

DISTINCT MECHANISMS UNDERLIE THE ONSET AND MAINTENANCE OF PAIN
LINKED TO CATECHOLAMINE DYSREGULATION

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ABSTRACT

Jane Elizabeth Hartung: Distinct mechanisms underlie the onset and maintenance of pain linked to catecholamine dysregulation.
(Under the direction of Andrea G. Nackley)

A body of evidence links decreased expression and activity of catechol-o-methyltransferase (COMT), an enzyme that metabolizes catecholamines, with idiopathic pain conditions including temporomandibular joint disorder and fibromyalgia. Parallel with clinical studies, COMT inhibition in rodents results in increased pain, but the mechanisms required for the onset and maintenance of pain remained unknown. The purpose of this dissertation was to determine the downstream mechanisms required for COMT-dependent pain as well as to understand how environmental triggers can lead to decreased COMT expression. Our lab previously identified that β_2 - and β_3 ARs are required for the onset of COMT-dependent pain, but the molecules downstream of β_2 - and β_3 AR activation remained unknown. Here we examined the mechanisms required for the onset and maintenance of COMT-dependent pain. During the onset of pain, we found that previously identified β_2 - and β_3 ARs, together with circulating pro-inflammatory molecules were required for the onset of pain. Furthermore, release of these pro-inflammatory molecules was β AR-dependent. We then sought to understand if β ARs and pro-inflammatory cytokines were required during the maintenance phase of COMT-dependent pain. We find that while β ARs are important during the onset phase, blockade of β ARs following the onset phase was not able to reverse COMT-dependent pain. Similarly, we did not observe differences in circulating pro-inflammatory molecules. Rather, the maintenance of COMT-dependent pain resulted in increased levels of activated mitogen activated

protein kinases (MAPKs) including p38 and extracellular regulated protein-kinase 1/2. Intrathecal inhibition of these molecules on day 14, after the onset of COMT-dependent pain, was able to reverse COMT-dependent pain, indicating that these molecules contribute to the maintenance of COMT-dependent pain. Finally, we sought to understand if environmental triggers could decrease COMT expression. We found that the inflammatory pathway nuclear factor- κ B (NF- κ B) is involved in prolonged inflammatory pain and decreased COMT expression in the brain. Taken together, these data point to COMT and downstream effectors play a central role in driving the onset and maintenance of pain.

*To my parents, who have encouraged me and provided infinite support throughout my life.
Thank you, Mom and Dad.
I love you.*

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variances
ATP	Adenosine triphosphate
CCL2	Chemokine (C-C motif) ligand 2
CFA	Complete Freund's Adjuvant
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular patterns
DRG	Dorsal root ganglion
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular regulated kinase
GABA	Gamma-aminobutyric acid
i.p.	Intraperitoneal
i.t.	Intrathecal
i.v.	Intravenous
IFN γ	Interferon- γ
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2

IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KO	Knockout mice
MAPK	Mitogen-activated protein kinase
MB-COMT	Membrane-bound catechol-O-methyltransferase
mRNA	Messenger ribonucleic acid
NF- κ B	Nuclear Factor- κ B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
P2X	Purinergic receptor
PGE2	Prostaglandin E2
PKA	Phosphokinase A
PKC	Phosphokinase C
S-COMT	Soluble catechol-O-methyltransferase
s.c.	Subcutaneous
TLR	Toll-like receptor
TMD	Temporomandibular joint disorder
TNF α	Tumor necrosis factor- α
TRP	Transient receptor potential
TTX-r	Tetrodotoxin-resistant
WT	Wild-type mouse

α AR	α -adrenergic receptor
β_2 AR	β_2 -adrenergic receptor
β_3 AR	β_3 -adrenergic receptor
β AR	β -adrenergic receptor

Chapter 1: Introduction

1. Introduction

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Mersky, 2012). Its importance in evolution is clear: pain is one of the first sensations organisms developed in order to protect them from injury to their surrounding environment. The range of environmental stimuli that organisms encounter reflects the variety of sensations experienced: dull or sharp, fast or slow, stinging or tingling, aching or pulsing. This complexity has attracted clinicians, researchers, and even philosophers to contemplate the means by which environmental stimuli linked to pain are transmitted and sensed as distinct sensations.

