INJURY ESTABLISHES CONSTITUTIVE µ-OPIOID RECEPTOR ACTIVITY LEADING TO LASTING ENDOGENOUS ANALGESIA AND DEPENDENCE

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INJURY ESTABLISHES CONSTITUTIVE µ-OPIOID RECEPTOR ACTIVITY LEADING TO LASTING ENDOGENOUS ANALGESIA AND DEPENDENCE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Gregory Franklin Corder
Lexington, Kentucky, U.S.A.

Director: Dr. Bradley K. Taylor
Lexington, KY
2013

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INJURY ESTABLISHES CONSTITUTIVE µ-OPIOID RECEPTOR ACTIVITY LEADING TO LASTING ENDOGENOUS ANALGESIA AND DEPENDENCE

Injury causes increased pain sensation in humans and animals but the mechanisms underlying the emergence of persistent pathological pain states, which arise in the absence of on-going physical damage, are unclear. Therefore, elucidating the physiological regulation of such intractable pain is of exceptional biomedical importance. It is well known that endogenous activation of µ-opioid receptors (MORs) provides relief from acute pain but the consequences of prolonged endogenous opioidergic signaling have not been considered. Here we test the hypothesis that the intrinsic mechanisms of MOR signaling promote pathological sensitization of pain circuits in the spinal cord. We found that tissue inflammation produces agonist-independent MOR signaling in the dorsal horn of the spinal cord, which tonically represses hyperalgesia for months, even after complete recovery from injury and re-established normal pain thresholds. Disruption of this constitutive activity with MOR inverse agonists reinstated pain and precipitated cellular, somatic and aversive signs of physical withdrawal. This phenomenon required N-methyl-D-aspartate receptor activation of calcium-sensitive adenylyl cyclase type 1. Thus, we present a novel mechanism of long-lasting opioid analgesia that regulates the transition from acute to chronic pain while, in parallel, generates physical dependence. In conclusion we propose that the prevalence of chronic pain syndromes may result from a failure in constitutive signaling of spinal MORs and a loss of endogenous analgesic control.

KEYWORDS: pain, NMDA receptor, adenylyl cyclase, allostasis, inflammation
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1.1 Introduction and Overall hypothesis

Maladaptive plasticity within the central nervous system (CNS) contributes to persistent pain states (Latremoliere and Woolf, 2009). Cellular models of chronic pain include facilitatory mechanisms such as long-term potentiation (LTP) of synaptic strength in dorsal horn neurons (Ikeda et al., 2006; Ruscheweyh et al., 2011) and opposing mechanisms driven by µ-opioid receptors (MORs) expressed on primary afferent terminals and spinal dorsal horn neurons that reduce neurotransmission (Heinke et al., 2011). Exogenous administrations of opiates such as morphine provide powerful pain relief in part by action in the superficial dorsal horn. Similarly, endogenous MOR signaling, driven by endorphin and enkephalin peptide release is recruited following tissue or nerve injury to reduce acute pain (Basbaum and Fields, 1984; Ossipov et al., 2010). MOR-mediated dissociation of inhibitory Gαi-βγ complexes reduces neurotransmitter release (Heinke et al., 2011) and neuronal excitability (Yoshimura and North, 1983) by several mechanisms, including modulation of inward-rectifying potassium channels (KIR) and voltage-dependent calcium channels (VDCC), as well as inhibition of adenylyl cyclase-mediated (AC) production of adenosine 3′,5′-cyclic monophosphate (cAMP).

Chronic administration of exogenous opiates leads to the development of compensatory neuroadaptations underlying opiate tolerance and dependence (Christie, 2008), including the selective upregulation of the calcium-sensitive AC isoforms, type 1
and type 8 (AC1 and AC8) (Avidor-Reiss et al., 1997; Lane-Ladd et al., 1997). Thus, cessation of opiates leads to cellular and behavioral symptoms of withdrawal including cAMP generation, synaptic plasticity, aversion and induces NMDA-Receptor (NMDA-R)-dependent LTP at C fiber synapses in the spinal cord to precipitate hyperalgesia (Nestler and Aghajanian, 1997; Angst and Clark, 2006; Kauer and Malenka, 2007; Drdla et al., 2009; Heinl et al., 2011). An intriguing hypothesis of drug addiction suggests that chronic opiates increase MOR constitutive activity (MOR\textsubscript{CA}) to preserve physical and psychological dependence (Wang et al., 1994; Liu and Prather, 2001; Wang et al., 2004; Shoblock and Maidment, 2006; Meye et al., 2012), which is further enhanced by enkephalin peptide release (Shoblock and Maidment, 2007). Whether MORs adopt constitutive signaling states in other disease syndromes, such as chronic pain, remains untested. Indeed, it is unknown whether endogenous opioidergic mechanisms can induce physical dependence. Furthermore, while exogenously applied opioids prevent (Terman et al., 2001; Benrath et al., 2004) and/or erase (Drdla-Schutting et al., 2012) spinal LTP, the ability of the endogenous opioid system to persistently repress pathological pain is unclear.

This dissertation tests the hypothesis that injury increases the proportion of MOR\textsubscript{CA} in the spinal cord to halt the transition from acute to chronic pain. We further reasoned that, with time after injury, tonic MOR\textsubscript{CA} would produce an endogenous cellular and physical dependence in the CNS, thereby creating an increased susceptibility to develop chronic pain.

1.2 General background

1.2.1. Pain: physiologic utility and chronic pathology

"Particles of heat" (A) activate a spot of skin (B) attached by a fine thread (cc) to a valve in the brain (de) where this activity opens the valve, allowing the animal spirits to flow
from a cavity (F) into the muscles that then flinch from the stimulus, turn the head and eyes toward the affected body part, and move the hand and turn the body protectively.”

- Descartes, Treatise of Man (1664)

René Descartes wrote that pain was transmitted directly from the periphery to the brain by a single, direct, dedicated pain pathway (Figure 1.1). His dualistic Cartesian mind-and-body model maintained the physical body as a mechanical system governed by a soul (mind). Today we recognize this tract as the nociceptive pathway, part of the larger somatosensory system, which integrates with higher-order brain circuits to create the experience of pain. In this modern materialist view, physical states influence mental states and vice versa. However, the transmission of nociceptive signals undergoes extensive modulation at various anatomical locations throughout the nervous system prior to cortical processing and conscious perception.
Figure 1.1. Illustration of “pain pathways” by Rene Descartes from Treatise of Man.
The dorsal horn of the spinal cord is a key site for modulation of noxious information. Thus, the relationship between detection of noxious stimuli at the periphery and pain perception in the brain is far from linear, but integrative and modulatory. According to the International Association for the Study of Pain (IASP), nociception is the “neural processes of encoding and processing noxious stimuli”; whereas pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. This definition expounds that pain involves both physical and psychological components. These sensory and affective qualities are mediated by the somatosensory and limbic systems, respectively. The sensory-reflexive component in humans and animals can be assessed utilizing reflexive tests (e.g. von Frey filaments, Hargreaves’s thermal assay, etc.). The response to an injury is determined by measuring the hypersensitivity of the limb to withdrawal from the sensory stimuli. While the aversive-spontaneous component is determined by patient self-reporting or motivation to seek pain relief in animals (i.e. Conditioned Place Preference for analgesics). Both aspects of pain (sensory and emotional) need further consideration, from the basic and clinical sciences, to develop effective therapeutics.

Acute or nociceptive pain is an evolutionarily conserved defense mechanism which protects an organism from exacerbating injuries by potentially harmful and damaging stimuli. Nociceptors, specialized sensory neurons, detect such threatening stimuli and project to the central nervous system so that an appropriate behavioral response can be made (e.g. withdrawal of a limb from a hot surface). Without such a protective mechanism, rampant injuries would reduce survival and quality of life. This is exemplified in rare human cases by individuals with genetic defects in nociceptor receptors/channels that preclude the detection of nociceptive signals, leading to extensive bodily damage, infection and shortened lifespan (Indo et al., 1996; Cox et al., 2006). Alternatively, pain may be associated with a specific underlying disease or injury
and can be a sign of illness that provokes appropriate measures such as rest or medication to be taken. Thus, pain is typically considered a “symptom of something”.

It is when the hypersensitivity of the nociceptive system does not remit with the healing or recovery of injury or illness that pain no longer serves a physiologic purpose, becoming a primary clinical problem itself. The IASP defines pain persisting for periods longer than four months as *chronic pain*. Here, chronic pain results from a dysfunction of the somatosensory system or limbic circuits that feed into the somatosensory system, brought about by inflammation or neuropathy for example, and are regarded as a pathological disease state.

Comorbidities such as depression, anxiety and sleep disorders are common. Chronic pain also reduces the ability of patients to socialize and maintain relationships and can have a significant negative impact on employment, exacerbating economic burden on businesses and state-run healthcare programs. The needs of chronic pain patients to date are largely unmet with a lack of good therapeutic drugs as well as a lack of recognition by physicians that their pain is more than a “symptom of something.” Thus, a greater appreciation for chronic pain as a pathological disease entity, improved diagnostic procedures and the development of novel pharmacological treatments are desperately required to combat the clinical problem of chronic pain.

While novel therapeutics are not directly investigated in this dissertation, a major goal of this research is to present several new targets for future therapies. Development of novel treatments will be aided by a greater understanding of the pathological mechanisms which convert acute pain to chronic pain and the endogenous counter-adaptations that attempt to restore normal signaling within the nociceptive circuitry.

1.2.2. Anatomy of nociceptive circuitry: systems and cellular level

1.2.2.1 Peripheral sensory afferents
The sensory experience begins in the peripheral nervous system, where the distal terminals of primary afferent fibers respond to a myriad of stimuli and translate this information through electrical signals into the dorsal horn of the spinal cord, where the central ends of these fibers terminate. There are three main types of sensory neuron fibers in the peripheral nervous system, Aβ-fibers, Aδ-fibers and C-fibers, each possessing different properties allowing them to respond to and transmit different types of sensory information. Aβ-fibers are large in diameter and highly myelinated, allowing rapid action potential conduction (30-80 m/s). These fibers have low activation thresholds and normally respond to light touch and joint movement and are responsible for conveying tactile information. Aδ-fibers are smaller in diameter and thinly myelinated; possess higher activation thresholds with a slower conduction velocity of 6-30 m/s. They respond to both thermal and mechanical stimuli. C-fibers have the smallest axonal diameter and are unmyelinated, thus making them the slowest conducting, at 0.5-2 m/s. They have the highest thresholds for activation and therefore detect selectively nociceptive or “painful” stimuli. There are two main classes of C-fibers, peptidergic and non-peptidergic. Collectively both Aδ-fibers and C-fibers can be termed as nociceptors or “pain fibers”, responding to noxious mechanical, thermal or chemical stimuli. It is assumed that Aδ-fiber nociceptors mediate “fast” pain which can be described as well-localized, acute, sharp pain, while C-fiber nociceptors mediate “secondary pain” which is delayed, more diffuse and dull (Basbaum et al., 2009).

1.2.2.2 Central innervation and signal transduction

The central terminals of primary afferent fibers enter the spinal cord via the dorsal root entry zones and terminate in specific regions of the dorsal horn, synapsing with second order dorsal horn sensory neurons. For the sciatic nerve, there are three distinct branches (tibial, common peroneal and sural nerves) which retain somatotopic
organization in the central innervation zones (Corder et al., 2010). All sensory fibers release glutamate, the predominant excitatory neurotransmitter in the central nervous system (CNS), to facilitate excitatory nociceptive transmissions. Peptidergic C-fibers as well as some Aδ-fibers release calcitonin gene related peptide (CGRP). In addition, peptidergic C-fibers may also release substance P, brain-derived neurotropic factor (BDNF), somatostatin and galanin (Ju et al., 1987). Action potentials arriving at the central terminal activate the vesicle-release machinery to induce neurotransmitter release, a process dependent on Ca\(^{2+}\) influx into the afferent terminal mediated by N-, P/Q- and T-type voltage-gated Ca\(^{2+}\) channels. Interestingly, the central terminals of primary afferent fibers closely resemble their peripheral terminals in that they express similar receptors and channels (e.g. TRPV1, TRPA1, P2X3 and BK2 receptors, etc.) (Snider and McMahon, 1998; Woolf and Ma, 2007). It is believed that activation of these pre-synaptic receptors in the dorsal horn acts to increase excitatory neurotransmitter release. In contrast, inhibitory signaling mediated by opioid receptors, γ-amino butyric acid (GABA) receptors, Neuropeptide tyrosine (NPY) receptors and cannabinoid (CB) receptors act to counter neurotransmitter release (discussed in greater detail below).

1.2.2.3 Anatomical arrangement of the dorsal horn of the spinal cord

Within the grey matter of the spinal cord, intrinsic spinal neurons can be anatomically organized into diverse laminae based on innervation and projections. In the early 1950s, Rexed first divided the spinal cord grey matter of the cat into a series of parallel laminae, numbered I to X, providing a uniform description of the arrangement of the spinal cord regardless of the particular spinal segment under investigation (Rexed, 1954). This scheme has since been applied to other species, including rodents. Together, laminae I and II are often described as the superficial dorsal horn or the substantia gelatinosa, and this is the main synaptic region for terminating nociceptive primary afferents. The remainder of the dorsal horn comprises four further laminae (III-
VI), with laminae V-VI often collectively referred to as the deep dorsal horn. Laminae VII-IX form the ventral horn of the spinal cord, while lamina X, surrounds the central canal.

1.2.2.4 Anatomy of ascending projections from the dorsal horn to the brain

The output from the dorsal horn to higher centers in the brain is carried by spinal projection neurons along ascending pathways. A large population of projection neurons are found superficially in lamina I. The majority of these neurons have axons which cross the midline and ascend in the contralateral white matter (Todd, 2002). It is estimated that 80% of these cells express the neurokinin 1 (NK1) receptor for substance P (Todd, 2002; Mantyh et al., 1997; Mantyh and Hunt, 2004). NK1+ cells in lamina I project to brain structures such as the thalamus, the periaqueductal grey (PAG) and brainstem structures such as the caudal ventrolateral medulla (CVLM) and the parabrachial nucleus (PBN). Projections to the thalamus are relayed to cortical regions of the brain, including the primary somatosensory cortex (S1), whereas the projections to the PBN subsequently reach limbic structures such as the amygdala and hypothalamus, thus contributing to the affective component of pain (Todd, 2002). From the PBN, signals are also transmitted to other brainstem areas such as the PAG and the rostroventromedial medulla (RVM), a region which has descending projections back to the dorsal horn. Therefore, lamina I NK1-expressing cells can modulate spinal processing by activation of descending pathways from the brainstem, thus forming a spino-bulbo-spinal loop. These descending pathways can be influenced by limbic regions in the brain and so can be altered by mood and attention.

Projection neurons contained in the deeper lamina of the dorsal horn, scattered throughout lamina III to VI, project predominantly to the thalamus, thereby making up a significant proportion of the spinothalamic tract. This ascending pathway carries primarily sensory information and so provides the sensory discriminative component of the pain experience. Many of these deep projection neurons have dendrites extending towards the
to the superficial dorsal horn, thus allowing them to make synaptic contacts with terminating C-fibers and receive noxious inputs (Woof and King, 1987; Todd, 2002).

1.2.2.5 Anatomy of descending projections from the brain to the dorsal horn

Descending pathways from supraspinal structures are able to influence nociceptive transmission in the dorsal horn of the spinal cord. Such descending influences may be either excitatory (descending facilitation) or inhibitory (descending inhibition) in nature and may equally engage primary afferent terminals or intrinsic dorsal horn cells, both interneurons and projection neurons. In this way, the brain influences the final output from the dorsal horn to cortical regions of the brain where pain is perceived. Several key supraspinal structures have been identified which can directly or indirectly modulate the dorsal horn, including the PAG in the midbrain and brainstem nuclei such as the PBN, RVM and locus coeruleus (LC). Such descending modulatory pathways access the spinal cord via the dorsolateral and ventrolateral funiculi. In addition, many of the structures implicated in descending modulation can exert both facilitatory and inhibitory influences in the dorsal horn, dependent on the context of the situation. Early studies demonstrated that electrical stimulation of the PAG could produce analgesia in the dorsal horn (Reynolds, 1969; Basbaum and Fields, 1984). However, direct projections from the PAG to the cord are few in number. Rather, the PAG projects to and modulates the output of brainstem nuclei and in this indirect way alters nociceptive processing in the dorsal horn. Similar connectivity exists from the PBN to the dorsal horn and these projections predominantly suppress responses of superficial dorsal horn neurons to both innocuous and noxious inputs (Millan, 2002). Numerous transmitters may be released by PAG and PBN projection neurons including substance P, cholecystokinin (CCK), GABA and endogenous opioids (Millan, 2002). The RVM in the brainstem receives the majority of its sensory inputs relayed through the PAG and PBN, and in turn, sends axons directly to both superficial and deep dorsal horn laminae.
(Millan, 2002). Initial work suggested that the descending influence from the RVM is inhibitory since electrical stimulation in this region produces analgesia via profound inhibition of noxious inputs to superficial and deep dorsal horn neurons (Basbaum et al., 1976; Fields et al., 1976; Fields et al., 1977). More recent work, however, has demonstrated that descending facilitatory pathways also originate from the RVM, particularly in chronic pain states (Burgess et al., 2002; Bee and Dickenson, 2007). This bidirectional control of spinal processing is mediated by two distinct populations of RVM cells. ON cells produce a burst of firing in response to peripheral noxious stimulation and are inhibited by endogenous opioids, thus they are believed to trigger descending facilitation. In contrast, OFF cells are inhibited by noxious inputs since they display a transient disruption in their firing immediately prior to a nociceptive reflex and they are disinhibited by endogenous opioids, thus they are implicated in descending inhibition (Fields et al., 1983; Heinricher et al., 1992; Bannister et al., 2009).

As illustrated in Figure 1.2, the integration of nociceptive signals through the peripheral, spinal, and supraspinal circuitries is required for the expression and perception of pain.
Axon terminals of primary afferent nociceptors transmit noxious signals to intrinsic projection neurons in the spinal cord dorsal horn. The ascending nociceptive fibers relay the information to several supraspinal nuclei for further processing. Projections to the thalamus relay signals to the somatosensory cortex providing information about location and intensity of the stimulus; projections to the parabrachial nucleus relay signals to the amygdala and other limbic circuits for valuation of pain affect; projections to the brainstem (e.g. periaqueductal gray and rostral ventral medulla) engage descending feedback systems that modulate spinal cord nociceptive processing. Adapted from Basbaum et al. Cell. 2009.
1.2.3. Spinal cord nociceptive plasticity

The spinal cord is an important relay site at which various incoming sensory and nociceptive signals undergo convergence and modulation. Spinal neurons are under ongoing control by peripheral inputs, interneurons and descending controls. One consequence of this modulation is that the relationship between stimulus and response to pain is highly plastic. The response of projection neurons is greatly altered following a peripheral afferent barrage, as produced by electrical stimulation, inflammation or nerve damage, via the influence of various neurotransmitter systems in the spinal cord, which are similarly subject to plasticity, particularly during pathological conditions. This prolonged pre-synaptic input can lead to spinal nociceptive plasticity such as long-term potentiation (LTP) and central sensitization of dorsal horn neurons. Central to the induction of these events is stimulation of dorsal horn glutamatergic NMDA receptors which can promote activation of intracellular signaling pathways that contribute to such pronociceptive plasticity.

Under pathological sensitization, neuronal responses to afferent inputs increase, as does receptive field size (also known as secondary pain), while spontaneous action potential firing is induced or enhanced. Together, these mechanisms of plasticity contribute to the establishment of chronic pain states which manifest clinically in patients as a heightened response to painful stimuli (hyperalgesia) and pain resulting from normally non-painful stimuli (allodynia), as well as spontaneous, ongoing pain in the absence of an evoking stimulus.

1.2.3.1. Central sensitization of dorsal horn neurons

According to the IASP, central sensitization corresponds to an enhancement in the functional status of neurons and circuits in nociceptive pathways throughout the neuraxis caused by increases in membrane excitability, synaptic efficacy, or a reduced
inhibition (Latremoliere and Woolf, 2009). The net effect is that previously subthreshold synaptic inputs generate an increased action potential output, a state of facilitation, potentiation, or amplification.

The demonstration of central sensitization in dorsal horn neurons provided the first evidence of a central component of nociceptive hypersensitivity in chronic pain (Woolf, 1983). In this early study, the responses from α-motor neuron efferents were recorded as a measure of the output of dorsal horn processing, while stimulating afferents both at the hindpaw and directly via the sural nerve. Prior to peripheral injury, the majority of efferents exhibited minimal spontaneous activity and responded exclusively to noxious, high threshold mechanical and thermal stimulation at the hindpaw. A thermal injury was then induced at the hindpaw (75°C for 60 s) which caused mild inflammation and produced significant changes in efferent properties. Spontaneous activity increased and mechanical thresholds to von Frey hairs were markedly reduced. The amplitude and duration of responses evoked by both noxious pinch and electrical stimulation of the sural nerve, at noxious intensities, were also increased. Interestingly, electrical stimulation of Aβ-fibers began to elicit responses in efferents which had been absent prior to peripheral injury indicating that dorsal horn neurons in the pathway had reduced thresholds. Furthermore, the cutaneous receptive fields expanded greatly and even extended to the contralateral hindpaw, providing definitive evidence that this sensitization could not be solely mediated by peripheral sensitization. Subsequent studies demonstrated that, in addition to peripheral thermal injury and electrical simulation, central sensitization of dorsal horn neurons could also be induced by peripheral injection of chemicals such as formalin (Dickenson and Sullivan, 1991), and capsaicin (Drdla-Schutting et al., 2012). Furthermore, it was demonstrated that central sensitization is an NMDA-dependent phenomenon and that activity of NMDA receptors
is required for both the induction and maintenance of central sensitization (Latremoliere and Woolf, 2009).

In this dissertation, we hypothesize that NMDA-R-dependent sensitization, within central nociceptive circuits, may underscore the mechanisms driving the emergence of pathological pain states. Thus, in Chapter 3, we will investigate the interactions of the endogenous opioidergic systems, with the spinal NMDA-R pathways, as they pertain to long-lived neural facilitation and sensitization (see Figure 3.4 for schematic diagram of signaling pathways involved in opioidergic masking of latent central sensitization).

It is important to note that plastic changes induced by peripheral injuries have been demonstrated in neurons at supraspinal sites, representative of the general phenomenon of central sensitization (Lee et al., 2008; Latremoliere and Woolf, 2009). In this dissertation, use of the term central sensitization is restricted to the spinal cord and specifically to central sensitization of dorsal horn sensory neurons. A multitude of studies have now demonstrated that central sensitization within the dorsal horn involves numerous mechanisms which work in concert to increase the excitability of spinal sensory neurons in chronic pain states. These mechanisms include signaling pathways which increase the intrinsic excitability of dorsal horn neurons, diminished spinal inhibition and increased facilitatory influences from supraspinal sites.

Downstream of NMDA receptor activation, various intracellular signaling cascades are activated, often via Ca\(^{2+}\) influx through the NMDA receptor channel, which promote the increased excitability of dorsal horn neurons, contributing to central sensitization. Such intracellular effectors include the extracellular signal regulated kinase (ERK), Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII), Ca\(^{2+}\)-sensitive adenylyl cyclases, PKA, PKC and the tyrosine kinase Src (Ji et al., 1999; Fang et al., 2002; Zou et al., 2002 Kawasaki et al., 2004; Kohno et al., 2008). These kinases can enhance activity of both AMPA and NMDA receptors during early-onset central sensitization by phosphorylating
key regulatory sites, as well as increasing receptor numbers by promoting membrane trafficking and insertion, thus increasing synaptic strength (Fang et al., 2003; Brenner et al., 2004). Late-onset central sensitization, like late-phase LTP, requires transcriptional changes driven by transcription factors such as the cAMP response element binding protein (CREB), which is phosphorylated during central sensitization (Ji and Rupp, 1997). This phosphorylation may be regulated by ERK and contributes to expression of genes such as c-Fos, a marker of neuronal activation following noxious stimulation (Hunt et al., 1987).

As we will propose in Chapter 3, that NMDA-R-dependent signaling is initiated upon the abrupt loss of endogenous opioid receptor signaling, we will also investigate the downstream cascades involved in persistent central sensitization. One hypothesis we will test is that specific components (e.g. adenylyl cyclase type 1) of excitatory cascades are upregulated or “primed” by injury. These primed pathways, when activated via disinhibition from the loss of opioidergic-mediated negative regulation, may trigger de novo LTP-like mechanisms to enhance synaptic strength in the spinal nociceptive circuitry (Figure 3.4).

1.2.3.2. Spinal long-term potentiation

LTP, first demonstrated in the hippocampus, is a ubiquitous mechanism throughout the central nervous system underlying a long-lasting localized increase in synaptic strength (Bliss and Lomo, 1973). Hippocampal LTP is believed to be the neuronal substrate of learning and memory (Bliss and Collingridge, 1993) and is dependent on activation of NMDA receptors, since NMDA antagonism prevents LTP without effect on normal synaptic transmission (Collingridge et al., 1983). Interestingly, spinal LTP related phenomena have also been reported in several animal pain models following both inflammation or nerve damage, and recent studies have demonstrated
that LTP can be induced in spinal and brain nociceptive pathways (Ji et al., 2003; Sandkuhler, 2007). As a result, it has become clear that similarities and probably common intracellular signaling pathways between central sensitization in the spinal cord and LTP in the hippocampus exist. The generation of spinal LTP may be one mechanism whereby acute pain is converted to chronic pain.

