [96,288]. These studies suggest that CNS insult may prime glia thereby increasing pronociceptive signaling that may counter opioid-induced analgesia and reduce analgesic efficacy. Foreign objects such as intrathecal catheters also induce spinal gliosis [64]. Intrathecal catheters are
extensively used to investigate the spinal effects of opioid induced analgesia [59,118,137,158,164,184], as they circumvent the need to anesthetize the animal for drug delivery. The implications of catheter-induced gliosis on opioid analgesia and tolerance are unknown. In light of the studies of nerve-injury and/or infection-induced gliosis, the potential effect of catheterization on tolerance warrants further investigation.

1.3.3.3 Gliosis and Opioid Tolerance

Even in the absence of previous CNS insult, opioids may induce spinal gliosis via direct activation of receptors (e.g. ORs) on glia [35,67,72,73,204,205,233] or indirect effects via release of excitatory transmitters such as ATP that activate glial P2X receptors. Chronic, but not acute, morphine administration induced activation of spinal astrocytes and microglia in rats [173,175,194,196,228]. Marked increases in cytokine mRNA (interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α)) and protein expression (IL-1β, IL-6 only) were also reported [194,196]. Other excitatory effects, including increased intracellular calcium and down-regulation of glutamate transporters, were observed in spinal glia with chronic morphine administration [157]. Antagonizing the activity of proinflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) attenuated the development of morphine tolerance and withdrawal [118,194]. In one study, chronic administration of a cocktail composed of morphine with IL-1 receptor antagonist (IL-1ra), soluble TNF receptor, and an IL-6 neutralizing antibody, prevented the development of antinociceptive tolerance measured by the tail-flick test in rats [194]. Others report that antagonism of IL-1β by treatment with IL-1ra potentiated and prolonged acute morphine antinociception, attenuated the development of tolerance when co-administered with morphine and reversed established tolerance; all three effects were assessed by changes in thermal withdrawal latencies in the tail flick test [118]. Shavit and colleagues [215] reported co-administration of IL-1β blocked morphine-induced analgesia in mice. Additionally, antagonism of IL-1 by multiple blockers (e.g. IL-1ra and IL-1 tripeptide antagonist) potentiated and
prolonged acute morphine analgesia and attenuated tolerance. Similar effects on morphine-induced analgesia were reported in mice with genetically impaired IL-1 signaling (over-expression of secreted human IL-1ra in CNS astrocytes) [215]. A novel study investigated the use of etanercept, a TNF-α antagonist clinically used for the treatment of rheumatoid arthritis and other inflammatory diseases, on opioid tolerance. Acute administration of etanercept following induction of morphine tolerance restored the antinociceptive effect of morphine in the hot water tail flick test [216]. These studies suggest that the production of proinflammatory cytokines by reactive glia significantly contributes to the development of opioid tolerance.

1.3.3.4 Inhibition of Gliosis Augments Opioid Tolerance

As described, antagonism of pronociceptive cytokine activity attenuates tolerance; in light of this evidence, others have investigated the role of spinal glia in opioid tolerance. Indeed, studies indicate glial inhibitors attenuate the development of antinociceptive tolerance to opioids in rodents [60,101,168,196,228]. Use of glial inhibitors to alter morphine tolerance was first reported by Song and Zhao [228], following their observation that chronic morphine induced activation of astrocytes in the brain and spinal cord. Co-administration of fluorocitrate, a glial metabolic inhibitor [181], attenuated the development of antinociceptive tolerance to morphine in the paw withdrawal test, a measurement of thermal nociception [228]. Investigation of the effects of other glial inhibitors on opioid tolerance soon followed this ground-breaking report.

Propentofylline (PPF) is a xanthine derivate that inhibits phosphodiesterases (PDEs), thereby augmenting cAMP signaling, and is an inhibitor of adenosine reuptake [179,220,221]; however, the mechanism by which PPF inhibits activation of microglia and astrocytes is unknown. Co-administration of PPF with chronic morphine blocked the development of antinociceptive tolerance in the hot water tail-flick (thermal) and paw-withdrawal (mechanical) nociceptive tests [196]. Furthermore, PPF attenuated morphine-induced gliosis, indicated by the attenuated increase in mRNA expression of GFAP (astrocytes), Mac-1 (microglia), and cytokines (IL-1β,
IL-6, TNF-α) compared to animals treated with morphine only. Minocycline is a second
generation tetracycline that selectively inhibits microglial activation and proliferation, via
inhibition of p38 MAPK [99], without effects on neurons or astrocytes [6,245,246]. Minocycline
attenuated the development of chronic morphine tolerance when co-administered intrathecally,
and partially reversed antinociceptive tolerance when administered in the early phase of tolerance
induction; however minocycline did not reverse established tolerance [60]. Likewise, minocycline
decreased glial reactivity (GFAP and CD11b labeling) when co-injected chronically with
morphine or if administered during the early tolerance-induction phase; only microglial reactivity
was reduced, with no effect on astrogliosis, when administered to morphine-tolerant rats. These
data suggest that microglia are involved in the early induction phase of opioid-tolerance, and
perhaps astrogliosis is more important to the maintenance of analgesic tolerance. Others have
confirmed morphine-induced microgliosis is inhibited by minocycline, and also report
potentiation of acute morphine analgesia [101]. Similarly, Mika et al., [168] reported minocycline
and pentoxifylline, also a PDE inhibitor and a non-specific cytokine inhibitor [150,154], delayed
the onset of analgesic tolerance to chronic morphine and attenuated CD11b/c upregulation in
spinal microglia measured by western blot. Overall, these studies indicate inhibition of opioid-
induced spinal gliosis as a potential mechanism by which the development of opioid tolerance
may be avoided in circumstances of chronic opioid use.

1.3.4 TLR4-Induced Gliosis

Spinal glia are uniquely equipped to monitor the CNS; in addition to ORs, glia express
receptors for a variety of signaling factors including ATP, prostaglandins, calcitonin gene related
peptide, substance P and glutamate [97,255,256,274,295]. Importantly, glia also express Toll-like
receptors (TLRs); these receptors recognize a number of exogenous (e.g. pathogen associated
molecular patterns) and endogenous (e.g. heat shock proteins) danger signals [23,100,242]. To
date, ten different TLRs (TLR1-TLR10) have been identified with each recognizing specific
components of pathogens and capable of eliciting unique inflammatory responses via their own
distinct signaling pathways (for review, see [241]). One member of this receptor family, TLR4, is
predominately expressed by spinal microglia and macrophages [140,141]. TLR4 mRNA has also
been detected in astrocytes following stimulation with LPS [23,77,109,129]. Activation of glial
TLR4 induces significant gliosis [1] and is implicated in opioid tolerance and hyperalgesia
[15,105,242].

1.3.4.1 TLR4 Structure & Signaling

TLRs are composed of a single membrane spanning region, an extracellular domain
containing leucine-rich repeats, and a highly conserved intracellular portion that is structurally
similar to the IL-1 receptor, thus named the Toll/IL-1 receptor (TIR) domain [241]. To activate
TLR4 signaling cascades, LPS must first be extracted from the bacterial cell by the lipid
transferase lipopolysaccharide binding protein (LBP) and transferred to CD14 [212,247,277].
CD14 is a membrane bound accessory protein that dimerizes upon LPS-LBP stimulation forming
a hydrophobic pocket containing LPS binding sites [130,169]. CD14 serves as a carrier for LPS,
transferring LPS to the TLR4/myeloid differentiation factor-2 (MD-2) receptor complex
[172,222]. MD-2 is an accessory protein that interacts with the extracellular domain of TLR4 and
is required for TLR4 signaling [70,170]. Recently, the crystal structure of the TLR4-MD-2
complex was resolved, supporting the previously hypothesized model of the ligand-induced
receptor complex [178]. Interaction of TLR4/MD-2 with LPS initiates aggregation of these
receptor complexes into dimers [263], activating two main intracellular signaling cascades via the
intracellular TIR domain. The first signaling pathway utilizes the adaptor molecule myeloid
differentiation factor-88 (MyD88) in combination with the MyD88 adapter-like (Mal)/TIR
domain-containing protein (TIRAP) to recruit IL-1R-associated kinases (IRAKs), ultimately
causing activation of the transcription factor NFκB and MAPKs inducing the production of
proinflammatory cytokines [3,24,169]. The MyD88-independent pathway is mediated by TIR

15
To activate TLR4 signaling, LPS is extracted from bacteria by LPS binding protein (LBP) and transferred to CD14, a membrane bound accessory protein that dimerizes upon LPS-LBP binding. CD14 transfers LPS to the TLR4/MD-2 receptor complex, initiating dimerization of these complexes. Two main intracellular signaling cascades are stimulated by TLR4 activation, both dependent on the intracellular TIR domain. Signaling via MyD88 and TIRAP leads to activation of the transcription factor NFκB and MAPKs, enhancing production of proinflammatory cytokines. The MyD88-independent pathway results in the production of IFN-β and increased proinflammatory cytokine production. Together, LPS-induced TLR4 signaling causes gliosis and enhanced production of pronociceptive chemicals that may induce hyperalgesia.
domain-containing adapter inducing IFN-β (TRIF)/TIR domain-containing adapter molecule-(TICAM)-1 and TRIF-related adapter molecule (TRAM)/TICAM-2; activation of this pathway results in the production of IFN-β in addition to proinflammatory cytokines [169,285]. The biological difference between the MyD88-dependent and independent pathways is not fully understood, however, a temporal correlation has been suggested with the former pathway responsible for the early phase of the inflammatory response and the latter involved in the late phase [123,151]. Thus, LPS-induced activation of TLR4 triggers profound gliosis and is capable of enhancing nociceptive transmission.

1.3.4.2 TLR4 and Opioids

Recent reports indicate opioid agonists and antagonists are also agonists and antagonists of TLR4, respectively [100,101,104,278]. Unlike ORs that are stereoselective for levo (-) opioid isomers [110], TLR4 exhibit no stereoselectivity responding to levo (-) and dextro (-) opioid isomers [101,104,278]. Hutchinson and colleagues [101,104] report (+) and (-)naloxone dose-dependently blocked LPS-induced TLR4 activation in human embryonic kidney cells transfected with human TLR4 and in a microglial cell line, as measured by secreted alkaline phosphatase (SEAP) production. These data are consistent with studies in which (-)naloxone attenuated the reduction in morphine analgesia observed following LPS pretreatment in rats [119,120]. Others report (+) and (-)naloxone blocked the reduction in morphine antinociception induced by LPS pretreatment in mice [280]. Also reported in mice was suppression of (-)morphine-induced antinociception by co-treatment with (+)morphine measured by the tail-flick test [279]. Likewise, Hutchinson et al. [105] found (+)morphine and (+)methadone induced significant hyperalgesia and allodynia, activation of spinal microglia and astrocytes, and increased production of proinflammatory cytokines and chemokines in rats. Collectively, these studies indicate a non-OR pathway, such as TLR4, may mediate opioid tolerance and hyperalgesia.
1.4 ULD Antagonists Attenuate Opioid Tolerance & Hyperalgesia

1.4.1 ULD Antagonists and the Bimodal Theory

The administration of sub-therapeutic (ultra-low dose; ULD) doses of opioid antagonists (e.g. naloxone, naltrexone) augmented opioid-induced analgesia and inhibited and/or reversed the development of tolerance and physical dependence [2,145,146,184,217,269,286]. The bimodal receptor model discussed above hypothesized analgesic tolerance following chronic opioid use is the consequence of enhanced stimulatory signaling via Gs-coupled μORs opposing the analgesic effect of Gi/o-coupled μORs [218,269]. Wang et al., [269] reported chronic morphine administration induced a switch in the Ga subunit coupled to μORs, from the normal Gi/o to the stimulatory Gs subunit demonstrated by co-immunoprecipitation experiments in the rat striatum and spinal cord. This data is in agreement with previous studies which have demonstrated that μORs normally couple to Gi/o [138] and that morphine exposure promotes Gs-coupling [29]. Co-administration of ULD naloxone (10ng/kg, s.c.) with morphine attenuated the morphine-induced μOR-Gs coupling and increased the Gi/o-coupling back to control levels [269]. Therefore, ultra-low dose antagonists may attenuate analgesic tolerance via modulation of G-protein coupling to μORs.

1.4.2 ULD Antagonists and Filamin A Hypothesis

More recently, Wang and colleagues [268] proposed that ultra-low dose naloxone attenuates opioid tolerance by preventing Gs-coupling to μORs via filamin A (FLNA). FLNA is a 300-kDa protein involved in cross-linking actin filaments into dynamic scaffolds [177]. It is involved in the maintenance of cytoskeleton integrity, cell motility and regulation of cell signaling [75,234]. FLNA binds to various membrane and signaling molecules [234] including GPCRs, such as D2 and D3 dopamine receptors [142–144]. FLNA also interacts with μORs, as discovered in a co-immunoprecipitation of μOR complexed with Ga proteins [177]. Interestingly, FLNA did not alter the binding affinity or function of μORs, but was required for agonist-induced
receptor downregulation, desensitization, and internalization [177]. A follow-up study investigated FLNA as a potential target of ULD opioid antagonists. A high-affinity binding site for naloxone was identified on FLNA, distinct from the μOR [268]. Co-treatment of rat striatal slices with a pentapeptide corresponding to the precise naloxone binding site identified on FLNA (FLNA<sub>2561-2570</sub>) blocked the ULD antagonist effects, such that the chronic morphine-induced G<sub>10</sub>-to-G<sub>s</sub> switch and downstream accumulation of cAMP was not attenuated by naloxone [268]. Wang and Burns [267] later reported that the G<sub>10</sub>-to-G<sub>s</sub> switch could be induced by acute morphine treatment without repeated exposure. This switch in G protein coupling was attenuated by co-treatment with ULD (-)-naloxone, (+)-naloxone, and (-)-naltrexone. The use of the OR-inactive (+)-naloxone stereoisomer confirmed that the ULD effect was mediated via a non-OR site of action. These studies were the first to identify an alternative high-affinity binding site which may be targeted by ultra-low doses of naloxone and naltrexone.

1.4.3 ULD Antagonists and Gliosis

Alternatively, ULD-antagonists may attenuate opioid tolerance and hyperalgesia via inhibition of opioid-induced spinal gliosis. As described, chronic opioid treatment activates microglia and astrocytes within the dorsal horn of the lumbar spinal cord evoking release of pronociceptive and proinflammatory molecules, including IL-1β, IL-6, TNF-α, and BDNF [49,134,194,195], which may counter opioid analgesia by enhancing nociceptive transmission. ULD opioid antagonists may block opioid-induced spinal gliosis directly via antagonism of glial receptors (e.g. ORs or TLR4), or through an alternate mechanism. Tsai et al., [251] reported ULD naloxone (15ng), but not high dose naloxone (15μg), inhibited activation of spinal microglia induced by PTX treatment. Moreover, ULD naloxone inhibited the increase in mRNA expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α); these cytokines co-localized with microglial CD11b in spinal sections [251]. Others report naloxone (1μM) non-stereoselectively inhibited LPS-induced microglial activation in rat mesenphalic neuron-microglia cultures and
blocked the release of proinflammatory IL-1β and TNF-α [148]. In vivo, (-) and (+)naloxone (1mg/day infused s.c.) blocked LPS-induced microgliosis in the rat substantia nigra [149]. Recent studies indicate ULD naloxone inhibited chronic morphine-induced expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and suppressed activation of spinal astrocytes and microglia, indicated by attenuation of GFAP and OX42 staining compared to morphine-only treated controls [145,146]. Taken together, these studies demonstrate ULD opioid antagonists inhibit spinal gliosis and indicate a potential mechanism by which these agents attenuate tolerance and hyperalgesia.

1.5 Research Statement and Objectives

The use of opioid analgesics for extended durations to relieve chronic pain is often limited by the development of analgesic tolerance and hyperalgesia. Concurrent administration of an ultra-low dose of opioid antagonist attenuates and/or reverses analgesic tolerance; however the mechanism by which this phenomenon occurs is unclear. The following thesis will address the GENERAL HYPOTHESIS that ultra-low dose opioid antagonists attenuate analgesic tolerance and opioid-induced hyperalgesia via inhibition of spinal gliosis.

Modulation of synaptic neurotransmission by glia may enhance nociceptive signaling via the release of proinflammatory cytokines. In addition to bacterial infection, nerve injury and chronic opioids, the implantation of spinal catheters are also associated with the induction of spinal gliosis [64]. Intrathecal catheters are extensively employed to study the spinal effects of opioids and other analgesics, therefore, OBJECTIVE 1 aimed to determine the impact of intrathecal catheter-induced spinal gliosis in the modulation of opioid analgesia and tolerance. Evaluation of spinal gliosis on the analgesic efficacy of opioids is relevant to the interpretation of pain literature and has important clinical implications as intrathecal catheters are used for the direct and repeated administration of opioid analgesics to patients suffering severe or chronic pain.
Activated spinal glia are implicated in the development of opioid-induced tolerance and hyperalgesia. In preclinical studies, glial inhibitors have prevented the onset of tolerance and the increased nociceptive signaling following chronic opioid administration in a manner similar to ultra-low dose opioid antagonists. Thus, OBJECTIVE 2 aimed to determine if ultra-low doses of the opioid antagonist naltrexone blocked the activation of spinal glia by repeated opioid administration.

The ability of ultra-low dose opioid antagonists to prevent or reverse analgesic tolerance to chronic opioids is intriguing. The sub-therapeutic doses of antagonist required to elicit these effects and the potentiation rather than inhibition of opioid analgesia indicates an alternative mechanism of action than simple antagonism of classical \( G_\text{i/o} \)-coupled \( \mu \)ORs. The recent discovery that opioid agonists and antagonists are ligands of TLR4 and the presence of TLR4 on spinal glia provides an alternate potential mechanism by which ultra-low doses of opioid antagonist may block tolerance and hyperalgesia. Therefore, OBJECTIVE 3 aimed to identify the receptor pathway targeted by opioid antagonists to modulate analgesic tolerance and opioid-induced hyperalgesia. The use of naloxone enantiomers and genetically modified mice lacking functional TLR4 will be used to determine the role of ORs and/or TLR4 in the modulation of opioid analgesia by ultra-low dose antagonists.

Together, these studies aim to identify the mechanism by which ultra-low dose opioid antagonists modulate opioid analgesia and the involvement of spinal glia in tolerance and hyperalgesia. Evaluation of the functional role of opioid-induced spinal gliosis in these phenomena will allow for a better understanding of how these cells modulate synaptic transmission of nociceptive neurons. Importantly, these studies will further our current understanding of opioid signaling and may identify novel drug targets for the treatment of chronic pain disorders.
CHAPTER 2:

INTRATHECAL CATHETERIZATION INFLUENCES TOLERANCE TO CHRONIC MORPHINE IN RATS

This work has been published in Anesthesia & Analgesia:

2.1 INTRODUCTION

Intrathecal catheters have been extensively used to investigate the spinal effects of opioid-induced analgesia and mechanisms of opioid tolerance [59,120,137,158,164,184]. Most rodent studies used polyethylene (PE) tubing for chronically indwelling spinal catheters. DeLeo et al.[64] demonstrated that this intrathecal catheterization induced spinal gliosis. Recent evidence demonstrated that immune cell activation is important in the etiology of morphine tolerance [161,193,196,228]. Thus, the effects of catheterization-induced gliosis on acute and chronic morphine tolerance were investigated.

2.2 METHODS

Experiments, complying with Canadian Council on Animal Care policies, were approved by the Queen’s University Animal Care Committee. Male Sprague-Dawley rats (200–250 g) were housed individually with ad libitum access to food and water, and maintained on a reverse 12/12-hour light/dark cycle.

Indwelling intrathecal catheters (7.5 cm, PE10) were implanted as previously described [284]. A surgical sham involving skin incision and muscle dissection, without breakage of the arachnoid membrane to cause leakage of cerebral spinal fluid was deemed appropriate to control for any postoperative pain. All animals were allowed to recover from surgery for 5 days before testing.
Drugs were injected intrathecally via catheter [185] or lumbar puncture (LP) as previously described [161]. Treatments were administered via either catheters (10 μL) or LP (15 μL). The tail-flick test was used to evaluate thermal nociception (2- to 3-second baseline responses, cutoff time of 10 seconds). All behavioral testing was performed blind to drug treatments.

### 2.2.1 Immunohistochemistry

Spinal cords were isolated and lumbar segments transected (40 μm) after cardiac perfusion with 4% paraformaldehyde. Free-floating sections were incubated in blocking solution, followed by overnight incubation at 4°C with antisera recognizing glial fibrillary acidic protein (1:2500; DakoCytomation, Mississauga, ON, Canada) to label astrocytes. Sections were incubated with a secondary antibody conjugated to an Alexa 488 fluorophore.

Imaging of immunoreactive cells was performed as previously described [95]. Briefly, immunofluorescence was captured using the Leica TCS SP2 multi-photon confocal microscope (Leica Microsystems, Buffalo Grove, IL). Serial images were captured at 100x magnification, at 0.75 μm increments throughout the z plane (4 series per section, 3 sections per animal). Images were stacked and reconstructed in 3 dimension using ImagePro Plus v5.0 software (MediaCybernetics, Bethesda, MD). Total cell volume was calculated for each cell. All data were collected by an experimenter blind to drug treatments.

### 2.2.2 Effect of Intrathecal Catheterization on Acute and Chronic Morphine Antinociception

Antinociception

Animals were divided into 3 groups: catheterized, sham-operated, or surgery-naive. After recovery, animals were injected intrathecally with a 50% effective dose of morphine (0.25μg) (Sabex, Canada) via LP. Nociceptive responses were measured before and after injection for 2 hours. Animals received morphine (1 μg) via LP on days 2 to 5, every 24 hours. On day 6, nociceptive responses were measured prior to morphine injection (0.5μg) and 30 minutes after drug injection.
2.2.3 Catheter- and Morphine-Induced Astrogliosis

Animals were divided into 2 groups: catheterized or surgery-naive. After recovery, rats were further divided into 4 groups receiving either vehicle (saline) or morphine (15 μg) via catheter or LP injection, once daily for 5 days. Spinal cords were collected on day 6 (see below) and volumes of 3-dimensional astrocytes were measured to determine hypertrophy, an indicator of astrogliosis.

2.2.4 Statistical Analyses

Tail-flick latencies were converted to a maximum possible effect: (post-drug latency - baseline)/(cutoff latency - baseline) x 100. All data are expressed as mean ± SEM. Statistical significance was determined using a 1- or 2-way repeated-measures analysis of variance, followed by Tukey or Bonferroni post hoc multiple comparisons test to determine between group differences. Mean area under the curve was determined for data presented in Figure 1A and analyzed for statistical significance by 1-way analysis of variance. P values < 0.05 were considered significant.