While acute pain is a healthy, physiological response to tissue damaging stimuli, chronic pain that outlasts its stimulus can have deleterious effects including tissue damage, altered immune system function, and a barrage of negative psychological effects. Most commonly, chronic pain patients exhibit exaggerated pain responses, which fall into two main categories, allodynia and hyperalgesia. Allodynia is defined as a painful response to a normally innocuous stimulus. Hyperalgesia is defined as increased pain to a normally painful stimulus. A lack of painful stimulus and resolution of injury, together with the presence of allodynia and/or hyperalgesia can begin to define chronic pain conditions. Clinically, chronic pain is defined by the frequency and duration of symptoms. Patients are diagnosed when they experience pain for more than 3 months (Mujakperuo et al., 2010).

When patients with chronic pain seek treatment, they find that there are few effective treatments available. As such, chronic pain conditions have become an increasingly large problem for our society, costing an estimated \$635 billion US dollars in treatments and lost wages (Gaskin and Richard, 2012). Therefore, it is imperative that researchers and clinicians work together to develop treatments that target the root cause of chronic pain.

2. The Pain System: Anatomy & Structure

2.1 Peripheral Nervous System Anatomy

The peripheral nervous system is the interface between our body and the environment, capable of sensing incoming inputs from our surroundings. As such, there are a variety of receptors that sense touch, pressure, vibration, temperature, and pain located on sensory neurons that innervate a variety of tissues: the epidermis, dermis, musculature and viscera. Upon their activation, sensory neurons relay information from these peripheral sites to the dorsal root ganglion and subsequently to the spinal cord (Basbaum et al., 2009, Purves, 2012).

Sensory neurons are subdivided into the modalities they detect in a non-pathological and healthy setting by the intensity of stimuli that they detect. Sensory afferents that detect low- and medium-intensity stimuli include proprioceptors (which relay information regarding body position in space), mechanoreceptors (which detect different modalities of touch such as vibration, pressure, and stretch), and thermoreceptors (which detect temperature). Generally these peripheral afferent neurons are categorized as the A β fibers, medium diameter, highly-myelinated peripheral afferents that are capable of relaying action potential at high velocities (Purves, 2012). In contrast, high-intensity mechanical, thermal, or chemical stimuli are detected by a subset of sensory neurons capable of detecting these noxious, painful stimuli, called nociceptors. Nociceptors are comprised of

two main groups: medium diameter, lightly myelinated A δ -fibers and small diameter, unmyelinated C-fibers. A δ -fiber afferent neurons are a group of neurons that detect and relay an action potential elicited by lower-threshold noxious stimuli quickly to the spinal cord. C-fiber afferent neurons are activated by higher-intensity noxious stimuli and because they are unmyelinated, more slowly conduct an action potential to the spinal cord. While A δ - and C- fibers play the most prominent role in the transmission of pain signals, it should also be noted that A β -fibers can be activated as a result of peripheral sensitization. While A β fibers normally sense low-threshold mechanical stimuli such as touch, they are capable of recruiting high threshold nociceptive neurons during a non-nociceptive event. As a result, a normally innocuous touch stimulus can result in a painful sensation. At present, pain researchers hypothesize that this phenomena could be a result of either strengthened excitatory output by A β fibers or A β fibers acting to lower the activation threshold for high-strength stimuli (Basbaum et al., 2009, Purves, 2012).

Nociceptors characterized as A δ fiber afferent neurons have been further classified based on the types of noxious stimuli they detect. Type I A δ fibers detect chemical, mechanical and relatively high thermal (50°C) noxious stimuli, while Type II A δ fibers detect noxious thermal stimuli and relatively high mechanical stimuli. Interestingly, Type I A δ fibers can be sensitized to noxious stimuli, while Type II A δ fibers cannot. Therefore, in the event of sensitization, Type I A δ fibers are more likely to be activated in the presence of lower-threshold mechanical and thermal thresholds (Basbaum et al., 2009).

C-fiber afferent neurons also include different groupings of nociceptors. While most C-fibers can detect both mechanical and thermal stimuli, a subset of C-fibers detects thermal stimuli. In the presence of a sensitization event such as injury, these C-fibers become capable of sensing mechanical stimuli as well. In general, C-fibers detect chemical stimuli such as menthol and

capsaicin as well as pruritogens such as histamine (Basbaum et al., 2009). Though most C-fibers detect nociception, some C-fibers are capable of detecting light touch and cooling temperatures as well (Burgess and Perl, 1967).