LTP could be induced in the superficial dorsal horn by high and low frequency tetanic electrical stimulation at C-fiber intensity (100 Hz) of dorsal roots using an in vitro spinal cord slice preparation (Randic et al., 1993; Ikeda et al., 2006), as well as in vivo by recording superficial C-fiber evoked field potentials (Liu and Sandkuhler, 1995). Natural stimuli, such as noxious heat and pinch applied to the skin, formalin or capsaicin injection into the hindpaw and acute physical injury to peripheral nerve also generate spinal LTP (Sandkuhler and Liu, 1998). Thus, spinal LTP was suggested to be a mechanism underlying afferent-induced hyperalgesia. This LTP in the superficial dorsal horn induced by both electrical HFS and natural noxious stimuli was blocked by NMDA antagonism.

Signal transduction pathways involved in spinal LTP are similar to those reported for hippocampal LTP. Specifically, inhibitors of calcium/calmodulin-dependent protein kinase II (CaMKII), PKA, PKC and PLC all have been shown to prevent induction of spinal LTP. Activation (phosphorylation) of mitogen-activated protein kinases (MAPKs) under different persistent pain conditions is involved in the induction and maintenance of pain hypersensitivity (Xin et al., 2006). In particular, nociceptive activity induces phosphorylation of spinal extracellular signal-regulated kinase (ERK) via multiple neurotransmitter receptors. Activated ERK, using different second messenger pathways, regulates the activity of glutamate receptors and potassium channels and induces gene transcription (Ji et al., 2009), and is therefore positioned to participate in both LTP induction and maintenance.
Recently, it was discovered that LTP at C-fiber synapses in the superficial dorsal horn of the spinal cord is induced during exogenous opioid withdrawal (Drdla et al., 2009), suggesting that LTP contributes to the hyperalgesia associated with opioid withdrawal (Angst and Clark, 2006). This novel form of LTP is induced postsynaptically, sharing mechanisms with stimulation-induced LTP, as it is abolished by preventing postsynaptic Ca\textsuperscript{2+} rise and by blocking postsynaptic G-protein coupled receptors or postsynaptic NMDA receptors. Although not yet tested it is possible that opioid-withdrawal LTP would exacerbate preexisting hyperalgesia or spontaneous pain by further strengthening nociceptive synaptic facilitation.

The research presented in this dissertation begins to investigate these open questions, as potential mechanisms for the emergence of chronic pain. 1. Does loss of tonic endogenous opioidergic signaling result in spinal NMDA-R-dependent LTP? 2. Is endogenous opioid withdrawal-LTP a causative mechanism in the development of chronic pain?

1.2.4. Loss of spinal inhibition contributes to mechanical hypersensitivity and allodynia

Low threshold inputs from Aβ-fibers contribute to an inhibitory control of lamina I neurons, decreasing spinal output of nociceptive information. This is the basis of the Gate Control Theory, which postulates that inhibitory interneurons within the spinal cord dorsal horn “gate” the flow of nociceptive information that is transmitted up to the brain (Melzack and Wall, 1965). Additionally, it has been suggested that low threshold inputs may activate inhibitory interneurons in the dorsal horn which prevent Aβ-fiber polysynaptic inputs reaching lamina I from deeper lamina (Torsney and MacDermott, 2006). Blockade of spinal inhibition, through the intrathecal administration of GABA receptor (bicuculline) or glycine receptor (strychnine) antagonists, resulted in pain evoked by light tactile stimulation, equivalent to mechanical allodynia observed in pain
patients (Yaksh, 1989). Therefore it was suggested that loss of spinal GABAergic and
glycinergic inhibition might underlie the clinical manifestation of mechanical allodynia in
chronic pain states. In the absence of spinal inhibition, a multi-synaptic pathway
involving polysynaptic, NMDA-dependent input from Aβ-fibers in lamina III is disinhibited
and can now activate lamina I projection neurons, effectively converting them to wide
dynamic range (WDR) neurons (Torsney and MacDermott, 2006). In this way, low
threshold stimulation may be processed and transmitted to higher centers to be
interpreted as painful. Spinal administration of strychnine alone produces behavioral
mechanical hypersensitivity (Yaksh, 1989) as well as enhanced neuronal
responsiveness to light mechanical stimuli (Sorkin and Puig, 1996). This neuronal
hypersensitivity is selectively blocked by NMDA antagonists (Sorkin and Puig, 1996).

Thus, it is likely that any reduction in spinal inhibition results from an increased
inhibitory or reduced excitatory control of inhibitory interneurons. Alternatively, Coull and
colleagues proposed that failure of GABAergic inhibitory control of low threshold inputs
in the superficial dorsal horn of neuropathic rats might arise from a loss of the Cl-
transporter protein KCC2 (Coull et al., 2003; Coull et al., 2005). This results in
accumulation of intracellular Cl- in lamina I cells which, when activated by GABA
released from inhibitory interneurons, are then depolarized rather than hyperpolarized
due to Cl- efflux, thus effectively making GABA excitatory. This mechanism would
contribute to the unmasking of a normally silent polysynaptic Aβ-fiber input to lamina I
cells, so that these cells can respond to innocuous mechanical stimulation, leading to
behavioral static mechanical hypersensitivity following nerve injury.

This logical line of thought, loss of endogenous inhibitory regulation, as a
mechanism for chronic pain is very intriguing, yet remains incomplete. The balance or
ratio between excitation and inhibition has recently been shown as a crucial factor in
determining abnormalities in cortical information processing and behavioral output
(Yizhar et al., 2011). In similar fashion to GABAergic signaling, opioidergic signaling provides inhibitory balance to curtail increased excitation. Thus, it stands to reason, that loss of this endogenous opioid inhibition may also result in an imbalance in excitatory transmission and promote the emergence of chronic pain. Chapter 6 will further discuss and analyze the current results presented in this dissertation within the context of excitation/inhibition balance.

1.2.5. Endogenous opioid system

The opioid receptors are widely distributed in the nervous system (Yaksh, 1987; Mansour et al., 1988) and their activation either by endogenous ligands (e.g. endorphins, enkephalins, dynorphins and endomorphins) or by exogenously administered agonists elicits powerful antinociception (Yaksh, 1987; Yaksh et al., 1988). All opioid receptor subtypes (μ, δ, and κ-, and the nociception/orphanin FQ peptide receptor) are members of the heterotrimeric guanosine 5'-triphosphate–binding protein (G protein)–coupled receptor (GPCR) superfamily, Class A rhodopsin subfamily.

In addition to expression in peripheral nerve endings and dorsal root ganglia, opioid receptors are highly expressed on the central terminals of primary afferents and second order spinal cord neurons. Opioids receptors are largely localized presynaptically on afferent terminals (70%), so substantial pain signal attenuation occurs before information reaches postsynaptic neurons in the dorsal horn. Agonists of the opioid receptors are known to modulate synaptic transmission through both pre- and postsynaptic mechanisms in the CNS. μ- and δ-opioid receptor agonists inhibit glutamatergic synaptic transmission presynaptically in the superficial spinal dorsal horn (Jeftinija, 1988; Hori et al., 1992; Glaum et al., 1994). Agonists dissociate Gαi/o which then inhibits adenyl cyclase production of cAMP, thus decreasing the opening of voltage-gated Ca2+ channels (VGCC) (Dickenson et al., 1987; Sullivan et al., 1989; Kohno et al., 1999; Kondo et al., 2005) (Figure 1.3). The dissociated Gαi/o subunit causes the opening of G
protein-coupled inwardly-rectifying potassium channels (GIRKs) to further hyperpolarize the neuron (Figure 1.3). Nociceptive transduction may be further attenuated by opioid activity on excitatory interneurons (Spike et al., 2002; Marker et al., 2006). The dissertation research will primarily focus on investigating opioidergic inhibition/regulation of spinal adenylyl cyclases, specifically the calcium-sensitive adenylyl cyclase type 1.
Figure 1.3. Opioidergic signaling. Opioid ligands bind to the extracellular binding pocket of opioid receptors producing activation of intracellular inhibitory G-proteins (Gαi/o–βγ). Dissociated G-proteins can reduce neuronal excitation and/or neurotransmitter release via inhibition of adenylyl cyclases (AC), voltage-gated calcium channels (VGCC), and activation of inward-rectifying potassium channels (GIRK). Red blunted lines indicate inhibition and blue arrow lines indicate activation.
Opioid signaling also occurs at numerous regions in the brain, including anterior cingulate cortex, nucleus accumbens, ventral tegmental area, locus coeruleus, amygdala, and prefrontal cortex to disrupt the perceptual modification of pain processing (Tracey, 2007; Baliki et al., 2010). Additionally, top-down descending inhibition involves actions of endogenous opioids on descending pathways that include the locus coeruleus and the periaqueductal gray, leading to inhibition of spinal pain transmission (Fields, 2004; Schepers et al., 2008a; Heinricher et al., 2009; Zhang and Hammond, 2010). For example, top-down activation from the periaqueductal gray induces endorphin and enkephalin release onto 5HT (serotonin)-producing nucleus raphe magnus neurons in the RVM. Raphe nuclei send axonal endings to the dorsal horn of the spinal cord to form excitatory connections with GABAergic and enkephalinergic interneurons in the spinal cord (Huang et al., 2008). Although not yet demonstrated, these interneurons, when activated, could release enkephalin to bind the pre-synaptic opioid receptors on incoming C and A-δ afferents from peripherally activated nociceptors to modulate the signal.

Although a great majority of the following studies presented in this dissertation focus on spinal mechanisms, supraspinal systems must be considered. Therefore, in Chapter 3, we have provided preliminary studies investigating the contribution of supraspinal opioidergic function in relation to persistent central sensitization.

1.3. Scope of thesis

Cutaneous noxious stimuli drive ascending pain transmission through the spinal release of glutamate and peptide neurotransmitters from presynaptic terminals of primary sensory neurons (Basbaum et al., 2009). Following repetitive or sustained noxious input, enkephalins, endomorphins and dynorphins are released within the dorsal horn (Yaksh and Elde, 1981; Basbaum and Fields, 1984; ladarola et al., 1986; Noguchi
et al., 1992; Song and Marvizon, 2003; Schepers et al., 2008b) and act at opioid receptors to inhibit spinal excitatory transduction and persistent pain (Cesselin et al., 1989; Zadina et al., 1997; Martin-Schild et al., 1998; Snyder, 2004; Trafton and Basbaum, 2004). For example, in animal models, during the acute stages (within the first 14 days of the injury) of ongoing inflammatory pain, opioid receptor antagonists increase mechanical and/or heat hypersensitivity (Millan et al., 1987; Herz and Millan, 1988; Ossipov et al., 1996; Hurley and Hammond, 2000, 2001; Schepers et al., 2008b). Thus, opioids orchestrate an adaptive compensatory response that modulates neuronal excitability and fine-tunes glutamatergic nociceptive transmission via pre- (Hori et al., 1992; Terman et al., 2001) and post-synaptic (Willcockson et al., 1984; Jeftinija, 1988; Aicher et al., 2000) mechanisms in the spinal cord.

Endogenous opioid inhibition of acute nociception persists even after the initial signs of hyperalgesia have subsided (Yu et al., 1994; Li et al., 2001; Guan et al., 2010; Joseph et al., 2010; Campillo et al., 2011a). This is demonstrated by the ability of opioid receptor antagonists to precipitate allodynia. These data led to the suggestion that tissue injury induces a long-lasting, adaptive opioidergic inhibitory system that serves to counterbalance latent nociceptive sensitization. My project extends these studies to investigate long-lived opioidergic signaling mechanisms and the cellular consequences on prolonged inhibition on the promotion of latent nociceptive sensitization.

The present studies were designed to investigate the molecular and neurophysiological mechanisms driving the transition from acute to chronic pain, and the mechanism by which this is inhibited by opioidergic activity. Specifically, we tested the overall hypothesis that spinal MOR signaling, while beneficial in reducing injury-induced hyperalgesia, becomes pathological, with time, in promoting the persistence of central nociceptive sensitization via the allostatic regulation of Ca^{2+}-sensitive adenylyl cyclase.
type 1. Thus, we will provide evidence that injured-induced tonic MOR signaling simultaneously provides endogenous analgesia and, paradoxically, increases the vulnerability to develop chronic pain.
The spinal endogenous opioidergic system opposes the transition from acute to chronic pain

2.1 Introduction

Cutaneous noxious stimuli drive ascending pain transmission through the spinal release of glutamate and peptide neurotransmitters from presynaptic terminals of primary sensory neurons (Basbaum et al., 2009). Following repetitive or sustained noxious input, enkephalins, endomorphins and dynorphins are released within the dorsal horn (Yaksh and Elde, 1981; Basbaum and Fields, 1984; Iadarola et al., 1986; Noguchi et al., 1992; Song and Marvizon, 2003; Schepers et al., 2008b) and act at opioid receptors to inhibit spinal excitatory transduction and persistent pain (Cesselin et al., 1989; Zadina et al., 1997; Martin-Schild et al., 1998; Snyder, 2004; Trafton and Basbaum, 2004). For example, in animal models of ongoing or persistent inflammatory pain, opioid receptor antagonists increase mechanical and/or heat hypersensitivity (Millan et al., 1987; Herz and Millan, 1988; Ossipov et al., 1996; Hurley and Hammond, 2000, 2001; Schepers et al., 2008b). Thus, opioids orchestrate an adaptive compensatory response that modulates neuronal excitability and fine-tunes glutamatergic nociceptive transmission via pre- (Hori et al., 1992; Terman et al., 2001) and post-synaptic (Willcockson et al., 1984; Jeftinija, 1988; Aicher et al., 2000) mechanisms in the spinal cord.

After traumatic injury, animals can develop long-lasting pain vulnerability: with subsequent injury or stressor they become more susceptible to enhanced allodynia.
(Mishima et al., 1997; Aley et al., 2000; Rivat et al., 2002; Parada et al., 2003; Rivat et al., 2007; Summer et al., 2007; Cabanero et al., 2009). This phenomenon is referred to as latent nociceptive sensitization and may reflect a critical mechanism responsible for the transition from acute to chronic pain (Rivat et al., 2007). Sensitization of pain pathways can persist in the absence of behavioral signs of hypersensitivity (Reichling and Levine, 2009; Asiedu et al., 2011b). Price and colleagues recently described a model of latent nociceptive sensitization, where an injury that is associated with a transient acute hypersensitivity produces a “priming” effect for enhanced allodynia that persists in the spinal cord (Asiedu et al., 2011a).

The present studies were designed to investigate the molecular and neurophysiological mechanisms driving the transition from acute to chronic pain, and the mechanism by which this is inhibited by endogenous opioidergic activity. We will test the hypothesis that injury-induced latent nociceptive sensitization is silenced by ongoing, tonic spinal MPR signaling.

2.2 Materials and Methods

2.2.1. Mice: At the beginning of all experiments the subjects were naïve, adult (6-10 weeks), male C57Bl/6 (Charles River). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky in accordance with American Veterinary Medical Association guidelines.

Mice were housed in plastic cages (bedding and enrichment) of 4 same-sex littermates in a temperature controlled environment (14 hr: 10 hr light-dark cycle; lights on at 7:00 am) with ad libitum access to food and water. Upon arrival to the University of Kentucky Division of Laboratory Animal Resources, mice were habituated to the colony housing room for 1 week prior to any experimentation.
<table>
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</tr>
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</table>

Unknown information is denoted by dashed lines (--).

Information that is not applicable is denoted as n/a
2.2.2. Complete Freund's Adjuvant (CFA) model of inflammatory pain: As previously described by Ren and Dubner (1999), immediately following baseline assessment of mechanical thresholds, mice were lightly restrained and injected with CFA (5 µl of 1mg/1ml mycobacterium to oil emulsion, non-diluted; Sigma-Aldrich) into the intraplantar surface (ventral-medial) of the left hindpaw. Sham injuries consisted of a saline injection which controlled for needle puncture and subcutaneous injectate, (5µl, 0.9% NaCl). To assess the development of tissue edema, we recorded the dorsal-ventral thickness of the injured paw with a fine digital caliper (Mitutoyo, Illinois).

2.2.3. Spared Nerve Injury variant (CPxSx) model of neuropathic pain: As previously described by Shields et al (2003), peripheral nerve injury was induced by the ligation and transection of the common peroneal (Cp) and sural (S) branches of the sciatic nerve (CpxSx), leaving the tibial (T) branch intact. This is a variant of the spared nerve injury (SNI) model (Decosterd et al., 2000; Bourquin et al., 2006). Anesthesia was induced with 5% isoflurane in oxygen and maintained with 2% isoflurane. The left hind-leg area was shaved and wiped clean with alcohol. An incision was made in the skin of the mid-thigh. The biceps femoris muscle was retracted, exposing the primary trifurcation of the sciatic nerve. After ligation with 6.0 silk (Ethicon, Somerville, NJ), the knot and adjacent nerves (2 mm) were transected. Care was taken to avoid contact with the T branch. The muscle was sutured with absorbable 5-0 sutures and then the wound was closed with two 7 mm sterile metal clips and wiped with Betadine. Sham operations were performed by exposure of the sciatic nerve trifurcation, and then closure of the wound with care to avoid nerve perturbation.

2.2.4. Mouse (in vivo) drug administration: For subcutaneous injections (200 µl), unanaesthetized mice were lightly restrained and injected with a 27 G needle under the skin of the back, above the lumbar region. For intrathecal injections (5 µl),
unanaesthetized mice were lightly restrained and a 30 G needle attached to a Hamilton microsyringe was inserted between the L5/L6 vertebrae, puncturing through the dura (confirmation by presence of reflexive tail flick) as previously described (Fairbanks, 2003). For intraperitoneal injections unanaesthetized mice were lightly restrained and a 27 G needle was inserted through the abdominal wall into the peritoneal space and drugs were administered in a 200 µl volume. For chronic drug infusions delivery, we implanted subcutaneous osmotic mini-pumps (ALZET Osmotic Pumps, Durect Corporation, Cupertino, CA, USA; model 2004, 0.25µl/hr for 14 d). Pumps were primed in 37 °C saline for 48 hr prior to surgical implantation according to the manufacturer’s instructions. Under isoflurane anesthesia (5% induction followed by 1.5-2.0% maintenance), a subcutaneous pocket was created above the lumbar region of the spinal column with a 1 cm skin incision above the scapulae, followed by blunt dissection of the skin from the connective tissues with blunt tipped scissors. The pump was then inserted with the flow moderator facing caudally, which rested just above the lumbar region of the spinal column. The skin incision was then sutured closed and wiped with Betadine. The pump was removed after 14d under anesthesia through the original incision site, followed by sterile wound re-closure and wiped with Betadine.

2.2.5. Drug dosing: The following drugs and doses were used for in vivo experiments: naltrexone HCl (NTX, gift of the NIDA Drug Supply Program; subcutaneous: 0.003 – 10.0 mg / kg / body weight, dissolved in 200 µl saline; intrathecal: 1µg in 5µl saline), naltrexone methobromide (NMB, gift of the NIDA Drug Supply Program; subcutaneous: 3 mg/kg in 200 µl saline; intrathecal: 0.3 µg in 5µl saline), naloxone (Sigma-Aldrich; intraperitoneal: 3 mg/kg in 200 µl saline), Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, Sigma-Aldrich; intrathecal: 100 ng in 5µl saline), pertussis toxin (Sigma-Aldrich;
intrathecal: 0.5 µg in 5µl water), [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO; Sigma-Aldrich; intrathecal:10-30 pmol in 5 µl saline), and lidocaine (4%; 100 µl).

2.2.6. Mechanical hyperalgesia testing: All testing was begun between 10:00 am - noon in a temperature- and light-controlled room. Mice were acclimated for 30 - 60 min in the testing environment within a custom rectangular plastic box (15x4x4 cm; 3 white opaque walls and 1 clear wall) on a raised metal mesh platform. Baseline testing was conducted prior to and after injury and drug injections at various time points shown in the figure panels. To evaluate mechanical hypersensitivity (hyperalgesia), we used a logarithmically increasing set of 8 von Frey filaments (Stoelting, Illinois), ranging in gram force from 0.007 to 6.0 g. These were applied perpendicular to the ventral-medial hindpaw surface with sufficient force to cause a slight bending of the filament. A positive response was characterized as a rapid withdrawal of the paw away from the stimulus fiber within 4 s. Using the modified up-down statistical method (Chaplan et al., 1994) originally described by Dixon (1980), the 50% withdrawal mechanical threshold scores were calculated for each mouse and then averaged across the experimental groups.

2.2.7. Heat hyperalgesia testing: As previously described by Hargreaves et al. (1988), mice were acclimated for 30 - 60 min in the testing environment within a circular clear plastic tube (15cm H x 4cm D) on a glass table top (Ugo Basile). A radiant infrared heat source (8V, 50W lamp, Ugo Basile) was directed to the ventral hindpaw (Hargreaves et al., 1988). Beam intensity was adjusted to yield a baseline paw withdrawal of 10-12 s. The heat source was calibrated throughout the study to maintain this intensity. Three paw withdrawal time scores were recorded, with a cut off of 30 s, and then averaged for analysis across the experimental groups.
2.2.8. *Cold allodynia testing*: As previously described by Choi et al. (1994), to evaluate hypersensitivity to a cool stimulus, a piece of PE-90 tubing, flared at the tip to a diameter of 3 ½ mm, was used to apply a drop of acetone to the plantar paw. Surface tension maintained the volume of the drop to 10 to 12 μl. Evaporation of the acetone cools the skin to approximately 6°C. The time spent lifting the ipsilateral paw was recorded, with a cut-off of 30 sec. This test was repeated 3 times and the results were averaged for analysis.

2.2.9. *Antinociception testing*: As previously described by Eddy and Leimbach (1953), to evaluate the antinociceptive effects of morphine mice were placed on a noxious 56 °C hotplate. The time for the mice to flick, lift or lick their hindpaws was recorded during a single trial, with a cutoff time of 30s to prevent tissue damage. Drug-induced effects on the latency to respond were calculated as the Maximal Possible Effect for each individual mouse: Maximum Possible Effect (MPE); calculated as: MPE = [(drug induced threshold – basal threshold) / basal threshold] X 100.

2.2.10. *Phosphorylated Extracellular regulated kinase (pERK) stimulation studies:*

2.2.10.1. *Light-touch*. A mechanical ‘Light-touch stimulation’ protocol was used as previously described (Gao and Ji, 2010). Briefly, mice were injected with vehicle or drug and then 30 min later anesthetized under isoflurane (5% induction and maintained at 1.5%). A cotton-tip was gently stroked across the plantar surface of the ipsilateral hindpaw, once every 5 s for 5 min. 5 min after the end of light-touch mice were transcardially perfused with 10% buffered formalin and the lumbar spinal cord was dissected. Control mice that did not receive the light-touch stimulation still underwent isoflurane anesthesia 30 min after the intrathecal injection.
2.2.10.2. Ambulation. The ‘Ambulation’ protocol consisted of an intrathecal injection immediately followed by unrestrained walking on a metal mesh grate for 30 min. Mice were then perfused and lumbar spinal cords dissected.

2.2.10.3. Anesthesia. For the ‘Anesthetized’ protocol, mice were first anesthetized under isoflurane (1.5%) to prevent walking and joint movement, followed by an intrathecal injection. Mice remained anesthetized for 30 min before they were perfused and lumbar cords dissected.

2.2.10.4. Sciatic nerve block. For the “sciatic nerve block” protocol, mice were first anesthetized under isoflurane (1.5%) followed immediately by an injection of 2% lidocaine (100 µl) into the popliteal fossa in order to target the sciatic nerve that innervates the hindpaw (protocol adapted from Okun et al. (2011), using popliteal fossa lidocaine injection in rats). Mice were then perfused and dissected 20 min later. This dose and volume of lidocaine were chosen based on a pilot study in conscious, awake mice which showed lack of reflexive responsiveness to noxious pin prick at the ipsilateral hindpaw, lasting approximately 25-30 min. We also found that this dose of lidocaine completely blocked the expression of spinal pERK following an intraplantar injection of 10% capsaicin.

2.2.11. Histology and Immunohistochemistry: As described previously (Corder et al., 2010), mice were deeply anesthetized with isoflurane or an intraperitoneal overdose injection of Fatal Plus (86 mg/kg, Vortech Pharmaceuticals). Mice were then perfused with ice-cold PBS/heparin followed by ice-cold 10% buffered formalin. Spinal cords (L3-L5) were postfixied in 10% formalin, cryoprotected in 30% sucrose, and sectioned at 30 µm on a sliding microtome.
Free-floating spinal cord sections were washed in 0.1M phosphate buffer, blocked in 3% normal goat serum containing 0.3% Triton-X, and incubated overnight in primary antibodies ((rabbit phospho-ERK1/2 antiserum (1:500, Cell Signaling), mouse NeuN-Alexa488 (1:200, Millipore), rabbit MOR (1:500-2000; Neuromics)). Sections were washed and incubated in Alexa 568-conjugated goat anti-rabbit antibody (1:700; Invitrogen)) for 2 hr at room temperature, serial washed, slide mounted, and coverslipped with ProLong Gold + Dapi counterstain (Invitrogen).