2.3 RESULTS

Catheterization or sham surgery did not affect the duration or peak antinociceptive effect elicited by a single morphine (0.25 μg) injection compared with nonsurgical rats (Fig.2.1A). The mean antinociceptive effects of intrathecal morphine (0.5 μg) injected via LP on day 6, in catheterized, sham-operated, or surgery-naive rats are represented in Figure 1B. Significantly greater analgesia was observed in sham-operated and surgery-naive rats compared with catheterized rats. No difference in antinociception was observed between sham-operated and surgery-naive animals.
Figure 2.1 Intrathecal catheterization modulates chronic but not acute morphine effects. (A) Catheterized, sham-operated, or surgery-naïve animals received intrathecal morphine (0.25 µg) via lumbar puncture. Nociception was measured before and for 2h following drug injection. No significant difference was observed between groups. A two-way repeated measures analysis of variance (ANOVA) revealed a significant effect of time ($F_{2,189} = 18.89$ p<0.0001), but not of treatment ($F_{2,189} = 0.271$ p=0.764). No significant difference between treatment groups was found using a 1-way ANOVA of the mean areas under the curve ($F_{2,25} = 1.024$, p=0.3739). The mean percentage of maximum possible effect (% MPE) at 30 minutes after morphine is presented on the right. A one way ANOVA revealed no significance between treatments ($F_{2,27} = 1.063$, p=0.359). (B) After chronic administration of intrathecal morphine (1 µg daily via lumbar puncture for 4 days), animals received intrathecal morphine (0.5 µg) via lumbar puncture. Nociception was measured before and 30 min after drug injection. Data are presented as mean ± SEM. A 1-way ANOVA revealed significance between treatments ($F_{2,27} = 6.186$, p<0.01). *Significant difference from catheterized rats; * = p < 0.05, ** = p < 0.01.
Figure 2.2 Intrathecal catheterization induced astrogliosis comparable to that induced by chronic morphine via lumbar puncture. (A) Representative 3-dimensional images of astrocytes from rats receiving chronic intrathecal saline (SAL) or morphine (15 µg; MS) via catheter or lumbar puncture once daily for 5 days. (B) Astrocyte cell volumes were measured. Data are presented as mean ± SEM. A 1-way analysis of variance revealed significance between treatments ($F_{3,433} = 49.96$, $p<0.001$). *Significant difference from rats administered saline by LP; *** = $p < 0.001$. #Significant difference from rats administered morphine by catheter; ##$p<0.01$, ###$p<0.001$
Mean volumes of astrocytes from rats treated with chronic vehicle (saline) or morphine (15 μg) via catheter or LP delivery are represented in Figure 2.2. Astrocytes reconstructed from animals administered saline via catheter showed significant hypertrophy, indicated by increased cell volume, compared with those receiving saline via LP. Astrocytes from animals receiving chronic morphine via LP were significantly larger compared with the LP saline group \((P < 0.001)\), but were not different from those receiving chronic saline via catheter \((P > 0.05)\). Astrocytes from animals receiving chronic morphine via catheter were significantly larger than those from animals receiving morphine via LP \((P < 0.001)\) or those treated with saline via catheter \((P < 0.01)\).

### 2.4 CONCLUSIONS

In previous reports, the onset of analgesic tolerance was more rapid in rats administered chronic morphine via intrathecal catheter [184] compared with those receiving morphine via LP [161]. In this study, we demonstrated that catheterization had no effect on acute morphine antinociception but augmented the analgesic tolerance to chronic morphine compared with sham or surgery-naive controls. A submaximal morphine dose was selected that would permit the detection of subtle differences in analgesic responses between groups. Similarly, Wu et al.[281] reported no difference in antinociception between catheterized and non-catheterized mice to acute morphine administration. Interestingly, Prado [189] reported decreased morphine potency in rats with intrathecal catheters inserted by the Yaksh and Rudy method [284] compared with those acutely catheterized via LP. A number of methodological differences may contribute to the differing results, including difference in rat strain, differences in protocols of tail-flick nociceptive testing (heated coil versus radiant light), and differences in route of morphine administration (via acute or indwelling catheter versus LP). Almeida et al. [5] demonstrated hyperalgesia after spinal sensitization by the administration of noxious treatments in rats with indwelling catheters. Therefore, prior sensitization, such as chronic morphine exposure, may be required to observe the effects of catheterization on changes in morphine-induced nociception.
Others have reported decreased antinociceptive potency of somatostatin [66] and dynorphin A [92,152] after catheterization surgery. The induction of spinal gliosis by intrathecal catheterization, in combination with that induced by repeated morphine, may oppose the analgesic effects, thereby contributing to the opioid-tolerant state. Our data suggest that catheter-induced inflammation does not alter sensitivity to acute morphine, but enhances the development of tolerance. Astrocyte volumes in morphine-tolerant animals receiving treatment via LP were comparable to those of opioid-naive, catheterized animals. Therefore, the gliosis observed in catheterized and morphine-tolerant animals, despite being comparable in magnitude, may be induced by independent mechanisms. When reviewing the literature in which treatments are administered via intrathecal catheters, potential implications should be considered: catheterization can induce significant morphologic changes in spinal processes [206], and thus alter nociceptive responses [5], drug potency, and the onset or the extent of tolerance to repeated drug administration [189,281].
CHAPTER 3:

Ultra-low dose naltrexone attenuates chronic morphine-induced gliosis in rats.

This work has been published in Molecular Pain:


3.1 INTRODUCTION

Opioid drugs, such as morphine, are widely used for the management of moderate to severe pain. Unfortunately, the usefulness of morphine and other opioid analgesics in the management of pain is limited due to the development of tolerance to the analgesic effects of these drugs with repeated exposure [51]. Clinically, the onset of tolerance necessitates increasing doses of opioids, which in turn typically increases the number and severity of adverse effects and compliance [13].

Morphine acts to inhibit nociception predominately via $G_{io}$ protein-coupled μ-opioid receptors [133,160] located in nociceptive pathways throughout the central nervous system including the dorsal spinal cord. Within the spinal cord, μ-opioid receptors are well recognized to localize on pre- and post-synaptic nociceptive neurons, but they are also present on astrocytes and microglia [35,67,72,73,205,233], however the function of μ-opioid receptors on glial cells remains elusive.

A number of factors appear to contribute to the development of analgesic tolerance. In general, the development of tolerance is thought to involve cellular adaptation/modulation that results in decreased analgesic potency. The precise mechanism(s) of action is not known;
however, investigators have been able to attenuate or reverse established analgesic tolerance to morphine by inhibiting either the release of neurotransmitters and/or inhibition of their receptors [158,166,186,187,191,250]. Within the last decade, activation of spinal glia has emerged as a novel mechanism underlying analgesic tolerance [193,196,228]. Relevant to the current study, the administration of sub-therapeutic (ultra-low) doses of opioid specific antagonists (e.g. naloxone, naltrexone) augmented opioid-induced analgesia and inhibited and/or reversed the development of tolerance and physical dependence [184]. Although this relationship was studied intensively in various in vitro and in vivo models [184,217,269], only recently have clinical trials been undertaken to investigate the improved therapeutic benefit of combining opioid analgesics with ultra-low dose opioid receptor antagonists. To date, clinical trials have confirmed that combinations of opioids and ultra-low dose antagonists both enhance and prolong opioid-induced analgesia, and prevent analgesic tolerance and physical dependence [38,275]. Precisely how ultra-low dose antagonists prevent/reverse tolerance to opioid analgesics is not fully understood, but spinal glia may play a crucial role. We demonstrate that one contributing mechanism is that ultra-low dose naltrexone blocks opioid-induced activation of spinal glial cells.

3.2 METHODS

3.2.1 Animals

Adult male Sprague-Dawley rats (180-200 g; Charles River, Québec, Canada), were housed in groups of two with ad libitum access to food and water, and maintained on a reverse 12/12 h light/dark cycle. All behavioural experiments were performed during the dark phase of the cycle, and animals were handled prior to experimentation in order to reduce stress-related analgesia. All experimental protocols were approved by the Queen's University Animal Care Committee, and complied with the policies and directives of the Canadian Council on Animal Care and the International Association for the Study of Pain.
3.2.2 Drug treatments

Morphine was purchased from Sabex, Kingston General Hospital, Kingston, Ontario, Canada. Naltrexone and 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma (St. Louis, MO, USA). Animals were separated into one of five groups receiving i) morphine (15 μg; n = 18), ii) morphine and naltrexone (5 ng; n = 19), iii) morphine and naltrexone (0.05 ng; n = 3), iv) naltrexone (5 ng) alone (n = 8), or v) saline (n = 15). Intrathecal (i.t.) administration of all drugs (diluted in saline to 30 μl volume) was accomplished by way of lumbar puncture between the L4 and L5 vertebrae under brief isoflurane anesthesia. Successful drug placement was confirmed by a vigorous tail flick upon injection.

To determine if chronic morphine treatment induced cell proliferation, animals received 5-bromo-2-deoxyuridine (BrdU, 100 mg/kg; prepared in a concentration of 25 mg/ml in 0.007 N NaOH and saline), injected intraperitoneally (i.p.) on days 1, 3, and 5. Animals were separated into two groups receiving intrathecal morphine (15 μg/15μl; n = 3) or saline (15 μl; n = 3) by lumbar puncture under brief isoflurane anaesthesia for 5 days. Saline or morphine was injected 30 minutes after BrdU injections.

3.2.3 Behavioural tail flick assay

The effects of drug administration on thermal nociceptive responses were assessed on Days 1, 3 and 5 of the study using the tail flick assay. In brief, a beam of radiant light was applied to a spot marked 5 cm from the tip of the tail, and the latency to a vigorous tail flick was measured. Three baseline latencies were measured prior to drug injection to determine the normal nociceptive responses of the animals. A cut-off time of three times the animal's average baseline was imposed to avoid tissue damage in the event that the animal became unresponsive following drug injection. Rats were then injected intrathecally with their respective treatments, and the thermal latency measured at 30 minutes post-injection, as previous studies have found that the peak antinociceptive effects of morphine occur at this time point [86]. Tail-flick values were
converted to a maximum possible effect (% MPE): \((\text{post-drug latency} - \text{baseline}) ÷ (\text{cut-off latency} - \text{baseline}) \times 100\). Statistical analyses were performed using a two-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc multiple comparisons test to determine between group differences. P values less than 0.05 were considered significant. All behavioural testing was performed by the experimenter blind to drug treatment.

3.2.4 Immunohistochemistry

On day 6, 24 h after the last injection, rats \((n = 3-6 \text{ per drug treatment})\) were deeply anesthetized with sodium pentobarbital \((75 \text{ mg/kg, i.p.; MTC Pharmaceuticals, Cambridge, Ontario, Canada})\) and transaortically perfused with 4% paraformaldehyde \((\text{PFA})\) in 0.1 M phosphate buffer \((\text{PB}; 500 \text{ ml, pH 7.4})\). The spinal cords were removed by spinal ejection and post-fixed in the above fixative for 1 hour on ice and cryoprotected in 30% sucrose in 0.1 M PB for 48 hours at 4˚C. Lumbar segments were isolated and cut into 40 μm transverse sections on a freezing sledge microtome and collected in 0.1 M Tris buffered saline \((\text{TBS; pH 7.4})\).

Free-floating sections were incubated in a blocking solution containing 5% NGS in TBS-T \((\text{TBS and 0.2% Triton X-100})\), followed by incubation with a rabbit polyclonal antisera recognizing glial fibrillary acidic protein \((\text{GFAP; 1:2500 working dilution; DakoCytomation, Ontario, Canada})\) to label astrocytes and a mouse monoclonal antisera recognizing OX42 \((1:1000 \text{ working dilution; Serotec, NC, USA})\) to label CD3/CD11b receptors on microglia. Spinal cord sections were incubated overnight at 4˚C with both primary antibodies, followed by incubation with goat anti-rabbit and goat anti-mouse secondary antibodies \((1:200 \text{ working dilution; Molecular Probes, Invitrogen, Ontario, Canada})\) conjugated to Alexa 488 and Alexa 594 fluorophores, respectively. To assess nonspecific labelling, control sections were processed in the absence of primary antibody. Sections were mounted on glass slides, air-dried and cover-slipped using Aquamount \((\text{Fisher Scientific, Ontario, Canada})\).
BrdU immunolabelling was performed as described by Suter et al [236]. Briefly, spinal cord sections were heated in solution containing 50% formamide, 50% 2x saline sodium citrate (SSC) for 2 h at 65°C. Sections were further incubated at 37°C for 30 min in 2N HCL then placed in 0.1 M borate buffer (pH 8.5). Sections were incubated in blocking solution, followed by incubation with a mouse monoclonal antibody against BrdU (1:500, Chemicon, Temecula, CA). To identify the phenotype of newly formed cells, sections were double labelled with one of the following antibodies: rabbit anti-GFAP (for astrocytes, 1:2500), rabbit anti-Iba1 polyclonal antibody (ionizing calcium-binding adaptor molecule, for microglia and macrophages, 1:1000; Wako, Richmond, VA), or rabbit anti-MAP-2 polyclonal antibody (microtubule-associated protein-2, for neurons, 1:1000; Chemicon, Temecula, CA). Sections were then incubated with goat anti-rabbit and goat anti-mouse secondary antibodies (1:200) conjugated to Alexa 488 and Alexa 594 fluorophores, respectively, and mounted as described above.

Imaging of immunoreactive cells was performed as previously described [95]. In brief, immunoreactive cells were imaged using the Leica TCS SP2 multi photon confocal microscope (Leica Microsystems Inc, Ontario, Canada). Images were taken within the dorsal horn (lamina III-V) at 63x magnification for quantification of intensity. Serial images (twenty-five to thirty-five) were captured at 100x magnification, at 0.75 μm increments throughout the z plane in the deep and superficial dorsal horn (4series per section, 3 sections per animal). For quantification of the intensity of antibody labelling, images were converted to gray scale using Adobe Photoshop 7.0. Using Image J (NIH), the mean gray values were measured and the average within each treatment group calculated and expressed as mean ± s.e.m. For quantification of GFAP, OX42 and BrdU-positive cells, immunolabelled cell bodies were counted for each section (150 μm × 150 μm) and the average within each treatment group calculated and expressed as mean ± s.e.m. To quantify astrocyte volume, images taken at 100x magnification were stacked and reconstructed in three-dimensions using ImagePro Plus v5.0 software (MediaCybernetics, MD, USA). Total cell volume
was calculated for each reconstructed cell. The average volume for cells within each treatment group was calculated and expressed as mean ± s.e.m. Mean intensities and 3D volumes were analyzed by one-way ANOVA followed by Tukey's posthoc multiple comparison test. Differences in cell numbers were analyzed by unpaired T-tests. P values less than 0.05 were considered significant. All quantification data was collected by experimenter blind to drug treatment.

3.3 RESULTS

3.3.1 Ultra-low dose naltrexone attenuated the development of tolerance to morphine antinociception

Animals chronically administered intrathecal morphine (15 μg; MS) by lumbar puncture displayed a loss in antinociception on day 5 (49.1% maximum possible effect; MPE) as compared to day 1 treatment (100% MPE; Figure 3.1). Attenuation of the loss in antinociception was observed in animals that were co-administered ultra-low dose naltrexone (5 ng; NTX) with morphine, maintaining 72.7% of the maximum possible antinociceptive effect (Figure 3.1). This effect is dose dependent, as animals co-administered 0.05 ng naltrexone with morphine showed a loss in antinociception similar to animals treated with morphine only, maintaining 41.6% of the maximum possible antinociceptive effect on day 5. Control animals administered vehicle (saline) or ultra-low dose naltrexone alone did not produce changes in thermal nociceptive thresholds compared to baseline values (Figure 3.1).

3.3.2 Ultra-low dose naltrexone attenuated morphine-induced increases in the expression of glial fibrillary acidic protein (GFAP) and CD3/CD11B (OX42)

Spinal cords collected from animals administered vehicle (saline), morphine (15 μg), combined morphine and ultra-low dose naltrexone (5 ng), or naltrexone alone were processed for immunohistochemical labelling. Representative images (Figure 3.2A.i-iv) illustrate the increase in GFAP labelling observed in animals chronically administered morphine alone (Figure 3.2A.ii)
Figure 3.1 Co-administration of ultra-low dose naltrexone (5 ng; NTX) with morphine (15 µg; MS) attenuated the loss in antinociception produced by morphine treatment alone in rats. The lower dose, 0.05 ng NTX did not attenuate the loss in antinociception produced by chronic morphine. Ultra-low dose naltrexone alone did not produce significant antinociception as compared to vehicle (saline) treated controls. A two-way repeated measures analysis of variance (ANOVA) revealed a significant effect of time ($F_{5,63}=21.83$, $p<0.001$) and treatment ($F_{5,63}=149.8$, $p<0.001$). The asterisk denotes a significant difference from morphine-treated rats. ** = $p < 0.01$, *** = $p < 0.001$. 
Figure 3.2 Intensity of astrocyte labelling in the dorsal lumbar spinal cord of rats. (A) Representative photomicrographs acquired by confocal microscopy of spinal cord sections labelled for the astrocytic protein, glial fibrillary acidic protein (GFAP). Spinal cord sections were collected from rats receiving intrathecal vehicle (saline; SAL) (i), morphine (15 μg; MS) (ii), morphine and naltrexone (5 ng; MS+NTX) (iii), or naltrexone (NTX) alone (iv). Photomicrographs were converted to gray scale and then analyzed to obtain mean gray values. A
1-way analysis of variance (ANOVA) revealed significance between treatments (F_{3,152}=18.40, p<0.001). Morphine treatment produced a significant increase in the amount of GFAP labelling as compared with saline-treated control. Attenuation of increased GFAP immuno-labelling was observed in animals co-administered ultra-low dose naltrexone with morphine (### = p < 0.001 compared to morphine treatment). Naltrexone alone had no significant effect on GFAP immuno-labelling compared to saline control (p > 0.05). Data represent means ± s.e.m. for n = 6-8 sections per rat from n = 5-6 per group. The asterisk denotes significant difference from saline-treated rats, *** = p < 0.001.
as compared to all other treatments. Quantification of GFAP labelling intensity (Figure 3.2B) revealed a significant increase in GFAP labelling in morphine only treated spinal cord sections as compared to saline controls. Co-administration of ultra-low dose naltrexone in combination with morphine did not produce a significant increase in GFAP expression as compared to saline-treated and naltrexone-treated controls (P > 0.05). No difference in GFAP expression was observed between saline-treated and naltrexone-treated sections. Similarly, increased CD3/CD11b expression was observed in morphine-treated animals compared to controls (Figure 3.3B), which was attenuated by co-administration of ultra-low dose naltrexone. Therefore, ultra-low dose naltrexone significantly attenuated the increase in GFAP and CD3/CD11b expression induced by chronic morphine administration.

### 3.3.3 Ultra-low dose naltrexone attenuated morphine-induced astrocyte hypertrophy

Astrocyte cells were reconstructed in three dimensions from spinal cord sections obtained from rats chronically administered drug treatments. Representative images of astrocytes reconstructed from morphine-treated animals (Figure 3.4A i-iv) demonstrate hypertrophy characteristic of astrogliosis. Measurement of astrocyte cell volume confirmed that chronic morphine treatment produced significantly larger volumes compared to saline-treated controls (Figure 3.4B). Co-administration of ultra-low dose naltrexone with morphine attenuated this increase in cell volume (p < 0.001 compared to morphine-treated), however, astrocytes were still significantly larger volumes than saline-treated controls but did not differ from naltrexone only controls. Moreover, naltrexone treatment alone did not significantly affect astrocyte volume compared to saline-treated controls. Thus, co-administration of ultra-low dose naltrexone significantly attenuated morphine-induced hypertrophy of astrocytes.

### 3.3.4 Chronic morphine does not induce cell proliferation

The number of GFAP and OX42-positive cells present in lumbar spinal cord sections from animals chronically administered intrathecal vehicle (saline) or morphine (15 μg) were
Figure 3.3 Intensity of microglial labelling in the dorsal lumbar spinal cord of rats. (A) Representative photomicrographs acquired by confocal microscopy of spinal cord sections labelled for the microglial marker, CD3/CD11b (OX42). Spinal cord sections were collected from rats receiving intrathecal vehicle (saline; SAL) (i), morphine (15 μg; MS) (ii), morphine and naltrexone (5 ng; MS+NTX) (iii), or naltrexone (NTX) alone (iv). Photomicrographs were
converted to gray scale and then analyzed to obtain mean gray values. A 1-way analysis of variance (ANOVA) revealed significance between treatments ($F_{3,155}=11.50$, $p<0.001$). Morphine treatment produced a significant increase in the amount of OX42 labelling as compared with saline control. Attenuation of increased OX42 immuno-labelling was observed in animals co-administered ultra-low dose naltrexone with morphine ($### = P < 0.001$ compared to morphine treatment). Naltrexone alone had no significant effect on OX42 immuno-labelling compared to saline control ($P > 0.05$). Data represent means ± s.e.m. for $n = 6-8$ sections per rat from $n = 3-6$ per group. Statistical analyses were performed by a one-way ANOVA followed by Tukey’s *post-hoc* multiple comparison test. The asterisk denotes significant difference from saline-treated rats, * = $P < 0.05$. 
Figure 3.4 Ultra-low dose naltrexone attenuates morphine-induced astrocyte hypertrophy.