Type	Diameter (μm)	Myelination	Activation	Spinal Innervation	Conduction velocity (m/s)
A β	6-12	Highly myelinated	Touch (can be involved in pain transmission)	Lamina III-IV; Lamina II after nociceptive event	35-75
A δ	1-5	Lightly myelinated	Medium intensity (mechanical (Type I), temperature (Type II))	Lamina I-V, X	5-30
C	0.2-1.5	None	High intensity (pain, temperature, itch, algogens)	Lamina I, II	0.5-2

Table 1. Description of fiber types capable of nociception.

Adapted from table published in Chapter 4 of Neuroscience, Edition 6 (Purves, 2012).

As seen in the table above, the conduction velocity of nociceptors differs depending on fiber diameter and axon myelination. Therefore, during a nociceptive event, A δ fibers are involved in what is considered “first pain,” which is immediate and localized, while C-fibers are involved in “second pain,” which is longer-lasting and more diffuse (Basbaum et al., 2009).

Nociceptors can also be biochemically characterized into peptidergic and non-peptidergic populations. Peptidergic neurons release calcitonin gene-related peptide (CGRP), substance P, and other neuropeptides and they respond to nerve growth factor (NGF) *via* TrkA receptors on their surface. Peptidergic neurons are capable of detecting heat, itch, and mechanical stimuli, largely through activation of transient receptor potential channels (TRP), which are capable of detecting these different modalities of pain (e.g. cold, heat, chemical) (Basbaum et al., 2009, McCoy et al.,

2013). Non-peptidergic neurons, which detect mechanical pain and itch, express c-Ret neurotrophin receptor, artemin, and neurturin. These non-peptidergic neurons are most-generally labeled using IB4 isolectin and include a further subset of neurons that express of the Mas-related G-protein receptors (Mrgprs) (Basbaum et al., 2009). Both A δ and C fibers can be either peptidergic or non-peptidergic. While research efforts have worked to further characterize the myriad populations of primary sensory neurons, ongoing studies continue to reveal the heterogeneity of nociceptors based on three main factors: the proteins they express, the stimuli they detect, and their firing patterns.

A β , A δ , and C-fiber sensory afferent fibers converge at the dorsal root ganglion (DRG), a structure comprised of sensory afferent cell bodies. DRGs line the spinal cord bilaterally and contain a collection of afferents adjacent to the body region they innervate.

2.2 Spinal Dorsal Horn Anatomy

Within the central nervous system, the spinal cord contains important architecture necessary for the transmission and modulation of pain signals. Nociceptors terminate in the spinal cord dorsal horn, synapsing with second-order neurons throughout lamina I-V depending on the fiber type. Peptidergic C fibers terminate in lamina I and II and non-peptidergic C fibers terminate in lamina II. A δ nociceptors primarily terminate in Lamina I, though they have also been found to terminate in lamina V and X as well. A δ hair follicles terminate in Lamina III and IV, but also have terminals in lamina III. Table 1 above and Figure 1 below lists the sites where these nociceptors terminate (Todd, 2010, Purves, 2012).

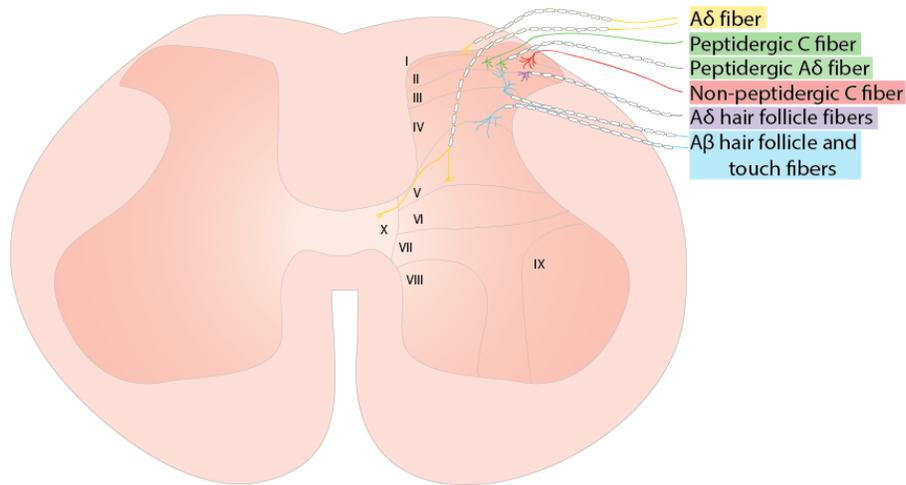


Figure 1.1. Sensory afferents terminate in different regions of the dorsal horn.