For pERK quantification, 5 spinal sections, separated by at least 120 µm, were randomly selected from each mouse. Images were taken on a Nikon TE2000 microscope (10x) and the number of pERK+ profiles were counted by a blind observer (Jennifer Grasch and Justin Pinson) and binned by Rexed lamina (I-II and III-V).

2.2.12. GTPγS binding assay: As previously described by Winter and McCarson (2005), the lumbar enlargement of the spinal cord was removed by hydrostatic pressure ejection (18 G needle was used to push ~5 ml cold saline through the spinal column to expel the spinal cord) and frozen on dry ice (performed by Greg Corder). Dr. Kenneth McCarson and Michelle Winter (University of Kansas) were given the spinal cords for further processing and analysis: 25 µm thick sections were cut and mounted on positively charged glass slides. Sections were equilibrated in assay buffer (3 mM MgCl₂, 100 mM NaCl, 0.2 mm EDTA, and 50mM Tris-HCl, pH 7.4) for 10 min and then in 1 mM (β-FNA studies) or 2 mM (DAMGO studies) GDP for 15 min both at room temperature. Agonist-stimulated binding was then performed with 1 mM or 2 mM GDP (β-FNA or DAMGO, respectively) and 0.1 nM GTPγS³⁵ (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and one of five serial dilutions ranging from 33 nM to 33 µM DAMGO (Sigma-Aldrich) or 100 nM to 10 µM β-FNA (Sigma-Aldrich) in water treated with a peptidase inhibitor cocktail containing 0.170 mg/ml bacitracin, 17 µg/ml leupeptin,
17 μg/ml chymostatin, and 0.850 mg/ml bovine serum albumin. Drug was omitted and replaced with either peptidase-treated water for basal determinations or 10 μM unlabeled GTPγS in water for nonspecific binding determinations (nsb). Slides were incubated in binding conditions at 24°C (room temp) for 2 hr and then rinsed twice with ice-cold 50 mM Tris-HCl, pH 7.4, for 2 min and twice with room temperature deionized water for 2 min. Slides were exposed to Kodak X-OMAT autoradiographic film (Sigma-Aldrich) for 8 h. Densitometry of images was performed by measuring the mean number of pixels in lamina I of each left and right dorsal horn and subtracting the mean dorsal column background value. Percent stimulation over basal was calculated using the following equation:

\[
\frac{(\text{dorsal horn} - \text{column}) - \text{nsb}}{(\text{dorsal horn basal} - \text{column basal}) - \text{nsb}} \times 100 - 100
\]

Concentration-response curves were generated using non-linear regression curve-fitting, and values for maximal stimulation (E_{max}) and half-maximal stimulating concentration (EC_{50}) were determined.

2.2.13. Statistics: Data were analyzed using one-way or two-way ANOVA, as indicated in the main text or figure captions, followed by Bonferroni post-hoc tests or Student’s t tests as appropriate (GraphPad Prism v6). For dose-response hyperalgesia studies the best-fit line was generated following non-linear regression analysis based on each individual mouse’s Maximum Possible Effect (MPE); calculated as: MPE = (drug induced threshold – basal threshold) / basal threshold; For GTPγS^{35} binding studies the best fit line was generated following non-linear regression analysis. All data are presented as mean ± the standard error of the mean (s.e.m.). Although standard deviation (S.D.) provides direct indication of variance, while s.e.m. does not, and thus S.D. is more appropriate, from a statistical standpoint presenting the s.e.m., is a
commonly used practice in the behavioral pharmacology and pain fields (communication from Drs. Bradley Taylor and Karin Westlund High).

2.2.14. Experimental blinding procedures: The experimenter, Greg Corder, was blind to drug treatments during all behavioral pharmacology experiments. This was accomplished by making each drug and vehicles in similar tubes and to the same volume. These tubes were then given to Renee Donahue, Jennifer Grasch, Ryan Griggs, Weisi Fu or Suzanne Doolen for tube coding. Greg was then given the tubes marked with distinguishing letters. The code was typically kept hidden by the blinder until completion of the experiment. After the experiment, the code was broken and Greg analyzed the data.

2.3. Rationale and Results

2.3.1. Opioid receptor signaling provides acute pain relief and long-lasting intrinsic pain suppression.

To model the progression of inflammatory pain following the unilateral hindpaw intraplantar injection of complete Freund’s adjuvant (CFA), we assessed the development of mechanical hypersensitivity in mice. Compared to saline injection, CFA produced mechanical hypersensitivity (Figure 2.1a; \( F_{1,19} = 13.36, P < 0.001 \)), evident as a decrease in mechanical response threshold to von Frey filament stimulation of the inflamed hindpaw. Mechanical hypersensitivity lasted 7d and fully resolved to a stable baseline within 10d, demonstrating that the inflammatory pain naturally recovers.

Recently our lab found that conditional knockdown of neuropeptide tyrosine (NPY) before the initiation of inflammation extended the time course of mechanical and heat hyperalgesia (Solway et al., 2011). Similarly, dual blockade of cannabinoid CB1 and CB2 receptor signaling prevented the resolution of postoperative allodynia (Alkaitis
et al., 2010). These studies suggest that endogenous NPY and endocannabinoids hasten the resolution of acute pain. Thus pain intensity and duration are regulated by numerous inhibitory systems, including spinally secreted opioid peptides and subsequent activation of opioid receptors (Basbaum and Fields, 1984; Ossipov et al., 2010). To determine whether endogenous opioid signaling promotes the intrinsic recovery of acute inflammatory pain, we implanted subcutaneous minipumps to chronically infuse naltrexone hydrochloride (NTX; 10 mg/kg/d), a non-selective opioid receptor antagonist. Compared to saline, NTX produced a robust, persistent hypersensitivity throughout the 14d infusion period (Fig. 2.1b; \( F_{10,110} = 4.425, P < 0.0001 \), 2-way ANOVA, Drug x Time interaction), while having no effect in sham mice (\( P > 0.05 \) compared to Sham + saline). Upon NTX-minipump removal at 14d, mechanical hypersensitivity gradually resolved, fully recovering in four days (\( P > 0.05 \), 18d compared to baseline, Student’s \( t \) test). This suggests that peripheral tissue injury increases adaptive opioidergic processes to relieve on-going pain. However, it is important to note that chronic treatment with opioid antagonists (NTX and naloxone) can increase the expression of \( \mu, \delta, \) and \( \kappa \) opioid receptors independent of nociceptive input (Yoburn et al., 1995; Lesscher et al., 2003; Patel et al., 2003). However, our dose of NTX (10mg/kg/d) appears sufficient to block all opioid signaling regardless of receptor expression modulation that may have occurred (either from the antagonist or from the CFA injury).

Hyperalgesic priming models, such as intraplantar IL-6 injection and the plantar paw incision model of post-operative pain, suggest that the spinal nociceptive pathways remain sensitized to subsequent injuries following recovery from the initial injury (Reichling and Levine, 2009; Asiedu et al., 2011a). Opioid systems provide an endogenous braking mechanism that exerts inhibitory control of acute pain intensity soon after tissue injury (Ossipov et al., 2010), but whether MOR signaling is maintained
for sufficient duration to oppose long-lasting nociceptive mechanisms is unknown. Therefore to determine if nociceptive sensitization processes are masked by opioid receptor signaling, we delivered NTX after the inflammatory pain had attenuated. When administered 21d after CFA, systemic NTX reinstated mechanical hypersensitivity (Fig. 2.1c; $F_{1,21} = 41.48$, $P < 0.0001$). This effect peaked from 30 - 60 min and lasted approximately 4 hr, a time course consistent with its reported plasma half-life in rodents (Shader, 2003). The degree of NTX-induced pain reinstatement was dose-dependent, with no observable effect in sham-injured mice (Figure. 2.1d).
Figure 2.1. Peripheral inflammatory injury initiates endogenous opioid analgesia to silence on-going nociceptive processes. (a) Time course for the development and resolution of inflammatory mechanical pain induced by CFA (5 µl), as measured by von Frey filaments (n = 10 mice per group). ★ P < 0.05 compared to Sham group. (b) Changes in mechanical pain resolution after continual minipump infusion of saline or NTX (10 mg / kg /d) for 14d in Sham and CFA mice (n = 4 - 7 per group). ★ P < 0.05 compared to CFA + saline group, ◆ P < 0.05 compared to Sham + NTX group. (c) Effect of a single subcutaneous injection of saline (n = 6) or NTX (3 mg /kg)(n = 13) on mechanical thresholds 21d after CFA. ★ P < 0.05 compared to Saline group. (d) Dose-response effects of NTX on mechanical thresholds 30 min after subcutaneous injection in Sham and CFA-21d mice (n = 6 per group per dose). Data shown as mean ± s.e.m.
2.3.2. *Tonic μ-opioid receptor signaling is present for months after injury.*

Residual ipsilateral tissue inflammation persisted for 49d after CFA and was absent by 77d (Figure 2.2a), therefore we examined if latent sensitization persisted beyond, not just the recovery of pain, but recovery of the physical tissue injury. Repeated injections of NTX but not saline reinstated mechanical hyperalgesia for at least 105d post-CFA (Figure 2.2b; 21d: $F_{1,80} = 8.51, P = 0.0154$; 49d: $F_{1,72} = 58.79, P < 0.0001$; 77d: $F_{1,72} = 76.36, P < 0.0001$; 105d: $F_{1,64} = 33.15, P < 0.0001$), and as late as 200d post-CFA (Figure 2.2e; n=2 per injection group, $P = 0.025$).

To control for possible context-dependent (associative) sensitization between the testing environment and multiple drug injections (Adams and Holtzman, 1990; Taiwo et al., 1989; Hummel et al., 2008), we injected CFA and left the animals undisturbed for 105d. Again, we found that a single injection of NTX or the MOR-selective antagonist CTOP, but not vehicle, elicited reinstatement of mechanical hypersensitivity (Figure 2.2c and d; NTX: $F_{1,14} = 28.85, P < 0.0001$; CTOP: $F_{3,10} = 9.87, P = 0.0025$). Taken together, these results indicate that both excitatory and μ-opioidergic inhibitory processes remain active for months, even in the absence of pain and tissue damage.
Figure 2.2. Endogenous opioid analgesia and the opponent nociceptive processes continue to operate for months beyond injury healing.

(a) Time course of paw edema after Sham and CFA injuries for 105d post injury initiation. The effect of repeated subcutaneous saline or NTX administration on paw edema is also noted. (b) Effect of repeated subcutaneous saline (n = 5 – 7 at each injection) or NTX (3 mg /kg) injections on mechanical thresholds over 105d after CFA (n = 5 – 7). These are the same mice shown in panel a. (c) Effect of a single subcutaneous injection of either Saline or NTX (3 mg/kg) at 105d post CFA in mice naïve to drug exposure and the behavioral testing environment (n = 7 – 11). (d) Effect of intrathecal saline or CTOP on mechanical thresholds in sham and CFA mice 105d post injury initiation (n = 3 - 5). (e) Effect of subcutaneous saline or NTX (3 mg/kg) at 200d post CFA (n = 2 per group). ★ P < 0.05 compared to Saline group. Data shown as mean ± s.e.m.
2.3.3. Tonic opioid receptor signaling modulates latent nociceptive sensitization in the spinal cord

Naltrexone hydrochloride (NTX) is lipophilic and therefore readily crosses the blood-brain barrier (BBB), antagonizing opioid receptors at supraspinal, spinal, and peripheral sites. To determine whether peripheral opioid receptor blockade can reinstate pain hypersensitivity, we subcutaneously administered naltrexone methobromide (NMB), an opioid receptor antagonist with a quaternary amine that restricts BBB permeability. Unlike NTX, systemic NMB did not alter mechanical thresholds at the ipsilateral (Figure 2.3a) or contralateral (Figure 2.3b) paws when injected 21 d after CFA.

Opioid receptors are highly expressed on the central terminals of primary afferents and second order spinal cord neurons (Besse et al., 1990; Kohno et al., 1999; Spike et al., 2002; Marker et al., 2005; Scherrer et al., 2009; Heinke et al., 2011). To determine whether spinal opioid receptor blockade can reinstate pain hypersensitivity, we administered receptor antagonists by direct lumbar intrathecal injection. We found that either intrathecal NTX or NMB, but not saline, reinstated mechanical hypersensitivity in both the ipsilateral (Figure 2.3c; \( F_{2,63} = 25.40, P < 0.001 \)) and contralateral paws (Figure 2.3d; \( P < 0.05 \)), with no effect in sham-injured mice (Figure 3.3e).

Opioid receptors are expressed on TRPV1 positive primary afferents in the spinal cord (Chen and Pan, 2006). Therefore we also investigated if the reinstatement effect of NTX is multimodal for heat hypersensitivity. As illustrated in Figure 2.3f, CFA decreased paw withdrawal latencies in response to noxious radiant heat (\( F_{1,15} = 14.94, P < 0.0002 \)). Heat withdrawal latencies returned to pre-injury values within 5 d after injury. On day 21 post-CFA intrathecal NTX reinstated heat hypersensitivity (Figure 2.3f; \( F_{2,12} = 13.42, P < 0.0001 \)), with no effect in sham controls (\( P > 0.05 \)). Together these data demonstrate that opioid receptors located in the spinal cord, and not the periphery,
regulate mechanical and thermal nociception as blockade of spinal opioid tonic signaling precipitates hyperalgesia.
Figure 2.3. Blockade of spinal opioid analgesia reinstates mechanical and heat hyperalgesia.

(a,b) Effect of subcutaneous injection of saline, NMB (3 mg/kg), and NTX (3 mg/kg) on mechanical thresholds at the (a) ipsilateral and (b) contralateral paws. (n = 7-10) ★ P < 0.05 compared to Saline, ◆ P < 0.05 compared to NMB. (c,d) Effect of intrathecal injection of saline, NMB (300 ng), and NTX (1 µg) on mechanical thresholds at the (c) ipsilateral and (d) contralateral paws. (n = 5-10). ★ P < 0.05 NTX compared to Saline, ◆ P < 0.05 NMB compared to Saline. (e) Effect of intrathecal NTX (1 µg) in sham injured mice. (n = 5-8). (f) Effect of intrathecal saline or NTX (1 µg) on thermal heat thresholds 21d after CFA or sham injuries as measured by the Hargreaves’ radiant thermal heat test. (n = 5-10). left: ★ P < 0.05 compared to Sham, right: ★ P < 0.05 compared to ‘CFA + saline’. Data shown as mean ± s.e.m.
2.3.4. Increased spinal μ-opioid receptor::G-protein signaling

MOR-triggered intracellular second messenger signaling cascades are relayed through coupled Gαi/o proteins. Therefore we next set to determine if MOR-G-protein coupling and signaling is augmented following injury to suppress on-going nociceptive processes:

First, we found that disruption of Gαi/o signaling with intrathecal injection of pertussis toxin (an agent that causes ADP-ribosylation of Gi proteins, thus preventing binding to GPCRs and loss of signal transduction), precipitated hyperalgesia in CFA-21d but not shams (Figure. 2.4a; \( P < 0.05 \)). This demonstrates the presence of injury-induced tonic inhibitory G-protein signaling in the spinal cord.

Second, we (Greg Corder, Bradley Taylor, Michelle Winter and Kenneth McCarson) assessed guanosine-5′-O-(3-[35S]thio)triphosphate (GTPγS\(^{35} \)) binding in fresh spinal cord slices (Figure. 2.4b-d). GDP-bound G-proteins bind activated GPCRs and are subsequently dissociated and activated by exchange of GDP from GTP. The GTPγS\(^{35} \) analogue contains a sulfur residue, in lieu of a third O\(^-\) molecule, on the γ-phosphate, which prevents hydrolysis to its di-phospho form. Thus GTPγS\(^{35} \) incorporation into membrane preparations serves as a steady direct indicator of GPCR-G-protein activity. We found that in control spinal cord slices derived from sham mice, the MOR selective agonist DAMGO elicited a stimulation of GTPγS\(^{35} \) binding with an \( E_{\text{max}} \) and EC\(_{50} \) of 58.02 ± 0.67% and 0.24 ± 0.01 µM, respectively (Figure. 2.4c). We discovered that \( E_{\text{max}} \) was potentiated in 21 d CFA slices, not only in the ipsilateral (Figure. 2.4c; 79.85 ± 7.35%; \( P < 0.05 \) compared to Sham) but also the contralateral (Figure. 2.4d; 74.05 ± 4.13%; \( P < 0.05 \) compared to Sham) dorsal horns, with no change in the EC\(_{50} \) (ipsilateral: 0.25 ± 0.07 µM, contralateral: 0.29 ± 0.04 µM; both \( P > 0.05 \)).
compared to Sham). These data illustrate that injury increases the membrane availability of functional MORs in the dorsal horn.

Third, the antinociceptive effects of intrathecal DAMGO were potentiated in CFA-21d mice (Figure 2.4e; \( P < 0.05 \), Student’s \( t \) Test), which further suggests increased MOR number. Fourth, as illustrated in Figure 2.3f, intrathecal injection of Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP), a MOR-selective antagonist, reinstated hyperalgesia in CFA-21d (\( F_{3,20} = 9.83, P < 0.0005 \)) and CFA-105d (\( F_{3,10} = 9.87, P = 0.0025 \); [same data as shown in Figure 2.2d]) mice but not sham controls. Taken together these findings demonstrate that upregulated tonic MOR-G-protein signal transduction persists after the resolution of inflammatory hyperalgesia, and mediates long-lasting endogenous antinociception that opposes the emergence of chronic hyperalgesia.
Figure 2.4. The functional capacity of spinal µ-opioid receptor coupling to G-proteins is increased after injury.

(a) Effect of intrathecal PTX (0.2 µg) on mechanical thresholds 21d after sham and CFA injuries. ★ P < 0.05 compared to Sham + PTX. (n = 4 - 6 per group) (b) Representative pseudo-color and (c,d) dose-response effects of DAMGO-stimulated GTPγS$^{35}$ binding as compared to basal binding in Sham and CFA-21d lumbar spinal cord slices; (c) ipsilateral and (d) contralateral dorsal horns; insets: group binding E$_{max}$. (n = 7 – 9). (e) Antinociceptive effects of intrathecal DAMGO in sham and CFA-21d mice on a 56ºC hotplate (n = 8 per group). ★ P < 0.05. (f) Effect of intrathecal CTOP (100 ng) on mechanical thresholds at 21d (left; n =5-7) and 105d (right; n = 3-5) post CFA and sham. ★ P < 0.05 compared to Sham. Data in panels b-d was collected by Greg Corder, Michelle Winter and Kenneth McCarson; see Methods for specific contributions. Data shown as mean ± s.e.m.
2.3.5. Spinal \( \mu \)-opioid receptors inhibit a latent central sensitization that persists in the post-hyperalgesic state.

Opioids inhibit central sensitization in the dorsal horn (Terman et al., 2001) (Benrath et al., 2004), but whether this can be maintained for long periods of time to impinge on chronic pain in unknown. Phosphorylated extracellular signal-regulated kinase (pERK) is implicated in the spinal transduction of nociceptive signals and serves as a marker of central sensitization during pathological conditions (Ji et al., 1999; Ji et al., 2009). We found that compared to shams, innocuous light-touch of the injured hindpaw did not increase pERK expression 21 days after CFA in the dorsal horns (Figure. 2.5a and 2.5b). However, intrathecal NTX increased touch-evoked pERK in lamina I-II (NTX, 15.1 ± 3.5 cells; saline, 5.2 ± 2.6 cells; \( P < 0.05 \); Figure. 2.5a and 2.5b) and III-V (NTX, 5.3 ± 1.7 cells; saline, 1.8 ± 1.3 cells; \( P < 0.05 \); Figure. 2.5a and 2.5b). Ipsilateral touch stimulation after NTX also increased pERK at the contralateral dorsal horn (7.34 ± 1.37 cells per slice, \( P < 0.05 \); Figure. 2.5b and 2.5c and 2.5d). Confocal microscopy revealed that pERK was expressed on many NeuN-positive profiles within the tibial nerve innervation zone of the medial dorsal horn (Figure. 2.5e). pERK positive structures were not co-localized with markers of spinal microglia or astrocytes (Figure 2.6). These results reveal the presence of sensitized spinal nociceptive neurons that outlast the resolution of hyperalgesia and remain under the control of endogenous MOR inhibitory mechanisms. These findings are consistent with previous work suggesting that stress (Rivat et al., 2007) or injury (Rivat et al., 2002) escalates opposing inhibitory and excitatory influences on pain processing.

We noted residual edema of the CFA paw at 21d (Figure 2.2a); therefore we hypothesized that the NTX-induced hyperalgesia and pERK results from disinhibition of on-going primary afferent activity from the inflamed tissue. To test this hypothesis, we systemically eliminated possible sources of activity coming into the spinal cord and then
quantified the number of pERK positive cells in the dorsal horn as a measure of spinal nociceptive pathway activity. To ensure mechanical stimulation of the injured paw, 21d-CFA mice were allowed to walk, unrestrained, on a metal-mesh grate for 30 min (Ambulation). We found no difference in pERK expression compared to sham mice allowed to ambulate on the same surface (Figure 2.5f). However intrathecal NTX prior to ambulation significantly increased pERK expression in the ipsilateral and contralateral dorsal horns (Figure 2.5f). This hints that NTX may disinhibit sensitized Aβ-fiber circuits since innocuous ambulation, just like the light-touch protocol above, produces activity in spinal laminas I and II. Therefore to eliminate stimulus-induced activity we anesthetized the mice with light isoflurane anesthesia, to prevent walking and joint-movement. This, however, did not prevent NTX-induced increases in spinal pERK levels (Figure 2.5f); suggesting NTX may disinhibit tonic, non-evoked, afferent activity. We further assessed afferent activity by combining anesthesia with a lidocaine sciatic nerve block prior to NTX. However, with afferent input removed we found significant pERK expression (Figure 2.5f; P < 0.05). Local anesthesia did not change pERK expression. The pERK profiles were clustered in lamina I neurons only, with little to no pERK+ cells in deeper laminas. Together, these data suggest that removal of inhibitory opioid receptor signaling produces neuronal activity, within spinal nociceptive pathways, independent of primary afferent drive.
Figure 2.5. Blockade of spinal opioid receptor signaling reveals sensitization in a subset of dorsal horn neurons that respond to innocuous light-touch stimulation or become active independent of primary afferent activity.

(a) Representative images and (b) laminar quantification (I-II and III-V) in ipsilateral and contralateral dorsal horn of immunoreactive cells for pERK in sham and CFA-21d mice given light mechanical touch of the injured paw 30 min after intrathecal saline or NTX (1µg). (n = 5 - 7). (c) Representative pseudo-colored light-microscope image and (d) confocal image of the same pERK positive cells in the ipsilateral and contralateral dorsal horns from a mouse given light touch + NTX. (e) From boxed region in panel d: Co-
localization of pERK with NeuN. (f) left: quantification of dorsal horn pERK cells from NTX-treated mice walking on a metal grate (Ambulation), not moving or walking (Anesthesia; 2% isoflurane), and/or injected with 2% lidocaine (100 µl) into the popliteal fossa (Sciatic nerve block). Right: effect of popliteal fossa lidocaine injection on intraplantar capsaicin-induced (10 µg in 5 µ) spinal pERK expression. All scale bars = 200 µm. ★ P<0.05; ◆ P < 0.05 compared to all groups. All data shown as mean ± s.e.m.
Figure 2.6. Light-touch evoked spinal pERK is co-localized within neurons but not astrocytes or microglia.

Representative spinal cord sections (30 μm) from CFA-21d mice given intrathecal NTX (1 μg) and an ipsilateral light-touch paw stimulation (see Supplementary Methods) were co-stained for phosphorylated extracellular regulated kinase (rabbit, 1:700 Cell Signaling) and either (A-C) NeuN::Alexa-488 (rat, 1:200, Millipore; confocal images taken at 5x; inset: 40x), GFAP (rat, 1:5000, Invitrogen; light microscope images taken 56
at 10x for panels D-F and 40x for panel G) or CD11b (rat, 1:5000, AbD Serotec; light microscope images taken at 10x for panels H-J and 40x for panel K). All scale bars are 200 μm. Panels A-C are from the same spinal cord slice shown in Fig. 1T-W.
2.3.6. Opioid receptor blockade reinstates hyperalgesia in a model of transient neuropathic pain

Up to this point we have demonstrated that opioid and nociceptive-facilitatory mechanisms persist in the complete Freund’s adjuvant (CFA) model of inflammation for up to 200 days after the initial injury. In many inflammation models opioid peptide and receptor expression are upregulated (Goff et al., 1998) (Calza et al., 2000). Yet studies using nerve injury models of neuropathic pain often report decreases in opioid receptor expression or de-coupling of inhibitory G-proteins from MORs, which would suggest decreased endogenous analgesic relevancy for spinal MORs. Therefore to determine whether NTX can reinstate hypersensitivity not only in the setting of inflammation but also in the setting of nerve injury, we performed comparable studies after transection of the common peroneal and tibial branches of the sciatic nerve (CPxSx) (Figure 2.7), as described by Solway et al. (2011). In contrast to the spared nerve injury model of neuropathic pain, which produces a long-lasting “floor” in mechanical von Frey thresholds, the CPxSx nerve injury model induces hyperalgesia that lasts for just a few weeks. As illustrated in Figure 2.7a, CPxSx produced mechanical hyperalgesia that began to resolve around day 14, and was fully resolved by day 21. Cp,Sx did not produce cold allodynia (Figure 2.7c). As detailed in Figures 2.6b and 2.6d, NTX (10 ug, i.t.) reinstated tactile hypersensitivity ($F_{3,28} = 4.95$, $P < 0.005$) and induced cold hypersensitivity ($F_{3,28} = 12.91$, $P < 0.0001$). By contrast, neither peripherally restricted NMB nor saline (s.c. or i.t.) produced alterations in tactile sensitivity ($P > 0.05$).
Figure 2.7. Blockade of spinal opioid receptor signaling reinstates hyperalgesia in a model of transient neuropathic pain.