(A) Representative three dimensional images of astrocytes from rats receiving intrathecal vehicle (saline, SAL) (i), morphine (15 μg, MS) (ii), morphine and naltrexone (5 ng, MS+NTX) (iii), or naltrexone (NTX) alone (iv). A 1-way analysis of variance (ANOVA) revealed significance between treatments ($F_{3,14}=46.09$, $p<0.001$). Morphine treatment produced a significant increase in astrocytic cell volume as compared with saline-treated and naltrexone-treated controls. Co-administration of ultra-low dose naltrexone with morphine attenuated this hypertrophy; astrocytes...
have significantly smaller volumes as compared to morphine only treatment (### = p < 0.001). Data represent means ± s.e.m. for n = 12-24 cells per rat from n = 5 per group. The asterisk denotes significant difference from saline-treated rats. * = p < 0.05, ** = p < 0.01. Scale bar, 30 μm.
Figure 3.5 The morphine-induced increase in astrocyte and microglial immuno-labelling is caused by cell hypertrophy, not proliferation. Lumbar spinal cord sections were collected from animals administered BrdU (100 mg/kg) by intraperitoneal injection on days 1, 3, 5, and either intrathecal vehicle (saline; SAL) or morphine (15 μg; MS) once daily for five days by lumbar puncture. Representative photomicrographs acquired by confocal microscopy of spinal cord sections double labelled with 5-bromo-2-deoxyuridine (BrdU) and the astrocytic protein GFAP (B, C), the microglial marker Iba1 (E, F) or the neuronal marker MAP2 (H, I). No co-localization
of BrdU-positive cells with GFAP or MAP-2-positive cells was observed. However, BrdU co-localized with a small number of Iba1 positive cells (arrow), suggesting a small portion of the newly formed cells were microglia or macrophages. (J). No difference in the number of BrdU-positive cells in the dorsal horn (lamina II-IV) of lumbar spinal cord sections from animals administered chronic intrathecal saline or morphine was revealed by unpaired t-test ($t=0.58$, $p=0.56$). Data represent means ± s.e.m. for $n = 6$ sections per rat from $n = 3$ per group. ns = no significance. Scale bars, 30 μm.
### Table 3.1: Glial cell counts.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Saline</th>
<th>Morphine</th>
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<tr>
<td>Astrocyte (GFAP)</td>
<td>6.8 ± 0.50</td>
<td>8.3 ± 0.56</td>
</tr>
<tr>
<td>Microglia (OX42)</td>
<td>8.1 ± 0.91</td>
<td>8.9 ± 0.50</td>
</tr>
</tbody>
</table>

The number of astrocyte (GFAP-positive) or microglia (OX42-positive) cell bodies in the dorsal horn of spinal cord sections collected from rats receiving intrathecal morphine (15 μg, MS), or vehicle (saline). No significant difference was observed between treatment groups by unpaired *t*-test for astrocytes (*t*=1.93, *p*=0.058) or microglia (*t*=0.84, *p*=0.40). Data represent means ± s.e.m. for *n* = 6-8 sections (150 μm × 150 μm) per rat from *n* = 4-6 per group.
counted (Table 3.1). The number of GFAP-positive (astrocytes) and OX42-positive (microglia) cell bodies observed in spinal cord sections from morphine-treated rats was not significantly greater than the number in spinal cord sections from saline-treated controls. To confirm this finding, cell proliferation was assessed via 5-bromo-deoxyuridine (100 mg/kg, i.p; BrdU) experiments. BrdU was injected on alternative days 30 minutes prior to intrathecal administration of saline or morphine (15 μg) for 5 days. Immunohistochemical labelling of spinal cord sections collected from these animals revealed no significant increase in the number of BrdU-positive cells in morphine-treated animals compared to saline controls (Figure 3.5J). Double labelling of sections with the astrocytic marker GFAP (Figure 3.5A-C), or the neuronal marker MAP-2 (Figure 3.5G-I) revealed no co-localization with BrdU-positive cells. Iba1, the microglial and macrophage marker, co-localized with a portion of the BrdU-positive cells (Figure 3.5D-F). The results of the BrdU experiments confirm the cell counts of astrocytes and microglia, demonstrating that the chronic morphine treatment employed in this study does not induce spinal cord cell proliferation.

3.4 DISCUSSION

The current study has provided additional evidence that ultra-low dose naltrexone attenuates the development of tolerance to the antinociceptive effects of morphine as previously demonstrated by Powell et al [184]. As the mechanism by which this phenomenon occurs is unknown, this study sought to investigate the contribution of glia in the actions of ultra-low dose opioid antagonists.

Intrathecal catheterization has been shown to induce gliosis [64], therefore the current study used lumbar puncture drug delivery to reproduce the original behavioural findings of Powell et al [184]. Preliminary experiments investigated the effects of different ultra-low doses of naltrexone on morphine tolerance. In this study, the dose of naltrexone (0.05 ng) used in experiments by Powell et al [184] did not attenuate the loss in antinociception observed with
chronic morphine administration. However, a hundred-fold greater dose (5 ng) preserved the analgesic effects of morphine throughout the treatment period, and thus was used to determine the effects on morphine-induced gliosis. In addition to the use of intrathecal catheters for drug delivery, another important difference in experimental protocol in the present study were the housing conditions; animals used in this study were housed in a room on a reverse light-dark cycle (lights off at 7:00 am), with all behavioural testing conducted during the animals’ active (dark) phase. It is well accepted that pain responsiveness and endogenous opioids have circadian fluctuations in rats [135] and that morphine-induced antinociception is greater during the active phase compared to during the inactive light phase. These fluctuations may account for the greater dose of opioid antagonist required to attenuate tolerance in the present study compared to what has been previously published.

Current research has demonstrated that spinal glia are not merely support cells within the CNS as previously hypothesized (i.e. responsible for the maintenance of neurons and CNS homeostasis); they also actively communicate with neurons, are involved in the modulation of synaptic signaling and may be involved in the development of opioid tolerance. Chronic, but not acute, morphine administration, induces gliosis characterized by cell hypertrophy, and is associated with increased expression of GFAP [173,175,194,228,243] in astrocytes and CD3/CD11b (OX42) in microglia [59]. Reactive glial cells (microglia and astrocytes) can release a variety of pro-nociceptive and neuroexcitatory substances (e.g. prostaglandins, excitatory amino acids, interleukins, nitrogen oxide species, ATP, glutamate etc.), which may enhance pain transmission by nociceptive neurons [134,194,195,228,237].

This is the first report to demonstrate that co-administration of ultra-low dose naltrexone prevents morphine-induced gliosis, demonstrated by normalization of GFAP and CD3/CD11b expression and attenuation of increased astrocyte cell volume. The observed increases in GFAP/CD3/CD11b expression and astrocyte cell volume in spinal cord sections from animals
chronically administered intrathecal morphine are consistent with gliosis and are in agreement with previous findings of astrocyte and microglial activation by chronic morphine administration [173,194,228,243]. As no significant difference was found in the number of immuno-positive cells or in the number of newly generated cells between morphine treated and saline controls, glial proliferation likely contributes very little to the observed increases in GFAP and CD3/CD11b expression. This finding is in agreement with that of Song and Zhao [228], in which chronic morphine resulted in increased astrocyte immunoreactivity with no difference in the number of cells from saline-treated controls. In contrast, Narita et al [173] reported that astrocyte proliferation was induced by chronic morphine administration; however, no quantification of the number of GFAP-positive cells was reported. Agents that modify [228] or inhibit [196] activation of astrocytes and microglia prevent the development of morphine tolerance; thus inhibition of gliosis by ultra-low dose naltrexone may prevent the development of analgesic tolerance. This evidence, taken in concert with the findings of the current study, supports the hypothesis that spinal glia are involved in the development of morphine analgesic tolerance and in the mediation of nociception. It has also been reported that ultra-low dose naltrexone augments morphine antinociception in a model of pertussis toxin-induced hyperalgesia [251]. While the present study provides strong support for the role of glia in the ultra-low dose effect, various molecular studies indicate that ultra-low dose antagonists may prevent opioid receptor coupling to stimulatory G-proteins (G\textsubscript{s}). Classically, opioid activation of \mu-opioid receptors results in coupling to inhibitory G-protein subunits (G\textsubscript{i/o}) and produces analgesia; however, following chronic opioid administration, increased coupling of \mu-opioid receptors to G\textsubscript{s} has been observed [269]. Therefore, increased excitatory stimulation via G\textsubscript{s}-coupled \mu-opioid receptors may oppose the analgesic effects mediated via G\textsubscript{i/o} signaling, and manifest as tolerance [55,269]. Wang et al [269] demonstrated that the switch in G-protein coupling to \mu-opioid receptors induced by chronic morphine could be prevented by co-administering an ultra-low dose of naloxone, further
supporting this hypothesis. Despite these advances, the switch in G-protein coupling to \( \mu \)-opioid receptors induced by chronic morphine treatment has not been localized to a specific cell population within the spinal cord, and therefore, may occur in glia and/or nociceptive neurons. On the contrary, the effects of ultra-low dose antagonists may not be mediated by \( \mu \)-opioid receptors but through a novel mechanism such as an interaction with filamin A [268] or Toll-like receptors [100]. Thus, future studies will aim to identify the mechanism by which ultra-low dose naltrexone alters gliosis.

3.5 CONCLUSION

The results of this study may have a significant impact on the clinical management of moderate to severe pain. Patients currently treated with chronic opioid therapy may benefit not only from increased efficacy of combined opioid treatment [38,262], but may also experience fewer and less severe adverse effects [108,275], as sufficient analgesia can be achieved and maintained at lower opioid doses. Additionally, an understanding of the mechanism of action of opioid drugs will provide insight toward the development of more selective and efficacious pharmacological treatments for pain management. Not the least of which could be for improving treatment of chronic pain conditions such as neuropathic pain where glial activation is also evident, with reactive gliosis being a key contributor to the painful neuropathy [81,116,238,254]. Additionally, reduced opioid analgesic efficacy has also been reported in patients with neuropathic pain [7,274], however, co-administration of ultra-low dose antagonists with opioid agonists increased analgesic efficacy in animal models of neuropathic pain [137] and in clinical trials [38,275]. Future research will be required to determine if ultra-low dose naltrexone is able to alleviate established chronic pain.
CHAPTER 4:

MORPHINE HYPERALGESIA GATED THROUGH MICROGLIA-MEDIATED DISRUPTION OF NEURONAL CI HOMEOSTASIS

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4.1 INTRODUCTION

Morphine and other opiates are indispensable in the treatment of moderate-to-severe postoperative and chronic pain, but the use of these drugs is plagued by the development of two major problems: tolerance and hyperalgesia [12]. Tolerance is characterized by a progressive lack of response to morphine that can be overcome by increasing the dose, whereas hyperalgesia is a sensitization process in which opioids, paradoxically, cause pain hypersensitivity [139]. Commonly held views are that tolerance and hyperalgesia reflect a single underlying cellular and molecular mechanism [159,260].

The spinal dorsal horn (SDH) is a primary site of action for the analgesic effects of morphine and other opiates, and has been implicated in morphine-induced hyperalgesia (MIH) and tolerance [90,293]. In the SDH, nociceptive information is received from sensory fibers, processed and relayed to brain areas involved in mediating the sensory and emotional aspects of pain [52,276]. Nociceptive processing involves neuron-neuron and neuron-glia interactions through multiple facilitatory and inhibitory signaling cascades regulating the final output of the pain signaling networks. But the manner in which morphine acts on these networks in the SDH to produce hyperalgesia or tolerance remains enigmatic.

In the SDH, lamina I neurons comprise one of the principal output pathways to the brain [52,69,208]. These neurons are central targets for opioid analgesia [90], which inhibit their
activity. Conversely, increasing the output in this pathway has been implicated as a neuronal substrate underlying morphine tolerance and hyperalgesia [69,293]. Spinal nociceptive output is increased by enhanced excitation and diminished inhibition [208], and the latter has recently been implicated as a substrate of several chronic pain conditions [292]. Although morphine induces analgesia via inhibition in the SDH, we examined the seemingly counterintuitive concept that morphine may also induce disinhibition, the latter being the neuronal substrate for hyperalgesia and/or tolerance.

We found that morphine induced hyperalgesia via a P2X4R-BDNF-KCC2 disinhibition cascade between microglia and SDH neurons. Interfering with the principal nodes in the cascade suppressed hyperalgesia, but had no effect on tolerance. The disinhibition was a result of impaired Cl- extrusion in lamina I neurons. Pharmacological blockade of P2X4Rs reversed hyperalgesia and mice lacking these receptors did not develop hyperalgesia. Similarly, hyperalgesia was reversed by blocking BDNF-TrkB signaling and did not develop in mice lacking BDNF in microglia. Finally, restoring hyperpolarizing inhibition reversed morphine hyperalgesia. Thus, our findings describe a signaling pathway underlying MIH, opening avenues to specifically prevent and reverse this highly deleterious effect of morphine without affecting its analgesic action.

4.2. METHODS

4.2.1 Animals

Adult male mice and rats (>postnatal day 60, P60) were used. Rats and mice were housed under a 12-h:12-h light/dark cycle. All experimental procedures were performed in accordance with guidelines from the Canadian Council on Animal Care.

4.2.2 Chronic morphine protocol and behavioral models

Morphine sulfate (morphine, Sandoz) was injected twice a day (9 a.m. and 6 p.m.) into Sprague-Dawley rats (10 mg per kg), P2x4+/+ or P2x4−/− C57BL/6 mice ([223], INSERM;
escalating doses from 10 to 40 mg per kg), CD11b-cre; Bdnfloxp/loxP or Bdnfloxp/loxP mice (escalating doses from 10 to 40 mg per kg), C3H/HeOuJ mice (TLR4 wildtype), or C3H/HeJ (functionally deficient TLR4 mutant) mice (escalating doses from 10 to 40 mg per kg, Jackson Laboratory[183]). Morphine was injected subcutaneously, unless otherwise stated. Thermal pain threshold was measured by the Hargreaves plantar test before and 1 h after morphine injection. Morphine antinociceptive effects measured 1 h after injection in chronically treated rats are not explained by altered metabolism [273]. Mechanical pain threshold was measured before morphine injection by von Frey hairs, as described previously [50]. Values are normalized to the control. Vocalizations were monitored during subcutaneous injections and differences in the relative number of vocalizing rats (%) or in the number of vocalizations (in mice) were analyzed. Licking time was measured during thermal or mechanical stimulation of the hindpaw. Motor coordination was measured by accelerating rotarod (IITC Life Science) before and 30 or 60 min after morphine injections. In a subset of experiments, a morphine cumulative dose-response curve was measured on day 6 to determine morphine ED50 value. Briefly, mice were given ascending doses of morphine every 30 min and the response to morphine was assessed by the thermal tail-flick test until a maximal level of antinociception was reached. In all behavioral studies, experimenters were blind to the drug treatments and genetic profile of rats and mice.

4.2.3 Behavioral assessment of naloxone-precipitated withdrawal

Mice received intraperitoneally ascending doses of systemic morphine at 8-h intervals (day 1, 10 and 20 mg per kg; day 2, 25 and 30 mg per kg; day 3, 35 and 40 mg per kg; day 4, 45 and 50 mg per kg). On day 5, mice received a morning injection of 55 mg per kg and 2 h later naloxone (2 mg per kg) to precipitate withdrawal. Control mice received saline and were challenged with naloxone on day 5. Mice were acclimatized to a clear Plexiglass testing chamber 1 h before naloxone. Signs of withdrawal were compiled as previously described [249]. Briefly, jumping, headshakes, wet-dog shakes and grooming behavior were evaluated at 10-min intervals.
for a total testing period of 30 min and a standardized score of 0 to 3 was assigned (0 = absent; 1 = 1–3 bouts; 2 = 4–6 bouts; 3 = 7 bouts and greater). Paw tremors, piloerection, salivation and ejaculation were also evaluated, with one point being given to the presence of each sign during each 10-min interval. The number of periods showing the latter signs were then counted (maximum score of 3 per behavioral sign) and the scores were added together to yield a final cumulative withdrawal score. Mice were also weighed before and after naloxone challenge and weight loss (an indicator of micturition and defecation) was calculated.

4.2.4 Generation of CD11b-cre; BdnfloxP/loxP mice

Mice with microglial lineage–specific excision of BDNF were generated using the Cre-loxP system. C57BL/6J mice, heterozygous for CD11b-cre were purchased from the European Mutant Mouse Archive (provided by G. Kollias, Alexander Fleming Biomedical Research Center) [21]. 129S4/SvJae mice homozygous for loxP-flanked BDNF (Bdnf<sup>tm3Jae/J</sup>) were purchased from Jackson (stock 00439). These mice have loxP sites flanking exon 5 of the Bdnf gene [201]. Homozygous Bdnf<sup>tm3Jae/J</sup> mice were crossed with heterozygous CD11b-cre mice and Cre-expressing progeny backcrossed with homozygous Bdnf<sup>tm3Jae/J</sup> mice to ensure all experimental mice were homozygous for Bdnf<sup>tm3Jae/J</sup> and matched for background strains. Mice were genotyped by PCR analysis. CD11b-cre; Bdnf<sup>loxP/loxP</sup> mice exhibit normal nociceptive responses under control conditions.

4.2.5 Intrathecal injections

In a subset of experiments (where indicated), rats were subject to drug administration via intrathecal catheters. Rats were anaesthetized with 4% isoflurane (vol/vol) and a catheter was inserted into the intrathecal space as described [283]. Unless otherwise stated, intrathecal injections were delivered 30 min before subcutaneous morphine or saline injections. Rats were tested 20 min after intrathecal injection (immediately before morphine or saline injection) and 1 h later. At the end of the experiment, the correct placement of the catheter was verified. Drugs
included saporin (20 μg) and saporin-conjugated antibody to Mac-1 (16–32 μg, Advanced Targeting Systems), antibody to TrkB (30 μg, R&D Systems), ACTZ (22.5 μg, Sigma), TNP-ATP (2’,3’-O-(2,4,6-trinitrophenyl)adenosine 5’-triphosphate, 30 nmol, Tocris), naloxone (5 ng, Sigma) and (+)-naloxone (5 ng, C.J. Evans, National Institute on Drug Abuse). All drugs were dissolved in a HEPES-buffered ringer (pH 7.8).

### 4.2.6 Rat spinal cord slice preparation

Parasagittal slices (300 μm) of the rat spinal cord were prepared, as described [50]. Slices were allowed to recover for 1 h in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃ and 10 mM glucose.

### 4.2.7 Patch-clamp recordings from rat lamina I neurons

Voltage-clamp recordings were performed as described previously [50]. For whole-cell experiments involving measurements of $E_{GABA}$ under Cl⁻ load, the intrapipette solution contained 115 mM potassium methylsulfate, 25 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 4 mM Na-ATP, 0.4 mM Na-GTP and 0.1% Lucifer Yellow (wt/vol), pH 7.2. For whole-cell experiments to study eIPSCs, the intrapipette solution also contained 135 mM potassium methylsulfate, 5 mM KCl, 0.5 mM EGTA. For perforated patch-clamp recordings, the intrapipette solution contained 115 mM potassium methylsulfate, 25 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 0.1% Lucifer Yellow and 30 μg ml⁻¹ gramicidin (Sigma), pH 7.2. Membrane potential measurements were corrected for liquid junction potential. Data were filtered at 5 kHz, digitized and acquired using the Strathclyde electrophysiology software (J. Dempster, University of Strathclyde). GABA (1 mM) was puffed locally for 30 ms. The puff pipette was aimed toward the center of the neuronal somata, approximately 5 μm from the recording pipette. Experimental $E_{GABA}$ was extrapolated from the $GABA_A I-V$ relationships. The difference between the experimental and the theoretical $E_{GABA}$ (according to the Hodgkin-Katz-Goldman equation) provides an estimate of Cl⁻ extrusion.
capacity as described [48]. Recordings of repeated eIPSCs were performed in the presence of bath-applied 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and D(-)-2-amino-5-phosphonovaleric acid (AP5, 40 μM). IPSCs were evoked by focal electrical stimulation (100 μA, 200 μs). Trains of stimuli (25 pulses, 20 Hz) were delivered every 20 s at 0 or −90 mV. Ten consecutive trains were averaged for subsequent analysis. In electrophysiological experiments, only neurons with resting membrane potential less than −50 mV and stable access resistance were included for subsequent analysis. No differences in access resistance were observed between morphine-treated (19.3 ± 0.9 MΩ) and untreated neurons (20.2 ± 1.1 MΩ). At the end of each recording session, a photograph of the recorded neuron was acquired. Data analysis was performed off-line with Clampfit 10.2 (Molecular Devices).

4.2.8 Imaging of reverse Cl⁻ transport in rat lamina I neurons

Rat spinal cord slices were labeled in ACSF containing 5 mM of the Cl⁻ indicator MQAE (N-6-methoxyquinolinium acetoylester, Molecular Probes) and 0.2% pluronic (wt/vol) in DMSO (Sigma) for 40–45 min at 20–25 °C. Slices were then transferred to a perfusion chamber (2 ml min⁻¹) and extracellular MQAE was washed out for 30 min in the presence of 1 μM tetrodotoxin, 10 μM CNQX, 40 μM AP5, 1 μM strychnine and 20 μM bicuculline to minimize KCC2-independent Cl⁻ transport. MQAE fluorescence was measured using a Zeiss LSM 510 laser-scanning microscope coupled to a femtosecond-pulsed Ti-Sapphire laser (Chameleon Ultra, Coherent) tuned at 750 nm. Fluorescence was acquired through a 40x water-immersion objective (Zeiss, 0.8 NA) and a band-pass filter (390–465 nm). Recorded cells were identified as lamina I cells merging transmitted light and MQAE fluorescence. MQAE images were acquired every 5 s. After a control period of 75 s, perfusion solution was switched to ACSF containing 15 mM KCl (osmolarity adjusted using mannitol) to reverse Cl⁻ transport [41]. The average fluorescence from each cell body was expressed as %ΔF/F₀. Fluorescence lifetimes were measured in control and after Cl⁻ equilibrium was achieved in 15 mM extracellular KCl to obtain quantitative estimates of
independent of [MQAE],[68]. To ensure that measurements of rates of intracellular
Cl\(^-\) loading were performed for comparable [Cl\(^-\)], we initiated exposure to 15 mM KCl after
sufficient incubation time so that the steady-state [Cl\(^-\)], was comparable in control and morphine
conditions. MQAE lifetime was recorded with a Becker & Hickl SPC-830 module through the
nondescanned port of the Zeiss LSM 510 using a band-pass filter (469/35 nm, Semrock) coupled
to a laser block (short-pass 750 nm, Semrock). Photon emission was detected using a PMC-100-I
photosensor (Hamamatsu). Lifetime in each cell was averaged over the cell body area and
extracted using SPCImage software (Becker & Hickl). Instrument response function of the
detection path was acquired using an 80-nm gold nanoparticle suspension to generate second-
harmonic signal. Absolute [Cl\(^-\)], was calculated from a calibration of the Cl\(^-\) dependence of
MQAE lifetime as described [68].