Adapted from Todd, 2010.(Todd, 2010)

2.3 *Ascending Spinal to Supraspinal Tracts*

Second order sensory neurons originate in the dorsal horn of the spinal cord, cross over the spinal midline and ascend contralaterally to brain regions that interpret different aspects of qualities of pain (e.g., discriminative, perceptual, emotional). Researchers have characterized three main ascending pathways that either sense pain or are involved in the sensorimotor integration of pain: the spinothalamic tract, the spinobulbar tract and the spinohypothalamic tract (Purves, 2012).

The spinothalamic tract ascends from the dorsal horn of the spinal cord and projects to various subregions of the thalamus, including the posterior portion of the ventral medial nucleus (VPN), the ventral posterior nuclei, the ventral lateral nucleus, the central lateral nucleus, the parafascicular nucleus, and the ventral caudal portion of the medial dorsal nucleus (Purves, 2012). While the anatomical locations of the projections of second order neurons to thalamic nuclei vary between species, or even between reference atlases within a species (Jones et al., 1987), their function remains largely the same: they integrate information from distinct spinal dorsal lamina and

project it to the brain so that both the character and intensity of pain can be perceived and modulated (Purves, 2012).

The spinobulbar tract projects to the brain stem and is primarily involved in homeostatic regulation and nociception. Unlike the spinothalamic tract, which projects contralaterally, the spinobulbar tract projects bilaterally. Ascending spinobulbar fibers terminate at four main nuclei involved in homeostatic regulation: the periaqueductal gray, the parabrachial nucleus, the reticular formation of the brain stem, and brain stem sites involved in catecholamine signaling (e.g. locus coeruleus, ventrolateral medulla). Activation of cells that terminate at sites on the spinobulbar tract can lead to modulation of pain, cardiovascular changes, and alterations in autonomic regulation (Purves, 2012).

Finally, the spinohypothalamic tract is proposed to play an important role in affective aspects of pain, including homeostatic regulation, emotional response, and endocrine regulation. The spinothalamic tract projects primarily to the hypothalamus, but also includes inputs to the pallidum and nucleus accumbens. At present, there is limited evidence suggesting the spinohypothalamic tract exists in primates (Zhang et al., 1999) and rodents (Zhang et al., 1995).

2.4 Non-neuronal Cells that Influence Pain

While primary and secondary sensory neurons in the nervous system play the starring role in the transmission of pain, non-neuronal cells including glia (i.e. microglia and astrocytes), immune cells (i.e. myeloid- and lymphoid-derived cells), and other peripheral cells (i.e. keratinocytes, adipocytes, endothelial cells) may play important supporting roles in regulating the intensity and character of a pain signal.

Microglia are one type of glia derived from erythromyeloid precursor cells (the immune-linked yolk sac cells) (Kierdorf et al., 2013, Prinz and Priller, 2014) and are considered the resident immune cells of the central nervous system. They play an important role in detecting and responding to local inflammatory mediators known as damage-associated molecular patterns (DAMPs) (e.g. immune-related molecule, excitatory neurotransmitter). In the presence of a DAMP, a microglial cell extends its processes to detect and potentially engulf the pro-damage molecule. In addition, microglia will release a cocktail of trophic factors to recruit more microglia to the affected site to remove debris and resolve any damage. These cells are considered the first responders during an inflammatory event because they constantly survey their local environment for potential dangers (McMahon et al., 2005, Grace et al., 2014).

Astrocytes are another type of glia located in the nervous system but, unlike microglia, these cells are derived from the neuroectoderm (De Leo et al., 2006). Their function reflects their derivation: astrocytes regulate glutamate release and neurotransmission by adjusting concentrations of local K^+ and Ca^{2+} (De Leo et al., 2006). Similar to microglia, astrocytes can also release small inflammatory mediators known as cytokines and chemokines. Astrocytes are considered the second responders to an inflammatory event, as they will often act in response to pro-inflammatory molecule release first initiated by microglia (Gao and Ji, 2010a).

Both microglia and astrocytes can act to affect the neurotransmission of an action potential at the site of the synapse. For example, microglia alter the local environment by adjusting local concentrations of K^+ and Cl^- near the synapse or by modifying their expression of Kv1.3 or K^+-Cl^- co-transporter KCC2 (De Leo et al., 2006, Ferrini et al., 2013). As a result, microglia can modulate local anion/cation concentrations that can alter homeostasis required for normal neuronal functioning. Astrocytes can also modulate local concentrations of potassium in the central nervous

system. These cells serve to remove and release potassium from the synaptic cleft through the action of astrocytic Na/K⁺ ATPases (De Leo et al., 2006).