(a and c) Progression and recovery of Spared Nerve Injury variant surgery (CPxSx) (n = 44) induced (a) mechanical hyperalgesia and (c) cold allodynia. (b and d) Effect of intrathecal NTX (1µg) or saline, and subcutaneous NMB (3 mg/kg) or saline on (b) mechanical thresholds and (d) duration of paw withdraw from an acetone drop. ★ P < 0.05 compared to all groups. All data shown as mean ± s.e.m. (Assistance with behavior was provided by Amanda Webb).
2.4 Discussion

2.4.1. Long-lasting endogenous opioid analgesia

Endogenous opioid inhibition of acute nociception persists even after the initial signs of hyperalgesia have subsided (Yu et al., 1994; Li et al., 2001; Guan et al., 2010; Joseph et al., 2010; Campillo et al., 2011a). This is demonstrated by the ability of opioid receptor antagonists to precipitate allodynia in the post-hyperalgesic state. In extension of these works, we found withdrawal frequencies to mechanical stimuli were unaltered by NTX in wild-type sham mice, consistent with observations in normal μ-opioid receptor knock-out mice that do not show changes in acute nociceptive processing (Fuchs et al., 1999). Similar studies in normal human subjects also report no modulation of pain perception upon opioid antagonist treatment, suggesting little to no tonically active endogenous opioid signaling in the absence of tissue or nerve damage (El-Sobky et al., 1976; Schoell et al., 2010). The contribution of endogenous opioid tone to transient pain or acute inflammatory pain is weak at best, as opioid receptor antagonists do not increase pain during cold, thermal or formalin acute testing (Taylor et al., 1997; Grevert and Goldstein, 1978; Kern et al., 2008). Under chronic pain conditions, however, opioid mechanisms are thought to be recruited in response to intense, sustained nociceptive signaling (Levine et al., 1978; Mansikka et al., 2004).

We found that CFA inflammatory pain progressively recovered. This is demonstrated by the return of mechanical withdrawal thresholds to pre-injury levels. This suggests that pain recovery results from either deactivation of pronociceptive systems or increased counter-adaptive endogenous inhibition. By ablating the initial pain recovery with continuous NTX infusion (Figure 2.1b), we demonstrated that the endogenous opioid system is activated after CFA to dampen persistent nociception.
Several explanations for this finding can be proposed: strong noxious input is accompanied by the release of opioid peptides, increased opioid receptor density, and enhanced G-protein coupling in the spinal cord (Yaksh and Elde, 1981; Basbaum and Fields, 1984; ladarola et al., 1986; Noguchi et al., 1992; Calza et al., 2000; Mousa et al., 2002; Song and Marvizon, 2003; Schepers et al., 2008b; Goff et al., 1998; Shaqura et al., 2004). Interestingly we find that augmentations in spinal MOR-G-protein coupling last for at least 3 weeks after CFA even though the initial bout of allodynia is completely absent, suggesting that opioid signaling did not dissipate.

In support of the hypothesis that increases in opioid receptor expression and function facilitate endogenous analgesia we show that disruption of $G_{\alpha i/o}$ activation with intrathecal pertussis toxin precipitated hyperalgesia. Moreover, pharmacological blockade of opioid receptor signaling with systemic or intrathecal NTX reinstated mechanical and thermal hyperalgesia. Remarkably we could reinstate pain behaviors for up to 105 d after the injury, while tissue inflammation had recovered by 49d. Others have demonstrated that opioid receptor density in the superficial spinal cord progressively increases over days to weeks during chronic inflammation states and is suggested to aid in the analgesic effectiveness of exogenously applied opiate drugs (Ji et al., 1995; Goff et al., 1998; Mousa et al., 2002). An autoradiographic study showed that opioid receptor density can remain greatly elevated 79 d after CFA tail-vein injection when pain scores had recovered to baseline (Calza et al., 2000). Collectively, our data illustrates the presence of persistent pronociceptive and opioidergic plasticity that outlasts not only acute pain, but the physical injury as well.

It is likely the case that the endogenous opioidergic system is not the sole promoter of endogenous analgesia. Other endogenous inhibitory systems, including the endocannabinoids (Alkaitis et al., 2010) and resolvins (Ji et al., 2011), reduce both nociception as well as inflammation. Additionally, our lab has recently shown that the
endogenous Neuropeptide Y system is also involved in recovery from inflammation and neuropathic pain (Solway et al., 2011). Therefore it is likely that endogenous MORs work in concert with numerous neuromodulatory systems to reduce nociception and aid in the resolution of pain.

2.4.2. No evidence for peripheral opioid effects in mediating long-lasting endogenous analgesia

Shortly after the induction of inflammation with intraplantar CFA, immune cells (granulocytes and monocytic cells) extravagation into the inflamed tissue is mediated, in part, by strong central nociceptive signal activation of intercellular adhesion molecules (e.g. ICAM-1 and β-integrin) (Machelska et al., 2002). Stimulation of Toll-like receptors causes the immune cells to release endorphin and enkephalin which act upon the µ- and δ- opioid receptors on nerve fibers innervating the inflamed tissue (Rittner et al., 2009). We found the paw thickness of CFA injured animals was maximal 7 days after CFA (50% increase from baseline thickness), while at the same time mechanical thresholds began to recover. On day 21, when pain behaviors had fully resolved, paw thickness decreased 11.25% from the day 7 measurements. It is likely that the mineral oil/mycobacterium emulsion was not cleared from the tissue as paw thickness remained elevated for 49 days compared to Sham controls.

With the use of the BBB-impermeable drug, NMB, we were able to localize opioid antagonism to either peripheral receptors in the nerve endings and DRG somata by subcutaneous injection, or to CNS receptors in the spinal cord by intrathecal injection. Lack of pain reinstatement with systemic NMB 21d after CFA suggests that peripheral opioid signaling does not contribute to the inhibition of latent nociceptive sensitization. Spinal intrathecal injection of NMB, however, produced robust reinstatement of mechanical hypersensitivity comparable to the systemic and intrathecal effects of NTX.
This suggests that it is the central opioid receptors that are responsible for long-term endogenous analgesia. Previous work has shown i.c.v. NMB was able to attenuate conditional analgesia in the formalin test but has no effect when delivered i.p. (Calcagenetti et al., 1987).

In contrast to the findings presented in this dissertation it was reported that peripheral opioids are exclusively responsible for endogenous analgesic mechanisms 96-144 hours after the induction of inflammation (which the authors describe as “later stage of inflammation”) (Stein, 2003). These studies evaluated spinal opioid contribution 6 days post-CFA by “low doses” (as claimed by the authors) of naloxone hydrochloride (1.125 μg) injected at the site of inflammation (e.g. injected directly into the inflamed tissue) that are presumed to “not enter systemic circulation or the central nervous system”. They do not, however, report using higher, centrally acting doses of naloxone HCl and therefore do not entirely validate their claim. Nonetheless, peripheral opioid signaling may be important for the immediate resolution of pain (several days after the injury, as shown by Stein and colleagues), but long-term maintenance of the endogenous analgesia (21 days and beyond, as we have demonstrated in this dissertation) appears to reside largely in the spinal cord and CNS.

2.4.3. Endogenous opioidergic analgesia occurs in the spinal cord

We further localized these persistent opposing processes (i.e. opioid signaling and latent nociceptive mechanisms) to the lumbar spinal cord by visualization of pERK in the dorsal horn nociceptive circuitry. pERK is typically observed following high-threshold (Aδ- or C-fiber) or noxious stimulation (Ji et al., 1999), however, during the maintenance phase of chronic pain (> 2 days post CFA) a low-threshold (Aβ-fiber) stimulation (Matsumoto et al., 2008) or light-touch (Gao and Ji, 2010) can induce spinal pERK expression, suggestive of injury-induced central sensitization (Ji et al., 2003). We found
that after pain resolution (21d), light-touch is unable to effectively activate superficial dorsal horn neurons unless the latent sensitization is disinhibited by NTX, indicating that opioid signaling actively represses signal amplification, similar to spinal GABAergic signaling (Baba et al., 2003; Torsney and MacDermott, 2006; Torsney, 2011). The expression of pERK, and thus central sensitization, was not reliant on on-going activity of the primary afferent as sciatic nerve block did not ablate neuronal activation in laminas I and II, providing additional support for this CNS site of action. The presence of pERK in the substantia gelatinosa suggests that the latent sensitization and tonic MOR signaling may exist in NK1-R expressing projection neurons or interneurons (inhibitory or excitatory). Future studies will be required to determine the exact neuronal population that MOR signaling occurs (immunohistochemical or electrophysiological phenotyping).

Either this latent nociceptive sensitization is maintained within the local spinal circuitry (Ruscheweyh et al., 2011) or is partially maintained by descending facilitatory signals from brainstem or cortical nuclei (Porreca et al., 2002). At this point we cannot rule out the contribution of supraspinal regions believed to store the memory of pain such as the anterior cingulate cortex or amygdala (please see Chapter 3 for evidence and discussion of supraspinal opioidergic circuits. Also see Chapter 2 Discussion section 2.4.4. Contralateral effects. However, hyperalgesic priming models suggest a “pain engram” is encoded in the spinal cord (Asiedu et al., 2011a), revealed as a potentiated pain response to a second injury or stressor. Our study suggests that intrathecal MOR antagonists disinhibit these excitatory spinal processes. This further illustrates a homeostatic reduction by increased endogenous opioid tone. In agreement, others have suggested this concept of counter-inhibition of nociceptive plasticity whereby opioid receptor antagonists precipitate pain-like responses under different pathological states (Yu et al., 1994; Back et al., 2006; Sevcik et al., 2006; Guan et al., 2010; Campillo et al., 2011b).
2.4.4. Contralateral effects

Interestingly, in the contralateral dorsal horn we also found increased MOR-G-protein GTPγS\textsuperscript{35} binding (Figure 2.4d) and NTX-induced neuronal activation (i.e. pERK+ cells; Figure 2.5b), illustrating the spread of central sensitization and increased opioid signaling, to uninjured areas of the CNS. This possibly accounts for the NTX-induced “mirror pain” in the uninjured paw (Figure 2.3b). The development of acute hindpaw hypersensitivity is not merely a side specific occurrence but rather a bilateral phenomenon. Contralateral mechanical sensitivity is also reported in numerous pain models (inflammation (Chillingworth et al., 2006), nerve injuries (Kim and Chung, 1992) (Seltzer et al., 1990), formalin (Aloisi et al., 1993), arthritis (Rees et al., 1996), sciatic inflammatory neuritis (Chacur et al., 2001), and muscle pain (Sluka et al., 2001; Ainsworth et al., 2006). Bilateral sensitivity has been discussed for years and several theories have been postulated involving spinal interneurons, astrocyte gap-junction networks, or top-down facilitation from the brainstem (For review see: (Koltzenburg et al., 1999) and (Cao and Zhang, 2008)), although a definitive cause remains elusive. However the lack of NTX-induced pERK (a marker of cellular activity and central sensitization) in microglia and astrocytes suggests that lasting sensitization does not occur in this cell type and these cells are not activated upon loss of tonic MOR signaling.

Whether this long-term neuroadaptation is strictly localized to the spinal cord or supraspinal regions remains unclear. Persistent inflammation increases the effectiveness of exogenously applied opioid agonists to inhibit contralateral nociceptive sensitivity in the RVM (Schepers et al., 2008a) and the spinal cord (Stanfa and Dickenson, 1995). Our opioid receptor antagonist injections into the spinal cord (Figure 2.3) or into the brain (i.c.v. drug injections: Chapter 3, Figure 3.9) also suggest adaptive enhancement modifications have taken place in both regions of the CNS.
2.4.5. *Endogenous opioidergic analgesia negatively regulates neuropathic pain*

Spinal intrathecal NTX injections increased mechanical and cold hypersensitivity in the CPxSx model of neuropathic pain. In the Spared Nerve Injury and Sciatic Nerve Ligation models of neuropathic pain, expression of spinal MORs are reported to be dramatically decreased in those areas where primary afferents have been injured, (Porreca et al., 1998; Kohno et al., 2005; Corder et al., 2010). It would seem that loss of MOR would be associated with hyperalgesia. But instead, we observed endogenous analgesia over our 21 day recovery period. DeGroot et al. (deGroot et al., 1997) saw a return of MOR expression 31 days following peripheral axotomy that was equal to the uninjured contralateral dorsal horn, which suggests possible recovery of spinal MOR inhibitory function. This could explain the natural resolution of neuropathic pain we observed.

Another study showed, two weeks after spinal nerve ligation, that a decrease in MOR immunostaining correlated with the development of hyperalgesia in rats, while non-allodynic rats did not exhibit a loss in spinal MOR expression. Those non-allodnic animals were given systmic naloxone HCl or intrathecal CTOP, which produced hyperalgesia. This suggests that loss of spinal MORs dictates the degree of neuropathic pain (Back et al., 2006). Similar suggestions on the importance of spinal MOR expression levels have been made when investigating the *severity* of injury to peripheral nerves. For example, the hyperalgesia accompanying damage to the larger tibial and common peroneal nerves does not recover (TxCPx). Whereas damage to the sural and common peroneal nerves displays a transient and recoverable neuropathic pain phenotype (SxCPx). Additionally, partial nerve ligation produces similar transient mechanical hyperalgesia compared to a full nerve ligation (Guan et al., 2010). The
shorter duration of hyperalgesia in the SxCPx model might represent a lesser decrease in MOR expression or function in the dorsal horn.

2.4.6. Future Studies

1. As illustrated in Figure 2.4f, spinal MORs mask latent sensitization: CTOP (i.t., 100ng) reinstated hypersensitivity 21 and 105d after CFA. However, the contribution of the δ- and κ- opioid receptor subtypes, in promoting long-lived endogenous analgesia, has not been tested. Future studies could administer selective blockers for μ (CTOP), δ (naltrindole), and κ (nor-binaltorphimine) opioid receptors to determine which receptors mediate long-lasting endogenous analgesia. It is important to note that the 100 ng of CTOP dose used in this dissertation is a high dose, and potentially interacts with δOR (personal communication with Dr. Gregory Scherrer). To test selectivity, a full dose-response curve should be generated for each opioid receptor blocker.

During inflammation and sustained pain conditions, δORs contained within large-dense-core-vesicles are functionally inserted into the plasma membrane (Cahill et al., 2003; Bao et al., 2003; Walwyn et al., 2005; Guan et al., 2005). Thus, δORs may contribute to long-term analgesia in our model. Therefore, a logical prediction is that naltrindole will reinstate hypersensitivity. In addition, blockade of spinal κORs with nor-BIN and genetic deletion of κOR enhanced CFA-induced hypersensitivity (Schepers et al., 2008) and nor-BIN reinstated mechanical hypersensitivity in a model of post-operative pain (Campillo et al., 2011). Therefore, a further prediction is that blockade of spinal κORs in our model will reinstate mechanical/heat hypersensitivity. These are important areas for future investigations.

2. In humans, males and females are differentially affected by chronic pain (Unruh, 1996; Greenspan et al., 2007). Male mice were exclusively used throughout all
studies in this dissertation. Therefore, the use of male and female mice to explore sex differences, in relation to prolonged endogenous analgesia, remain a critical hypothesis to test.

It will be important to consider the effects of the estrous cycle on endogenous opioid analgesia. Human female studies have shown that low estradiol (E2) correlates with decreased MOR activity and increased pain sensitivity (Smith et al., 2006), whereas experimental high E2 conditions correlate with enhanced opioid peptide release and receptor activation (Zubieta et al., 2002). E2 administration, in ovariectomized rodents, has been shown to increase MOR expression (Hammer et al., 1987; Dondi et al., 1992; Quinones-Jenab et al., 1997), opioid peptide expression (Broad et al., 1993; Hammer et al., 1993) and release (Eckersell et al., 1998). On this basis, we would predict that female mice will not recover sensory thresholds as quickly, or fully, as males. We could also predict that spinal NTX will be effective at unmasking latent sensitization females. However, this prediction is tentative for several reasons such as: a large body of literature argues that estrogens are pronociceptive (Levine et al., 1989; Aloisi et al., 2000; Craft et al., 2004); there are no sex differences in spinal MOR expression (Ji et al., 2006); male basal MOR expression is higher in the PAG (Loyd et al., 2008); and exogenous opioids are more effective at relieving pain in male rodents and humans (Cepeda et al., 2003; Miller et al., 2004).

Study limitations or confusion in data interpretation could result from findings demonstrating that, indeed, physical insult (e.g. i.pl CFA (Battisto et al., 1967)) and stress compromises menstrual cyclicity (Harlow et al., 1991; Bonen, 1994; Carpenter, 1994). The CFA could arrest female mice in diestrous, a period of low estrogens (Battisto et al., 1967).
3. In several pilot studies, myself and other members of the Taylor lab (Justin Pinson, Jennifer Grasch, Amanda Webb, and Ryan Griggs), attempted to visualize the spinal MOR population using immunohistochemistry. However, the anti-MOR antibodies from Neuromics (product # RA10104 and GP10106; 1:250 – 1:2000) and ImmunoStar (product # 24216; 1: 500 – 1:1000) produced high background and staining patterns not fully consistent with MOR in situ hybridization studies (i.e. intense positive staining throughout the spinal cord gray matter) (Matthes et al. 1996). Future studies should attempt a broader range of antibody concentrations (1:2000 and greater), to test selectivity. However, via a personal communication, these same antibodies have been reported to show some positive staining in MOR KO mouse brains and large amounts of diffuse staining within the dorsal root ganglia cell cytoplasm of wild-type mice (MOR, like other GPCRs, is present mostly on the cell membrane) (personal communication with Gregory Scherrer and Allan Basbaum). Therefore new antibodies should be investigated or created, and tested against MOR KO mice to ensure selectivity. Additionally, future studies could involve the use of alternative quantitative methods to assess MOR expression levels throughout the course of CFA hyperalgesia and into the post-hyperalgesic state. More sensitive techniques that could be explored include radiographic studies utilizing radio-tagged ligands (the radio-tag signal can be quantified as a measure of functional binding pockets (e.g. more binding pockets indicates increased MOR levels)), qRT-PCR, or in situ hybridization.

4. The predominant presence of pERK in the substantia gelatinosa (laminas I-II) suggests that the latent sensitization and tonic MOR signaling may exist in NK1-R expressing projection neurons, excitatory (PKCγ or calbindin populations), or inhibitory (GABAergic) interneurons. Future studies, using immunohistochemical co-expression experiments, will be required to determine the exact neuronal subpopulations in which tonic MOR signaling and latent sensitization occur.
5. The lack of NTX-induced pERK in astrocytes or microglia (Fig 2.6) suggests that the maintenance of lasting sensitization does not occur in these cell types. However this does not exclude astrocytes or microglia from participating in the induction of latent sensitization. Future studies could pharmacologically block glia function during the first 3 weeks of the CFA injury (e.g. minipump infusion of minocycline or fluorocitrate), then assess the ability of NTX to produce hyperalgesia and spinal pERK.
Tonic opioid receptor signaling contributes to the persistence of central sensitization by producing psychological and physical opioid dependence

3.1. Introduction

The data provided in Chapter 2 suggests that NTX disinhibits an underlying nociceptive sensitization, arguing that both opioidergic and pronociceptive systems did not subside after injury recovery. Indeed, a long-lasting balance between opioidergic and NMDA-R activity was demonstrated following chronic environmental stress (Le Roy et al., 2011) or injury (Rivat et al., 2002; Rivat et al., 2007). Although never fully investigated beyond mere behavioral pharmacology studies, this excitation/inhibition balance was suggested to result from a pathological consequence of increased endogenous opioid tone after the stress or insult.

How might long-lasting MOR signaling contribute to the persistence of pathological nociceptive plasticity or the latent central sensitization? Neuroadaptations result from prolonged exogenous opioid exposure, specifically compensatory increases in the expression of Ca\(^{2+}\)-sensitive adenylyl cyclases, AC1 and AC8 (Lane-Ladd et al., 1997). These cellular mechanisms are hypothesized to promote drug addiction and prolonged physical dependence. Such augmentations are revealed during opioid withdrawal and produce cAMP overshoot (AC superactivation), leading to excitatory synaptic plasticity.

AC1 is expressed in the post-synaptic density of central neurons (Xia et al., 1991; Conti et al., 2007), in the brain and the superficial dorsal horn (Wei et al., 2002), and is subject to negative regulation by G\(_{ai}/o\)-coupled receptors \textit{in vivo} (Nielsen et al.,
1996). Functionally, AC1 links NMDA-R-derived Ca\(^{2+}\) increases to cAMP signaling pathways (Chetkovich and Sweatt, 1993; Wong et al., 1999) necessary for LTP initiation (Wang and Zhuo, 2002), opiate physical dependence (Zachariou et al., 2008) and spinal synaptic facilitation (Wei et al., 2006; Wang et al., 2011). AC1 has a prominent role in chronic pain (Wei et al., 2002) and, mechanistically, may facilitate the phosphorylation and insertion of synaptic GluR1 (Xu et al., 2008) via PKA and/or PKM\(\zeta\) (Li et al., 2010; Asiedu et al., 2011a; Zhuo, 2012) to increase synaptic strength during LTP. It has been demonstrated that withdrawal from exogenous opioids results in synaptic LTP in spinal neurons and relies on post-synaptic NMDA-R Ca\(^{2+}\) mobilization to mediate the hyperalgesia associated with physical withdrawal (Drdla et al., 2009).

I hypothesize that prolonged endogenous-mediated MOR signaling drives the upregulation of the spinal adenylyl cyclase system in an analogous manner to opiate cellular dependence, thereby promoting the maintenance of latent central sensitization.

3.2. Methods and Materials

3.2.1. Mice: At the beginning of all experiments the subjects were naïve, adult (6-10 weeks for behavioral and biochemical studies; 3-4 weeks for Ca\(^{2+}\) imagining studies), male C57Bl/6 (Charles River), AC1\(-/-\), or littermate AC1\(+/+\) wild-type controls. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky in accordance with American Veterinary Medical Association guidelines.

AC1\(-\) mice and AC1\(+/+\) mice, bred congenic onto a C57Bl/6 background, were provided by Dr. Daniel Storm (University of Washington, Seattle, WA, USA). Renee Donahue maintained the line using heterozygous breeding and the genotype was confirmed by a tail-snip PCR.
As originally characterized by Xu and Storm (1995), generation of AC1 KO mice was performed as follows: A gene targeting vector was constructed and used for homologous recombination in ES cells. A mouse AC1 genomic clone, ZX3 (14.5 kb), encoding the N-terminal 210 amino acids of I-AC was isolated from a genomic library (OLA 129). The gene-targeting vector, 1126, was constructed by replacing a 0.5-kb Nar I-Pst I fragment of ZX3, which includes the coding region for the first 140 amino acids of AC1, with neo’ cassette. Then, a herpes simplex virus thymidine kinase gene was added to one end of the vector. An ES cell clone was injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeric mice bred with C57BL/6 females to produce heterozygotes that were subsequently inbred to produce homozygous mutant mice.

Mice were housed in plastic cages (bedding and enrichment) of 4 same-sex littersmates in a temperature controlled environment (14 hr: 10 hr light-dark cycle; lights on at 7:00 am) with ad libitum access to food and water. Upon arrival to the University of Kentucky Division of Laboratory Animal Resources, mice were habituated to the colony housing room for 1 week prior to any experimentation.
Table 3.1. Animal use quantification for figures 3.1 – 3.11.