4.2.9 Microglia primary culture preparation

Primary culture was prepared as described [255]. Briefly, mixed glial culture was isolated
using P1–3 rat cortex and maintained for 10–14 d in DMEM medium containing 10% fetal
bovine serum (vol/vol, Invitrogen). Microglia separated by gentle shaking were plated and treated
with morphine (100 nM), morphine/naloxone (1 μM), morphine/(+)naloxone (1 μM),
morphine/LPS-RS (1, 10 and 100 ng ml\(^{-1}\), Invitrogen), morphine/TNP-ATP (10 μM, Sigma),
morphine/PPADS (pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonate, 10 μM, Sigma),
morphine/TrkB-Fc (5 μg/ml, R&D Systems) or morphine/IgG-Fc (5 μg ml\(^{-1}\), R&D Systems) once
daily for 5 d. Control cultures were treated with either phosphate-buffered saline (PBS) or the
above drugs in the absence of morphine once daily for 5 d.

4.2.10 Microglia calcium imaging

Cells were incubated at 20–25 °C for 30 min with Fura-2 a.m. (2.5 μM, Molecular
Probes) in ACSF containing 140 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl\(_2\), 10 mM HEPES and 33
mM glucose (pH 7.35, osmolarity 315–320 mOsm). ATP (50 μM, Sigma) was bath-applied.
Excitation light was generated from a 75-W xenon arc lamp and passed alternatingly through 340- or 380-nm band-pass filters [207](Omega Optical).

4.2.11 Microglia whole-cell recording.

ACSF was as above, except for 1 mM MgCl$_2$ and 2 mM CaCl$_2$. Pipettes contained 140 mM CsCl, 1 mM MgCl$_2$, 10 mM BAPTA, 10 mM HEPES, pH 7.2. ATP (50 μM) was applied for 2 s using a fast-step perfusion system (SF-77B, Warner Instruments) and recorded using an Axopatch 1-D amplifier (Molecular Devices). The electrical signals were digitized with a DigiData 1200 (Molecular Devices) and filtered at 2 kHz.

4.2.12 Intrathecal injections of microglia.

These experiments were performed as described previously [136]. Briefly, before intrathecal injection, microglia cultures were washed out in PBS, removed from the dish surface using a cell scraper and collected in 100 μl of PBS. Cell density was measured using a cell counter and the volume of PBS was adjusted to give a final density of 1,000 cells per 10 μl. This preparation was injected via lumbar puncture. Paw withdrawal threshold was tested before injection and after 1, 3 and 5 h. In a previous set of experiments with intrathecal catheter implants, we found that microglia produced a significant effect ($P < 0.01$) on withdrawal reflex only when administered dorsally, confirming a selective action at the SDH level (Supplementary Fig. 3, Appendix).

**Western blotting.** Cultured microglia were harvested and collected in 100 μl of PBS containing a phosphatase inhibitor cocktail (2%, Sigma) and a broad spectrum protease inhibitor (2%, Sigma). After centrifugation, the pellet was resuspended in 3% sodium dodecyl sulfate (SDS, vol/vol) containing 15% glycerol (vol/vol) and 75 mM Tris-base. Total protein was measured using a BCA protein assay reagent kit (Pierce). Samples were heated at 95 °C for 10 min in 2× sample buffer (Pierce), electrophoresed on a precast SDS polyacrylamide gradient gel (4–12% Tris-HCl, Bio-Rad) and transferred onto a nitrocellulose membrane. After blocking, membranes were
incubated with rabbit antibody to P2X4R (1:1,000, Alomone, #- APR-002) or mouse antibody to actin (1:5,000, Sigma, #-A5316), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Bio-Rad, #-170-6515, #-170-6516), ECL detection (Amersham) and densitometric quantification (ImageJ, US National Institutes of Health). Rat spinal cords were rapidly dissected and frozen in liquid nitrogen. Spinal cord homogenates were separated on a precast SDS polyacrylamide gradient gel (4–12% Tris-HCl, Bio-Rad) or 4–12% Tris glycine gel (Invitrogen) and transferred onto nitrocellulose membrane. P2X4R detection was performed as described above. For KCC2 detection, gel loading was done in a Laemmli sample buffer (Bio-Rad) containing 0.5% lithium dodecyl sulfate (wt/vol) to solubilize KCC2. Membranes were incubated in PBS/milk (pH 7.4) and incubated overnight with the polyclonal rabbit antibody to KCC2 (1:1,000, Millipore, #-07-432) [17] at 4 °C. Membranes were washed and incubated for 2 h at 20–25 °C in rabbit-specific secondary antibody then quantified by direct detection of secondary antibody fluorescence at 700 and 800 nm (Odyssey Licor). The relative amount of monomeric or oligomeric KCC2 was calculated as described [17]. Full-length blots are shown in Supplementary Figure 4 (Appendix).

**Histological procedures.** Rats or mice were anesthetized and perfused transcardially with 4% paraformaldehyde (wt/vol) in 0.1 M phosphate buffer (pH 7.4). Spinal cord sections were obtained as described [153]. Sections were incubated overnight at 4 °C in rabbit antibody to KCC2 (1:1,000), guinea pig antibody to μ-receptor antibody (1:5,000, Neuromics, #-GP10106), mouse OX-42 antibody to CD11b (1:500, Millipore #-CBL1512; 1:1,000, Serotec, #-MCA275R). After washing, sections were incubated at 20–25 °C in a solution containing appropriate fluorochrome-conjugated secondary antibodies. Images were obtained with an Olympus FV300-IX71 confocal microscope (Olympus America) for KCC2 staining or with a Leica TCS SP2 confocal microscope (Leica) for CD11b immunocytochemistry (ICC). Images of CD11b ICC from intrathecal saporin/saporin-Mac1 experiments were acquired with a fluorescence
microscope (Olympus). Laminar boundaries were identified as described [153]. Quantification was performed using ImageJ for CD11b ICC and using locally designed software for KCC2 (MathWorks). β-galactosidase activity in P2rx4−/− mouse spinal cords was revealed by incubation with a solution of X-Gal (1 mg ml⁻¹), 5 mM potassium ferricyanide, 2 mM MgCl₂, 0.2% Triton X-100 (vol/vol) in PBS overnight at 37 °C. X-gal staining was quantified using ImageJ. No staining was detected in wild-type mice.

4.2.13 ELISA.

Recovery of BDNF released from microglia was achieved using a Microcon YM-10 centrifugal filter device (Millipore). As described previously [49], measurement of microglial BDNF was performed using a specific ELISA kit (detection range: 7.8–500 pg ml⁻¹) with BDNF standards (7.82–500 pg ml⁻¹) and 100 μl of supernatant sample run in triplicate (Chemicon). Samples were washed and incubated with biotinylated mouse monoclonal antibody to BDNF (1:1,000, Chemicon, #-CYT306), incubated with streptavidin-HRP conjugate, treated with 3,3′,5,5′-tetramethylbenzidine substrate and read by a spectrophotometer plate reader (Molecular Devices) at 450 nm. Samples were considered BDNF positive if their signal was higher than the background signal and was in the range of the standard curve. Data were normalized to the control.

4.2.14 Statistics.

All data are given as the mean ± s.e.m. As data distributions were poorly fit by a Gaussian distribution, non-parametric tests were used (unless otherwise stated). Differences between groups were tested by Mann-Whitney test or by Kruskal-Wallis test with post hoc Dunn test. Repeated measures were analyzed by Friedmann test with post hoc Dunnet test or Wilcoxon test. Fisher's exact test was used for contingency tables. Bi-exponential fittings were compared by F test. Sample sizes are consistent with those reported in similar studies. Differences were considered to be significant at \( P < 0.05 \).
4.3 RESULTS

To determine whether there is a common or separate mechanism for tolerance and hyperalgesia, we used a differential testing procedure in rats treated with morphine sulfate (10 mg per kg of body weight, subcutaneous) twice daily over 7 d. Morphine antinociception was measured by testing thermal withdrawal threshold 1 h after each morning injection (n = 14 rats, P < 0.001; Fig. 4.1a). Although morphine induced a significant increase in thermal withdrawal threshold at that time point on day 1, the antinociception was significantly reduced within 3 d of treatment (P < 0.001; Fig. 4.1a). By day 5, morphine had no effect on withdrawal threshold, indicating that the rats were tolerant to the antinociceptive effects of morphine. Development of hyperalgesia was assessed separately by testing rats just before each morning injection of morphine (Fig. 4.1b,c). We found a progressive decrease in withdrawal threshold over the course of 5–7 d of morphine treatment (n = 14 rats, P < 0.001), but not with saline injections (n = 10, P > 0.05; Fig. 4.1b,c), indicating the development of pain hypersensitivity in these animals. Pain hypersensitivity was also observed by increased responses to nociceptive stimulation: vocalizations in response to the subcutaneous injections (n = 7, P < 0.05; Fig. 4.1d) and licking behavior after thermal stimulation (n = 7 rats, day 7, P < 0.05; Fig. 4.1e) were increased by repeated morphine treatment. In contrast, no change in motor performance was observed using the accelerating rotarod test (Fig. 4.1f), indicating that the decrease in withdrawal thresholds was not a result of altered motor activity. That the time course for the development of hyperalgesia was different from that for tolerance raised the possibility that the two processes have distinct underlying mechanisms.

4.3.1 MIH resulted from altered Cl− homeostasis

Disinhibition through disrupting Cl− homeostasis is one mechanism for increasing the output of lamina I neurons in the SDH[50,124]. We investigated whether morphine alters Cl− homeostasis in lamina I neurons. We measured the Cl− extrusion capacity of these neurons in
Figure 4.1 Repeated morphine administration causes hyperalgesia and tolerance. a-c. Time course of morphine tolerance and pain hypersensitivity assessed by Hargreaves plantar test and von Frey filament in rats: (a) Thermal pain threshold 1 h after morphine (days 3–7 versus day 1, $\chi^2 = 61.5$, $^*P < 0.001$) or saline injection (morphine versus saline at days 1–4, $^*P < 0.05$, $***P < 0.001$). CTR, saline control; MS, morphine sulfate; PWT, paw withdrawal threshold. (b) Thermal pain threshold before morphine (days 5–7 versus day 1, $\chi^2 = 20.7$, $^*P < 0.01$) and saline injection (morphine versus saline at days 4–7, $^*P < 0.05$, $**P < 0.01$). (c) Mechanical pain threshold before morphine or saline injection. At day 7, the threshold of morphine-treated rats ($n = 7$) was significantly reduced as compared with the threshold at day 1 ($\chi^2 = 13.58$, $##P < 0.01$)
and the saline group \( n = 6, **P < 0.01 \). (d,e) Progressive increase in nociceptive behaviors in morphine-treated rats as compared with saline-treated controls. (d) Percentage of vocalizing rats during subcutaneous injections (day 5–9 versus day 1, *\( P < 0.05 \), **\( P < 0.01 \)). (e) Licking time after thermal stimulation (day 0, \( P > 0.05 \); day 7, \( U = 7, *P < 0.05 \)). (f) Maximal running speed at day 0 and day 7 of saline or morphine injections assessed by rotarod before the morning injection (\( U = 21, P > 0.05 \)). All threshold values were normalized to the baseline. Error bars in all panels represent s.e.m.
spinal cord slices isolated from rats receiving either saline or morphine injections for 7 d (Fig. 5.2a,b)[48]. Following application of an intracellular Cl− load, we found that the reversal potential for GABA_A currents, \( E_{GABA} \), in lamina I neurons of morphine-treated rats (−42.3 ± 1.3 mV, \( n = 6 \) cells) was significantly more depolarized than that in lamina I neurons of saline-treated rats (−50.1 ± 2.2 mV, \( n = 5 \) cells, \( P < 0.05 \); Fig. 5.2). Because hyperalgesia may also develop within hours of high-dose treatments [224], we incubated spinal cord slices from naive rats with 1 \( \mu \)M morphine for 3 h. In vitro morphine treatment also resulted in a significant shift in \( E_{GABA} \) (\( P < 0.01 \); Fig. 5.2c,d). Thus, both repeated and high-dose morphine treatment impair Cl− extrusion in lamina I neurons.

To separately test whether morphine weakens Cl− extrusion, we used an established procedure involving collapse of the Cl− gradient during trains of evoked inhibitory postsynaptic currents (eIPSCs) [93]. The contribution of Cl− accumulation to activity-dependent depression of synaptic activity was determined by stimulating inhibitory transmission while holding the membrane potential either above (0 mV) or below (−90 mV) the Cl− reversal potential, \( E_{Cl} \). When holding at −90 mV, the rate of decrease of eIPSC amplitude depends on activity-dependent synaptic depression, whereas the rate of decrease at 0 mV reflects synaptic depression and postsynaptic Cl− accumulation [93]. Morphine did not affect the rate of synaptic depression at −90 mV (\( P > 0.05 \); Fig. 5.2e), which is consistent with the lack of presynaptic μ receptor expression in spinal inhibitory interneurons [125]. In contrast, the decrease in eIPSC amplitude at 0 mV occurred more quickly after morphine (\( P < 0.05 \); Fig. 5.2f). The differential effect of morphine at 0 mV versus −90 mV indicates that morphine treatment resulted in increased Cl− accumulation. Thus, morphine weakened Cl− extrusion, as measured with Cl− loaded either tonically through the patch pipette or phasically through synaptic receptors.

Further evidence of a collapse in Cl− gradient in neurons exposed to morphine was found in the responses to exogenous GABA applied in the presence of physiological [Cl−]i in cells held
Figure 4.2 Morphine disrupts Cl− homeostasis in lamina I neurons. (a,b) Decrease in Cl− extrusion capacity in lamina I neurons after in vivo morphine treatments in rats. (a) Responses to 30-ms GABA puffs following saline or morphine treatments in the presence of a Cl− load (29 mM). The response obtained at −55.5 mV is shown in gray. (b) I-V relationships for GABA_{A} currents obtained from morphine-treated rats (n = 6 cells) is right-shifted as compared with controls (n = 5 cells). The dashed line indicates the I-V relationship when Cl− extrusion capacity was blocked by the KCC2 antagonist furosemide. (c) Effect of in vitro morphine (1 μM, >3 h, n = 7 cells) on I-V relationships for GABA_{A} currents versus saline control (n = 6 cells). (d) Pooled $E_{\text{GABA}}$ of neurons shown in b (U = 2) and c (U = 6, *P < 0.05). (e,f) Cl− accumulation under repetitive inhibitory input. (e) Representative traces (average of ten repetitions) from a control
(black) and an in vitro morphine-treated lamina I neuron (gray) clamped at −90 mV (top). The rate of eIPSC amplitude depression during repetitive stimulation (20 Hz) is shown below. Amplitude values were normalized to the first eISPC. No differences were observed (CTR, n = 6 cells; MS, n = 4 cells; F = 0.8, P > 0.05). (f) Data presented as in e for a neuron clamped at 0 mV. Note the larger and faster eIPSC depression in morphine-treated neurons. Differences between morphine and controls were significant (CTR, n = 6; MS, n = 4 cells; F = 21.98; *P < 0.05). (g) Gramicidin-perforated patch-clamp recording of GABA responses obtained at −60 mV in control (light gray dotted line) and from a morphine treated lamina I neuron before (black) and after ACTZ (50 μM, dark gray) Traces are scaled to the peak amplitude; arrow indicates the GABA puff. Note the biphasic response in the morphine neuron and the monophasic response following ACTZ. Below, I-V curves measured 1 s after the pulse (black arrowhead). Note the increased $E_{\text{GABA}}$ difference before and after ACTZ. (h) Effect of intrathecal ACTZ (22.5 μg, day 7–9) on morphine-induced mechanical (saline, n = 5; ACTZ, n = 5; U = 0, **P < 0.01) and thermal (saline, n = 11; ACTZ, n = 9; U = 15.5 *P < 0.05) pain hypersensitivity in rats. All threshold values were normalized to the baseline. The dashed line represents the baseline threshold. Error bars in all panels represent s.e.m.
near the resting potential (−60 mV; Fig. 5.2g). In these neurons, GABA evoked a biphasic response, an initial outward current followed by a shift to an inward current (Fig. 5.2g), as previously described in neurons with impaired Cl− extrusion capacity [48,225]. In contrast, in lamina I neurons that were not exposed to morphine, the response to GABA remained outward at −60 mV (Fig. 5.2g). The inward component of the response to GABA has been associated with outward flux of HCO3− becoming predominant when the Cl− gradient collapses, yielding a progressive depolarizing shift in $E_{GABA}$[48,68,232]. To determine whether this was the case in neurons exposed to morphine, we bath-applied acetazolamide (ACTZ, 50 μM), a cell-permeant inhibitor of carbonic anhydrase, the enzyme responsible for generating HCO3− intracellularly [232]. In the presence of ACTZ, the GABA response remained outward, yielding a 10-mV hyperpolarizing shift in $E_{GABA}$, measured 1 s after the onset of the GABA puff (Fig. 5.2g). These findings are consistent with ACTZ lowering the generation of HCO3−, resulting in an activity-dependent depletion of HCO3− and ensuing loss of HCO3− efflux, during the GABA response[232] (Supplementary Fig. 1, Appendix).

Preventing the progressive shift in $E_{GABA}$ in neurons with impaired Cl− extrusion substantially restores inhibition[68,190]. Thus, ACTZ may mitigate the effect of the morphine-induced collapse in Cl− gradient, rescuing the impairment of inhibition. We tested the effects of intrathecal administration of ACTZ in rats with established hyperalgesia after 7–9 d of morphine treatment. ACTZ administration reversed morphine-induced mechanical and thermal hypersensitivity (Fig. 5.2h). We conclude that ACTZ reversed the hyperalgesia by reducing HCO3−-mediated inward current, thereby restoring $E_{GABA}$. Taken together, our findings indicate that altered anion homeostasis underlies MIH.
Figure 4.3 Morphine effects on activity and expression of KCC2. (a) KCC2 immunostaining in rat lamina I and lamina II following 7 d of saline or morphine injections. Scale bar represents 50 μm. (b) Western blot showing the total KCC2 expression in the dorsal and ventral horn from...
saline- and morphine-treated rats. Histograms show quantification of KCC2 expression normalized to the actin level in the dorsal (one-tail \( t \) test, \( t = 2.1, *P < 0.05 \)) and ventral horn (one-tail \( t \) test, \( t = -1.1, P > 0.05 \)). (c) Western blot performed without β-mercaptoethanol to preserve KCC2 oligomerization. The histograms show quantification of KCC2 oligomer/monomer ratio in the dorsal (\( n = 8 \) control rats, \( n = 9 \) morphine-treated rats, one-tail \( t \) test, \( t = 2.1, *P < 0.05 \)) and the ventral horn (one-tail \( t \) test, \( t = 0.6, P > 0.05 \)). (d,e) Chloride imaging from rat spinal cord slices loaded with the Cl−-sensitive dye MQAE. (d) Pseudocolor images showing lifetime maps from a control lamina I neuron in the presence of 2.5 or 15 mM KCl. Lifetimes from lamina I neurons in control conditions (3.44 ± 0.34 ns, \( n = 11 \) cells) were significantly shortened (reflecting quenching of MQAE fluorescence) 15 min after exposure to high extracellular KCl to reverse KCC2 transport (3.02 ± 0.24 ns, \( n = 11, P < 0.001 \)). Scale bar represents 20 μm. (e) Representative traces of fluorescence intensity in control (black) and morphine-treated neuron (gray) showing the rate of intracellular Cl− accumulation (%ΔF/F0) in the presence of 15 mM KCl. Steady-state lifetime measurements just before 15 mM KCl solutions were not different (control, 3.37 ± 0.06 ns, 7.0 ± 0.7 mM Cl−; morphine, 3.23 ± 0.06 ns, 8.6 ± 0.8 mM Cl−; \( U = 38, P > 0.05 \)). Error bars in all panels represent s.e.m.
4.3.2 Morphine reduces Cl− transport and KCC2 expression

In adult lamina I neurons, Cl− homeostasis is mainly regulated by the K+-Cl− co-transporter, KCC2 [49,50]. We examined the effect of repeated morphine treatment on spinal KCC2 expression. After 7 d of morphine treatment, there was a significant decrease in KCC2 immunoreactivity in lamina I of morphine-treated rats (103.4 ± 5.8 intensity units (i.u.), n = 5 rats) compared with that of saline-treated rats (191.2 ± 5.8 i.u., n = 5 rats, P < 0.01; Fig. 5.3a). In addition, KCC2 protein levels in SDH homogenates were reduced by morphine (Fig. 5.3b). As KCC2 oligomerization may be critical for transporter function[17], we analyzed the KCC2 oligomer to monomer ratio in the absence of detergents and found that the ratio was significantly reduced in the SDH of morphine-treated rats (P < 0.05; Fig. 5.3c). In contrast, morphine had no effect on KCC2 expression or oligomer to monomer ratio in the spinal ventral horn (Fig. 5.3b,c). Thus, the morphine-induced impairment in Cl− homeostasis in lamina I neurons may result from a loss of the oligomeric form of KCC2 in these cells.

To test whether total KCC2 activity in lamina I neurons is affected by morphine, we examined the transporter activity in reverse mode[41] by measuring K+-driven uptake of Cl−. We performed intracellular Cl− imaging and stepped extracellular K+ from 2.5 to 15 mM in control or morphine-treated slices (1 μM for 3 h; Fig. 5.3d,e). The rate of Cl− accumulation was significantly lower in morphine-treated neurons (n = 8 cells, 0.32 ± 0.04 %ΔF/F per s) than in control neurons (n = 8 cells, 0.54 ± 0.2 %ΔF/F per s, P < 0.05; Fig. 5.3e). These data indicate that morphine impairs Cl− homeostasis by reducing KCC2-mediated Cl− transport.

4.3.3 MIH, but not tolerance, requires spinal microglia

Because microglia have been implicated in disrupting Cl− homeostasis in lamina I neurons[11,49], we asked whether MIH may differentially depend on spinal microglia. We found that repeated morphine treatment induced an increase in CD11b immunoreactivity, which is specifically expressed by microglia in the CNS (Fig. 5.4a), indicating that spinal microglia
respond to morphine treatment. To determine whether spinal microglia are required for morphine tolerance or hyperalgesia, we depleted microglia in the spinal cord of morphine-treated rats using intrathecal injection of a saporin-conjugated antibody to Mac1 (ref. 26) (Fig. 5.4b). Intrathecal injections were begun on day 7, when both pain hypersensitivity and morphine tolerance had been established. Within 2 d, saporin-conjugated antibody to Mac1 (20–36 μg), but not saporin alone (20 μg), reversed morphine-induced thermal pain hypersensitivity \( (n = 6 \text{ saporin-treated rats}, \ n = 7 \text{ antibody to Mac1– and saporin-treated rats}, \ P < 0.01; \ \text{Fig. 5.4c}) \). Saporin-conjugated antibody to Mac1 also reversed morphine-induced mechanical allodynia (mechanical threshold at day 7 of morphine injection, 0.4 ± 0.1 of the baseline, \( n = 5 \); mechanical threshold at day 8 of morphine injection following intrathecal antibody to Mac1 and saporin administration, 1.2 ± 0.3 of the baseline, \( n = 5 \) rats; \( P < 0.05 \)). In contrast, saporin-conjugated antibody to Mac1 had no effect on morphine tolerance (\( P > 0.05 \); Fig. 5.4d). Thus, we conclude that microglia in the SDH are necessary for the ongoing expression of hyperalgesia, but not for tolerance caused by repeated morphine treatment.