Thus far, I have only described the non-neuronal cells located in the peripheral and/or central nervous systems that affect pain. However, an ever-growing body of evidence now suggests that peripheral cells play a key role in the transmission and modulation of pain. The following review of various cell types in the periphery is not comprehensive, but rather, should serve as a starting point from which to understand the variety of non-neuronal cell types that may be involved in pain transmission, including immune cells, keratinocytes, adipocytes and endothelial cells.

The immune system is divided into two main cell types (myeloid and lymphoid cells), which is based on the progenitor cell type from which they arise. Myeloid cells are derived from progenitor blood cells originating in the bone marrow and lymphocytes true to their name, are derived from lymphoid cells originating in the lymphatic system. Both cell types are essential for host defense against pathogens: myeloid cells regulate innate immunity and lymphoid cells regulate adaptive immunity. Innate immune system activation is an immediate, non-specific response that serves to dampen a pathogen's strength. As the first line of defense, the innate immune system can respond quickly to an infection or injury by constantly circulating cells in the bloodstream that can be converted to immune-active cells. Myeloid-derived cells include granulocytes, such as neutrophils, eosinophils, basophils, dendritic cells, and monocytes. In the event of an infection that requires a more targeted response, the adaptive immune system is recruited to remove specific pathogens. The lymphoid-derived cells associated with the adaptive immune response include B-cells, T-cells, and natural killer cells. B- and T-cells are responsible for mounting pathogen- or damage-specific responses to fight off infection and resolve injury (Murphy, 2008). Both myeloid- and lymphoid-derived cells are important to pain because they can express and release key molecules involved in the amplification or reduction of pain. These include a variety of small molecules, proteins, and

receptors important for inflammation, healing and resolution. These molecules can act directly or indirectly to modulate a pro-pain signal (Grace et al., 2014).

Other cell types, including keratinocytes, adipocytes, and endothelial cells can also influence pain. Keratinocytes, or skin cells, directly interface with nociceptor terminals and can result in highly localized chemical and electrical signals to these terminals. For example, keratinocytes can release pro-inflammatory molecules including cytokines to these nerve terminals, which influence the release of related molecules (Li et al., 2013). Furthermore, recent evidence suggests that keratinocytes communicate with nerve terminals through electrical signals as well. Baumbaer and colleagues found that activation of keratinocytes by channelrhodopsin is capable of eliciting action potentials from sensory neurons, including peptidergic nociceptors (Baumbauer et al., 2015). Adipocytes may also indirectly influence the transmission of pain through the release of IL-6, a known modulator of neuropathic pain (Ramer et al., 1998). Finally, emerging research implicates that viscerally located endothelial cells, which release ATP which can increase firing of nociceptors innervating visceral tissues (Xanthos and Sandkuhler, 2014). While this is not an exhaustive list of non-neuronal cell types involved in pain transmission, it should be noted that more and more cell types are being implicated in their role in pain, particularly those cells proximal to nerve terminals.

3. Pathological Pain Processing

3.1 General Overview of Pain Conditions

Up to this point, I have described the anatomy and molecular processes that are responsible for acute pain. Typically, once the stimulus is removed or the tissue heals, acute pain resolves and the organism will return to baseline. In some instances, however, pain outlasts the stimulus as well as any chemical factors required for proper healing (e.g. local release of trophic factors, inflammatory

molecules, H⁺ ions) (Basbaum et al., 2009). Pain that lasts at least twelve weeks is defined as chronic pain. Chronic pain conditions have been subdivided into three basic categories based largely on their etiologies: neuropathic, inflammatory, and idiopathic pain. Neuropathic pain is defined as pain caused by damage to either the peripheral or central nervous system, including pain that develops as a result of amputation, nerve injury, cancer, and/or cancer treatment. Inflammatory pain is defined as pain associated with tissue injury and inflammation, caused by activation of the immune system. Examples of conditions categorized as inflammatory pain include arthritis (e.g. rheumatoid arthritis, osteoarthritis), autoimmune diseases (e.g. lupus), and colitis (Basbaum et al., 2009). Finally, idiopathic pain conditions do not have a clear etiology: these pain conditions are largely collected together by physicians because they have no clear origin, but often present a common set of symptoms. These symptoms include increased sensitivity to a painful mechanical and thermal stimuli (Gracely and Schweinhardt, 2015). Pain may be evoked or spontaneous in nature and manifest at multiple body sites. Based on their symptoms and their lack of etiology, the following are considered idiopathic pain conditions: fibromyalgia, temporomandibular joint disorder, irritable bowel syndrome, and vulvodynia, which will be described below based on their commonalities (Diatchenko et al., 2006).