<table>
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<tr>
<th>Figure</th>
<th>Group n at start of experiment</th>
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<td>3.1a</td>
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<td>Naive: 2 3d Sham: 4 3d CFA:5 21d Sham:5 21d CFA:7 1-2 spinal cord slices were used from each animal.</td>
<td>Only slices that displayed a consistent control response to 1 mM glutamate at the beginning and end of the experiment (showing a less than 40% decrease in glutamate-evoked Ca2+ transients) were included in this study.</td>
<td>2012</td>
</tr>
<tr>
<td>3.1b</td>
<td>--</td>
<td>Sham+NTX: 3 CFA+NTX: 4 Sham+CTOP: 4 CFA+CTOP: 5 Sham+NTX+MK-801: 5 CFA+NTX+MK-801: 6</td>
<td>n/a</td>
<td>2012</td>
</tr>
<tr>
<td>3.1d,e</td>
<td>--</td>
<td>Vehicle+vehicle: 5 Vehicle+NTX: 5 MK-801+vehicle: 7 MK-801+NTX: 9</td>
<td>n/a</td>
<td>2011</td>
</tr>
<tr>
<td>3.2a,b</td>
<td>Sham: 6 CFA: 6</td>
<td>Sham: 6 CFA: 6</td>
<td>n/a</td>
<td>2012</td>
</tr>
<tr>
<td>3.2c,d</td>
<td>Sham: 5 CFA: 5</td>
<td>Sham: 5 CFA: 5</td>
<td>n/a</td>
<td>2012</td>
</tr>
<tr>
<td>3.2e</td>
<td>Exp. 1: Sham+vehicle: 6 Sham+CTOP: 6 CFA+vehicle: 6 CFA+CTOP: 6 Exp. 2: Sham+vehicle: 8 Sham+NTX: 6 CFA+vehicle: 11 CFA+NTX: 10</td>
<td>Sham+vehicle: 14 Sham+CTOP: 6 Sham+NTX: 6 CFA+vehicle: 17 CFA+CTOP: 6 CFA+NTX: 10</td>
<td>Control groups (‘Sham+vehicle’ and ‘CFA+vehicle’) were combined from the two experiments</td>
<td>2012</td>
</tr>
<tr>
<td>3.3a-c</td>
<td>AC1-/-: 9 AC1+/+: 4</td>
<td>AC1-/-: 9 AC1+/+: 4</td>
<td>These were the only available mice provided by Dr. Daniel Storm (U. Washington)</td>
<td>2012</td>
</tr>
<tr>
<td>3.3d</td>
<td>Vehicle+NTX: 5 NB001+NTX: 7</td>
<td>Vehicle+NTX: 4 NB001+NTX: 7</td>
<td>--</td>
<td>2012</td>
</tr>
</tbody>
</table>
Table 3.1 (continued)

| 3.3e  | -- | Saline: 6  NTX: 8  NB001+NTX: 9 | n/a | 2012 |
| 3.5a,b| Sham+naloxone: 6  CFA+saline: 6  CFA+naloxone: 6 | Sham+naloxone: 6  CFA+saline: 6  CFA+naloxone: 6 | n/a | 2012 |
| 3.5c,d| Naloxone: 6  Naloxone+NB001: 6 | Naloxone: 6  Naloxone+NB001: 6 | n/a | 2012 |
| 3.8   | 8  | CFA 1d:8  CFA 3d: 8  CFA 21d: 7  CFA 49d: 7  CFA 77d:6 | - loss of mice resulted from death by littermate fighting | 2009 |
| 3.9b, c-e | -- | CFA+vehicle: 14  CFA+CTOP 100ng: 7  CFA+CTOP 300 ng: 12  CFA+B-FNA: 9 | n/a | 2012 |
| 3.10a-e | Sham: 8  CFA: 8 | Sham: 7  CFA: 8 | Mouse found dead in home cage on day 3. Exact cause unknown. Possible effect of morphine. | 2012 |

Unknown information is denoted by dashed lines (--).

Information that is not applicable is denoted as n/a
3.2.2. *Complete Freund’s Adjuvant (CFA) model of inflammatory pain:* please refer to section 2.2.2, page 30.

3.2.3. *Mouse (in vivo) drug administration:* For subcutaneous injections and intrathecal injection methods please refer to section 2.2.4, page 30. For intracerebroventricular injection of drugs, guide cannula (Plastics one, Roanoke, VA) were placed 14d after CFA. Surgical anesthesia was achieved with isoflurane (5% for induction; 1.5e2% for maintenance). Anesthetized mice were placed in a stereotaxic apparatus fitted with blunt ear bars (Stoelting, Kiel, WI). After an incision to expose the cranium, the dorsal surface of the skull was leveled by zeroing the dorso-ventral coordinate at lambda and bregma. A 26-G stainless steel guide cannula (Plastics One, Roanoke, VA) was lowered to the right lateral brain ventricle using the following stereotaxic coordinates: 0.7 posterior to bregma, 1.5 mm lateral from midline and 3.3-4.0 mm below the skull surface (Paxinos and Watson, 1997). Initial placement of the cannula was verified by slow downward movement of saline when the tubing was opened and raised (Taylor et al., 1994). The cannula was fixed to the skull with 2-3 small screws and dental cement. After hardening of the cement and suturing of the incision, a 30G stylet (Plastics One, Roanoke, VA) was secured within the guide.

3.2.4. *Drug dosing:* The following drugs and doses were used for *in vivo* experiments: naltrexone HCl (NTX, gift of the NIDA Drug Supply Program; subcutaneous: 0.003 – 10.0 mg / kg / body weight, dissolved in 200 µl saline; intrathecal: 1µg in 5µl saline), naltrexone methobromide (NMB, gift of the NIDA Drug Supply Program; subcutaneous: 3 mg/kg in 200 µl saline; intrathecal: 0.3 µg in 5µl saline), naloxone (Sigma-Aldrich; intraperitoneal: 3 mg/kg in 200 µl saline), Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, Sigma-Aldrich; intrathecal: 100 ng in 5µl saline), pertussis toxin (Sigma-Aldrich; intrathecal: 0.5 µg in 5µl water), (+)-MK-801 maleate (Tocris; intrathecal: 1 µg in 5µl water).
water) [this dose of MK-801 was devoid of overt motor effects], NB001 (Sigma-Aldrich; intrathecal: 2.5 µg in 5µl water), N-Methyl-D-aspartic acid (NMDA, Sigma-Aldrich; intrathecal: 30 pmol in 5µl water), forskolin (Sigma-Aldrich; intrathecal: 1.5 µg in 5µl 1% DMSO), and [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO; Sigma-Aldrich; intrathecal: 10-30 pmol in 5 µl saline).

3.2.5. Mechanical hyperalgesia testing: Please refer to section 2.2.6, page 32.

3.2.6. Antinociception testing: Please refer to section 2.2.9, page 33.

3.2.7. Conditioned Place Preference: The rationale and logic of these sets of experiments were designed by Greg Corder, Zaijie Wang (University of Illinois at Chicago) and Bradley Taylor. Ying He (University of Illinois at Chicago) performed the experiments. The CPP apparatus (San Diego Instruments, San Diego, CA) consists of 3 Plexiglas chambers separated by manual doors. A center chamber (6 1/4”W X 8 1/8” D X 13 1/8” H) connects the 2 end chambers, which are identical in size (10 3/8” W X 8 1/8” D X 13 1/8” H) but can be distinguished by texture of floor (rough versus smooth) and wall pattern (vertical versus horizontal stripes). Movement of mice and time spent in each chamber were monitored by 4 X 16 photobeam arrays and automatically recorded in San Diego instruments CPP software.

Preconditioning was performed 18 d after CFA injury across 3 days (days 1-3) for 30 min each day when mice were exposed to the environment with full access to all chambers. On day 3, a preconditioning bias test was performed to determine whether a preexisting chamber bias existed. In this test, mice were placed into the middle chamber and allowed to explore the open field with access to all chambers for 15 min. Data were collected and analyzed for duration spent in each chamber. Animals spending more than
80% or less than 20% of the total time in an end chamber were eliminated from further testing.

We used a single conditioning protocol. On day 4 (21 d after CFA), mice first received naloxone (i.p.) + saline (i.t.) paired with a randomly chosen chamber in the morning. 4hr later, naloxone (i.p.) + lidocaine (0.04% i.t.) was paired with the other chamber in the afternoon. Hyperalgesia resolved within 4 hr. During conditioning, mice remained in the paired chamber, without access to other chambers, for 15 min immediately following saline or drug injection. On the test day, 20 hr after the afternoon pairing (22 d after CFA), mice were placed in the middle chamber of the CPP box with all doors open, allowing free access to all chambers. Movement and duration of time spent in each chamber were recorded for 15 min for analysis of chamber preference.

The plasma half-life of NTX is approximately 4 hr and we have found behavioral effects of NTX lasting 210 min after subcutaneous administration. Based on this fact we used naloxone for all CPP experiments, due to the reported shorter half-life (~90 min), so that two conditioning sessions could be performed, 4 hrs apart, in one day.

3.2.8. Spontaneous nocifensive behavior: Following intrathecal injection of NMDA or forskolin, we recorded (15 min) the duration of nocifensive behavior, defined as caudally-directed licking or biting of the limbs, tail and trunk. Time spent on genital licking or grooming above the trunk mid-line was not scored. The number of spontaneous paw lifts/flinches was quantified separately.

3.2.9. Phosphorylated extracellular regulated kinase (pERK) stimulation: Please refer to section 2.2.10.1, page 33.

3.2.10. Histology and Immunohistochemistry: Please refer to section 2.2.11, page 34.
3.2.11. cAMP ELISA assay: Greg Corder and Dr. Brandon Jutras performed all cAMP ELISA studies. Greg Corder analyzed all data and generated the tissue. For in vivo detection of intracellular cAMP levels, lumbar spinal cords were rapidly dissected 30 min after intrathecal injections. L3-L5 segments were quickly blocked, frozen on dry ice, and stored at -80 Celsius for later use. cAMP measurements were performed using a direct cAMP enzyme immunoassay (Enzo Life Sciences) according to the manufacturer's protocol (non-acetylated protocol with sensitivity = 0.39 pmol/ml).

We utilized entire lumbar spinal cord section (dorsal and ventral) for the following reasons: 1) it was necessary to use the dorsal and ventral aspects to achieve sufficient sample (protein) quantities to run the cAMP ELISA in duplicate as well as run a concurrent Bradford protein assay in duplicate for each spinal cord sample; 2) only dorsal horn neurons exhibited increased pERK expression, suggesting that the latent central sensitization processes exist in this region; and 3) The large majority of MOR expression is in the dorsal horn as well (Abaddie et al., 2000). Therefore, we believe that changes in cAMP reflect events in the dorsal horn and not the ventral horn.

3.2.12. GTPγS<sup>35</sup> binding assay: Please refer to section 2.2.12, page 35.

3.2.13. Calcium imaging: As previously described by Doolen et al. (2012), Dr. Suzanne Doolen (University of Kentucky) sectioned transverse spinal cord slices (300-450 μm), derived from 6 week old mice (CFA and sham injuries, and nociceptive behavior performed by Greg Corder), and incubated them for 60 min at room temperature with Fura-2 AM (10 μM) and pluronic acid (0.1%) in oxygenated aCSF containing (in mM): NaCl 127, KCl 1.8, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.4, MgSO<sub>4</sub> 1.3, NaHCO<sub>3</sub> 26, glucose 15. This was followed by a 20 min de-esterification period in normal aCSF. Slices were perfused at 1–2 ml/min with normal aCSF in an RC-25 recording chamber.
(Warner Instruments) mounted on a Nikon FN-1 upright microscope fitted with a 79000 ET FURA2 Hybrid filter set (Nikon Instruments) and a Photometrics CoolSNAP HQ2 camera. Relative intracellular Ca\textsuperscript{2+} levels were determined by measuring the change in ratio of fluorescence emission at 510 nm in response to excitation at 340 and 380 nm (200 ms exposure). Paired images were collected at 1–1.5 seconds/frame. Relative changes in Ca\textsuperscript{2+} levels were evaluated using Nikon Elements software by creating a region of interest over the cell body and calculating the peak change in ratio. The peak magnitude of Ca\textsuperscript{2+} transient was expressed as difference in exposure to exogenous glutamate compared to baseline before glutamate. The criteria for a Ca\textsuperscript{2+} response required at least a 10% increase above the baseline ratio. Ca\textsuperscript{2+} transients were in response to a 10 s exposure to 0.3 mM glutamate. In antagonist studies, the slice was perfused with the antagonist for 15 min prior to and during glutamate stimulation. Only cells that displayed a consistent control response to 1 mM glutamate at the beginning and end of the experiment (showing a less than 40% decrease in glutamate-evoked Ca\textsuperscript{2+} transients) were included in this study.


3.2.15. Experimental blinding procedures: The experimenter, Greg Corder, was blind to drug treatments during all behavioral pharmacology experiments. This was accomplished by making each drug and vehicles in similar tubes and to the same volume. These tubes were then given to Renee Donahue, Jennifer Grasch, Ryan Griggs, Weisi Fu or Suzanne Doolen for tube coding. Greg was then given the tubes marked with distinguishing letters. After the experiment, the code was broken and Greg analyzed the data. For i.c.v. experiments, Dr. Felipe Porto helped with animal handling and blinding.
3.3. Rationale and Results

3.3.1. Endogenous opioidergic cellular withdrawal relies on NMDA-R activity

Our lab recently reported that peripheral nerve injury produces a potentiated increase in glutamate-evoked Ca$^{2+}$ mobilization in lamina II dorsal horn neurons, coinciding with the manifestation of neuropathic pain (Doolen et al., 2012). To determine if spinal glutamatergic sensitization resolves alongside inflammatory pain, Dr. Suzanne Doolen and I assessed Ca$^{2+}$ transients in spinal neurons in response to brief (10 s) exogenous bath-application of glutamate (0.3 mM). Fura-2 ratiometric analysis of lumbar spinal cord slices revealed that glutamate induced a small rise of intracellular [Ca$^{2+}$] in neurons from uninjured naïve mice (Figure 3.1a; 340 nm/380 nm ratio = 0.0221 ± 0.0014). However, the Ca$^{2+}$ response was significantly greater 3d after CFA compared to sham mice (340 nm/380 nm ratio = 0.0492 ± 0.0062 in CFA vs. 0.0311 ± 0.0027 in Sham, $F_{3,17}=15.23$, $P<0.0001$), consistent with observations 24 hr after CFA (Luo et al., 2008). The potentiated Ca$^{2+}$ signals were not observed 21d after CFA (340 nm/380 nm ratio = 0.0254 ± 0.0073 in CFA vs. 0.0303 ± 0.0011 in Sham), matching the temporal onset and resolution of inflammatory hyperalgesia.

We next tested the hypothesis that similar NMDA-R and Ca$^{2+}$-dependent mechanisms which induce central sensitization and long-term potentiation (LTP) during the induction of inflammatory hyperalgesia (Latremoliere and Woolf, 2009; Luo et al., 2008) are required for the induction of pain reinstatement. Perfusion of either CTOP or NTX markedly increased the peak amplitude of the glutamate-evoked [Ca$^{2+}$] in CFA-21d but not sham slices (Figure 3.1b-d; CTOP: Sham, 96.09 ± 3.47 % vs. CFA-21d, 145.3 ± 28.03 %; NTX: Sham, 112 ± 3 % vs. CFA 21d, 157 ± 16 %, all values are relative to pre-drug control responses; $P<0.05$). To determine the contribution of NMDA-R activity to potentiated [Ca$^{2+}$] levels, we delivered MK-801, an activity-dependent NMDA-R blocker.
MK-801 prevented the NTX-mediated rise in \([\text{Ca}^{2+}]_i\) (Figure 3.1d; Sham: 107 ± 7 % over control vs. CFA 21d: 104 ± 5 % over control; \(F_{1,16} = 4.6, P < 0.05\)).

NMDA-R-derived \(\text{Ca}^{2+}\) flux is necessary for ERK activation (Krapivinsky et al., 2003; Lever et al., 2003; Sindreu et al., 2007), specifically under pathological conditions or noxious stimulation (Ji, 2004; Matsumoto et al., 2008). We found that pre-treatment with intrathecal MK-801 (3 nmol) resulted in prevention of NTX-induced hyperalgesia (Figure 3.1g; \(F_{3,88} = 5.4, P < 0.005\)) and loss of bilateral increases in dorsal horn pERK levels (Figure 4.1e-f, ipsilateral and contralateral; both \(P < 0.05\) compared to Vehicle + NTX). Together these results reveal that loss of tonic MOR signaling initiates an NMDA-R—\(\text{Ca}^{2+}\)—pERK pathway to generate the reinstatement of pain.
Figure 3.1. NMDA-receptor-mediated Ca\(^{2+}\) signaling is required for pain reinstatement and dorsal horn neuron activity during endogenous opioid cellular withdrawal.

(a) Time course of glutamate-evoked (0.3 mM) intracellular calcium concentrations in adult spinal cord slices from naïve, sham or CFA mice. \((n = 4 – 7)\). (b) Glutamate-evoked calcium levels in the presence of vehicle, CTOP (1 µM), NTX (1 µM) or NTX+MK-801 (100 µM). \((n = 3-6 \text{ mice}, 1-2 \text{ slices per mouse})\). ★ \(P < 0.05\) compared to Sham. (c) Representative F\(_{380}\) nm image of dorsal horn neurons responding to glutamate stimulation before (control, top row panels) and 15 min after continuous NTX (10 µM, bottom row panels) perfusion in a spinal cord slice from a CFA-21d mouse. A decrease
in fluorescence intensity corresponds to increases in intracellular [Ca\(^{2+}\)] since basal Fura-2 fluorescence is decreased by 380nm light. The red traces illustrate the Δ F340/F380 for the glutamate-evoked rise in [Ca\(^{2+}\)], in the indicated cell (red arrow). Vertical Δ F340/F380 scale = 0.02, horizontal time scale = 3 min; scale bar =100 µm. Insets at upper left depict area in white box; scale bar = 10 µm. The F380nm, in lieu of the ratiometric F340/380 image was selected for the sake of clarity. Fura-2 AM is excited at both 340nm and 380 nm light. The magnitude in fluorescence decrease following 380nm exposure is greater (and therefore easier to see by eye) than the magnitude of fluorescence intensity following 340nm exposure. Thus, to adequately show a significant rise in intracellular calcium levels the 380nm is displayed. (d-f) Effect of intrathecal MK-801 (1 µg) on NTX-precipitated (d) mechanical hyperalgesia and (e and f) light-touch evoked pERK in lamina I-li or III-IV. (n = 5-10). ★ P < 0.05 compared to ‘Vehicle + vehicle’; ◆ P < 0.05 compared to ‘MK-801 + NTX’. Data shown as mean ± s.e.m. Dr. Suzanne Doolen performed the calcium imagining experiments.
3.3.2. Endogenous opioidergic withdrawal precipitates an NMDA-R-dependent adenylyl cyclase superactivation

Opioids produce their acute actions in part through inhibition of adenylyl cyclases (ACs), whereas chronic opiate exposure produces a homeostatic upregulation of ACs (Nestler and Aghajanian, 1997; Christie, 2008). In this opioid-dependent state, opioid receptor antagonists produce cellular withdrawal, characterized by a cAMP overshoot response. We hypothesized that similar homeostatic mechanisms operate in the setting of tonic opioid receptor signaling after injury. To address this novel concept, Dr. Brandon Jutras and Greg Corder sampled intracellular cAMP content from ex vivo lumbar spinal tissue using an ELISA assay. While basal spinal cAMP levels were comparable in sham and CFA 21d mice (Figure 3.2a), intrathecal CTOP or NTX dramatically increased cAMP levels in CFA-21d mice (Figure 3.2a; P < 0.05), indicative of AC superactivation.

Because the Ca^{2+} stimulated isoforms of ACs are activated by NMDA-Rs (Chetkovich and Sweatt, 1993) and we have shown that NMDAR signaling is necessary for hyperalgesia reinstatement, we next hypothesized that NMDA-R signaling contributes to this cAMP overshoot during opioid receptor blockade. Pretreatment with intrathecal MK-801 abolished the NTX-precipitated increases in cAMP (Figure 3.2b; P < 0.05 compared to NTX group). Moreover, direct activation of spinal NMDARs with intrathecal NMDA (3 pmol) increased spontaneous nocifensive behaviors (Figure 3.2c; P < 0.05) and spinal cAMP levels (Figure 3.2d; P < 0.05) in CFA mice as compared to shams. Similarly, direct activation of the spinal AC system with intrathecal forskolin (2.5 μg) produced enhanced spontaneous nocifensive behaviors (Figure 3.2e; P < 0.05) and intracellular cAMP (Figure 3.2f; P < 0.05). Thus, injury-induced tonic MOR signaling is accompanied by latent up-regulation in the spinal cord of NMDA-R and AC functional capacity.
Figure 3.2. Endogenous opioid withdrawal initiates NMDA-R-dependent AC superactivation.

(a) Effect of forskolin (i.t.; 1.5 µg) on spontaneous nocifensive behaviors (left) and paw flinches (right) over 15 min in Sham (n = 6) and CFA (n = 6) mice. *P < 0.05 compared to Sham group. (b) Lumbar spinal cord cAMP levels from the same mice in (a) collected 30 min after i.t. injection. (c) Effect of NMDA (i.t.; 3 pmol) on spontaneous nocifensive
behaviors (left) and paw flinches (right) over 15 min in Sham (n = 5) and CFA (n = 5) mice. ★ P < 0.05 compared to Sham group. (d) Spinal cAMP levels from the same mice in panel c. ★ P < 0.05 compared to Sham group. (e) Spinal cord cAMP content measured by ELISA at 30 min after intrathecal vehicle (Sham, n=14; CFA-21d, n=18), CTOP (100 ng; Sham, n=6; CFA-21d, n=6) or NTX (1 µg; Sham, n=6; CFA-21d, n=10). ★ P < 0.05. Mice in control groups (Sham+vehicle and CFA-21d+vehicle) across CTOP and NTX studies were combined. (f) Effect of MK-801 (3 nmol) pretreatment on NTX-mediated cAMP superactivation (n = 5 CFA-21d + saline, n = 4 CFA-21 + MK-801, n = 5 CFA-21d + NTX, n = 5 CFA-21d + MK-801 + NTX). ★ P < 0.05. Student’s t test, two-tailed. Data shown as mean ± s.e.m. Greg Corder and Dr. Brandon Jutras worked side by side to perform the cAMP ELISA studies.
3.3.3. Adenylyl cyclase type 1 is required for endogenous opioidergic cellular dependence

Ca^{2+} sensitive adenylyl cyclase type 1 (AC1) is intricately linked to morphine dependence in the locus coeruleus (Lane-Ladd et al., 1997; Zachariou et al., 2008; Mazei-Robison and Nestler, 2012) and the maintenance of chronic pain in the anterior cingulate cortex (Wei et al., 2002). While in the spinal cord AC1 contributes to activity-dependent high-frequency-stimulation-induced LTP (Wang et al., 2011). However, the regulation and contribution of AC1 to injury-induced plasticity in the spinal cord is less clear (Zhuo, 2012). Therefore, we tested the hypothesis that spinal AC1 is the specific isoform responsible for the development of cellular dependence to tonic endogenous opioidergic signaling. To test this hypothesis we intrathecally administered NB001, a potent selective inhibitor of AC1 (Wang et al., 2011), prior to NTX. NB001, but not saline, prevented spinal cAMP overshoot (Figure 3.3e; $P < 0.05$ compared to NTX group) and hyperalgesia reinstatement (Figure 3.3d; $F_{1,9} = 6.612$, $P = 0.03$). To further demonstrate the contribution of AC1 to opioid dependence we utilized AC1 knockout mice. As reported previously (Wei et al., 2002), baseline mechanical thresholds were similar in wild-type and AC1 knockout mice (AC1$^{-/-}$) (Figure 3.3a), indicating AC1 does not facilitate acute nociception. However, AC1 gene deletion significantly reduced inflammatory hyperalgesia (Figure 3.3a; 3d vs. baseline: $P < 0.05$, t test; $F_{1,11} = 31.5$, $P = 0.0002$, 2-way ANOVA, Genotype X Time), despite the presence of an intact inflammatory response (Figure 3.3c). Importantly, the ability of NTX to reinstate mechanical hyperalgesia in wild-type mice was lost in AC1$^{-/-}$ mice (Figure 3.3b; $F_{1,7} = 20.30$, $P = 0.002$, 2-way ANOVA Gene x Time). We conclude that long-lasting tonic MOR signaling generates opposing mechanisms (i.e. NMDA-R-AC1 signaling pathways) that create a state of opioid cellular dependence in the spinal cord. This is manifested as
cellular withdrawal (increased cAMP) and increased neuronal excitability to facilitate the reinstatement of hyperalgesia (Figure 3.4).
Figure 3.3. Superactivation of spinal AC1 facilitates pain reinstatement and opioid cellular withdrawal.

(a) Progression of CFA-induced mechanical hyperalgesia in AC1<sup>−/−</sup> (n = 9) and littermate AC1<sup>+/+</sup> mice (n = 4). (b) Effect of intrathecal NTX (1 µg) in AC1<sup>−/−</sup> and AC1<sup>+/+</sup> mice 21 days after CFA. (c) Progression of CFA-induced paw edema in AC1<sup>−/−</sup> and AC1<sup>+/+</sup> mice. (d,e) Effect of intrathecal NB001 (1.5 µg) on NTX-precipitated (d) mechanical hyperalgesia (n=4-7) and (e) spinal cAMP content (n=6-9). ★ P < 0.05. Data shown as mean ± s.e.m.
Figure 3.4. Cellular signaling pathways underlying endogenous opioid withdrawal.