To determine whether morphine-treated microglia are sufficient to produce pain hypersensitivity, we used an \textit{in vivo} microglia transfer approach\[49,255\]. Microglia in primary culture were chronically treated with morphine and then injected intrathecally at the lumbar spinal level in naive rats (Fig. 4.5a). We found that mechanical withdrawal threshold was markedly decreased after intrathecal administration of morphine-treated microglia (\( n = 7 \) rats, \( P < 0.001 \); Fig. 4.5a). In contrast, mechanical withdrawal threshold was unaffected by saline-treated microglia. Thus, morphine-treated microglia are sufficient to cause pain hypersensitivity in naive animals.
Figure 4.4 Morphine-induced hyperalgesia depends on microglia activation. (a) CD11b expression in rat SDH following 5 d of saline or morphine treatment (scale bar represents 30 μm) and fluorescence intensity quantification (CTR, 0.84 ± 0.06 i.u., n = 28 sections; MS, 2.78 ± 0.22 i.u., n = 34 sections; U = 8, P < 0.001). (b–d) Effects of intrathecal injection of saporin-conjugated antibody to Mac-1 (Mac-1–saporin; versus saporin alone) on SDH CD11b expression,
morphine-induced hyperalgesia and tolerance (intrathecal injections were performed from day 7 to 9 of morphine treatment). (b) CD11b expression in SDH after microglia depletion with antibody to Mac1 (saporin, 10.12 ± 1.32 i.u., n = 17 sections; Mac-1–saporin, 7.84 ± 2.23 i.u., n = 16 sections; U = 55, P < 0.01). Scale bar represents 50 μm. (c) Thermal pain threshold before morphine injection (Mac-1–saporin versus saporin alone at day 9, U = 1, **P < 0.01). (d) Thermal pain threshold 1 h after morphine injection (day 9, U = 12, P > 0.05). All threshold values were normalized to the baseline. Error bars in all panels represent s.e.m.
Figure 4.5 P2X4Rs in microglia are required for morphine-induced hyperalgesia. (a) Rat mechanical pain threshold 5 h after intrathecal injections of cultured microglia treated with morphine (100 nM, n = 7, H = 19.98, *P < 0.01), morphine + TNP-ATP (n = 5, P > 0.05), morphine + PPADS (n = 8, **P < 0.01), versus CTR (n = 7). (b-d) Lack of hyperalgesia in P2rx4−/− mice. (b) Thermal (n = 5 mice per group) and mechanical (n = 7 mice per group) pain threshold after 5 d of subcutaneous morphine injections in P2rx4+/+ mice and P2rx4−/− mice (mechanical, U = 5; thermal, U = 2; *P < 0.05). (e) Licking time following mechanical stimulation of the paw (morphine-treated P2rx4+/+ versus morphine-treated P2rx4−/−, *P < 0.05; # significant differences compared with the baseline, P < 0.05). (d) Vocalizations produced by subcutaneous injections (morphine-treated P2rx4+/+ versus other groups, *P < 0.05; # significant differences compared with the baseline, P < 0.05). a.u., arbitrary units. (e-g) Effect of intrathecal TNP-ATP (30 nmol) on MIH in rats. (e) Thermal pain threshold before morphine injection in vehicle (n = 8) versus TNP-ATP–injected rats (n = 8, day 7, U = 13, *P < 0.05). (f) Thermal pain threshold 1 h after morphine injection. (g) Mechanical withdrawal threshold following TNP-ATP in morphine-treated rats measured before morphine injection (day 7; CTR, n = 6; TNP-ATP, n = 7; U = 0, **P < 0.01). (h) X-gal staining in P2rx4−/− mice. The percentage SDH staining area of control (3.6 ± 0.3%, n = 5 sections) and morphine-treated mice (4.8 ± 0.3%, n = 12).
sections, $U = 10$, $P < 0.05$). Scale bar represents 30 μm. (i,j) Increased expression and function of P2X4R in morphine-treated (100 nM) microglia cultures. (i) Expression of P2X4R after morphine treatment normalized to the saline controls for 1 d ($n = 7$ trials), 3 d ($n = 7$) and 5 d ($n = 7, H = 19.35, ***P < 0.001$). (j) ATP-mediated currents in morphine-treated microglia over 5 d (peak current normalized to the pre-ATP baseline, $1.6 \pm 0.2; n = 12$ cells, $U = 91, *P < 0.05$) versus control ($1.0 \pm 0.1, n = 28$ cells). All threshold values in the figure are normalized to the baseline. Error bars in all panels represent s.e.m.
4.3.4 Microglial P2X4 receptors are required for MIH

Pain hypersensitivity produced by microglia critically depends on P2X4 receptors (P2X4Rs)[255], suggesting that morphine-induced pain hypersensitivity may require microglial P2X4Rs. We found that the decrease in withdrawal threshold produced by transferring morphine-treated microglia was prevented by TNP-ATP, an antagonist of P2X1R, P2X2R, P2X3R and P2X4R, but not by PPADS, an antagonist of P2X1R, P2X2R, P2X3R, P2X5R and P2X7R[27], implicating P2X4Rs in the expression of the pain hypersensitivity evoked by morphine-treated microglia (Fig. 4.5a). Moreover, we found that repeated morphine treatment did not induce a change in thermal and mechanical withdrawal threshold, licking time or vocalization in P2rx4−/− mice (Fig. 4.5b–d); however, P2rx4+/+ mice developed multiple signs of pain hypersensitivity in response to morphine (Fig. 4.5b–d). Thus, P2X4Rs are required for the development of hyperalgesia. To test whether P2X4Rs are also necessary for the ongoing expression of hyperalgesia, we administered TNP-ATP (30 nmol) intrathecally in morphine-treated mice. We found that TNP-ATP reversed the decrease in thermal withdrawal threshold that had developed after 7 d of morphine treatment (Fig. 4.5e) without affecting morphine tolerance (Fig. 4.5f). In another series of experiments, intrathecal TNP-ATP administration also reversed established mechanical allodynia in morphine-treated rats (Fig. 4.5g).

As our findings suggest that P2X4Rs are required for the development and ongoing expression of hyperalgesia resulting from morphine treatment, we questioned whether morphine treatment may increase P2X4R expression, which is normally present at low levels in the naive CNS[255,256]. In P2rx4−/− mice, in which P2rx4 was replaced with lacZ, we found a greater X-gal (5-bromo-4-chloro-indolyl-galactopyranoside) signal in the spinal cord of morphine-treated than in saline-treated mice (Fig. 4.5h). Moreover, we found that repeated morphine treatment increased P2X4R protein expression in wild-type mice (see below). To determine whether morphine may act directly on microglia, we used primary microglia cultures and found that chronic morphine
treatment resulted in an increase in P2X4R protein expression (Fig. 4.5i). These receptors were functional, as indicated by morphine-induced increases in P2X4R-mediated currents (Fig. 4.5j) and Ca2+ responses (see below). Thus, MIH depends on P2X4Rs, and morphine treatment induces an increase in *P2rx4* gene expression autonomously in microglia.

### 4.3.5 MIH, but not tolerance, requires microglial BDNF

Stimulation of P2X4Rs in microglia evokes the release of BDNF[49,248,259], which is known to downregulate KCC2 expression in adult neurons[132]. Thus, we asked whether morphine treatment causes a P2X4R-dependent release of BDNF from microglia. We found that chronic morphine treatment resulted in the release of BDNF from primary microglia cultures (Fig. 4.6a). The morphine-evoked release of BDNF was blocked by TNP-ATP, but was unaffected by PPADS (Fig. 4.6a). BDNF release was also blocked by the ATP-degrading enzyme apyrase (Fig. 4.6a), indicating that morphine causes the release of BDNF through ATP-mediated stimulation of P2X4Rs. The mechanical hypersensitivity evoked by morphine-treated microglia was prevented by a BDNF-sequestering fusion protein (TrkB-Fc)[155], but not by the control peptide IgG-Fc (Fig. 4.6b). Together, our findings indicate that morphine treatment results in P2X4R-dependent release of BDNF from microglia, which is sufficient to induce pain hypersensitivity.

We then addressed whether BDNF and signaling through its cognate receptor TrkB are necessary for morphine-induced impairment of the Cl− extrusion capacity of lamina I neurons and ensuing hyperalgesia. The shift in $E_{\text{GABA}}$ induced by morphine treatment was prevented by co-incubation with a function-blocking antibody to TrkB (Fig. 4.6c,d). To determine whether BDNF-TrkB signaling is required for the ongoing expression of MIH, we tested the effects of intrathecal injections of a function-blocking antibody to TrkB. The antibody produced a gradual reversal of thermal hyperalgesia over 3 d in rats that had been treated with morphine for 7 d, comparable to that obtained by ACTZ treatment (Fig. 4.6e); however, neither antibody to TrkB
Figure 4.6 Altered Cl⁻ homeostasis in spinal neurons and morphine-induced hyperalgesia are dependent on P2X4R-BDNF-TrkB signaling. (a) Enzyme-linked immunosorbent assay
(ELISA)-based measurement of BDNF release from cultured microglia treated with 100 nM morphine (n = 14 trials, H = 30.17, **P < 0.001), morphine + TNP-ATP (n = 5, P > 0.05), morphine + PPADS (n = 5), morphine + apyrase (n = 5, P > 0.05) or saline (n = 14). (b) Rat mechanical withdrawal threshold 5 h after intrathecal injection of morphine-treated microglia (n = 7, H = 23.66, **P < 0.01), morphine + TrkB-Fc (n = 7, P > 0.05), morphine + IgG-Fc (n = 7) or saline (n = 7). (c,d) Effect of antibody to TrkB (1 μg ml−1) on morphine-induced shift of $E_{\text{GABA}}$ in lamina I neurons in vitro. Shown are I-V relationships for GABA$_A$ currents after incubation with morphine (n = 12 cells) or morphine + antibody to TrkB (n = 7) or in control (n = 6) (c) and the respective $E_{\text{GABA}}$ values (H = 11.16, *P < 0.05; d). (e,f) Effect of intrathecal injections in rats of ACTZ and antibody to TrkB on morphine-induced pain hypersensitivity and tolerance by Hargreaves plantar test. (e) Thermal withdrawal threshold before morphine in ACTZ-treated (22.5 μg, n = 9), antibody to TrkB–treated (30 μg, n = 7) and vehicle-injected rats (n = 11, day 9; arrows indicate injections). (f) Thermal pain threshold 1 h after morphine treatment in ACTZ-, TrkB antibody– and vehicle-injected groups (ACTZ versus control group at days 8–9; arrows indicate injections). All threshold values were normalized to the baseline. Error bars in all panels represent s.e.m.
nor ACTZ affected morphine antinociception (Fig. 4.6f), indicating that BDNF and the associated disrupted Cl– homeostasis are required for ongoing expression of MIH, but not of tolerance.

To determine whether the requisite BDNF is released from microglia, we used gene-targeted mice in which BDNF was deleted in microglia. These mice were generated by crossing mice expressing Cre recombinase under the control of the CD11b (also known as Itgam) promoter (CD11b-cre) with those in which exon 5 of the Bdnf gene, which encodes the BDNF protein, was flanked by loxP sites (BdnfloxP/loxP) yielding CD11b-cre; BdnfloxP/loxP mice[21,201]. Lumbar spinal activity of the CD11b-cre transgene was verified by crossing mice with Rosa26 mice, which ubiquitously express the β-galactosidase (β-gal) transgene[21]. Microglia, but not neurons or astrocytes, expressed β-gal in Rosa26; CD11b-cre mice. In addition, Cre expression was detected only in cells that expressed the microglial markers Iba-1 and CD11b (S.B., T.T., J. Alexander and M.W.S., unpublished observation). No differences in mechanical nociceptive behavior were observed between CD11b-cre; BdnfloxP/loxP mice (1.5 ± 0.2 g, n = 7 mice) and their littermate BdnfloxP/loxP controls (1.5 ± 0.2 g, n = 7 mice; P > 0.05). However, only BdnfloxP/loxP mice developed robust hyperalgesia following repeated morphine treatment, and we found no change in mechanical withdrawal threshold, licking time or vocalization in CD11b-cre; BdnfloxP/loxP mice (Fig. 4.7a–c). CD11b-cre; BdnfloxP/loxP mice were indistinguishable from BdnfloxP/loxP mice with regard to a peak antinociceptive response to a single dose of morphine (Fig. 4.7d), progressive increase in median effective dose (ED50) to escalating morphine doses, shift in the morphine dose-response curve following 5 d of morphine treatment (Fig. 4.7e) or expression of naloxone-precipitated signs of withdrawal (Fig. 4.7f). Thus, acute morphine antinociception, tolerance and withdrawal behavior were not altered in the CD11b-cre; BdnfloxP/loxP mice. We conclude that BDNF from microglia is required specifically for the hyperalgesia induced by morphine.
Figure 4.7 Genetic deletion of Bdnf from microglia abrogates development of morphine-induced hyperalgesia, but not tolerance. (a–c) Assessment of morphine-induced hyperalgesia in CD11b-cre; BdnfloxP/loxP mice (n = 7 mice) and BdnfloxP/loxP mice (n = 7). (a) Effect of 5 d...
of morphine on mechanical threshold \((F = 3.499)\). (b) Licking time following mechanical stimulation of the hindpaw \((H = 16.21)\). (c) Vocalizations produced by subcutaneous injections. Legend in inset applies to b and c. (d,e) Assessment of morphine antinociception in CD11b-cre; BdnfloxP/loxP mice \((n = 7)\) and BdnfloxP/loxP mice \((n = 7)\). (d) Time course of antinociceptive response to a single dose of morphine in morphine-naive mice. (e) Morphine dose-response curves following 5 d of morphine or saline injections in CD11b-cre; BdnfloxP/loxP mice \((n = 7)\) and BdnfloxP/loxP mice \((n = 7)\). The rightward shift in morphine-treated mice indicates the development of morphine tolerance in both CD11b-cre; BdnfloxP/loxP and BdnfloxP/loxP mice, with no significant differences between the two genotypes (ED50 of saline-injected controls: BdnfloxP/loxP, 3.5 ± 0.3 mg per kg; CD11b-cre; BdnfloxP/loxP, 3.3 ± 0.3 mg per kg; \(P > 0.05\); morphine treated: BdnfloxP/loxP, 16.5 ± 1.0 mg per kg; CD11b-cre; BdnfloxP/loxP, 14.3 ± 1.1 mg per kg; \(P > 0.05\)). (f) Naloxone-precipitated withdrawal syndrome in CD11b-cre; BdnfloxP/loxP mice (CTR, \(n = 3\); MS, \(n = 6\)) and BdnfloxP/loxP mice (CTR, \(n = 5\); MS, \(n = 5\)). Withdrawal cumulative score was significantly higher in both morphine-treated groups as compared with the saline controls, but no differences were detected between CD11b-cre; BdnfloxP/loxP and BdnfloxP/loxP mice \((F = 10.20, P < 0.01)\). n.s., not significant \((P > 0.05; *P < 0.05)\). All threshold values were normalized to the baseline. Error bars in all panels represent s.e.m.
Figure 4.8 Activation of two separate signaling pathways is necessary for morphine-induced hyperalgesia. (a–c) Effect of intrathecal injections of low doses of (+)naloxone (5 ng, (+)NL) on morphine-induced pain hypersensitivity, tolerance and microglia activation. (a) Thermal withdrawal threshold before morphine or saline injections in (+)naloxone- or vehicle-treated rats ((+)-naloxone, n = 8; morphine + (+)-naloxone, n = 7; morphine, n = 8). (b) Thermal pain threshold 1 h after treatment. (e) CD11b immunostaining in rat SDH following 5 d of intrathecal morphine or morphine and (+)-naloxone and quantification (CTR, n = 28 sections; MS, n = 34 sections; morphine + (+)-naloxone, n = 34 sections). Scale bar represents 30 μm. (d) Lamina I $E_{GABA}$ following 3 h in vitro with (+)-naloxone alone (n = 6 cells) versus morphine and (+)-naloxone (n = 6, U = 14, P > 0.05), and with (+)-naloxone (n = 6) versus morphine and (+)-naloxone (n = 8, U = 26, P > 0.05). (e) Mechanical withdrawal threshold 5 h after intrathecal injections of microglia cultures treated with morphine and (+)-naloxone (n = 8, H = 12.97), morphine and (+)-naloxone (n = 5), or morphine alone (n = 7). (f) Western blot analysis of P2X4R protein expression in microglia cultures following treatment with morphine (100 nM, n = 10 trials, H = 26.49), morphine and (-)naloxone (P > 0.05), morphine and (+)-naloxone (n = 5), (-)naloxone (n = 3, P > 0.05), (+)-naloxone (n = 6, P > 0.05), or saline (n = 10). (g) ATP-evoked rise in intracellular $[Ca^{2+}]$ in cultured microglia treated with morphine (100 nM, n = 54 cells, H = 67.98), morphine and (-)naloxone (n = 38, P > 0.05), morphine and (+)-naloxone (n = 40), (-)naloxone (n = 15, P > 0.05), (+)-naloxone (n = 15, P > 0.05), or saline (n = 35). (h) Western blot of P2X4R protein from spinal cords isolated from rats treated for 5 d with intrathecal saline, morphine, morphine and (-)naloxone, or morphine and (+)-naloxone. (i) ELISA-based measurement of BDNF release from microglia treated with morphine and (-)naloxone (n = 5 trials, H = 14.29), morphine and...
(+)-naloxone \((n = 5)\), or morphine \((n = 14)\). (j) ELISA-based measurement of BDNF release from microglia treated with morphine \((n = 4\) trials, \(H = 15.84)\), morphine and 1 ng ml\(^{-1}\) LPS-RS \(1\) \((n = 4)\), morphine and 10 ng ml\(^{-1}\) LPS-RS \((n = 4)\), morphine and 100 ng ml\(^{-1}\) LPS-RS \((n = 4)\), 100 ng ml\(^{-1}\) LPS-RS \((n = 4)\), or control \((n = 4)\). (k) Effect of morphine (escalating doses from 10 to 40 mg per kg intraperitoneally twice a day for 7 d) on mechanical sensitivity of TLR4-deficient C3H/HeJ mice \((n = 11)\) and wild-type C3H/HeOuJ mice \((n = 8)\). Mechanical sensitivity index was calculated as \((10 - PW)/PW\) \((10 =\) stimulations with 3.41 g–calibrated filament; \(PW =\) number of paw withdrawals). No differences were observed in the development of mechanical allodynia between mice groups \((U = 30, P > 0.05)\). All threshold values were normalized to the baseline. Error bars in all panels represent s.e.m. n.s., not significant \((P > 0.05); *P < 0.05; ***P < 0.001)\).
4.3.6 Both μ-dependent and μ-independent signaling are necessary

The simplest cascade of events that could account for our findings is that the hyperalgesia induced by morphine is driven by increased expression of P2X4Rs in microglia leading to a release of BDNF that acts through TrkB receptors to downregulate functional expression of KCC2 with subsequent disruption of Cl− homeostasis in lamina I neurons. This raises the question of what are the direct molecular targets of morphine and their location, or locations, in this core pathway. The direct molecular target for morphine-induced analgesia are the well-known seven transmembrane isoforms of the μ opioid receptor[160]. Expression of μ receptors is evident in spinal microglia[97] (Supplementary Fig. 2, Appendix). On the other hand, there is increasing evidence that the actions of morphine or its metabolites may be dependent on concurrent activation of μ receptor–independent pathways[103,282]. To test this possibility, we examined the effects of stereoisomers of the opioid receptor antagonist naloxone. (−)Naloxone blocked both μ-mediated and non–μ-mediated signaling, and (+)naloxone blocked only the non–μ-mediated mechanisms[103]. We found that spinally administered (+)naloxone prevented morphine hyperalgesia (Fig. 4.8a), but did not affect the development of tolerance (Fig. 4.8b). Moreover, (+)naloxone reduced morphine-induced upregulation of CD11b (Fig. 4.8c) and prevented the shift in $E_{\text{GABA}}$ in lamina I neurons in spinal slices treated with morphine ($n = 8$ cells, $P > 0.05$; Fig. 4.8d). Finally, (+)naloxone prevented the hyperalgesic action of intrathecal administration of morphine-treated microglia (Fig. 4.8e). Thus, μ receptor–independent signaling is essential for the microglia-neuron interactions that cause hyperalgesia.

To examine whether morphine targets microglia specifically, we tested naloxone stereoisomers in cultured microglia that are competent to transfer hyperalgesia. Morphine-induced increases in P2X4R expression and function in microglia cultures were blocked by (−)naloxone, but were unaffected by (+)naloxone (Fig. 4.8f,g). Similarly, the increase in spinal P2X4R expression caused by repeated morphine administration in vivo was prevented by
(−)naloxone, but not by (+)naloxone (Fig. 4.8h). The morphine-induced increase in microglial P2X4Rs therefore required μ opioid receptors. However, morphine-evoked release of BDNF in microglia cultures in which P2X4R expression was already upregulated was prevented by (+)naloxone (Fig. 4.8i). Thus, we conclude that, in microglia, μ receptors mediate the upregulation of P2X4Rs and a μ receptor–independent mechanism is required for the morphine-stimulated release of BDNF.

The mechanisms of μ receptor–independent signaling by morphine are not fully understood. It has been suggested that Toll-like receptor 4 (TLR4) mediates morphine-induced neuroinflammatory responses[271]. To test the potential involvement of TLR4, we measured morphine-induced release of BDNF from microglia cultures in the presence of the TLR4 antagonist LPS-RS (lipopolysaccharide from *Rhodobacter sphaeroides*)[271]. Across a range of concentrations, LPS-RS had no effect on morphine-induced release of BDNF (Fig. 4.8j), consistent with our previous finding that LPS treatment does not induce BDNF release from microglia[248]. In addition, we found that mice lacking functional TLR4 (C3H/HeJ mice) developed tactile hypersensitivity with morphine treatment that was indistinguishable from that of wild-type controls (C3H/HeOuJ mice; Fig. 4.8k). These findings indicate that TLR4 is not required for morphine hyperalgesia.