Left to Right: Spinal cord MOR signaling, through inhibitory Gαi/o proteins, tonically represses AC1 production of cAMP, thereby reducing nociceptive signal transduction. Blockade of MOR results in the disinhibition of AC1, allowing for NMDA-R-derived, Ca2+-mediated activation and downstream increases in neuron excitability, signal transduction, and consequently, hyperalgesia. Gray text indicates pharmacological inhibitors that act upon the indicated protein of interest.
3.3.4. Unmasking psychological and physical dependence to supraspinal endogenous opioidergic signaling

Opioid receptors are widely distributed throughout all axes of the nervous system (Mansour et al., 1995), including the spinal cord and brain. Chronic exposure of these receptors to opiate drugs leads to physical dependence, such that abstinence or decrease in dose produces negative physical and affective symptoms. Experimentally, injection of an opioid receptor antagonist such as naltrexone precipitates a withdrawal syndrome in opiate-dependent animals. Manifestations of physical withdrawal in rodents include: autonomic dysfunction; motor abnormalities such as ptosis, paw flutters, body shakes, and rearing (Maldonado et al., 1992; Kest et al., 2002b); and hyperalgesia (Sweitzer et al., 2004; Bie et al., 2005; Vera-Portocarrero et al., 2011). Peripheral tissue inflammation is associated with increased CNS expression of opioid receptors (Ji et al., 1995; Goff et al., 1998; Calza et al., 2000; Mousa et al., 2002) and increased release of opioid peptides in pain-modulatory regions of the brain, thereby leading to intrinsic pain relief (Fields, 2004; Ossipov et al., 2010). Given that opioid peptides and receptor expression can remain significantly elevated in the brain for several weeks after severe peripheral inflammation (Hurley and Hammond, 2001), we hypothesized that tissue injury induces endogenous opioid dependence in the brain, as well as the spinal cord.

3.3.4.1. Psychological withdrawal

Pain comprises sensory (hyperalgesia) and affective (aversiveness) components; the latter can be identified by changes in the rewarding property of analgesics and associated motivational behavior. For example, in a conditioned place preference paradigm (King et al., 2009; He et al., 2012), the negative reinforcing capacity of intrathecal lidocaine (motivation to seek pain relief) demonstrates the presence of
aversive spontaneous pain 1d after CFA (He et al., 2012), in addition to the reflexive pain measured by traditional von Frey and Hargreaves’s assays.

The following studies were designed by Greg Corder, Bradley Taylor and Zaijie Wang and were performed by Ying He at the University of Illinois at Chicago. We found that this inflammatory injury-mediated spontaneous pain was completely absent 21 days after CFA (Figure 3.5a; ‘CFA-21d+saline’ group). This enabled us to determine whether NTX-reinstated hyperalgesia is accompanied by symptoms of aversive somatic and psychological withdrawal as well as spinally-driven spontaneous pain. To test these hypotheses we assessed the aversion associated with somatic endogenous opioid withdrawal as well as spontaneous pain mediated by the spinal (not supraspinal) circuitry. During the one-day conditioning session, sham and CFA mice received ‘i.p. naloxone + i.t. saline’ paired with a randomly chosen chamber in the morning and, 4 hr later, ‘i.p. naloxone + i.t. lidocaine’, paired with the other chamber (see Methods, page 71, for rationale on use of naloxone in CPP studies). Chamber preference was tested 20 hr later. We found no chamber preference in sham-injured mice treated with naloxone or in 21d-CFA mice treated with systemic saline (Figure 3.5a and c; P > 0.05), confirming lack of spontaneous pain or on-going aversion. In contrast, CFA-21d but not sham mice responded to systemic naloxone by spending significantly more time in the chamber paired with intrathecal lidocaine (538 ±39 s) than with intrathecal saline (Figure 3.5a and c; 283±28 s; P < 0.001). We can infer two conclusions from this finding: 1. systemic naloxone increases aversive spontaneous pain in CFA21d-mice only and 2. blocking neural transmissions in the spinal cord produces pain relief induced by endogenous opioid withdrawal.

We next tested the hypothesis that opioid withdrawal-induced alterations in motivational behavior (i.e. drive to seek pain relief after opioid receptor blockade) rely on spinal AC1 superactivation. To do this we pre-administered intrathecal NB001 prior to
systemic naloxone during the one-day conditioning phase for intrathecal lidocaine (Figure 3.c and 3.5d). We found that CFA 21d-mice given only naloxone spent significantly more time in the intrathecal lidocaine paired chamber (P < 0.05), whereas mice receiving intrathecal NB001 prior to naloxone did not show a preference for either paired chamber. This demonstrates that the rewarding aspects of intrathecal lidocaine in opioid-withdrawing mice are blocked by inhibition of spinal AC1.
Figure 3.5. Endogenous opioid psychological dependence.

(a,b) Behavioral signs of psychological withdrawal (aversion associated with spontaneous pain), reflected by place preference for intrathecal lidocaine upon naloxone administration. (a) Intragroup chamber analysis for intrathecal saline (5 µl) or lidocaine (0.04%) in sham and CFA-21d mice treated with intraperitoneal saline or naloxone (3 mg/kg). (b) Intergroup difference score analysis illustrating time spent in intrathecal lidocaine-paired chambers. (n = 6 per group). (c,d) Effect of AC1 inhibition on naloxone-induced psychological withdrawal. (c) Intragroup chamber analysis for intrathecal saline (5 µl) or lidocaine (0.04%) in CFA-21d mice treated with intraperitoneal naloxone (3 mg/kg) or intrathecal NB001 (1.5 µg) + intraperitoneal naloxone. (d) Intergroup difference score analysis illustrating time spent in intrathecal lidocaine-paired chambers. (n= 6 – 12). ★ P < 0.05. Data shown as mean ± s.e.m. Studies designed by Greg Corder, Bradley Taylor and Zaijie Wang. Studies performed by Ying He.
3.3.4.2. Somatic withdrawal

To determine whether cutaneous inflammation induced an endogenous opioid physical dependence, we subcutaneously administered naltrexone (NTX; 3 mg/kg), a non-selective opioid receptor antagonist that precipitates withdrawal symptoms in morphine-dependent mice (Wang et al., 2004). When administered 24 hr or 3 d after intraplantar sham or CFA injection, NTX did not produce extensive escape/aversive behaviors (Figure 3.6a-f). Astonishingly, when administered 21 d after CFA, NTX precipitated pronounced somato-motor behaviors that were analogous with classical morphine physical withdrawal symptoms (Koob et al., 1992; Kest et al., 2002b). Symptoms began within 5 min of drug administration and lasted approximately 60 min. As illustrated in Figure 3.6a-f, NTX but not saline precipitated mechanical hypersensitivity ($F_{2,154} = 5.4, p < 0.05$), jumping ($F_{2,88} = 28, p < 0.0001$), rearing ($F_{2,88} = 36, p < 0.0001$), and paw flutters ($F_{2,88} = 70, p < 0.0001$).
Figure 3.6. Endogenous opioid physical dependence.

Incidence of opioid receptor antagonist-induced somatic withdrawal behaviors during a 60 min observation session following subcutaneous injection of saline, NTX (3mg/kg) or NMB (3mg/kg). (a and b) jumping frequency and total, (c and d) rearing frequency and total, (e and f) paw flutter frequency and total. 2-way ANOVA for behavioral frequency plots (a,c,e) and Student's t Test, two-tailed for cumulative plots (b,d,f). ★ $P < 0.05$. Data are s.e.m.
Additionally NTX, but not saline, precipitated ptosis, body shakes, teeth chattering and hyperlocomotion (Figure 3.7a-d; all \( P < 0.05 \)). No such effects were produced by naltrexone methobromide (NMB), a peripherally-restricted opioid receptor antagonist (\( P > 0.05 \)).
Figure 3.7. Additional somatic withdrawal behaviors indicative of endogenous opioid physical dependence.

Incidence of opioid receptor antagonist-induced somatic withdrawal behaviors, in the same mice from Figure 4.6, during a 60 min observation session following subcutaneous injection of saline, NTX (3mg/kg) or NMB (3mg/kg): (a) ptosis, (b) teeth chatters, (c) body shakes, and (d) locomotor activity. ★ P < 0.05. Data are s.e.m.
3.3.6. Endogenous opioidergic dependence intensifies over time

Tissue edema completely subsided within 77 d after CFA (Figure 3.1e). Therefore, to determine if endogenous opioid physical dependence persists beyond tissue healing, we gave periodic injections of NTX during the course of recovery. In mice, jumping is frequently considered a sensitive and reliable index of physical withdrawal intensity (Way et al., 1969; Saelens et al., 1971; Smits, 1975; Bilsky et al., 1996; Kest et al., 2001; Wang et al., 2004). One-way ANOVA analysis of NTX-induced escape jumps at 1d, 3d, 21d, 49d and 77d after CFA revealed a significant effect of Time (Figure 3.8; $F_{4,29} = 14.37, P < 0.0001$), suggesting that the magnitude of the physical withdrawal intensifies with the duration of tonic opioid receptor signaling. Taken together these data establish that intensifying opioidergic and compensatory neuroadaptations create a physical and psychological dependence that outlasts early inflammatory pain and tissue injury.
Figure 3.8. Endogenous opioid physical dependence strengthens with time after injury.

Effect of repeated NTX (3 mg/kg) on the number of precipitated escape jumps over 77 days after CFA. \((n = 6-8)\). Repeated measures one-way ANOVA. test. \(\star P < 0.05\). All data shown as mean ± s.e.m.
3.3.7. Endogenous opioidergic dependence within the supraspinal circuitry

Numerous signs of opiate physical withdrawal result from receptor blockade in the CNS (Hand et al., 1988). Therefore, to investigate the selective activity of supraspinal MORs we administered NTX, CTOP and β-funaltrexamine (β-FNA) directly into the brain 21d after CFA (Figure 3.9). As expected, intracerebroventricular NTX (i.c.v., 1 μg), CTOP (100 and 300 ng), nor β-FNA (1 μg) did not produce signs of physical withdrawal in sham injured control mice (Figure 3.9a-e). By contrast all three antagonists but not saline elicited somatic withdrawal behaviors: mechanical hyperalgesia (Figure 3.9a and b; F₃,₁₂₀ = 5.0, p < 0.0001), jumping (Figure 3.9c; F₃,₈₀ = 69, p < 0.0001), rearing (Figure 3.9d; F₃,₈₀ = 151, p < 0.0001), and paw flutters (Figure 3.9e; F₃,₈₀ = 83, p < 0.0001). Importantly, intrathecal NTX or CTOP only precipitated hyperalgesia; all other somato-motor behaviors were never observed.
Figure 3.9. Endogenous opioid physical withdrawal symptoms are precipitated by µ-opioid receptor blockade in the brain.

(a) Effect of i.c.v. injection of saline or NTX (1 µg) on mechanical thresholds in sham and CFA-21d mice. (n = 6 per group). ★ P < 0.05 compared to all groups. (b) Effect of i.c.v. injection of saline, CTOP (100 and 300 ng), or β-FNA (1 µg) on mechanical thresholds in CFA-21d mice (n = 7 – 14). ★ P < 0.05 compared to saline. Incidence of antagonist-induced physical withdrawal behaviors. (c) jumping, (d) rearing, and (e) paw flutters. ★ P < 0.05 compared to Sham + the respective antagonist. ♦ P < 0.05 compared to CFA-21d + saline. All data shown as mean ± s.e.m. Data on effects of NTX were collected by Greg Corder. Data on effects of CTOP and β-FNA were collected by Julio Morales Medina. Greg Corder analyzed all data.
3.3.8. Enhanced antinociception and delayed analgesic tolerance to morphine in the post-hyperalgesic state

One of the major disadvantages of exogenous opiate drugs as pain killers is that their analgesic efficacy is subject to tolerance (i.e. over time increasing doses of the drug are required to maintain analgesia). In Figure 2.2b, we showed that opioid receptor signaling persists for at least 105 days after CFA, thus providing endogenous analgesia for months. Interestingly we did not detect any general indications of analgesic tolerance (i.e. spontaneous return of hypersensitivity). Behavioral tolerance to an endogenous analgesic system is difficult to test directly due to the fine-tuned temporal control of receptor expression levels, receptor post-translational modifications, receptor recycling and desensitization rates, and endogenous agonist release. However, we can use an indirect measure of endogenous MOR tolerance by testing the cross-tolerance effects of other µ-agonists. To do this, we tested cross-tolerance between endogenous MOR activity and morphine-induced MOR activity.

Morphine-induced antinociception was evaluated by measuring response latencies in the hot-plate test (Figure 3.10). We used a dose of morphine (10 mg/kg body weight, s.c.) that is known to produce sub-maximal analgesia in mice in order to test the hypothesis that morphine antinociception might be increased. As illustrated in Figure 3.10a and b, the analgesic effect of morphine was significantly potentiated in CFA-21d mice compared to sham mice (AUC: P < 0.001). Morphine analgesia was more efficacious, and lasted longer in CFA-21d mice. Basal responses to the hot plate did not differ between the groups.

Analgesic tolerance to morphine develops with repeated administration. Mice were injected daily with morphine, and paw-withdrawal latencies were recorded (Figure 3.10c). Although the sham mice had significantly diminished responsiveness to morphine by day 3, the CFA-21d mice continued to experience enhanced analgesia for 5
days (Figure 3.10 and d: AUC: P < 0.001). The analgesic tolerance, in CFA-21d mice, appeared by day 10. Together, these data demonstrate enhanced and prolonged acute analgesic effects of morphine (similar to the enhanced analgesic effects on intrathecal DAMGO; Fig. 2.3e). They also demonstrate a delay development of tolerance in injured mice, suggesting that tonic endogenous MOR signaling lessens cross-tolerance.
Figure 3.10. Enhanced acute morphine antinociception and delayed morphine tolerance in mice with a history of injury.

(a) Timecourse and (b) Area-under-curve analysis of the acute antinociceptive effects of subcutaneous morphine (10 mg/kg) in sham and CFA-21d mice placed on a 56°C hotplate. (c) Daily timecourse and (d) Area-under-curve analysis of the antinociceptive efficacy for once-daily subcutaneous morphine (10 mg/kg) in sham and CFA-21d mice placed on a 56°C hotplate. (e) Daily pre-morphine baseline latencies in sham and CFA-21d mice during 10 days of morphine administration. (n = 7 – 8). ★ P < 0.001. All data shown as mean ± s.e.m.
3.4. Discussion

3.4.1. Pain reinstatement is the result of endogenous opioid cellular withdrawal in the spinal cord

Earlier we showed evidence that NTX disinhibits an underlying nociceptive sensitization (Chapter 1), arguing that neither opioidergic nor pronociceptive systems have abated in the post hyperalgesia state. Based on these results we hypothesize that these long-lasting adaptations serve to stabilize the level of sensory information transmitted through the central pain circuitry. The parallel upregulation of these opposing systems represents a new physiological set-point for dynamic excitation/inhibition balance, adhering to the principles of allostasis (Koob and Le Moal, 2001) (Figure 3.11, page 112; discussed further in Chapter 5: Final Discussion). During pharmacological blockade of spinal MOR signaling we found that the pronociceptive processes relied on NMDA-R signaling for the full expression of pain reinstatement and nociception transduction (spinal pERK, cAMP and potentiated [Ca^{2+}]i). Indeed, allostasis of opioidergic and NMDA-R activity has been suggested following environmental stress (Le Roy et al., 2011) or injury (Rivat et al., 2002; Rivat et al., 2007), as a pathological consequence of increased endogenous opioid tone. How might long-lasting MOR signaling contribute to the persistence of pathological nociceptive plasticity? Neuroadaptations result from prolonged exogenous opioid exposure, specifically compensatory increases in the expression of Ca^{2+}-sensitive AC1 and AC8 (Lane-Ladd et al., 1997). These augmentations are revealed during opioid withdrawal and produce cAMP overshoot, leading to excitatory synaptic plasticity via protein kinases and MAPK/ERK pathways (Christie, 2008). Indeed, Ca^{2+} influx via NMDA-R is essential for ERK phosphorylation (Lever et al., 2003; Sindreu et al., 2007). Ca^{2+} can elevate
intracellular cAMP levels leading to the activation of PKA. PKA then activates ERK pathway through Rap1, then B-Raf (Impey et al., 1999).

Consequently we proposed that prolonged endogenous-mediated MOR signaling drives the upregulation of the adenylyl cyclase system. We found that intrathecal forskolin or N-methyl-D-aspartic acid both produced enhanced pain behaviors and cAMP levels in CFA mice (Figure 3.2), indicating that Ca^{2+}-sensitive AC expression is increased. MOR blockade disinhibited spinal ACs leading to cAMP overshoot that was abolished by MK-801 and NB001. Together our data suggest that a population of spinal neurons is physically dependent on endogenous opioidergic activity to prevent the expression of pronociceptive NMDA-R–AC1 neuroadaptations (Figure 3.11 and Figure 3.4).
Figure 3.11. Allostatic load between tonic µ-opioid receptor activity and opponent excitatory processes increase the potential for chronic pain.

Under naïve conditions nociceptive and opioidergic systems have little to no activity, resting at a basal set point (homeostasis). Following injury or strong nociceptive input the pro-nociceptive systems are sensitized to facilitate the experience of pain (nociceptive sensitization). In order to reduce rampant nociception and pain counter-adaptive, endogenous inhibitory systems are enhanced (opioidergic counter-adaptation). A state of allostasis is generated once a new equilibrium or set point between pro-nociceptive and endogenous analgesic processes is reached. The intensity and degree of sensory information transmitted through the nociceptive system returns to pre-injury levels (i.e. pain has resolved) while the opponent processes are potentiated but functionally cancel out one another; thus the nociceptive system is dependent on the counterbalancing signaling of tonic MOR (allostasis). The allostatic state is only detectable when one of the potentiated systems is removed leaving the other potentiated system unchecked. Blockade of tonic MOR signaling produces a superactivation of the NMDA-R–AC1 pathways and subsequent re-emergence of pain (pharmacological reinstatement).
3.4.2. Adenylyl cyclase type 1 facilitates endogenous opioid dependence

We found that AC1 gene deletion resulted in reduced acute inflammatory pain and total loss of NTX pain reinstatement, demonstrating that lack of AC1 upregulation eliminates the endogenous opioid withdrawal symptoms. However, taken together these results suggest that tonic MOR signaling, simultaneously, represses AC1 activity and perpetuates its upregulation, similar to mechanisms observed during opiate-dependence.

Furthermore, in our studies, pharmacological inhibition of AC1, with NB001, prevented the NTX-induced pain reinstatement and cAMP overshoot response, displaying the selectivity of AC1 to mediate endogenous opioid withdrawal. AC1 is necessary for the development of chronic pain (Wei et al., 2002) and the expression of LTP (Xu et al., 2008). Withdrawal from exogenous opioids results in NMDA-R-dependent LTP (Drdla et al., 2009). Possibly explaining the hyperalgesia associated with physical withdrawal. Thus the increased functional activity of NMDA-R and AC1 during MOR withdrawal suggests that loss of opioidergic signaling results in spinal LTP.

NTX-induced pain reinstatement (e.g. abrupt withdrawal from tonic MOR signaling) could result from either: 1) disinhibition of tonically active primary afferent terminals; 2) disinhibition of AC1 superactivation in dorsal horn neurons; 3) potentiation of descending facilitatory signals from the brainstem; and/or 4) induction of de novo spinal NMDA-R-dependent withdrawal. While we have no direct evidence of withdrawal-induced de novo LTP, several of our data and other studies suggest this as a promising area of future investigation:

1) Exogenous opiate withdrawal in the spinal cord initiates de novo NMDA-R-dependent LTP (Drdla et al., 2009; Zhuo, 2010);
2) NMDA-R signaling and post-synaptic spinal neuron Ca\(^{2+}\) rise are required in the induction, rather than maintenance, of spinal LTP and hyperalgesia (Weyerbacher et al. 2010; Sandkuhler, 2012);

3) AC1 is activated by NMDA-R-derived Ca\(^{2+}\) and is necessary for LTP induction (Wang et al., 2011; Liauw et al., 2005; Wei et al., 2006);

4) Intrathecal NMDA (3 pmol) increased pain behaviors and spinal cAMP levels in CFA-21d mice compared to shams (Figure 3.2). This suggests that spinal NMDA-R–adenylyl cyclase signaling pathways are not occluded (saturated); and

5) Intrathecal MK-801 alone did not increase mechanical threshold (Figure 3.1), decrease basal pERK expression (Figure 3.1), or decrease basal cAMP levels (Figure 3.2) when administered during the post-hyperalgesia state. In addition, MK-801 perfusion did not reduce basal spinal intracellular calcium levels from CFA-21d slices (Figure 3.2). Together, these findings suggest that spinal NMDA-Rs are not active prior to NTX-induced withdrawal.

We propose that the incidence of long-lasting, tonic MOR signaling within the spinal nociceptive system promotes a physical and cellular dependence on tonic MOR signaling. Therefore, it stands to reason that withdrawal from MOR would result in spontaneous activation of those dependent-cells (possibly NK1R positive projection neurons or PKCγ excitatory interneurons in lamina I and II), through de novo generation of LTP, leading to subsequent reinstatement of pain (see Figure 5.2 for a schematic representation of potential intracellular signaling cascades that would facilitate endogenous opioidergic withdrawal-LTP).
3.4.3. Endogenous opioid dependence occurs in supraspinal circuitry

Humans and other mammals develop tolerance and physical dependence to exogenous opiate drugs. By contrast, endogenously-released peptides are not generally thought to produce tolerance or physical dependence. Therefore, we found it remarkable to observe that, when administered 21 days after the induction of inflammation, NTX produced robust behaviors (jumping, paw flutters, rearing, ptosis, teeth chattering, body shakes, hyperlocomotion, and mechanical hypersensitivity) identical to withdrawal from exogenous opiates. This is the first set of experiments to describe a physical dependence to endogenous opioid activity in the setting of persistent inflammation.

3.4.3.1. Endogenous opiodergic dependence occurs during other forms of chronic physiologic stress.

We found that NTX did not change sensory thresholds and did not produce aversive behaviors in sham-injured mice. This is consistent with other studies using opioid receptor antagonists in opiate-naive or uninjured rodents and humans (El-Sobky et al., 1976; Wang et al., 2001; Wang et al., 2004; Schoell et al., 2010). These data indicate that endogenous opioid dependence is causally initiated by the cutaneous inflammatory insult. However, many chronic pain models are also accompanied by signs of increased HPA-axis activity and stress, such as increased circulating glucocorticoids and pro-inflammatory cytokines, which could initiate opioid receptor signaling in brain regions not directly activated by ascending nociceptive signals. In fact other environmental or dietary factors have been previously suggested to induce CNS manifestations of endogenous dependence. The first account was described in rats repeatedly stressed for 10 days and then given naloxone, which produced “wet-dog” (body) shakes indistinguishable from opiate withdrawal (Morley and Levine, 1980).
Second, naloxone precipitated piloerection, ptosis, and body shakes following a stressful chronic schedule of warm water swimming (Christie and Chesher, 1982). Third, naloxone produced very mild symptoms of forepaw tremor and decreased body weight following the chronic delivery of RB101, an enkephalinase inhibitor (Noble et al., 1994). Fourth, naloxone precipitated somatic indices of withdrawal (teeth chattering, forepaw flutters, and head shaking) and anxiety (reduced time on the exposed arm of an elevated plus maze), as well as a dopamine/acetylcholine imbalance in the nucleus accumbens, following excessive ingestion of sugar (Colantuoni et al., 2002). Therefore, it is difficult to distinguish whether the initiation of opioid physical dependence in the brain is a direct result of inflammatory injury/nociception or that of an elevated stress response.

3.4.3.2 Possible cellular mechanisms driving endogenous opioidergic dependence.

We found that endogenous opioid dependence persists after the cutaneous edema has subsided (Figure 3.8). We propose that endogenous opioid dependence develops and strengthens gradually over time following the induction of inflammation but that dependence is not maintained by injury. We propose two mechanisms to explain the long-lasting nature of endogenous opioid dependence. First, inflammation causes the release of enkephalins and dynorphins in several brain areas including the RVM (Hurley and Hammond, 2000, 2001; Schepers et al., 2008a), locus coeruleus, arcuate nucleus and periaqueductal gray (Porro et al., 1991; Zangen et al., 1998) to provide intrinsic pain relief (Fields, 2004; Ossipov et al., 2010). Because these brain regions are also implicated in the expression of somatic withdrawal during opiate dependence (Maldonado et al., 1992; Vera-Portocarrero et al., 2011), we speculate that blockade of this ligand-dependent opioid receptor signaling with NTX reverses the actions of endogenous opioids, leading to physical withdrawal. Second, inflammation might
sensitize opioid receptors such that receptor signaling continues in the absence of peptide binding and ligand activation. As suggested by dependence studies using exogenous opiate drugs, continual receptor stimulation might transform the opioid receptor into a constitutively active state (Liu and Prather, 2002; Wang et al., 2004). We speculate that injury-induced release of endogenous opioid peptides, released throughout the 21-day recovery time course in our studies, may have a similar effect on intrinsic signaling. If true, then NTX would act as an inverse agonist to decrease basal signaling (see Chapter 4 for studies investigating constitutive activity of spinal opioid receptors).

3.4.3.3. Possible supraspinal structures involved in endogenous opioidergic dependence.

Brainstem areas thought to mediate endogenous opioid dependence in the setting of stress or high sugar diet include mesolimbic reward circuits. Other candidates include CNS noradrenergic systems such as the locus coeruleus. The locus coeruleus contributes to the locomoter hyperactivity associated with opiate withdrawal, such as jumping, rearing and forepaw flutters, (Aghajanian, 1978; Llorens et al., 1978; Koob et al., 1984; Maldonado et al., 1992). Of particular interest is the rostral ventral medulla (RVM), identified as a key regulator of chronic inflammatory nociception (Hurley and Hammond, 2001; Finnegan et al., 2004; Zhang and Hammond, 2010) and mediator in the manifestation of opiate withdrawal (Vera-Portocarrero et al., 2011). In opiate-dependent animals, lidocaine inactivation of the RVM diminished naloxone-precipitated hyperalgesia and other aversive withdrawal symptoms such as teeth chattering, body shakes, and writhing. Further studies are required to pinpoint critical cerebral structures involved in the endogenous opioid physical dependence associated with tissue inflammation.
3.4.3.4. **Intensity of opioid somatic withdrawal may depend on route of administration**

Compared to subcutaneous administration, we found that i.c.v. NTX produced less jumping, paw flutters, teeth chattering and body shakes. One explanation is that only after systemic administration can sufficient concentrations of NTX reach critical hindbrain structures. Consistent with this idea, more intense withdrawal is produced following injection of naloxone into the fourth ventricle as compared to the third ventricle (Herz et al., 1972; Laschka et al., 1976). This may also explain why intrathecal NTX does not produce overt jumping, paw flutters, body shakes behaviors, etc. (anecdotal observations).

3.4.4. **Delayed morphine analgesic tolerance in injured mice**

In Figure 3.10, we demonstrated that the occurrence of analgesic tolerance to morphine is delayed in mice with a prior CFA-injury. Unlike tolerance to exogenous opioids, endogenous opioid mechanisms do not readily undergo antinociceptive tolerance. For example, enkephalinase inhibitor drugs (i.e. RB101), which prevent degradation of released enkephalin opioid peptides, produce analgesia that does not undergo tolerance or cross-tolerance to morphine (Noble et al., 1992). Furthermore, the presence of tissue inflammation reduces peripheral morphine tolerance. This relied on injury-induced increased opioid peptide stimulation of MORs in the dorsal root ganglia, increased MOR endocytosis, intact G-protein coupling and cAMP inhibition (Zollner et al., 2008). These studies suggest that opioid peptides and exogenous opiate drugs differentially regulate MOR desensitization and internalization mechanisms. In fact, Robert Lefkowitz and Marc Caron’s groups found increased morphine antinociception (Bohn et al. Science 1999) and decreased tolerance (Bohn et al. Nature 2000) in β-arrestin2 KO mice (a regulatory protein responsible for MOR internalization). Importantly,
their findings were later found to rely on MOR constitutive activity (Lam et al. Mol Pain 2011), which will be further discussed in Chapter 4: µ-Opioid receptors display in vivo constitutive activity after injury.

3.4.5. Future Studies

1. NTX-induced pain reinstatement (e.g. abrupt withdrawal from tonic MOR signaling) could result from either: 1) disinhibition of tonically active primary afferent terminals; 2) disinhibition of AC1 superactivation and/or tonically active NMDA-Rs in dorsal horn neurons; 3) potentiation of descending facilitatory signals from the brainstem; and/or 4) induction of de novo spinal NMDA-R-dependent withdrawal. While we have no direct evidence of withdrawal-induced de novo LTP, several of our data and other studies suggest this as a promising area of future investigation. This evidence is discussed in section 3.4.2 above.

Altogether our evidence suggests that spinal NMDA-Rs are not active prior to NTX-induced withdrawal. Therefore, we propose that the incidence of long-lasting, tonic MOR signaling within the spinal nociceptive system promotes a physical and cellular dependence on tonic MOR signaling. This mechanism would also imply that withdrawal from MOR would result in spontaneous activation of those dependent-cells (possibly NK1R positive projection neurons or PKCγ excitatory interneurons in lamina I and II), through de novo generation of LTP, leading to subsequent reinstatement of pain (Figure 5.2).

In a collaborative pilot study with Dr. Min Zhuo (University of Toronto), using a multi-electrode array recording technique that stimulates and records field potentials across 64 channels overlaying a dorsal horn slice, NTX (1 µM) increased excitatory synaptic responsiveness in these spinal cord circuits. These findings of enhanced synaptic signaling may share common mechanisms with spinal LTP induced by electrical
stimulation. Future studies should use electrophysiological techniques, such as patch-clamp recording of spinal neurons or in vivo field potential recordings using tetanic stimulation of dorsal roots, to test the hypothesis that NTX induces LTP.

2. As illustrated in Figure 3.9, i.c.v. NTX, CTOP and β-funaltrexamine reinstate hyperalgesia in the hindpaw. This suggests that descending facilitation is disinhibited by the opioid receptor antagonists. In certain injury states, the RVM maintains spinal sensitization (Fields, 2004; Vanegas et al., 2004; Ossipov et al., 2010; Urban et al., 1999; Mansikka et al., 1997). MOR agonists into the RVM produce their antinociceptive effects through opening of Kir channels to hyperpolarize “on-cells” (Pan et al., 1990; Heinricher et al., 1992; Chieng et al., 1994) and disinhibiton of GABAergic “off-cells” (Heinricher et al., 1992; Heinricher et al., 1994). If “on-cell”- MORs tonically inhibit RVM-pain-facilitation, then CTOP into the RVM may reinstate hypersensitivity. The δOR antagonist naltriben in the RVM of CFA-treated rats enhanced hindpaw hyperalgesia (Hurley et al., 2001). Increased levels of met- and leu-enkephalin in the RVM were also noted 2 weeks post-CFA. Therefore, MOR and/or δOR blockade, within the RVM, may also reinstate hindpaw hyperalgesia in our model. Additionally, future studies could replicate the use of cAMP ELISAs, NB001, ex vivo Ca$^{2+}$ imagining, and GTPγS$^{35}$ binding experiments on RVM tissue to determine if the cellular opioid receptor, AC1 and NMDA-R mechanisms, in the spinal cord, exist in the brainstem or other nuclei within the CNS nociceptive circuitry (see Figure 2.2 schematic circuitry diagram of CNS pain pathways).
µ-Opioid receptors display *in vivo* constitutive activity after injury

4.1. Introduction

G-Protein-Coupled-Receptors (GPCRs), also known as 7-Transmembrane Receptors (7TMRs), represent the largest class of receptor drug targets for clinical pathologies. All three opioid receptors, µ-, δ- and κ-, belong to the rhodopsin family (Class A) of 7TMRs and have been extensively investigated as potential therapeutic targets for disorders ranging from depression to severe pain (Lutz and Kieffer, 2013). For example, morphine, a ligand for the µ-opioid receptor (MOR), reduces pain by binding to the extracellular binding domain of MORs which causes a conformational change in the receptor which allows for G-protein binding and intracellular signal transduction. Thus the positive efficacy of morphine broadly classifies this drug ligand as an *agonist*. This simple two-state model expounds that the receptor rests in an *inactive* conformation (Ri) and with the addition of an agonist (A), the receptor becomes active (Ra) and subsequently binds to a G-protein (ARaG) (Figure 4.1a).

The actions of agonists can be blocked by addition of antagonists which prevent binding of the agonist to the receptor. This can occur through competition for the binding pocket or at an allosteric site. Regardless of mechanism, the definition of a true antagonist describes its action in terms of blocking an agonist without effecting receptor conformations that might alter signaling (i.e. antagonists do not have efficacy at a given receptor for either the active or inactive state) (Figure 4.1b).
Figure 4.1. Modeling GPCR constitutive activity.

(a) The Extended Ternary Complex model of GPCR signaling which predicts that modulating the allosteric constant (red arrows) produces a spontaneously active receptor (Ra). Ra can then bind to G-proteins (G) independent of agonist (A) facilitation (pink arrows). (b) Effect of various ligands on 7TMR/GPCR activity.
It was assumed that most GPCR drugs were either agonists or antagonists acting on basally inert GPCRs until it was experimentally demonstrated by Costa and Herz (1989) that overexpression of recombinant delta opioid receptors in NG-108-15 neuroblastoma cells could assume an active signaling state in the absence of a ligand. Some antagonists were able to reduce basal GTPase activity of pertussis toxin sensitive G-proteins, while other antagonists did not. Thus the term inverse agonist was used to describe ligands that display negative efficacy (Figure 4.1b). Negative efficacy is the idea that an antagonist binding to its receptor can be ‘active’ in repressing the spontaneous activity of the receptor. In fact, in 1980 De Lean et al. had postulated the theoretical existence of agonist-independent GPCR activity in their Ternary Complex model (De Lean et al., 1980), which was later expanded to the Extended Ternary Complex (ETC) model (Costa and Cotecchia, 2005) (Figure 4.1a). Based on the thermodynamics of the fluid biophysical nature of the seven transmembranes of GPCRs within a lipid bilayer, it was predicted that GPCRs exist in equilibrium of spontaneous Ra and Ri states, known as an allosteric constant. This constant represents the unique energy barrier for a given GPCR in a specific cellular environment which dictates the formation of spontaneous Ra states. Thus, lowering the allosteric constant (e.g. receptor point mutations, removal of Na⁺ ions, loss of negative regulatory/internalizing/desensitizing proteins) increases the formation of Ra.

In the past few decades most GPCRs have been shown to display constitutive activity, including MORs. However the physiologic influence of constitutive signaling has not been fully explored. Constitutively active α₁B-adreno-receptors enhance the transforming potential of quiescent fibroblasts, suggesting that mutations in GPCRs may be a possible cause of disease. In fact, mutations in several GPCRs causing constitutive activity have been linked to ovarian cancer (Zeineldin et al., 2010) and other human
diseases, such as hyperthyroidism and precocious puberty (Shenker et al., 1993; Mueller et al., 2010).

To date, approximately 30 different studies using site-directed point-mutations in the oprm1 gene encoding MOR have found numerous amino acids that affect ligand binding, G-protein coupling efficiency, and receptor phosphorylation and internalization; all of which increase constitutive activity of MORs (MOR\text{CA}). Importantly, genetic mutations are not necessary for constitutive activity to occur. Liu and Prather demonstrated that prolonged exposure of morphine can induce constitutive activity that persists after agonist washout (Liu and Prather, 2001), suggesting that post-translational modifications to MOR or decreased interactions with negative regulatory proteins may induce constitutive activity. In whole animal studies, MOR\text{CA} is detectable in the brainstem of morphine dependent mice, which is hypothesized to preserve opioid dependence and addiction (Wang et al., 2004; Shoblock and Maidment, 2006). Whether MOR\text{CA} is important in disease states other than drug addiction, such as chronic pain, is unclear. What would be the potential physiological benefits and drawbacks of acquiring tonic MOR\text{CA} after suffering an injury?

In previous chapters we have demonstrated that spinal MORs become tonically active after injury and continue to signal beyond wound healing; the consequence of which appears to be endogenous opioid dependence. Here we investigate potential receptor mechanisms by which MORs remain active for such long periods of time.
4.2. Methods and Materials

4.2.1. Mice: For species, sex, and age, please refer to section 2.2.1, page 27.
Table 4.1. Animal use quantification for figure 4.2.

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<td>Exp. 1: Sham+vehicle: 5 Sham+NTX: 5 CFA+vehicle: 10 CFA+NTX: 10 Exp. 2: Sham+vehicle: 2 Sham+NTX: 1 CFA+vehicle: 1 CFA+NTX: 5 Sham+6B-naltrexol: 5 CFA+6B-naltrexol: 5 Sham+6B-naltrexol+NTX: 5 CFA+6B-naltrexol+NTX: 5</td>
<td>Sham+vehicle: 7 Sham+NTX: 6 CFA+vehicle: 11 CFA+NTX: 15 Sham+6B-naltrexol: 5 CFA+6B-naltrexol: 5 Sham+6B-naltrexol+NTX: 5 CFA+6B-naltrexol+NTX: 5</td>
<td>Exp. 1 and 2 were combined.</td>
<td>2012</td>
</tr>
<tr>
<td>4.2f,g</td>
<td>Sham: 10 CFA: 10</td>
<td>Sham: 9 CFA: 7</td>
<td>Spinal cords were damaged during dissection</td>
<td>2011</td>
</tr>
</tbody>
</table>

Unknown information is denoted by dashed lines (--).

Information that is not applicable is denoted as n/a
4.2.2. Complete Freund’s Adjuvant (CFA) model of inflammatory pain: Please refer to section 2.2.2, page 30.

4.2.3. Mouse (in vivo) drug administration: Please refer to section 2.2.4, page 30.

4.2.4. Drug dosing: Please refer to section 2.2.5, page 31.

4.2.5. Mechanical hyperalgesia testing: Please refer to section 2.2.6, page 32.

4.2.6. cAMP ELISA assay: Please refer to section 3.2.11, page 78.

4.2.7. GTPγS$^{35}$ binding assay: Please refer to section 2.2.12, page 35.

4.2.8. Calcium Imaging: Please refer to section 3.2.13, page 78.

4.2.9. Statistics: Please refer to section 2.2.13, page 36.

4.2.10. Experimental blinding procedures: Please refer to section 2.2.14, page 37.

4.3. Results

4.3.1. MOR inverse agonists, but not neutral antagonists, block constitutive receptor signaling in vivo.

Tonic GPCR signaling arises from either continuous agonist binding to and/or constitutive activity (spontaneous signal transduction that occurs in the absence of agonist stimulation) of MOR (Costa and Herz, 1989; Kenakin, 2001; Seifert and Wenzel-Seifert, 2002; Kenakin, 2004). MOR constitutive activity (MOR$_{CA}$) is typically studied using in vitro systems. Important clues come from a few in vivo studies suggesting that MOR$_{CA}$ develops with chronic morphine administration, leading to physical and affective signs of opiate dependence and addiction (Wang et al., 1994; Wang et al., 2004; Shoblock and Maidment, 2006, 2007; Meye et al., 2012). Although β-arrestin2 deletion
mutant mice exhibit elevated $\text{MOR}_{\text{CA}}$ that is linked to a decrease in transient heat pain (Lam et al., 2011), the existence and physiologic significance of $\text{MOR}_{\text{CA}}$ in pathological pain processing is unknown. To address this question we used the neutral antagonist 6β-naltrexol (10 µg in 5 µl 1% DMSO), a structural analog of NTX (Raehal et al., 2005). Neutral antagonists have little or no intrinsic effect on receptor activity themselves, and competitively block not only the effects of agonists, but importantly, the effects of inverse agonists as well (Wang et al., 1994; Bilsky et al., 1996; Raehal et al., 2005; Sally et al., 2010). Accordingly, we found that intrathecal 6β-naltrexol alone did not change Ca$^{2+}$ levels in sham or CFA-21d spinal slices (Figure 4.2a), and failed to precipitate a cAMP overshoot (Figure 5.2b) or hyperalgesia (Figure 4.2c). 6β-naltrexol abolished the ability of NTX to produce Ca$^{2+}$ mobilization (Figure 4.2a; $P < 0.05$), cAMP overshoot (Figure 4.2b; $P < 0.05$) and hyperalgesia (Figure 4.2c; $P < 0.05$). These data are consistent with the assumption that NTX behaves as an inverse agonist to inactivate basal signal transduction (Sadee et al., 2005; Lam et al., 2011).

4.3.2. β-funaltrexamine blocks MOR constitutive activity in vivo and reduces basal G-protein coupling.

Intrathecal administration of an alternative µ-selective inverse agonist, β-funaltrexamine (β-FNA; 2.5 µg in 5 µl water) (Liu et al., 2001), reinstated hyperalgesia in injured but not sham mice (Figure 4.2d; $P < 0.05$). Since $\text{MOR}_{\text{CA}}$ results in elevated basal G-protein cycling (Liu et al., 2001; Wang et al., 2004), we determined whether β-FNA could promote the MOR-inactive state and thereby decrease spontaneous basal GDP/GTPγS$^{35}$ exchange. In CFA-21d spinal cord slices, β-FNA concentration-dependently reduced basal GTPγS$^{35}$ binding in ipsilateral (Figure 4.2e and f; $E_{\text{min}}$ of -30.94 ± 0.74% and an $E_{50}$ of 0.55 ± 0.05 µM) and contralateral dorsal horn (Figure 4.2g; $E_{\text{min}}$ of -18.89 ± 1.19% and an $E_{50}$ of 0.3 ± 0.07 µM). β-FNA also decreased basal
binding in sham-injured slices, but to a significantly lesser degree (Figure 4.2e-g; Ipsilateral: $E_{\text{min}}$: -15.44 ± 0.31%; EC$_{50}$: 0.23 ± 0.04 µM; Contralateral: $E_{\text{min}}$: -14.51 ± 1.57%; EC$_{50}$: 0.38 ± 0.11 µM). These data establish that inflammation causes a population of spinal MORs to adopt a constitutively active state that allows for tonic intracellular G-protein signaling that is independent of ligand activation.
Figure 4.2. Spinal µ-opioid receptors acquire constitutive activity after injury. (a,b,c) Effects of NTX (10µM or 1 µg), 6β-naltrexol (1µM or 10 µg) or co-administration of 6β-naltrexol+NTX in sham and CFA-21d mice on (a) intracellular calcium concentration in spinal cord slices (n = 5 - 6), (b) spinal cAMP content (n = 5 - 15) and (c) hyperalgesia (n = 6 - 7). (d) Effect of intrathecal β-funaltrexamine (β-FNA; 2.5 µg) on hyperalgesia in sham and CFA-21d mice (n = 6 - 7). (e) Representative pseudo-colored radiograms and dose-response effects of β-FNA on basal GTPγS35 binding in lumbar (f) ipsilateral and (g) contralateral dorsal horns of Sham or CFA-21d mice; inset: group binding E_{max} (n = 7 - 9). ★ P<0.05. All data shown as mean ± s.e.m.
4.4. Discussion

4.4.1. Injury increases the proportion of spinal MORs that display constitutive activity.

Here we have demonstrated, for the first time, in vivo acquisition of constitutive MOR signaling that naturally develops after injury, without chronic drug dosing (Sadee et al., 2005) or genetic manipulations (Chavkin et al., 2001). NTX, CTOP and β-FNA had no effect on pain thresholds in uninjured mice but all produced significant pain reinstatement in mice with a history of prior injury. This result was recapitulated as enhanced Ca\textsuperscript{2+} signaling and cAMP superactivation after inverse agonist treatment, all from ex vivo spinal tissue of CFA-mice. One major limitation in the interpretation of our results is that all of our opioid receptor inverse agonists (NTX, CTOP, and β-FNA) do not reduce constitutive activity, but block the actions of opioid peptide agonists. First, opioid peptides that are continually released in vivo or in the ex vivo spinal cord slices, or, second, the trace presence of endogenous agonists that are either not sufficiently removed during our spinal cord slice washing protocols may give rise to apparent constitutive activity. However, the lack of effect of 6β-naltrexol, and blockade of NTX inverse effects by 6β-naltrexol, in all our behavioral and molecular assays, clearly argues that agonist-independent signaling is present in a population of spinal MORs. In strong support we demonstrated that β-FNA knocked down the basal GTPγS\textsuperscript{35} binding levels in the spinal cord. Interestingly we did observe a small degree of GTPγS\textsuperscript{35} knockdown in sham mice, which argues for the presence of constitutive MORs under normal physiological conditions (Wang et al., 2004; Meye et al., 2012); although inverse agonists did not produce hypersensitivity on their own.

The chronic administration of opioid agonists, such as morphine or DAMGO, have been shown to cause an increase in the number of constitutively active opioid receptors (increased basal GTPγS\textsuperscript{35} binding, increased B\textsubscript{max} of the agonist-responsive high-affinity GTPγS\textsuperscript{35} binding site and suppressed forskolin-stimulated cAMP
accumulation) during the period of opioid dependence (Wang et al., 2001; Sade´e et al., 2005; Walker and Sterious, 2005; Xu et a., 2007; Sally et al., 2010). Many opioid antagonists have been demonstrated to be inverse agonists in times of opioid tolerance and dependence and are quite effective at precipitating withdrawal to differing degrees (Wang et al., 2007; SirohWe et al., 2007; Sally et al., 2010). These in vitro studies used exogenous opioid agonists in an attempt to model opiate analgesic use that leads to development of tolerance and dependence. While constitutive activity is usually difficult to detect outside of in vitro heterologous cell expression systems, as described above (Costa and Herz, 1989; Liu et al., 2001) (Wang et al., 2007), basal MOR signaling was discovered in the striatum (Wang et al., 2004), and recently the Ventral Tegmental Area (Meye et al., 2012) of morphine-dependent mice. Inverse agonists decreased basal GTPγS binding, produced cAMP superactivation and disinhibited GABAergic signaling, indicative of acquired state-dependent activity following chronic receptor stimulation. Additionally, naloxone has been shown to induce excitatory post-synaptic responses in frontal cortex and striatal single neurons representing withdrawal to a presumed latent hyperexcitability of post-synaptic membrane in opiate tolerant rats (Fry, Herz and Zieglgansberger, 1980). In light of this literature, our data further describe a mechanism whereby loss of MORCA signaling, in a cellular dependent-state, can spontaneously cause neuronal depolarization. Depolarization of neurons within the nociceptive circuitry could lead to the propagation of ascending excitatory signals to facilitate spontaneous pain transmissions.

4.4.2. Mechanisms promoting MOR constitutive activity

As MOR signaling persists for several months after minor tissue damage this raised questions as to what mechanisms might maintain tonic signal transduction. Receptor signaling is initiated by either ligand binding or spontaneous acquisition of the
active conformation. The former scenario is susceptible to antagonists that outcompete for the receptor binding pocket, while the latter case is blocked by inverse agonists with preferential affinity for the inactive state of the receptor and, therefore, reverse basal responses attributed to constitutive activity (Kenakin, 2007). The concept of constitutive activity has yet to be fully understood but some postulate that in the presence of increased exposure to agonists a structural modification, perhaps in the carboxyl tail or an intracellular loop, leads to either increased G-protein coupling and/or prevents receptor internalization/desensitization by blocking CaMKII association. Additionally, constitutive activity can be produced by lowering the energy barrier to spontaneously form the active state (i.e. decreasing the allosteric constant). This could be achieved through possible post-translation modification of the receptor (e.g. hyper-phosphorylation (Wang et al., 1994) or reduction in the association with a negative regulatory protein, such as β-arrestin2 (Lam et al., 2011). It remains an interesting question as to what mechanisms underlie constitutive signaling during our in vivo model and warrants further investigation in the future.

4.4.3. Future Studies

1. We have demonstrated that the basal MORs signaling is increased and is reduced by NTX, indicating intrinsic constitutive activity of the receptor. However, we have not definitively ruled out possible parallel receptor signaling mechanisms that involve opioid peptide-mediated signaling. I propose that future studies could test the hypothesis that opioid peptides are not being continually released in the spinal cord to mediate the prolonged endogenous antinociception by intrathecal injection of sequestering antibodies specific for opioid peptides (leu- and met-enkephalin).

If opioid receptors exclusively signal via constitutively active conformations then the prediction is that intrathecal enkephalin antibodies will not reinstate pain
hypersensitivity. However, if hyperalgesia is observed then that would suggest that opioid peptides are still actively being released in the spinal cord. Alternatively, mRNA and peptide levels of dynorphin (Chavkin et al., 1982) are markedly increased in the ipsilateral dorsal horn during peripheral inflammation (Iadarola et al., 1988; Parra et al., 2002). Spinal dynorphin, however, is reported to be pro-nociceptive by interaction with bradykinin-R (Lai et al., 2006) and NMDA-R (Tan-No et al., 2002) during chronic sensitization states (Wang et al., 2001; Gardell et al., 2004). Therefore antiserum for dynorphin may be analgesic or have no observable effect. An additional experiment using microdialysis or a push-pull perfusion method for peptide sampling can be employed to measure active peptide release (Lisi et al., 2006).

Careful attention must be taken to avoid type II negative data and misinterpretation due to a false positive result. This could arise due to non-specificity of the antisera or the inability of the antisera to fully sequester the released opioids. Therefore as a control for antibody binding the antisera (i.t.) should be injected prior to formalin injection into the paw. It has already been demonstrated that i.t. leu and met enkephalin antibodies potentiate the 2nd phase of the formalin test, suggesting that opioid peptides are released. If we can replicate this finding with our antisera then we can conclude specificity and sufficient diffusion of the antibodies to the target tissues in the spinal cord.