**4.4 DISCUSSION**

Our findings suggest a pathway by which morphine produces hyperalgesia, which ultimately occurs via the dysregulation of Cl− homeostasis in pain-signaling neurons in spinal lamina I. The most parsimonious interpretation is that this pathway is initiated by morphine acting on μ-opioid receptors in SDH microglia, which, through release of BDNF, signal the lamina I neurons, inducing Cl− dysregulation by reducing the expression of KCC2. Although, as with hyperalgesia, morphine tolerance is dependent on μ receptors[18], our results indicate that microglia and the subsequent components of the hyperalgesia pathway are not required for
tolerance. That hyperalgesia and tolerance are mechanistically distinct is consistent not only with the observation that tolerance and hyperalgesia have differing clinical characteristics[156], but also with recent findings uncovering the signaling pathways underlying morphine tolerance, which do not affect hyperalgesia[272]. Our results also suggest that the mechanism that we uncovered represents a substrate of ongoing hyperalgesia induced by morphine rather than withdrawal hyperalgesia.

A key concept emerging from our findings is that morphine causes disinhibition by disrupting neuronal Cl− homeostasis. However, morphine is currently perceived as causing inhibition either pre- or postsynaptically. Thus, morphine-induced spinal disinhibition is a previously unknown mechanism for the actions of this drug and other opiates. We found that, in the SDH, this disinhibition led to the paradoxical hyperalgesic action of morphine. A therapeutic avenue that follows from this finding to prevent or reverse selectively the hyperalgesia is to restore GABA_A and/or glycine receptor–mediated inhibition, which could be achieved by enhancing GABA_A receptor activation through positive modulation, such as with benzodiazepines[131]. This strategy may be of limited efficacy, however, given our finding that disinhibition results from dysregulation of Cl− homeostasis. Indeed, if E_GABA is depolarized beyond a certain point, enhancing GABA_A transmission may become counterproductive[190]. In such conditions, restoring E_GABA by blocking the carbonic anhydrase to attenuate the depolarizing HCO3− component of GABA_A− and glycine-mediated currents could potentially reverse morphine hyperalgesia, as has been done to improve the anti-hyperalgesic potency of benzodiazepines[8]. Activity-dependent accumulation of Cl− through GABA_A or glycine channels resulting from compromised Cl− extrusion capacity will nevertheless provoke some breakdown of inhibition with the above approaches[68]. Thus, restoring Cl− extrusion capacity through enhancing KCC2 expression or activity[132] will likely be more effective as an adjuvant therapy to chronic morphine treatment.
Disinhibition and hyperalgesia caused by morphine treatment are mediated by upregulation of P2X4Rs in the spinal cord microglia in vivo, driving synthesis and release of BDNF[248,259]. Morphine has been found to upregulate P2X4Rs[96] and to induce BDNF transcription[239] via μ receptors in microglia in vitro. These findings point to a role for μ receptor activation in the microglia signaling cascade leading to hyperalgesia. However, we found that, although μ receptor activation induced P2X4R upregulation, μ receptor–independent signaling was necessary for the subsequent BDNF release. In contrast with previous findings[271], our results do not support an involvement of TLR4 in this mechanism. This apparent discrepancy may be a result of the much lower doses of morphine that we used, although more work is needed to define the underlying pharmacology. P2X4R-dependent and μ receptor–independent pathways represent strategic targets for the pharmacological prevention of MIH, as their activation can be blocked without interfering with μ receptor–mediated analgesia[103].

Our results also reveal that the spinal disinhibition underlying MIH results from BDNF-TrkB signaling[167]. Inflammatory and neuropathic pain also depend on BDNF-TrkB signaling. Yet, in inflammatory pain, BDNF arises from primary afferents[294], whereas neuropathic pain hypersensitivity depends on BDNF being produced by microglia[49,259]. In both MIH and neuropathic pain, the synthesis and release of BDNF from microglia requires P2X4R upregulation[248,259], in contrast with inflammatory pain. Thus, our results indicate that MIH shares a common etiology with neuropathic pain.

Our results establish an unexpected commonality in mechanisms between MIH, pain hypersensitivity after peripheral nerve injury[49] and the sequelae of spinal cord injury[22]. Altered Cl− homeostasis, which causes depolarizing GABA_A-mediated events, may also contribute to NMDA receptor plasticity[11,50], thereby favoring the onset of opioid-induced long-term potentiation in spinal neurons[69]. Moreover, our findings may extend to mechanisms involved in morphine-induced drug dependence in critical reward centers of the brain. For
example, BDNF causes a switch from GABA_A-mediated inhibition to excitation, suggestive of altered Cl− homeostasis, in the ventral tegmental area in opiate-dependent rats[261]. Neuronal dysfunction in the mesolimbic reward pathways is considered to be an important mechanism underlying addiction. Thus, our discovery may provide a new perspective on drug dependence, in that it may involve cross talk between microglia and neurons leading to neuronal disinhibition in key brain regions that underlie reward and addiction.

Morphine hyperalgesia can no longer be seen as an inevitable consequence of morphine analgesia or tolerance. Of particular importance to therapeutic development, we found that continuous activation of this signaling pathway is necessary to specifically maintain MIH, whereas there was no effect on tolerance or withdrawal. We found that it is possible to reverse what causes the established pain hypersensitivity, potentially alleviating opiate use liability. Notably, this can be accomplished by targeting non-classical opioid receptors or by restoring Cl− homeostasis in SDH neurons, sparing morphine analgesia mechanisms. Taken together, our findings and the recent demonstration of a distinct mechanism underlying morphine tolerance [272] overturn the traditional dogma of the common mechanism underlying both morphine tolerance and hyperalgesia and establish a basis for a new approach to enhancing the utility of morphine in treating chronic pain.
CHAPTER 5:

Modulation of opioid tolerance and hyperalgesia by ultra-low dose naloxone is independent of Toll-like receptor-4.

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5.1 INTRODUCTION

A number of pharmacological interventions are used to treat moderate to severe pain including opioids, such as morphine. Conversely, chronic administration of morphine and other opioid analgesics is limited by the development of analgesic tolerance [51] and opioid-induced hyperalgesia [139]. Morphine analgesia is produced predominantly via activation of G\textsubscript{i/o} protein-coupled \(\mu\)-opioid receptors (\(\mu\)ORs)[133,160] localized throughout the central nervous system. Within the dorsal spinal cord, \(\mu\)ORs are found on pre- and post-synaptic nociceptive neurons, but have also been identified on glial cells, including astrocytes and microglia, within the nervous system [35,67,72,73,205,233]. The function of \(\mu\)ORs on these non-neuronal cells remains elusive. Opioid tolerance and hyperalgesia are explained on the basis of augmented glial reactivity, as chronic morphine treatment leads to increased gliosis. Glial inhibitors, such as fluorocitrate and propentofylline, prevent the development of, and reverse established, opioid analgesic tolerance in animal models [193,196,228]. We reported that ultra-low dose (ULD) naltrexone attenuates the development of morphine tolerance, and this behaviour is positively correlated with inhibition of spinal gliosis [161]. It remains unknown whether ULD naltrexone modulates these behavioural effects directly, by activating glial surface receptors, or indirectly, by altering the release of
It is also unclear if ULD naltrexone blocks tolerance and inhibits morphine-induced gliosis via opioid receptors or other membrane receptors, such as Toll-like receptors (TLRs) [23,242]. TLRs are single transmembrane receptors that recognize a number of endogenous (e.g. heat shock proteins) and exogenous (e.g. lipopolysaccharides) substances that signal danger and initiate immune responses [23,100,242]. Recent evidence also suggests morphine and naloxone are respective agonists and antagonists of TLRs [100,148]. Unlike opioid receptors, which are selective for the (-) isomers of opioids, TLRs are not stereoselective and may be activated or antagonized by either (+) or (-) isomers [100,278]. One member of this receptor family, TLR4, is widely expressed in spinal microglia and macrophages [140,141]. TLR4 mRNA was also detected in astrocytes following stimulation with LPS [23,77,109,129]. Activation of glial TLR4 induces significant gliosis [1] and is implicated in opioid tolerance and hyperalgesia [15,105,242].

Recently, we reported that the opioid-receptor inactive (+)naloxone blocked chronic morphine-induced thermal hyperalgesia, but not tolerance, in the Hargreaves plantar test in rats [76]. Contrary to our findings, others report (+)naloxone potentiated morphine analgesia [105], and morphine and oxycodone analgesia were potentiated in TLR4 null mutant mice [102,271]. Therefore, the current study aimed to extend our previous findings and address these conflicting data by using transgenically mutated mice lacking functional TLR4 receptors to assess the involvement of TLR4 in the modulation of opioid tolerance, hyperalgesia, and spinal gliosis by ULD opioid antagonist enantiomers.

5.2 METHODS

5.2.1 Animals

Adult male TLR4-mutant C3H/HeJ (TLR4) and wildtype C3H/HeOuJ (WT) mice (20-25g) were obtained from Jax Laboratories (Bar Harbor, Maine, USA). Mutant TLR4 mice possess
a missense mutation (A→C) of the TLR4 gene at position 2342 of the cDNA sequence resulting in a substitution of histidine for proline, thus producing non-functional TLR4-protein [98,183]. Mice were housed 3-4 per cage with ad libitum access to food and water, and maintained on a 12/12 h light/dark cycle with lights on at 7:00 h. Animals were housed for at least 1 week prior to testing, and habituated to testing environment for 4-5 days in order to reduce stress-related analgesia. All experimental protocols were approved by the Queen's University Animal Care Committee, and complied with the policies and directives of the Canadian Council on Animal Care and the International Association for the Study of Pain.

5.2.2 Drug Treatments

Morphine was purchased from Sabex, Kingston General Hospital, Kingston, Ontario, Canada. Levo (-)naloxone and lipopolysaccharide (LPS) from Escherichia coli O111:B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextro (+)naloxone was a kind gift of the National Institute on Drug Abuse. All drugs were prepared in 0.9% sterile saline solution and administered via intraperitoneal (i.p.) injection.

5.2.3 Thermal Nociception

The effect of drug administration on thermal nociceptive responses was assessed using the tail flick assay. Briefly, the distal portion of the tail is immersed in hot water (50°C) and the latency to a vigorous tail flick was measured. Three baseline latencies were measured prior to drug injection to determine the normal nociceptive responses of the animals. A cut-off time of 10s was imposed to avoid tissue damage in the event that the animal became unresponsive following drug injection. Following drug administration, thermal latencies were measured following injection as indicated within each study. Tail-flick latencies were converted to a maximum possible effect (% MPE): (post-drug latency – baseline) ÷ (cut-off latency – baseline) x 100. All behavioural testing was performed by an experimenter blinded to drug treatment.
5.2.4 **Mechanical Withdrawal Thresholds**

The effect of drug administration on mechanical nociceptive responses was assessed by measurement of withdrawal responses to von Frey filament stimulation. Briefly, animals were placed in Plexiglas boxes on a wire grid through which a 3.61g von Frey filament was applied to the plantar surface of the hind paw. The number of paw withdrawals to 10 repeated stimulations was recorded for each animal prior to drug administration (before morning injection) or 24h post-treatment. All behavioural testing was performed by an experimenter blinded to drug treatment.

5.2.5 **Analysis of gene expression by quantitative real-time polymerase chain reaction.**

Mice were sacrificed by decapitation under light halothane anesthesia and their spinal cords were immediately removed by spinal ejection. Total RNA was isolated, using the TRIZOL method, from the dorsal lumbar spinal cord (approximately the L4/L5 level) of WT and C3H/HeJ mice from Study 2.5.3. Spectrophotometric analysis was performed using a Biotek Synergy HT plate reader to monitor RNA purity and concentration and only RNA with a 260nm/280nm ratio of 1.8 to 2.0 was considered acceptable. RNA samples were then converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad). cDNA was quantified and assessed similar to RNA samples. cDNA was combined with iQ SYBR Green mix (Bio-Rad), autoclaved water and the necessary primers according to protocol to evaluate expression of CD11b, GFAP and GAPDH. Primers were purchased from Invitrogen (San Diego, CA), with the following sequences:

CD11bF, CAG ATC AAC AAT GTG ACC GTA TGG G and CD11bR,CAT CAT GTC CTT GTA CTG CCG CTT G [202]; GFAPF, GAT CGC CAC CTA CAG GAA AT and GFAPR, GTT TCT CGG ATC TGG AGG TT [91]; mGAPDHF, TCA TGA CCA CAG TGG ATG CC and mGAPDHR, GGA GTT GCT GTT GAA GTC GC. The Bio-Rad thermocycler was programmed to perform a 5 min 95°C hot-start followed by 50 cycles of 95°C for 15 s, 65°C for 15 s and 72°C for 30 s. Annealing temperature was optimized using primer efficiency curves for all primer pairs using a 7-point 2x dilution series of cDNA pooled from control samples. All primer efficiencies
were in the acceptable range of 90-100%.

5.2.6 Study Design

5.2.6.1 Acute Morphine-induced Antinociception

The involvement of TLR4 receptors in acute morphine-induced antinociception was assessed by measurement of responses to a thermal noxious stimulus (tail flick test) in WT and TLR4-mutant mice. After determination of baseline withdrawal thresholds, mice were administered a single injection of morphine (3 mg/kg, i.p.) and tail-flick latencies were measured every 15 minutes for 1h post-injection.

5.2.6.2 Lipopolysaccharide-induced Hyperalgesia

To assess the functionality of TLR4 receptors in TLR4-mutant mice, the TLR4 agonist LPS was used to induce mechanical hyperalgesia. Paw withdrawal thresholds were measured prior to and 24h after LPS (1mg/kg, i.p.) injection in WT and TLR4-mutant mice.

5.2.6.3 Antinociceptive Tolerance to Chronic Morphine Treatment and Spinal Gliosis

The role of TLR4 receptors in both the development of analgesic tolerance to chronic morphine and the ULD opioid antagonist effect was investigated. Both WT and TLR4 mutant mice were chronically administered either morphine (10 mg/kg, i.p.), morphine with (+) or (-)naloxone (1ng/kg, i.p.), or vehicle (saline) only, once daily for five days. Thermal nociception was assessed on days 1, 3 and 5 prior to drug injection and 30 minutes post-injection by tail flick test. Mice were euthanized and spinal cord tissue collected for quantitative real-time PCR analysis to evaluate treatment effects on spinal microglia and astrocytes.

5.2.6.4 Opioid-induced Hyperalgesia

To determine the involvement of TLR4 receptors in opioid-induced hyperalgesia, WT and TLR4-mutant mice were treated with morphine (10mg/kg i.p. day 1, 20mg/kg i.p. day 2, 30mg/kg i.p. day 3, 40mg/kg i.p. days 4-7), morphine with (+) or (-)naloxone (1ng/kg i.p.), or
with vehicle (saline) only, twice daily for 7 days. Mechanical withdrawal thresholds were measured prior to the morning injection each day and 12h after the last injection.

5.2.7 Statistical Analyses

All values are expressed as the mean ± standard error of mean (s.e.m.). Statistical analyses were performed with GraphPad Prism 5 software (San Diego, CA, USA). Statistical significance for multiple group comparisons was determined by a two or three-way analysis of variance (ANOVA) followed by Tukey’s, Bonferroni’s or Dunnett’s post-hoc test to determine between group differences. QRT-PCR data was analyzed by one–way repeated measures ANOVA followed by Tukey’s post-hoc test. Effects of mouse genotype were analyzed by paired or unpaired t-tests, where appropriate. A p-value less than 0.05 was considered significant.

5.3 RESULTS

5.3.1 Acute Morphine-induced Antinociception

The acute antinociceptive responses to morphine were measured in the TLR4-mutant and WT mice (Figure 5.1a). No difference was observed in baseline nociceptive responses between genotypes. A submaximal antinociceptive dose of morphine was assessed in both genotypes in the event that morphine-induced effects were augmented in the TLR4 mutant mice. Following morphine administration (3mg/kg, i.p.), the maximal antinociceptive response occurred at 15 minutes post-injection for both TLR4-mutant (20.2% MPE) and WT (33.4% MPE) mice. Neither the peak morphine-induced antinociceptive responses nor the mean area under the curve (Figure 5.1b) differed significantly between genotypes.
Figure 5.1 No difference in acute morphine-induced antinociception between C3H/HeOuJ wildtype (WT) and C3H/HeJ TLR4-mutant mice. Animals were administered morphine (3mg/kg, i.p.) and thermal nociceptive responses were measured every 15 minutes post-injection. A. No difference in antinociception was observed between genotypes at any time point ($F_{1,18} = 0.97$, $p=0.34$). B. The mean area under the curve was not significantly different between WT and TLR4-mutant mice ($p =0.35$). Data represent means for $n = 10$ per group. Statistical analyses were performed using a two-way repeated measures ANOVA or unpaired t-test.
Figure 5.2 TLR4-mutant (C3H/HeJ) mice are insensitive to lipopolysaccharide (LPS)-induced mechanical hyperalgesia. Mechanical thresholds in WT (C3H/HeOuJ) or TLR4-mutant mice were measured prior to and 24h after LPS (1mg/kg, i.p.) injection. No difference between baseline withdrawal thresholds was observed between genotypes (t-value=0.22, df=18, p=0.83). The number of paw withdrawals significantly increased in WT mice 24h post-LPS injection (t-value=12.82, df=9, p<0.001). No difference in mechanical sensitivity was observed between baseline and 24h post-injection responses in TLR4-mutant mice (t-value=0.00, df=9, p=1.0). Data represent means for n = 10 per group. Statistical analyses were performed using a paired or unpaired t-test. The asterisk denotes a significant difference from baseline WT response. ***=p<0.001.
5.3.2 Lipopolysaccharide-induced Hyperalgesia

Baseline mechanical withdrawal thresholds did not differ between WT and TLR4-mutant mice (Figure 5.2). Following LPS (1mg/kg, i.p.) treatment, a significant increase in the number of paw withdrawals to mechanical stimulation was observed in WT mice, increasing from 2.0 ± 0.30 to 6.7 ± 0.30 withdrawals per 10 stimulations (p < 0.001). The number of withdrawals to mechanical stimulation following LPS administration was not different compared to baseline responses in the TLR4-mutant mice suggesting that mechanical sensitivity was not affected by LPS administration in the mutant mice.

5.3.3 Effects of Naloxone Enantiomers in Attenuating Antinociceptive Tolerance to Chronic Morphine

Baseline thermal nociceptive responses were measured prior to drug administration and 30 minutes post-injection for 5 days in WT (Figure 5.3A) and TLR4-mutant (Figure 5.3B) mice. No difference was observed in baseline thresholds between treatment groups or genotypes (data not shown). There was no main effect of saline (F2,14=0.64, p=0.54), nor interaction with time (F2,14=0.33, p=0.73) or strain (F1,7=3.18, p=0.118), therefore saline was removed from further analysis. The antinociceptive responses to drug treatments on day one were not different between treatment groups (Figure 5.3A,B). As expected, daily administration of morphine significantly reduced thermal antinociceptive responses by day 5 in WT controls (Figure 5.3A) compared to day 1 (41.5 vs 94.2% MPE), consistent with the onset of analgesic tolerance. Likewise, TLR4-mutant (Figure 5.3B) mice receiving morphine also exhibited decreased antinociception on day 5 compared to their initial responses on day 1 (47.6 vs 91.7 % MPE). The simple main effect of strain was examined within each level of time and treatment. A significant difference between strains was present on day 5 for MS and (-)NLX (F2,13=4.79, p=0.039), with TLR4-mutant mice exhibiting greater antinociceptive responses compared to WT. The day 5 responses of morphine-only treated mice were not different between genotypes. In WT controls, the ability of ULD
Figure 5.3 Ultra-low dose naloxone stereoselectively attenuated the loss in morphine-induced antinociception following chronic administration independent of TLR4. A. Wildtype (C3H/HeOuJ) and B. TLR4-mutant (C3H/HeJ) mice were administered morphine (MS; 10mg/kg i.p.), or MS and (-) or (+)naloxone (NLX; 1mg/kg i.p.) once daily. Thermal nociception was measured by tail flick test 30 minutes post-injection on days 1 and 5. There was no main effect of saline ($F_{2,14}=0.64$, $p=0.54$), nor interaction with time ($F_{2,14}=0.33$, $p=0.73$) or strain ($F_{1,7}=3.18$, $p=0.118$), therefore saline was removed from further analysis. A significant interaction of time with strain ($F_{2,46}=4.65$, $p=0.014$) and time with treatment ($F_{4,46}=5.86$, $p=0.001$) was revealed. Post hocs revealed that antinociception was significantly reduced in all treatment groups (except MS + (+)NLX on day 3 vs day 1 in TLR4-mutant mice (B), and MS + (-)NLX on day 5 vs day 1 in both strains (A,B)) with time ($p < 0.001$). MS-induced antinociception was significantly reduced in both WT (A) and TLR4-mutant (B) mice following chronic MS treatment. Co-treatment with (-)NLX significantly attenuated the loss in antinociception compared to MS-only treated mice in both genotypes. (+)NLX did not significantly reduce the loss of MS-induced antinociception on Day 5 compared to mice treated with MS alone in either genotype. Data represent means of $n = 4-6$ per group, with each $n$-value comprising 3-5 animals.
Statistical analyses were performed using a three-way ANOVA followed by Bonferroni post hoc test. The asterisk denotes a significant difference from the day 1 response of respective treatment. *** = p < 0.001. The hash denotes a significant difference from morphine-treated mice # = p < 0.05.
naloxone to attenuate the development of morphine-induced antinociceptive tolerance was stereoselective as co-administration of (-)naloxone (58.4% MPE) but not (+)naloxone (48.0% MPE) was significantly different compared to morphine-only treatment (41.5% MPE). In TLR4-mutant mice co-administration of (-)naloxone (76.0% MPE), but not (+)naloxone (63.2% MPE), was also able to significantly block the development of antinociceptive tolerance compared to morphine-only treatment (47.6% MPE).

5.3.4 Effects of Naloxone Enantiomers in Attenuating Chronic Morphine-Induced Gliosis

To assess the stereoselective effects of ULD naloxone and the role of TLR4 receptors in opioid-induced glial activation, the mRNA levels of microglial (CD11b) and astrocytic (GFAP) markers in the dorsal lumbar spinal cord were measured by qRT-PCR (Figure 5.4). Animals from the opioid tolerance studies (see Section 5.3.3) were used for this study. Chronic morphine treatment induced a significant increase in CD11b and GFAP mRNA expression in WT mice that was attenuated by co-administration of either (-) or (+)naloxone (Figure 5.4A,C). In TLR4-mutant mice, chronic morphine treatment did not significantly alter CD11b or GFAP mRNA expression compared to saline treated mice (Figure 5.4B,D). Co-treatment with (-) or (+)naloxone did not alter CD11b or GFAP mRNA expression in TLR4-mutant mice compared to saline controls. However, a genotype effect was evident when comparing the expression of CD11b and GFAP mRNA in saline-treated animals (Figure 5.4E,F). Hence, a significant increase in the expression of CD11b and GFAP mRNA was observed in TLR4-mutant mice compared to saline-treated WT control mice (Figure 5.4E,F).

5.3.5 Opioid-induced Hyperalgesia

Baseline mechanical withdrawal thresholds measured prior to drug treatment were not significantly different between WT (Figure 5.5A) and TLR4-mutant (Figure 5.5C) mice. No main effect of strain ($F_{1,66}=0.40, p=0.53$) was evident, and there was no interaction of strain with time ($F_{7,462}=1.18, p=0.31$) or treatment ($F_{3,66}=0.401, p=0.75$). As the responses were not different
Figure 5.4 Effect of ULD naloxone and morphine treatment on CD11b and glial fibrillary acidic protein (GFAP) mRNA expression in mouse spinal cord. Wildtype (C3H/HeOuJ) and TLR4-mutant (C3H/HeJ) mice were treated (i.p.) with vehicle (saline; SAL), morphine (MS; 10mg/kg), MS and (-)naloxone (NLX; 1ng/kg), or MS and (+)NLX (1ng/kg), once daily for five days. Total RNA was extracted from the dorsal lumbar spinal cord, converted to cDNA templates and the expression levels of CD11b and GFAP were then assessed by quantitative real-time polymerase chain reactions as described. Values for each target gene were measured in triplicate (n = 4-5), and expressed as mean percentage expression ± SEM relative to respective vehicle-treated controls (A-D) or vehicle-treated WT mice (E,F). A one-way analysis of variance (ANOVA) revealed a significant effect of treatment on CD11b expression in (A) WT (F3,11=18.28, p<0.001) and (B) TLR4-mutant mice (F3,11= 4.24, p=0.046). A significant effect of treatment on GFAP expression was revealed by 1-way ANOVA in (C) WT (F3,11=9.10, p=0.006), but not (D) TLR4 mutant mice (F3,11=0.85, p=0.50). CD11B (A) and GFAP (C) mRNA expression were significantly up-regulated in MS-treated WT mice; this increase was non-
stereoselectively blocked by naloxone. No difference was observed between MS and (-) or (+)NLX and saline-treated controls. Unpaired t-tests revealed significant differences in the expression of (E) CD11b (t=12.68, p=0.0002) and (F) GFAP (t=6.58, p=0.0028) mRNA in saline-treated TLR4-mutant mice compared to WT controls. The asterisk denotes a significant difference from morphine-treated mice. *=P<0.05, **=p<0.01, ***=p<0.001.
Figure 5.5 ULD naloxone non-stereoselectively inhibited morphine-induced hyperalgesia in C3H/HeOuJ wildtype (WT) and C3H/HeJ TLR4-mutant mice. WT (A) and TLR4-mutant (C) mice were administered treatments (i.p.) twice daily, with mechanical thresholds measured daily prior to the morning injection. The mean number of paw withdrawals per treatment group on Day 7 in WT (B) and TLR4-mutant (D) mice. No significant main effect of strain ($F_{1,66}=0.40, p=0.53$) and no interaction of strain with time ($F_{7,462}=1.18, p=0.31$) or treatment ($F_{3,66}=0.401, p=0.75$) were revealed, so data were collapsed across this factor (E,F). Post hoc analysis of the significant time x treatment interaction ($F_{21,462}=8.05, p<0.001$) revealed that morphine (MS) significantly increased mechanical hyperalgesia. Co-administration of (-) or (+)-naloxone (NLX) with MS blocked morphine-induced hyperalgesia. Data represent means for $n=7-11$ per group. Statistical
analyses were performed using a three-way ANOVA followed by Tukey’s post hoc tests. The asterisk denotes a significant difference from morphine-treated mice. ***=p<0.001. The hash tag denotes a significant difference from saline-treated mice. ##=p<0.01.
between strain, data were collapsed across this factor (Figure 5.5E,F). The number of paw withdrawals in saline-treated controls increased slightly, but significantly (p<0.001) over the study duration (Figure 5.5A,C,E). Mice chronically treated with escalating doses of morphine over 7 days exhibited increased mechanical sensitivity, as indicated by an increase in the number of paw withdrawals compared to saline-treated mice (from day 4 onward p<0.05, Figure 5.5E). Day 7 mechanical withdrawal responses (Figure 5.5F) are significantly increased in morphine-only treated mice compared to saline controls (p < 0.001). Co-administration of either ULD (-) or (+)NLX significantly attenuated the morphine-induced increase in mechanical sensitivity (p < 0.001); although responses of mice co-treated with (+)NLX were different from saline-treated mice (p < 0.05).

5.4 DISCUSSION

These studies demonstrate ULD naloxone stereoselectively attenuated morphine-induced antinociceptive tolerance and non-stereoselectively blocked opioid-induced mechanical hyperalgesia and gliosis. Furthermore, these data indicate TLR4 receptors do not directly mediate acute morphine-induced analgesia, chronic analgesic tolerance, or morphine-induced hyperalgesia, as these effects were present in both TLR4-mutant and WT mice. No differences in the thermal nociceptive responses were observed between TLR4-mutant and WT mice when administered acute or chronic morphine, and comparable mechanical hyperalgesia developed in both genotypes following chronic opioid treatment. To the best of our knowledge, this report is the first to demonstrate TLR4 receptors are not the target through which ULD opioid antagonists mediate chronic morphine-induced analgesic tolerance or morphine-induced hyperalgesia. Moreover, these data are consistent with opioid tolerance and hyperalgesia occurring as distinct phenomena mediated by independent mechanisms, as recently proposed [76,122].

The present study evaluated opioid-induced antinociceptive responses in TLR4-mutant mice in which a single point mutation within the highly conserved intracellular domain results in
non-functional TLR4 protein [98,183]. LPS, a potent TLR4 agonist that triggers gliosis and hyperalgesia [229,288], was used to confirm TLR4 receptors are non-functional in C3H/HeJ mice. LPS induced significant hyperalgesia in WT (C3H/HeOuJ) mice but TLR4-mutant mice were LPS-insensitive, consistent with this genotype having non-functional TLR4 receptors. In agreement, Hoshino et al. [98] reported responses to LPS in TLR4 knockout and C3H/HeJ mice were similar with neither genotype capable of LPS-induced signaling.

TLR4 receptors do not mediate acute morphine-induced antinociception in the hot-water tail-flick test; no difference in withdrawal latencies was observed between WT and TLR4-mutant mice. Contrary to this finding, acute analgesia induced by oxycodone and morphine was potentiated in TLR4 null (TLR4−/−) mutant mice compared to wildtype Balb/c mice in the hot-plate paw withdrawal assay [102,271]. A number of methodological differences may account for these conflicting results, including the agonist, nociceptive assay, and mouse genotype used. The behavioural assay used to determine antinociception may contribute to the different results, as specific agonist-induced antinociception is reportedly varied between specific assays, particularly between spinal (e.g. tail flick) versus supraspinally (e.g. hot plate) mediated responses [10,43,47,71,127,171,182]. Also, large variations in sensitivity to opioid-induced analgesia are reported between mouse strains [53,88,127,213,244]. The cumulative effect of these differences on acute opioid-induced analgesia is difficult to quantify: here, acute morphine-induced analgesia was not different in mice lacking functional TLR4 from WT controls.

Recently, Fukagawa and colleagues [78] reported no difference in the development of morphine tolerance in TLR4-mutant C3H/HeJ and WT C3H/HeN mice, nor between WT (C57BL/6) and TLR4−/− mice. In agreement, our results also demonstrate that morphine tolerance develops to a similar extent in TLR4-mutant and WT C3H/HeOuJ mice. It is widely recognized that spinal gliosis is associated with analgesic tolerance following chronic opioid administration, but is not observed following acute opioid exposure [194,228]. We have shown that co-
administration of ULD naltrexone inhibited glial activation, in addition to attenuating tolerance, following chronic morphine treatment [161]. It was unknown if tolerance modulation was a direct result of glial inhibition or if glial inhibition was secondary to tolerance prevention. In the present study, naloxone non-stereoselectively inhibited activation of spinal microglia and astrocytes, indicated by the attenuation of morphine-induced CD11b and GFAP mRNA (markers indicative of activated microglia and astrocytes, respectively) expression; however, these cellular effects do not correlate with the stereoselective inhibition of antinociceptive tolerance by (-)-naloxone. In agreement, Ferrini et al [76] also reported (+)-naloxone blocked morphine-induced activation of spinal microglia without preventing analgesic tolerance in rats. One potential mechanism for these observations is that ULD opioid antagonists target non-opioid glial receptors, such as TLRs, which have been shown to bind levo and dextro opioid enantiomers [100,278]. In the present study, chronic opioid tolerance still developed in TLR4-mutant mice, suggesting these receptors are not required for tolerance and are, therefore, not the target of ULD antagonist action. Furthermore, the stereoselective effect of ULD naloxone in attenuating opioid tolerance is consistent with the effects being attributed to a mechanism involving opioid receptors. Indeed, the opioid-induced increase in P2X4R and BDNF expression in microglia was also inhibited by only (-)-naloxone indicating a stereoselective effect [76]. Interestingly, morphine did not induce up-regulation of CD11b or GFAP in the spinal cord of TLR4-mutant mice compared to saline-treated controls. These data disagree with a previous report in which chronic morphine induced increased CD11b expression in TLR4−/− mice compared to controls [78]. The sizable difference in morphine dose (10mg/kg vs 60mg/kg in the Fukagawa study) and the different mouse strains employed may explain the discord. Importantly, in the present study, we report a significant increase in CD11b and GFAP mRNA expression in vehicle-treated TLR4-mutant mice compared to vehicle treated WT mice indicating a genotype effect without pharmacological treatment. These data are in agreement with increased macrophage and astrocyte
labeling in TLR4-mutant mice compared to WT mice previously reported [129]. Given the immune-compromised state of these TLR4-mutant mice, their glia may be in some state of reactivity rather than the normal resting state found in wildtype controls. If such were the case, it is possible that opioid-induced gliosis was masked by an already alerted immune system.

The involvement of TLR4 receptors in mediating ULD opioid antagonist effects on tolerance and hyperalgesia was also evaluated. ULD (-)NLX attenuated the onset of tolerance when co-administered with morphine, in agreement with previous reports [57, 145, 269]. However, the OR-inactive (+)naloxone did not significantly alter the development of tolerance, conflicting with the hypothesis that ULD antagonists mediate tolerance and opioid-induced hyperalgesia via TLR4 receptors. These data are consistent with the bimodal theory of tolerance initially proposed by Crain and Shen [55] which hypothesized chronic opioid exposure induced a switch in G-protein coupling to μ-ORs from inhibitory G_<sub>i/o</sub> proteins that induce analgesia, to excitatory G_<sub>s</sub> proteins that result in enhanced nociception and anti-analgesia. Indeed, increased G_<sub>s</sub> and decreased G_<sub>i/o</sub>-coupling to μORs following chronic morphine administration were reported in vitro [29] and in vivo in rat brain and spinal cord [29, 251, 269]. Co-administration of ULD naloxone (10ng/kg, s.c.) attenuated the morphine-induced μOR-G_<sub>s</sub> coupling and increased the G_<sub>i/o</sub>-coupling back to control levels [269]. Here, the stereoselectivity of the ULD antagonist effect on tolerance suggests an OR-dependent mechanism, such as modulation of G-protein coupling to μORs.

In contrast to the stereoselective effect of ULD naloxone on tolerance, the antagonist non-stereoselectively blocked opioid-induced hyperalgesia in TLR4-mutant and WT mice. These data are in agreement with that of Ferrini et al. [76], which reported (+)NLX blocked chronic morphine-induced thermal hyperalgesia, but not tolerance, in the Hargreaves plantar test. ULD antagonists prevented opioid-induced hyperalgesia via inhibition of morphine-induced μOR-dependent microglial P2X4R up-regulation and also by OR-independent release of BDNF from
spinal microglia. Morphine-induced release of BDNF from microglia was not blocked by the TLR4 antagonist LPS-RS, confirming our finding that TLR4 receptors do not mediate opioid-induced hyperalgesia [76]. Juni et al. [122] provide further evidence that an OR-independent mechanism underlies opioid-induced hyperalgesia, demonstrating hyperalgesia in OR triple knockout mice lacking all three opioid receptor genes when chronically infused with morphine or oxymorphone. Taken together with the results of the present study, ULD naloxone mediates opioid-induced hyperalgesia independently of TLR4 and μORs by a mechanism distinct from that of tolerance. The non-stereoselective effect suggests the ULD antagonist blocks hyperalgesia via an alternate pathway, such as inhibition of BDNF release from morphine-activated microglia.

5.5 CONCLUSION

We demonstrated that tolerance to chronic morphine treatment and opioid-induced hyperalgesia occurred in wildtype and in genetically mutated mice with non-functional TLR4 receptors, indicating these receptors are not involved in these effects. Likewise, ULD naloxone blocked the development of opioid tolerance and hyperalgesia in both mouse genotypes; thus, TLR4s do not mediate these ULD antagonist effects. Importantly, opioid tolerance was stereoselectively attenuated by (-)-naloxone suggesting an OR-mediated mechanism of action. Conversely, opioid-induced hyperalgesia was blocked by both (-) and (+)naloxone enantiomers and, therefore, mediated by a non-opioid pathway that is also independent of TLR4. Taken together, these data contribute to the growing evidence that indicates opioid tolerance and opioid-induced hyperalgesia occur via distinct mechanisms.
6.1 SUMMARY

Chronic pain is expected to become more prevalent as the baby-boomer generation ages and so too will the use of analgesics used to treat such pain. Opioids are commonly prescribed for the treatment of a variety of chronic pain conditions [9]. Chronic use of opioids is associated with analgesic tolerance and opioid-induced hyperalgesia, whereby an opioid drug initiates the genesis of a pain state that is independent of the pain for which the opioid was originally prescribed; these occurrences severely limit opioid use and compromise patient compliance. Thus, understanding the mechanisms underlying these phenomena is necessary, with the goal of identifying prophylactic treatments to mitigate these side effects. One such pharmacological treatment for chronic pain management is the combination of ULD antagonists with opioid agonists. The experiments presented within this thesis aimed to identify the mechanism by which ULD opioid antagonists modulate opioid-induced analgesia and hyperalgesia and to elucidate the role of opioid-induced spinal gliosis in these effects. The general hypothesis of this thesis was that ULD opioid antagonists attenuate opioid-induced analgesic tolerance and hyperalgesia via inhibition of opioid-induced spinal gliosis.

The activation of spinal glia is associated with a number of chronic pain pathologies (e.g. neuropathic pain) and is implicated in the development of opioid tolerance and hyperalgesia [242,253,255]. Activated spinal glia release pronociceptive chemicals that may enhance nociceptive neurotransmission and/or oppose analgesia. The vital role these cells play in the modulation of synaptic neurotransmission indicate their potential importance in mediating opioid analgesia. Others have reported glial inhibitors, including propentofylline and fluorocitrate,
prevented and reversed analgesic tolerance to chronic morphine treatment [196,228], thus, the involvement of gliosis in the development of tolerance and inhibition of morphine-induced activation of these cells by ULD antagonists was considered. More specifically, the hypothesis tested in Chapter 2 was that spinal gliosis induces an opioid-tolerant-like state in opioid-naïve animals and that reactive glia enhance the development of analgesic tolerance with chronic opioid exposure. Understanding the relationship between gliosis and analgesic tolerance has profound clinical implications and may provide valuable insight into developing novel strategies for the treatment of chronic pain. Preclinical investigations of the spinal effects of analgesics utilize indwelling polyethylene catheters to administer treatments; intrathecal catheters are capable of inducing spinal gliosis [64]. Thus, the effects of intrathecal catheterization-induced gliosis on acute and chronic morphine-induced antinociception were measured and responses were compared to those of animals that underwent a sham surgery or were surgery-naïve. Catheter-induced gliosis did not alter acute morphine analgesia, with no difference observed between treatment groups. Interestingly, the decrease in morphine-induced antinociception with repeated opioid administration was potentiated in catheterized animals. These data show pre-existing gliosis does not diminish sensitivity to opioid analgesia; however reactive glia enhance tolerance development as a consequence of their primed state of reactivity. Therefore, the hypothesis that reactive glia induce opioid-tolerance in opioid-naive animals must be rejected, but gliosis does enhance the development of analgesic tolerance with chronic opioid treatment. Others report priming isolated microglia in vitro with morphine followed by exposure to inflammatory agonists, such as lipopolysaccharide (LPS; bacterial endotoxin that induces glial activation via TLR4 found on microglia and other immune cells [96,288]), caused enhanced release of the pronociceptive cytokine TNF-α [33]. Furthermore, opioid analgesia was reduced in rats pre-treated with LPS 24h prior to receiving morphine, an effect that was blocked if fluorocitrate (a glial inhibitor) or intracerebroventricular administration of a therapeutic (antagonist) dose of
naloxone was administered prior to LPS injection [120]. These latter studies are consistent with the hypothesis that glial reactivity modulates opioid analgesia. The results presented in Chapter 2 demonstrating catheter-induced spinal gliosis does not attenuate acute opioid antinociception but exacerbates the development of analgesic tolerance to chronic opioid treatment should be considered when evaluating and interpreting pain literature, as intrathecal catheters are frequently used to study the analgesic effects of many agents, including opioids. The convenience of catheterization for the direct and repeated administration of opioid analgesics in both basic science studies and in clinical practice may be offset by the potentiated rate of tolerance development causing reduced analgesic efficacy.

The potentiation of analgesic tolerance to chronic morphine by reactive spinal glia prompted us to consider whether ULD opioid antagonists attenuate the development of analgesic tolerance by inhibiting morphine-induced gliosis. In Chapter 3, we tested the hypothesis that ULD naltrexone blocked opioid-induced spinal gliosis and attenuated the development of analgesic tolerance. Animals were administered chronic intrathecal morphine via lumbar puncture, measuring antinociceptive responses over the study duration. Activation of microglia and astrocytes in the dorsal lumbar spinal cord was assessed using immunohistochemical techniques to label and quantify expression of specific factors that are upregulated in activated glia. The null hypothesis is accepted, as we report that ULD naltrexone blocked morphine-induced activation of spinal microglia and astrocytes in addition to attenuating the development of analgesic tolerance in rats. These results are consistent with previous studies in which glial inhibitors, such as propentofylline [196] and fluorocitrate [228], blocked opioid-induced analgesic tolerance. Moreover, these data are also in agreement with other reports that ULD naloxone attenuated morphine-induced analgesic tolerance and gliosis [145,146]. These experiments provided the rationale to further investigate the cellular mechanism(s) through which
these antagonists inhibit opioid-induced gliosis and attenuate analgesic tolerance and opioid-induced hyperalgesia.

In Chapters 4 and 5, we sought to elucidate the underlying mechanism(s) that contribute to chronic morphine-induced analgesic tolerance and hyperalgesia. Novel antagonism of glial TLR4s by the OR-inactive (+) enantiomer of naloxone has been reported and may be a potential mechanism by which ULD antagonists modulate opioid analgesia. These experiments tested the null hypothesis that ULD opioid antagonists will modulate analgesic tolerance and opioid-induced hyperalgesia via a non-stereoselective mechanism and that such effects are mediated via activity at TLR4 proteins to inhibit opioid-induced gliosis. TLR4 mutant mice and (+)naloxone were employed to determine if ULD antagonists mediate tolerance and opioid-induced hyperalgesia via ORs, or an alternate pathway such as TLR4. Co-treatment with an ULD of the OR-inactive (+)naloxone did not attenuate morphine tolerance, but did block morphine-induced activation of spinal microglia. Thus, ULD antagonists modulate tolerance via an opioid-receptor mediated mechanism that occurs independently of its effects on glial reactivity. On the contrary, ULD (+)naloxone effectively blocked opioid-induced hyperalgesia when co-administered with chronic morphine in rats, indicating a non-OR pathway is involved in this effect. Subsequently, opioid-induced hyperalgesia was studied in genetically mutated mice in which TLR4s are non-functional. No difference in the baseline mechanical nociceptive thresholds was observed between TLR4-mutant and wildtype mice and following chronic morphine treatment the genotypes developed comparable degrees of opioid-induced hyperalgesia. Therefore, TLR4 receptors are not essential for the development of hyperalgesia following chronic opioid treatment and suggests that (+)naloxone is not producing its effects through this receptor system.

The final set of experiments described in this thesis is a continuation of the work presented in Chapter 4. The main objective was to further evaluate the role of TLR4 in acute morphine-induced analgesia, in the development of tolerance and hyperalgesia, and to determine
if these receptors are the target through which ULD antagonists mediate these effects. Here, we again utilized the TLR4 mutant mice to determine if acute morphine-induced analgesia was altered, and if tolerance and hyperalgesia developed in the absence of functional TLR4. To identify if TLR4 is the mechanism by which ULD naloxone mediates tolerance and opioid-induced hyperalgesia, naloxone enantiomers were co-administered with chronic morphine in TLR4-mutant and wildtype mice. Morphine-induced antinociception did not differ between TLR4-mutant and wildtype mice when administered as an acute submaximal dose. These effects were contrary to observations reported in TLR4 null (TLR4\(^{-/-}\)) mice treated with morphine [271] or oxycodone [102], in which analgesia was potentiated compared to control mice. Methodological differences likely contribute to the contradictory results, including differing measurements of nociception, differing mouse strains, genetic alterations, and agonists. Variability in nociceptive measurements to specific agonists is reported between test paradigms, and may depend on the type of nociceptive stimulus (e.g. thermal versus mechanical) and/or the site (e.g. spinal versus supraspinal) at which the effect is mediated [10,43,47,71,127,171,182]. Both studies measured thermal nociceptive responses, however, the present study utilized the tail flick test (spinal mediated responses), whereas Hargreaves’ paw-withdrawal test (supraspinally mediated effects) was employed in the other reports [102,271]. Differing sensitivities to opioid-induced analgesia between murine strains are widely reported [53,88,127,213,244], and thus, the genetic backgrounds may account in part for the conflicting responses. Furthermore, the use of different opioid agonists, such as oxycodone versus morphine, may also contribute to the discord in acute opioid-induced analgesia reported. Opioid receptors are capable of existing in multiple conformations and can form unique receptor-effector complexes dependent upon the specific agonist, known as ligand-directed signalling or biased agonism [188].