2. In addition to the biochemical and intracellular mechanisms maintaining constitutive activity, it may be of clinical relevance to elucidate the initiating mechanisms. Shoblock et al. (2007) demonstrated the endogenous encephalin release is necessary for morphine-mediated MOR\textsubscript{CA}, suggesting that opioid peptides may be involved in the first steps toward ligand-independent signaling. While it is difficult to speculate on how enkephalins would promote the development of MOR\textsubscript{CA}, their involvement can be tested.
For example, a future study testing the hypothesis that endogenously released spinal enkephalins drive the creation of MOR\textsubscript{CA} can be tested by implanting an intrathecal osmotic minipump, continuously secreting the neutral antagonist 6β-naltrexol, for the first 21d after a CFA injury. If the interaction of the peptide and receptor can be blocked with the antagonist, then the intracellular signaling mechanisms that lower the allosteric constant may not be triggered. On day 21 post injury and drug infusion, MOR\textsubscript{CA} can be assessed measuring GTPγS35 knockdown by β-funaltrexamine. The prediction is that the antagonist and subsequent block of opioid peptide signaling would prevent the development of MOR\textsubscript{CA}. While this procedure is expected to increase hyperalgesia through the drug infusion, just as NTX mini-pump infusion extended the duration of mechanical hyperalgesia, it is a useful study to understand the initiating mechanism of MOR\textsubscript{CA}. The potential pitfalls of this study however may preclude any useful information: on their own, chronic opioid receptor antagonists increase MOR expression in the spinal cord. Constitutive activity is generated in in vitro cell-based assays by overexpressing the receptor. Thus, we predict that chronic 6β-naltrexol infusion may increase spinal MOR expression and produce constitutive activity via an alternate mechanism similar to what occurs in these cellular systems. To circumvent this issue, preproenkephalin knockout mice (\textit{penk1} null) could be generated and tested for the presence of MORCA after injury.
5.1. General Discussion

The main aims of this thesis were to investigate novel mechanisms that contribute to or facilitate the transition from acute to chronic pain. Working from the experimental framework of drug addiction theory and allostasis, we have designed and implemented studies that implicate a dual role of the endogenous spinal μ-opioid receptor system to chronic pain. In our model, the endogenous μ-opioid receptor system produces both a long-lasting analgesia and a paradoxical sensitization of nociceptive pathways.

Opioid drugs, such as morphine, remain the gold-standard for clinical pain relief but are severely limited by tolerance, dependence and addiction. However, the utility of the intrinsic opioid system has been overlooked in the scientific literature. In this dissertation, we demonstrate, for the first time, that injury produces a unique signaling state for the μ-opioid receptor (MOR\textsubscript{CA}). This signaling conformation allows for tonic inhibitory signal transduction, independent of endogenous ligand stimulation. Inverse agonists for the μ-opioid receptor precipitate somatic and aversive withdrawal symptoms, indicative of endogenous physical and psychological dependence on μ-constitutive activity. We further show that allostatic counter-adaptions occur, including functional sensitization of NMDA-receptor and AC1 signaling pathways, which facilitate escalating endogenous opioid dependence. To support these conclusions we used controlled and blinded behavioral pharmacology, genetic knockout mice, molecular and biochemical assays, and \textit{ex vivo} spinal cord slice Ca\textsuperscript{2+} imagining studies.
In conclusion, we have discovered a novel mechanism of long-lasting opioid analgesia that regulates the transition from acute to chronic pain while, in parallel, generates opioid dependence. Thus, the prevalence of chronic pain syndromes may result from a failure in constitutive signaling of spinal MOR.

5.1.1 Allostatic mechanism for the emergence of chronic pain.

5.1.1.1. Persistent activity of inhibitory and excitatory opponent processes.

A single tissue injection of complete Freund’s adjuvant (CFA) to mice caused a transient acute nociceptive hypersensitivity that resolved within several days. From this timecourse we inferred that spontaneous pain recovery resulted from either deactivation of pronociceptive systems or increased counter-adaptive endogenous inhibition. Using behavioral pharmacology we blocked the endogenous spinal opioidergic system to rekindle hyperalgesia, a phenomena we have termed “pain reinstatement.” We found that pain reinstatement could be induced as early as 21 days after the injury when inflammation was still present (Figure 2.1c), and as late as 200 days (Figure 2.2e) after the injury when no signs of the physical injury were remained (discussed thoroughly in Chapter 2, section 2.4.1.).

A long history of scientific literature has demonstrated that strong noxious input into the spinal cord is accompanied by the release of opioid peptides, increased opioid receptor density and enhanced opioid receptor—G-protein coupling within the dorsal horn (Yaksh and Elde, 1981; Basbaum and Fields, 1984; Iadarola et al., 1986; Noguchi et al., 1992; Mousa et al., 2002; Song and Marvizon, 2003; Schepers et al., 2008b; Mousa et al., 2002; Calza et al., 2000; Goff et al., 1998; Shaqura et al., 2004). Here we have presented similar evidence that the opioidergic systems are activated or “turned on” after CFA to promote the resolution of acute hypersensitivity (i.e. naltrexone (NTX) osmotic pump prevents pain recovery). Furthermore, at the 21d timepoint, we found
significant increases in the functional capacity for spinal µ-opioid receptors (MORs) to couple G-proteins (i.e. increased DAMGO-stimulated GTPγS binding) by a ligand-independent mechanism. Which when disrupted by intrathecal pertussis toxin, naloxone, NTX, CTOP or β-funaltrexamine (β-FNA) induced pain reinstatement. Therefore, from this data one possible conclusion is that spinal MORs provide substantial endogenous analgesia by tonically suppressing on-going nociception, central sensitization or a spinal “pain engram”. While this is the simplest, most straightforward interpretation of these results (discussed thoroughly in Chapter 2), we have discovered divergent evidence to suggest that long-lived MORCA becomes pathological in promoting this persistent sensitization of the nociceptive pathway (i.e. endogenous opioid dependence).

During the initial recovery of acute CFA-induced hyperalgesia opioid peptides are released in the spinal cord to activate opioid receptors. I believe that the stimulation of opioid receptors by endogenous ligands during this recovery period promotes the acquisition of MORCA, which appears to last for months after the injury. This chronic endogenous signaling in turn drives the upregulation of AC1, making the system dependent on MORCA to continue to inhibit the now potentiated AC1 system. An analogous mechanism occurs with long-term exposure of opiate drugs: Chronic administration of exogenous opiates leads to the development of compensatory neuroadaptations underlying opiate tolerance and dependence (Christie, 2008), including the selective upregulation of calcium-sensitive AC isoforms (Avidor-Reiss et al., 1997; Lane-Ladd et al., 1997). Likewise, these same opiate dosing paradigms can induce MORCA, which is enhanced by the presence of endogenously released enkephalin peptides (Shoblock and Maidment, 2007). Therefore, this dissertation proposes that opioidergic signaling loses its physiological utility in countering nociception when the receptor cannot “turn off” and enters a vicious cycle of ever increasing counter-adaptive processes, described here further as an allostatic load.
5.1.1.2. Principles of allostasis relating to chronic pain.

Pain has been described as a homeostatic emotion, involving sensory and cognitive control of nociceptive information that travels through the spinal cord, reflecting an adverse condition that requires a behavioral response to maintain stability (Craig, 2003). *Homeostasis* is a core principle of physiology that corresponds to the mechanisms that maintain stability within a system: deviation from a specific set-point is corrected by negative feedback loops that regulate physiologic parameters in order to ensure survival of the organism. Based on this viewpoint one assumes that as physical injuries heal, pain resolves in parallel, and both pro-nociceptive and anti-nociceptive processes return to their pre-injury levels in a homeostatic manner. However, chronic pain syndromes may spontaneously re-occur months or years after an injury (Andersson, 1999). The mechanisms that drive the reemergence of chronic pain are not well understood, but clearly argue that some facet of the nociceptive system has undergone stable adaptations that facilitate the expression of persistent pain states and do not comply with the principles of homeostasis.

In contrast, the principles of *allostasis* state that the processes of maintaining stability are achieved through change of integrative systems that create a new balanced set-point (McEwen, 2003). Thus, an allostatic state can be defined as a state of chronic deviation of the regulatory systems from their normal state of operation with the establishment of this new set-point (McEwen, 2003). The persistence of an allostatic state is hypothesized to undergo a collapse or breakdown. This occurs when the opposing systems reach a maximum and become stressed such that new set-points cannot be maintained (e.g. loss of stability). This chronic state is referred to as an *allostatic load*, defined as “the cost or the price the body may have to pay for being forced to adapt to an adverse or deleterious psychological or physical situation, and it
represents the presence of too much demand on the operation of the regulatory systems - mainly the primary mediators of the physiological response — or their failure to relax when the demand is over.” (Koob and Le Moal, 2001). The allostatic load hypothesis has been proposed to underlie several disease states, including drug addiction (Koob and Le Moal, 2001), migraine (Borsook et al., 2012), and latent pain sensitization (Celerier et al., 2001).

5.1.1.3. Overarching allostatic theory of chronic pain

Thus, while the physical tissue may heal, we propose that the neural composition of the nociceptive system does not homeostatically return to a pre-injury state. Rather, a new, elevated balance (set point) between inhibitory (opioidergic) and excitatory (NMDA-Rs and AC1) systems is created (i.e. endogenous opioid physical dependence), thereby "priming" the system for recurring pain should the inhibitory system fail. Therefore, we propose that the emergence of chronic pain is the result of a breakdown in the allostatic load between MOR_{CA} and the opposing systems that maintain endogenous opioid dependence (Figure 5.1).
• homeostasis
• nociceptive sensitization
• opioidergic counter-adaptation
• allostasis
• allostatic load
• allostatic load breakdown
• chronic pain

endogenous dependence intensifies

• demand too great on opioid system
• stress / depression / menopause

→ opioid system dysfunction (endogenous tolerance)

NMDA-R – AC1

Pain

MOR_{CA}

injury

time: 0 day → 21 day → permanent (?)
Figure 5.1. Breakdown of the allostatic load between MOR\textsubscript{CA} and pronociceptive mechanisms establishes chronic pain.

The allostatic state (i.e. physical dependence) persists for months after the injury has recovered. The degree of opioid physical dependence continues to intensify and we hypothesize that over time the high demands on MOR\textsubscript{CA} will cause a breakdown in the allostatic load (a natural withdrawal state). This natural withdrawal could theoretically occur during menopause when estrogen levels drop or during states of chronic stress or depression. At the cellular level this breakdown may induce de novo LTP that further strengthens synaptic facilitation and promotes the full emergence of chronic pain (Figure 5.2).
5.1.2. MOR\textsubscript{CA} dysfunction: a mechanism of chronic pain?

It is inviting to speculate that the transition to chronic pain involves dysfunction of endogenous MOR signaling, possibly via mechanisms involving cellular tolerance, anxiety or stress. While we did not detect the presence of analgesic tolerance here, studies have shown non-nociceptive stress can precipitate hyperalgesia after injury recovery (Le Roy et al., 2011), which may rely on the anti-opioid effects of cholecystokinin (Zhang et al., 2009). A few clinical chronic pain studies have suggested that a transient up-regulation of endogenous opioid analgesia, followed by opioid dysfunction is associated with the onset of chronic pain (Bruehl et al., 2004; Bruehl et al., 2010). Future therapeutic strategies for chronic pain designed to strengthen endogenous opioid signaling will have to consider the pitfalls of countering pro-nociceptive systems while concomitantly creating an allostatic state.

5.1.2.1. Clinical evidence of MOR\textsubscript{CA}: Endogenous opioidergic analgesia and dependence

Opioid receptor antagonists, such as naloxone can produce hyperalgesia in humans, indicating the presence of endogenous opioid analgesia (Buchsbaum et al., 1977; Jungkunz et al. 1983; Frid et al. 1981; Levine et al. 1979; Gracely et al., 1986; Anderson et al., 2002). Furthermore, tonic pain causes the release of endogenous substances acting at MORs in the brain (Zubieta et al., 2001), and induces long-lasting activation of spinal MOR (Tambeli et al., 2009). Our data suggest that endogenous activation of MORs will initiate both analgesic signaling as well as a standard compensatory opponent-processes that can lead to a rebound effect when an inverse agonist is given (i.e. withdrawal symptoms). Anecdotal evidence supports the idea that endogenous opioidergic mechanisms lead to physical dependence in humans. For
example, naltrexone can induce withdrawal-like symptoms including nausea and jitteriness (Kaur et al., 2006).

5.1.2.2. Therapeutic strategies to target the $\text{MOR}_{\text{CA}}$ and AC1 allostatic load

If the persistent allostatic load between $\text{MOR}_{\text{CA}}$ and AC1 signaling pathways is indeed responsible for priming individuals to develop chronic pain then clinically efficacious and novel treatments (pre-emptive) may stem from preventing the formation of the load or eliminating the load. Specifically, targeting the upregulation and/or signaling components of spinal AC1 may prove most beneficial. Treatments should be targeted to knockdown (RNAi or other gene silencing technologies) or pharmacologically inhibit AC1 in order to erase the persistent sensitization, while retaining the benefits of endogenous constitutive opioid signaling.

5.2. Conclusions

5.2.1. $\text{MOR}_{\text{CA}}$ silences persistent AC1 central sensitization

We have identified an injury-induced $\text{MOR}_{\text{CA}}$ that promotes both endogenous analgesia and dependence. Our data suggest that long-term $\text{MOR}_{\text{CA}}$ inhibition of AC1-mediated central sensitization drives a counter-adaptive, homeostatic increase in pronociceptive AC1 signaling cascade, thereby paradoxically promoting the maintenance of latent central sensitization. Thus, injury produces a long-lasting dependence on $\text{MOR}_{\text{CA}}$ that tonically prevents withdrawal hyperalgesia, consistent with proposed mechanisms of dependence to opiate drugs such as morphine (Zahariou et al., 2008; Li et al. 2006). These data indicate that blockade of $\text{MOR}_{\text{CA}}$ unmask a silent AC1 central sensitization pathway that persists beyond the resolution of pain and inflammation, reflective of hyperalgesic priming (Asiedu et al. 2011).
The presence of contralateral spinal \( \text{MOR}_{\text{CA}} \) and neural sensitization illustrates the spread of this pathology to areas of the CNS beyond those directly innervated by the injured tissue. Thus \( \text{MOR}_{\text{CA}} \) might tonically repress wide-spread hyperalgesia. Unilateral injuries often produce bilateral hyperalgesia, and the current data extends this to latent pain sensitization in CFA 21-day mice. NTX produced bilateral hyperalgesia and bilaterally increased stimulus-evoked pERK expression. Leading explanations for bilateral hyperalgesia include descending facilitatory fibers from the brainstem (Chai et al. 2011; Tillu et al., 2008) and reactive spinal astrocytes communicating through gap-junction networks (Gao et al. 2010; Hatashita et al., 2008). We found evidence for bilateral expression of \( \text{MOR}_{\text{CA}} \), namely contralateral increases in DAMGO-induced MOR-G-protein coupling and \( \beta \)-funaltrexamine-induced decreases in basal MOR-G-protein coupling. We suggest that \( \text{MOR}_{\text{CA}} \) is intricately involved in the regulation of contralateral sensitization, and thus chronic pain phenotypes that display widespread hypersensitivity, such as fibromyalgia. If true, then loss of \( \text{MOR}_{\text{CA}} \) antinociception (e.g. during stress) could lead to the emergence of rampant chronic pain (De Felice et al., 2010; Rivat et al., 2007).

5.2.2. \( \text{MOR}_{\text{CA}} \) promotes the existence of AC1 central sensitization to establish cellular dependence

While \( \text{MOR}_{\text{CA}} \) provides lasting antinociception, our findings indicate that prolonged \( \text{MOR}_{\text{CA}} \) promotes cellular consequences of dependence including potentiated NMDA-R–derived \( \text{Ca}^{2+} \) mobilization and downstream activation of AC1 leading to cAMP overshoot. Upregulated AC1 function facilitates morphine dependence (Li et al., 2006; Zachariou et al., 2008) and chronic pain (Wei et al., 2002; Zhuo, 2012), in part by promoting phosphorylation and trafficking of AMPA receptors via PKA (Lu et al., 2003; Xu et al., 2008) or PKMζ (Li et al., 2010), and promoting phosphorylation of NMDA-Rs
via PKA–Src kinase (Qiu et al., 2013) to increase synaptic strength during LTP. Spinal LTP at C-fiber synapses occurs during opiate withdrawal (Drdla et al., 2009). Similar synaptic mechanisms may underlie withdrawal from MORCA.

The overall conclusion from this body of work is that loss of MORCA, and the ensuing reinstatement of pain, reflects a process of spinal cellular withdrawal and AC1 superactivation to enhance pronociceptive synaptic strength (Figure 5.2). This raises the prospect that the opposing interactions between MORCA and latent NMDA-R–AC1 sensitization create a lasting susceptibility to develop chronic pain.
Figure 5.2. Endogenous MOR$_{CA}$ withdrawal facilitates the reinstatement of pain by induction of $de$ $novo$ NMDA-R-dependent LTP in spinal nociceptive circuits.

*Left:* MORs in the spinal nociceptive system acquire constitutive activity (MOR$_{CA}$) after injury which chronically inhibit AC1. Due to this chronic inhibition of the AC-cAMP-PKA
pathway there is a compensatory increase in AC1 and possibly PKA expression. This increase in spinal AC1 maintains a state of endogenous MOR\textsubscript{CA} dependence.

Right: When MOR\textsubscript{CA} is disrupted by the inverse agonists, such as NTX, CTOP or β-FNA, the upregulated AC1 population becomes superactivated by NMDA-R/VGCC-derived calcium, producing a cAMP overshoot response. Elevated levels of cAMP act upon the potentiated PKA population which would drive several mechanisms to induce LTP: increased glutamate neurotransmitter release from presynaptic boutons, augmented gene expression, phosphorylation and synaptic insertion of postsynaptic AMPA-Rs and phosphorylation of NDMA-Rs.
Appendix 1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>7TM-R</td>
<td>Seven transmembrane receptor</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AC1</td>
<td>Adenylyl cyclase type 1</td>
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<tr>
<td>AMPA</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazoleprionic acid</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AUC</td>
<td>Area under Curve</td>
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<tr>
<td>β-FNA</td>
<td>Beta-funaltrexamine</td>
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<tr>
<td>CaMKII</td>
<td>Ca^{2+}/calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3′,5′-cyclic monophosphate</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CONTRA</td>
<td>Contralateral</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>CPxSx</td>
<td>Common peroneal nerve transection, sural nerve transection</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CTOP</td>
<td>Phe-Cys-Tyr-Trp-Orn-Thr-Pen-Thr-NH2</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>E&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>Maximum possible effect</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinases</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein linked inward rectifying potassium channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPγS&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Guanosine-5'-O-(3-[35S]thio)triphosphate</td>
</tr>
<tr>
<td>HFS</td>
<td>High frequency stimulation</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>IPSI</td>
<td>Ipsilateral</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MK-801</td>
<td>(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>NK1</td>
<td>Neurokinin receptor 1</td>
</tr>
<tr>
<td>NMB</td>
<td>Naltrexone methobromide</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide tyrosine</td>
</tr>
<tr>
<td>NTX</td>
<td>Naltrexone</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductual gray</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBN</td>
<td>Parabrachial nucleus</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pERK</td>
<td>Phosphorylated extracellular-signal-regulated kinases</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKCζ</td>
<td>Atypical protein kinase zeta</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventral medulla</td>
</tr>
<tr>
<td>SNI</td>
<td>Spared nerve injury</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid receptor-1</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated calcium channel</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range neuron</td>
</tr>
<tr>
<td>Y1R</td>
<td>Neuropeptide tyrosine receptor 1</td>
</tr>
</tbody>
</table>
References


Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR (1999) Calcium-stimulated adenylyl cyclase activity is critical for
hippocampus-dependent long-term memory and late phase LTP. Neuron 23:787-798.


Vita

Gregory F. Corder

Education

2003 – 2007 Bachelor of Science (Cell and Molecular Biology), Tulane University, New Orleans, LA

Professional experience
2002 Summer Research Assistant, Department of Medicine, University of Maryland, Baltimore, MD

2005 Science Educator (6-8th grade biology), Terrace Community Middle School, Tampa, FL

2007 Undergraduate Honors Independent Researcher, Dept. of Pharmacology, Tulane University, New Orleans, LA

2007-2008 Lab Technician, Department of Pharmacology, Tulane University, New Orleans, LA

2008-2009 Visiting Scientist, Department of Physiology, University of Kentucky, Lexington, KY

2009-2013 Doctoral Student, Department of Physiology, University of Kentucky, Lexington, KY
- Advisor: Bradley K. Taylor, PhD

2013- Postdoctoral fellow, Stanford Institute for Neuro-innovation and Translation Neurosciences and Department of Anesthesia, Stanford University, Palo Alto, CA
- Advisor: Grégory Scherrer, Pharm D, PhD
Other professional experience

2006-2008 Operating Room Volunteer Assistant, Dept. of Surgery/Anesthesiology, Children’s Hospital, New Orleans, LA

2009 Integrated Biomedical Sciences Rotation WE (fMRI: human face processing), Dept. of Anatomy and Neurobiology, University of Kentucky, Lexington, KY

2009 Integrated Biomedical Sciences Rotation IWE (molecular biology: prion diseases), Dept. of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, KY

Honors

2006-2007 Senior Class Representative and Student-Faculty Liaison, School of Science and Engineering Council of Students, Tulane University, New Orleans, LA

2010 1st Place Graduate Student Poster, Bluegrass Chapter of the Society for Neuroscience, Lexington, KY

2010 1st Place Graduate Student Poster, Dept. of Physiology 50th Anniversary Gala, University of Kentucky, Lexington, KY

2009 & 2011 Travel Award ($400/each) for domestic conference presentation, The Graduate School of University of Kentucky, Lexington, KY

2010 & 2012 Travel Award ($800/each) for international conference presentation, The Graduate School of University of Kentucky, Lexington, KY

2009-2010 Integrated Biomedical Sciences Research Fellowship ($29,940), University of Kentucky College of Medicine, Lexington, KY

2012 Trainee Travel Award (€700), IASP 14th World Congress, Milan, Italy

2012-2013 Individual Predoctoral Kirschstein National Research Service Award (NRSA F31), Impact/Priority Score: 23, Percentile: 11th

Professional Society Affiliations

2010 – present International Association for the Study of Pain (IASP), Trainee Member

2008 – 2010 American Pain Society, Trainee Member

2008 – present Society for Neuroscience, Student Member
2009 – present  Bluegrass Chapter of the Society for Neuroscience (BGSFN), Member

Peer Reviewed Publications


Manuscripts in preparation

Corder, G*., Morales-Medina, J.C*., and Taylor, B.K. CNS manifestations of injury-induced opioid dependence. (In preparation ). * These authors have contributed equally to this work


Corder, G.*, Ianitti, T.*, Morales-Medina, J., Pinson, J., Telling, G., and Taylor, B. Dismutase properties of Cellular Prion Protein regulate the severity of neuropathic pain. (In preparation) * These authors have contributed equally to this work
Selected Abstracts

**Corder, G.**, Doolen, S., Winter, M., McCarson, K., and Taylor, B. Spinal μ-opioid receptor signaling tonically inhibits NMDA receptor-dependent activation of adenylyl cyclase 1 after injury. 42nd Neuroscience Meeting of Society for Neuroscience (2012), New Orleans, LA

**Corder, G.**, Doolen, S., Winter, M., Hu, X., He, Y., Wang, Z., McCarson, K., and Taylor, B. Opioid inhibition of NMDAR-dependent spinal sensitization persists long after the resolution of inflammatory hyperalgesia. 14th World Congress on Pain, International Association for the Study of Pain (2012), Milan, Italy

**Corder, G.**, Winter, M., Chen, W., Donahue, R., McCarson, K., Marvizon, J-C., and Taylor, B. Activation of NPY Y1 and Y2 receptors reduce spinal presynaptic Substance P release. 41st Neuroscience Meeting of the Society for Neuroscience (2011), Washington, D.C.

**Corder G.** and Taylor B.K. Prolonged activation of endogenous opioid analgesia after inflammation. 40th Neuroscience Meeting of the Society for Neuroscience (2010), San Diego, CA

**Corder G.** and Taylor B.K. Reinstatement of inflammatory and neuropathic pain with naltrexone: Endogenous CNS opioids mask latent pain sensitization.
- 13th World Congress on Pain, International Association for the Study of Pain (2010), Montreal, Canada
- 50th Anniversary Celebration of the Department of Physiology (2010); 1st Place Graduate Student Poster

**Corder G.** and Taylor B.K. Naltrexone unmasks an endogenous opioid physical dependency after injury. Spring Neuroscience Day, BGSFN (2010), Lexington, KY; Best Graduate Student Poster Winner

Soignier, R.D., Zadina, J.E., **Corder, G.**, Siegel, A., Intondi, A.B., and Taylor, BK. Correspondence of neuropathic pain with neurochemical changes in dorsal horn and
dorsal root ganglia for 6 months after peripheral nerve injury. 38th Neuroscience Meeting of the Society for Neuroscience (2008), Washington D.C

Collaborations

Zaijie Jim Wang, University of Illinois at Chicago
- Conditioned Place Preference
Kenneth McCarson, University of Kansas
- GTPγS binding assay
Glen Telling, Colorado State University
- Role of prions in neuropathic pain

Mentoring and Teaching Experience

2012 Justin Pinson, Eastern Kentucky University (undergraduate)
2012 Josh Bose, University of Kentucky (undergraduate)
2012 Jennifer Grasch, Vanderbilt University (undergraduate)
2011 Michael Bechard, University of Kentucky (undergraduate)
2010 Amanda Webb, University of Kentucky College of Medicine (medical student)
2005 Terrace Community Middle School – Tampa, FL