Our studies demonstrate that TLR4 receptors are not required for the induction of analgesic tolerance or hyperalgesia following chronic morphine treatment, both effects
developing to a similar extent in TLR4 deficient and wildtype mice. In agreement, others reported no difference in morphine-induced tolerance in the same TLR4-mutant mouse strain (C3H/HeJ) compared to wild type [78]. Consistent with our previously reported data in rats (Chapter 4; Ferrini et al., 2013), ULD (-)-naloxone attenuated tolerance in both mouse genotypes while (+)-naloxone had no significant effect. Therefore, TLR4 cannot be the molecular target through which ULD antagonists mediate tolerance. Likewise, TLR4 is not the mechanism by which ULD naloxone prevents opioid-induced hyperalgesia; hyperalgesia was non-stereoselectively blocked in both mouse genotypes. These data also indicate that ULD antagonists mediate tolerance via an opioid-receptor mediated mechanism, where as inhibition of opioid-induced hyperalgesia occurs by a distinct mechanism independent of opioid receptors and TLR4.

In summary, we aimed to determine if ULD of opioid antagonists attenuate analgesic tolerance and opioid-induced hyperalgesia via inhibition of spinal gliosis. We have demonstrated that analgesic tolerance and opioid-induced hyperalgesia occur through distinct mechanisms. Based on the stereoselectivity of ULD naloxone’s ability to attenuate analgesic tolerance, this effect likely occurs via an OR-mediated mechanism that is TLR4-independent. Furthermore, the non-stereoselective inhibition of morphine-induced spinal gliosis by ULD antagonists indicates glial inhibition is not the mechanism by which these antagonists prevent tolerance. However, inhibition of gliosis by ULD antagonists likely contributes to their ability to attenuate opioid-induced hyperalgesia, with this effect occurring in an OR- and TLR4-independent manner.

6.2 JUSTIFICATION OF IN VIVO TLR4 MODEL

The recent identification of a novel opioid target, TLR4, has garnered much attention and supposition regarding this receptor’s potential involvement in opioid tolerance and hyperalgesia. Agonist-induced activation of these receptors triggers activation of glia resulting in the production and release of pronociceptive molecules [1]. To evaluate the role of TLR4 in opioid-induced tolerance and hyperalgesia, we used genetically mutated mice (C3H/HeJ) that express
non-functional TLR4 [98,183]. These mice possess a single point mutation, adenosine in place of cytosine at position 2342 of the cDNA sequence, which was mapped to the *tlr4* gene on chromosome 4 and resulted in the substitution of histidine for the normal proline residue at position 712 in the highly conserved TIR domain of C3H/HeJ mice [98,183]. This mutation (*Lps-d*, with *Lps-n* representing the normal allele) resulted in significantly reduced sensitivity to LPS [183,192], and endotoxins from *E. coli* 0127:B8 and *Salmonella typhosa* 0-901 [235]. *In vitro* and *in vivo* studies confirm the TLR4 receptors expressed in these mice are non-functional. LPS-induced responses in macrophages isolated from TLR4 null (TLR4<sup>−/−</sup>) mutant and C3H/HeJ mice were similar, with neither capable of LPS-induced signaling nor activation of NF-κB [98]. *In vivo*, acute treatment with LPS failed to induce mechanical hyperalgesia in C3H/HeJ mice compared to the robust effect observed in wild type controls [27]. Similarly, we report an absence of mechanical allodynia in C3H/HeJ mice following administration of LPS (Figure 5.2). Therefore, although TLR4 receptors were not deleted in our murine model, this model is still an appropriate investigative tool to study the involvement or lack thereof of TLR4 in opioid tolerance and hyperalgesia.

### 6.3 CLINICAL RELEVANCE

Based on the numerous preclinical studies in which co-administration of ULD opioid antagonists produce enhanced analgesia with decreased side effects, including tolerance, hyperalgesia and dependence [184,217,269], clinicians are investigating the effects of combined therapy for the treatment of acute and chronic pain states in humans. Although the findings of animal studies have been predominately positive, many clinical studies have produced conflicting results. A number of randomized, double-blind placebo controlled studies have examined the benefits of combining naloxone (either low or ultra-low doses) with morphine to treat post-operative pain. Many report no change in analgesia, opioid requirements, or the frequency and/or severity of side effects with combined treatment compared to placebo [16,28,209], although a
number found a reduction in the incidence of nausea and pruritis [39,162,227]. More recently, substance abuse dependent patients with moderate chronic pain who were treated with naloxone (0.02 mg, i.v.) and buprenorphine (0.3 mg) experienced neither enhancement of analgesia nor decreased number and/or severity of side effects compared to those treated with buprenorphine alone [147].

Contrary to the discouraging clinical reports, other clinical studies report ULD naloxone reduced opioid-related side effects and decreased opioid use without affecting analgesia [80,108,262]. Positive results have also been reported regarding the use of Oxytrex, a combination of oxycodone and ULD naltrexone, in phase II and phase III clinical studies. Enhanced analgesia, decreased side effects, and reduced physical dependence and withdrawal upon cessation were observed in patients with chronic osteoarthritis or low back pain [38,275].

Several variables may contribute to the discrepancies between these studies, including the type of pain treated (e.g. acute post-operative vs. chronic osteoarthritic pain) and the dose of antagonist selected. Susceptibility to opioid analgesia differs between pain states depending on the pathophysiological mechanisms underlying the pain; analgesic efficacy to opioids is often reduced in neuropathic and idiopathic pain states compared to primary nociceptive pain [7]. Therefore, it is possible that the beneficial effects of combining ULD antagonists with agonists are most efficacious in the relief of particular types of pain. Alternatively, determining the appropriate dose of antagonist to combine with an opioid agonist in human patients has troubled many investigators. The ULD antagonist effects do not follow a typical dose response curve; very low doses potentiate opioid analgesia and higher doses antagonize analgesia. Further complicating dose determination, the efficacy of the ULD antagonist effect appears to fluctuate with sex, strain, age, pain, and agonist combination [25]. Some propose that a particular range of agonist:antagonist dose ratios may be defined, within which ULD effects are observed [262]. As a result of the conflicting outcomes reported by the clinical investigations conducted to date, use of
multiple dose ratios in future studies is warranted in order to avoid false negative results.

Several pilot studies and clinical trials are evaluating the use of low or ultra-low doses of the opioid antagonists, naloxone and naltrexone, alone for the treatment of a variety of disorders. One pilot study reported low dose naltrexone improved mental health quality of life effects, including improvement on the pain effects scale, in patients with multiple sclerosis, a debilitating autoimmune disease [58]. In another pilot study, low dose naltrexone reduced fibromyalgia symptoms and improved thermal and mechanical pain thresholds [289]. Others investigated its use to treat inflammatory disorders such as Crohn’s disease [226].

In addition to potentiating opioid analgesia and preventing analgesic tolerance and opioid-induced hyperalgesia, ULD opioid antagonists may also prevent the development of psychological and physical dependence that can occur with chronic opioid use. Indeed, ULD naltrexone blocked the aversion associated with morphine withdrawal and also blocked condition place preference to morphine, a paradigm used to evaluate a drug’s reinforcing effects and its propensity for addiction [176]. The results of this study dissociate the analgesic effects from the rewarding properties of opioid agonists, suggesting combined treatment of opioid agonists with antagonists may reduce opioid dependence and abuse. Several studies have been conducted or are currently underway to investigate the potential of ULD naloxone and naltrexone to treat a number of other addictions, including drug (e.g. methadone and heroin, amphetamines, and cocaine) dependence [4,112–114,197,264], alcohol dependence (for review, see Hillemacher, Heberlein, Muschler, Bleich, & Frieling, 2011), and to aid in smoking cessation (for review, see Berrendero, Robledo, Trigo, Martin-Garcia, & Maldonado, 2010).

6.4 POTENTIAL MECHANISMS OF ULD ANTAGONIST EFFECTS

6.4.1 Modulation of G-protein coupling to μOR

Recently, Largent-Milnes et al [137] demonstrated increased analgesic efficacy to oxycodone when it was co-administered with ULD naltrexone to nerve injured animals. Nerve
injury increased $G_s$-coupling to $\mu$ORs in the ipsilateral dorsal horn of the spinal cord, whereas chronic oxycodone treatment increased $G_s$-coupling both ipsilateral and contralateral to the nerve injury. ULD naltrexone attenuated the opioid-induced increase in $G_s$-coupling and reduced $G_s$ coupling caused by the nerve injury. Thus, the ability of ULD antagonists to increase opioid efficacy in neuropathic pain states may result from modulation of $G$-protein coupling to $\mu$OR. These data are in agreement with a previous report that ULD antagonists attenuate tolerance and prevent the morphine-induced switch in $\mu$OR coupling to $G_s$ from $G_i/o$ proteins [269]. Consistent with these data, the stereoselective nature by which ULD antagonists mediate tolerance suggest they do so via an OR-dependent mechanism.

6.4.2 Modulation of G-protein coupling to $\mu$OR by filamin A

ULD antagonists may attenuate analgesic tolerance through modulation of $G$-protein coupling to $\mu$ORs via filamin A (FLNA). Blockade of the high-affinity binding site for naloxone identified on FLNA prevented ULD naloxone from attenuating the morphine-induced $G_i/o$-to-$G_s$ switch and downstream accumulation of cAMP [268]. Moreover, the binding site was non-stereoselective for naloxone isomers, such that (+)naloxone also prevented the switch in $G$-protein coupling to $\mu$ORs induced by acute morphine [268]. The data presented within Chapter 4 and Chapter 5 of this thesis are inconsistent with this proposed mechanism of action, as (+)naloxone did not alter the development of tolerance to chronic morphine. The discordance in these results may be a result of the distinct mechanisms that underlie the development of tolerance to acute and chronic opioid exposure (Song & Zhao, 2001; reviewed by Christie, 2008); ULD opioid antagonists may mediate these effects via distinct pathways. Furthermore, the experiments by Wang and colleagues were conducted ex vivo in striatal slice cultures, whereas the present experiments examined the effects of chronic ULD (+)naloxone in vivo in an intact animal. It is also possible that ULD (+)naloxone partially attenuated tolerance development, but did not reach statistical significance due to limitations of the study design. Antinociceptive
responses of mice co-treated with MS and (+)-naloxone were intermediate between the morphine treated (tolerant) and morphine and (-)-naloxone treated group, although not significantly different from either (Figure 5.3). If the latter is true, mediation of μOR coupling via FLNA by ULD naloxone may contribute to the overall mechanism by which these antagonists attenuate tolerance. Extension of the studies by Wang and Burns has led to the development of a novel ligand, PTI-609, that is selective for the high affinity binding site on FLNA and also binds ORs via a distinct binding domain from classic opioid agonists (Burns and Wang). Preclinical experiments indicate PTI-609 shares similar analgesic efficacy to morphine with no condition place preference, thus lacking the propensity for addiction of typical opioid agonists (Burns and Wang, 2010). Further testing of this compound and investigation into the mechanism by which FLNA modulates μORs may lead to improved and more consistent analgesia compared to the currently available therapies used for the management of chronic pain.

### 6.4.3 Inhibition of opioid-induced microglial activation: BDNF release

Our experimental outcomes on modulating opioid-induced hyperalgesia compliment the mechanism proposed to underlie hyperalgesia induced by neuropathic pain (Coull et al., 2005). Microglia are capable of modulating synaptic transmission of lamina I neurons in the dorsal horn of the spinal cord [49]. Activation of microglial P2X4 receptors (P2X4R) by ATP activates these cells, stimulating increased production and release of BDNF. Subsequent activation of neuronal Trk-B receptors by BDNF down-regulates potassium chloride co-transporter-2 (KCC2) expression, a protein responsible for actively maintaining the chloride gradient, resulting in a slightly depolarized membrane potential. Consequently, in some of these cells reversal of the normally inhibitory GABA_A receptor that hyperpolarizes the cell occurs following BDNF exposure, causing enhanced excitation. This disinhibition is thought to be the reason for hyperalgesia observed following nerve injury, and may explain opioid-induced hyperalgesia and
Figure 6.1 Modulation of microglial BDNF signaling by ULD antagonists to prevent and/or reverse opioid-induced hyperalgesia. Disinhibition of lamina I neurons in the dorsal lumbar spinal cord is thought to underlie opioid-induced hyperalgesia. (1) ATP or chronic morphine (MS) induces up-regulation of P2X4 receptor expression in microglia; antagonism of P2X4 by TNP-ATP blocked increased receptor expression. (2) Increased BDNF synthesis and release occurs in reactive microglia. (3) BDNF activates Trk-B receptors on Lamina I neurons; anti-Trk-B antibody or BDNF fusion proteins (BDNF-Fc) block Trk-B activation. (4) Trk-B activation down-regulates KCC2 transporter expression, resulting in anion imbalance, neuronal disinhibition and possible reversal of GABA_A. (5) ULD (-)naloxone prevents morphine-induced P2X4 receptor up-regulation. (6) ULD (-) or (+)naloxone inhibit morphine-induced BDNF release from microglia.
tolerance. In Chapter 4, it was reported that chronic morphine induced P2X4 receptor expression, which was stereoselectively blocked by (-)naloxone but not (+)naloxone, consistent with opioid receptor-mediation. Moreover, morphine-induced BDNF release was blocked by both naloxone isomers, indicating a non-opioid receptor-mediated mechanism is involved following glial activation. Blockade of P2X4Rs or TrkB (BDNF receptor) prevented and/or reversed hyperalgesia without altering tolerance. Likewise, in a conditional knockout mouse model in which BDNF was removed from microglia, the development of opioid-induced hyperalgesia was prevented, however tolerance was again unaffected. These findings fit well with our hypothesis that ULD opioid antagonists block opioid-induced hyperalgesia via inhibition of opioid-induced glial activation, and indicate these antagonists likely do so by inhibiting BDNF-release from reactive microglia (Figure 6.1). Although our data fit well with this proposed mechanism of opioid-induced hyperalgesia and attenuation by ULD antagonists, this theory cannot explain how ULD antagonists attenuate the development of analgesic tolerance to chronic morphine.

6.5 FUTURE DIRECTIONS

The studies presented here aimed to identify whether one of the mechanisms underlying the ability of ULD antagonists to modulate the development of analgesic tolerance and hyperalgesia induced by chronic morphine treatment is via modulation of glial reactivity. Initially, it was thought that these antagonists alter tolerance and opioid-induced hyperalgesia by inhibiting opioid-induced activation of spinal glia, an occurrence present in both effects. However, based on our experiments, tolerance and opioid-induced hyperalgesia occur via distinct mechanisms and are affected by ULD antagonists acting via unique molecular pathways.

The original hypothesis is valid in the case of opioid-induced hyperalgesia, such that ULD antagonists non-stereoselectively inhibited morphine-induced microglial activation, thereby preventing and/or reversing the development of hyperalgesia. These data indicate that hyperalgesia may be mediated by both opioid receptor-dependent (e.g. P2X4R-regulation) and
opioid receptor-independent (e.g. microglial BDNF release) mechanisms. Activation of spinal microglia induced hyperalgesia in opioid-naive animals and blocking the activation of these cells effectively prevented or reversed established hyperalgesia, thereby confirming the importance of glial reactivity in opioid-induced hyperalgesia. Future work may aim to elucidate the precise target through which ULD antagonists block opioid-induced hyperalgesia. In P2X4 null (P2X4−/−) mutant mice, tolerance but not hyperalgesia developed with chronic morphine treatment; evaluation of P2X4Rs as a target for ULD antagonists would be worthwhile. Additionally, opioid-induced hyperalgesia is reported here in TLR4-mutant mice and is also present in triple opioid receptor knockout mice [122]. To date, there is no data regarding the state of glial reactivity in these models, thus future experiments may investigate 1) if opioid receptor expression is required for opioid-induced gliosis, 2) if ULD antagonists block opioid-induced hyperalgesia in opioid receptor knockout mice.

Inhibition of opioid-induced spinal gliosis is not the mechanism by which ULD antagonists attenuate analgesic tolerance to chronic morphine treatment. The precise mechanism of action is unknown, however, this series of experiments demonstrates that the antagonists mediate tolerance independently of TLR4 and likely through an opioid receptor-dependent mechanism. Future experiments may investigate the role of other possible targets of ULD antagonists, such as the scaffolding protein FLNA. To date, the role of FLNA in chronic opioid tolerance has not been studied. Determining if ULD naloxone enantiomers prevent the chronic opioid-induced switch in G-protein coupling to μOR via FLNA would be a logical experiment. Additional studies may include investigating chronic opioid-induced effects in FLNA knockout mice to establish if tolerance and/or opioid-induced hyperalgesia develop in the absence of FLNA.
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APPENDIX


Supplementary Text and Figures.
Supplementary Figure 1 Effects of the carbonic anhydrase (CA) inhibitor acetazolamide (ACTZ) on responses to prolonged activation of GABA\(_A\) receptors analysed both experimentally and by computer simulations.
Indeed, the slope conductance is holding potential. (morphine + ACTZ) Hodgkin in membrane potentials as in (cells to maintain inhibition under sustained input. (current collapses faster than the GABA - ions 1 X 10 g - KCl, (HOD) model neuron in the same conditions as in (activity). (response. Yet, anion homeostasis is activity-dependent. (d-I) Computer simulations: (d-f) Time course of ion concentrations of Cl- and HCO3 - during a response to a puff of GABA in a model neuron in control condition (d), after morphine (e) and after morphine in the presence of ACTZ (f) at a holding potential of -60 mV. Ion concentrations were calculated by:

\[
\frac{d[Cl^-]}{dt} = \frac{I_{Cl}}{F \cdot Vol} + g_{KCC}(E_K-E_{Cl})
\]

and

\[
\frac{d[HCO_3^-]}{dt} = \frac{I_{HCO_3}}{F \cdot Vol} + ca([Cl^-],-[HCO_3^-]_eq)
\]

where \( g_{KCC} \) (0-5 nS) is the level of KCC2 activity, \( E_K \) (-90 mV) is the potassium reversal potential, \( Vol \) (1X10^-10 cm^3) is the equivalent intracellular volume, \( ca \) (0-1X10^6 s^-1) is an index of CA activity and \( [HCO_3^-]_eq \) (12 mM) is the equilibrium intracellular HCO3- concentration. Inset in (d) shows the time course of the GABA_A conductance taking into account the following permeabilities:

\[
P_{Cl} = P_{Cl,mat}(1 + 0.1e^{(t-0.5t,0.2)}) \quad \text{and} \quad P_{HCO_3} = 0.25P_{Cl}.
\]

ACTZ had a limited impact on the baseline value of \([HCO_3^-]_i\), (e.g., at the onset of GABA_A response). Yet, by blocking CA activity, ACTZ compromised the stability of \([HCO_3^-]_i\), during the response to GABA, allowing activity-dependent HCO3 depletion (as seen after 1 s of GABA_A activity). (g-I) Ionic currents carried by Cl- and HCO3- during a response to a puff of GABA in the model neuron in the same conditions as in (d-f). Ionic currents were computed with the Goldman-Hodgkin-Katz (GHK) flux equation at 293 K given a maximal permeability to Cl- ions \( P_{Cl,\text{max}} = 1 \times 10^{-15} \text{ m3/s}. \) (h) In conditions of impaired Cl- extrusion, the maintenance of \([HCO_3^-]_i\), allows the HCO3- current to parallel the GABA_A conductance while the Cl- current collapses faster, yielding an inversion of the resulting GABA_A current. (i) In the presence of ACTZ, the HCO3- current collapses faster than the GABA_A conductance. The parallel collapse in Cl- and HCO3- currents prevents the development of the rebound inward GABA_A current, restoring the ability of cells to maintain inhibition under sustained input. (j-I) The above simulations with different steps in membrane potentials as in (a); measurements were made at 1 s after the onset of the GABA puff. Values of ECl, EHCO3 and EGABA were calculated from the Nernst and Goldman-Hodgkin-Katz equations for each membrane potential step in conditions of morphine + ACTZ (k). (j) The time dependent collapse in \([HCO_3^-]_i\), varies as a function of the holding potential. (k) This dependency is stronger under ACTZ application. (l) A side effect of the change in EHCO3 (and hence EGABA) as a function of time and holding potential (Vh) is an apparent change in GABA_A conductance consistent with experimental observations in (b-e).

Indeed, the slope conductance is given by \( g_{\text{slope}} = \Delta I/\Delta V_h \). Given that \( I = g_{\text{true}}(V_h - E_{\text{ion}}) \) and that \( E_{\text{ion}} \) is independent of \( V_h \), we have \( g_{\text{slope}} = g_{\text{true}} \). However, when \( E_{\text{ion}} \) depends on \( V_h \) (as in the present case), the conductance estimated from the slope between two holding potentials \( V_1 \) and \( V_2 \) is given by \( g_{\text{true}} - g_{\text{true}}(E_{\text{ion}}(V_2) - E_{\text{ion}}(V_1))(V_2 - V_1)^{-1} \), underestimating the true conductance.
Supplementary Figure 2. Expression of μ opioid receptor in spinal microglia. (a). Spinal microglia are double immunolabelled for the microglial marker CD11b (in blue). (b). Spinal μ-receptors are expressed in spinal neurons (e.g., star) as well as in identified microglial cells (e.g., arrows). (c) Merged fluorescent image. The insets show an enlargement of one of the doubled labeled microglial cells (arrows). Scale bar: 50 μm in the picture and 25 μm in the inset.
Supplementary Figure 3. Activated microglia has a differential effect when injected dorsally or ventrally to the spinal cord. Paw withdrawal threshold (PWT, g) to mechanical stimuli before and after injection of activated microglia via intrathecal catheter. Data were separated based on whether the intrathecal catheter was found dorsal (n = 6) or ventral (n = 6) to the spinal cord after post-mortem examination.
Supplementary Figure 4. Full length western blots. a-b. Expression of KCC2 in the dorsal and ventral horn with and without betamercaptoethanol shown in Figure 3b,c. c. Expression of P2X4R in microglia cultures after morphine treatment shown in Figure 5i. d. Expression of P2X4R from spinal cords isolated from rats treated for 5 days with intrathecal saline, morphine, morphine-(−)NL, morphine+(+)NL shown in Figure 8h.