3.3 Idiopathic pain conditions

While the etiology of idiopathic pain conditions remain largely unknown, they are characterized by dysfunction in the neuroendocrine system, the autonomic system, sleep, and/or motor function (Diatchenko et al., 2006). Researchers hypothesize that certain environmental and genetic factors may predispose an individual to become more vulnerable to one or more idiopathic pain conditions (Diatchenko et al., 2006). Because these disparate conditions may share similar underlying factors, diagnosis of one idiopathic pain condition increases risk of the

development/diagnosis of another (Lim et al., 2011). For example, patients with fibromyalgia are reported to be diagnosed with irritable bowel syndrome at a rate of 42-70%, while the average incidence rate for IBS in patients without fibromyalgia is approximately 10-16% (Veale D, 1991).

To understand how chronic idiopathic pain conditions are established and maintained, research efforts are focused on identifying genetic and environmental factors that disrupt sympathetic tone and stress response (Mannisto and Kaakkola, 1999a, Diatchenko et al., 2005, Nackley et al., 2006, Orrey et al., 2012). Indeed, these potential contributors to idiopathic pain conditions may alter pain processing as a result of abnormal sympathetic tone during normal and stress-inducing situations. Given that there are few consistent and specific phenotypic and/or molecular markers to distinguish idiopathic pain patients, understanding the etiology of these diseases will serve to better treat patients. Here, I will detail how abnormalities in the sympathetic nervous system may affect peripheral and central sensitization of nociceptors and immune cells in peripheral and central nervous systems.

3.4.1 The Sympathetic Nervous System & Pain Sensitivity: A Historical Perspective

The autonomic nervous system, which drives the “fight or flight” response in the presence of stress-inducing stimuli, consists of the sympathetic and parasympathetic systems. The sympathetic nervous system, which primes the body for activity (to “fight”), includes peripheral and central nervous system inputs that drive the release of epinephrine and norepinephrine. Short-term effects of sympathetic nervous system activation include pupil and blood vessel dilation, redistribution of the blood supply from the digestive system to skeletal muscle, and increased sweating. In addition, short-term activation of the sympathetic nervous system generally leads to an analgesic state. However, long-term abnormalities in the sympathetic nervous system are associated with increased

pain and chronic pain conditions (McMahon, 1991, B.L. et al., 1992, Schott, 1994, Baron et al., 1999, Pertovaara, 2006, 2013).

Established literature has linked sympathetic nervous system activity to the sensation of pain. When the sympathetic nervous system was first characterized, scientists and clinicians argued whether or not the sympathetic fibers innervating the periphery were only efferent, or if the system included both efferent and afferent fibers (Procacci and Maresca, 1987). Not until clinicians observed dramatic pain relief in patients with sympathetic nerve blocks or complete sympathectomies did the idea of a “sympathetic afferent system” gain clout in the medical and scientific community. Since these initial studies, a growing body of literature has linked pain, including chronic pain conditions such as rheumatoid arthritis, fibromyalgia, and chronic regional pain syndrome with altered sympathetic tone. For example, in early reports of fibromyalgia, originally termed “reflex sympathetic disorder,” affected individuals exhibited elevated levels of norepinephrine in blood plasma at baseline and following a painful stimulus (Drummon et al., 1991). These patients were often subject to the same sympathectomies, in the painful, affected area, which provided only limited relief to patients, since they were performed at discrete bodily sites. These were also unfavorable because blockade of the sympathetic nerve resulted in loss of function of sudoriferous glands in the affected area and, as a result, often led to the loss of the ability to sweat at the site of the sympathectomy (Bandyk et al., 2002). While these studies were first to provide a link between the sympathetic nervous system and pain, the pharmacological and molecular mechanisms that resulted in increased pain remained unknown.

In an experimental setting, pioneering research spearheaded by Jon Levine in the mid- to late-1980s demonstrated the importance of sympathetic nerve terminals in the transmission of pain signals (Levine et al., 1985, Levine et al., 1986a, Levine et al., 1986b, Levine et al., 1988). Studies in rodents mirrored observations made in patient populations: increased levels of either epinephrine or

norepinephrine result in decreased pain thresholds (Levine et al., 1988), increased pain responsiveness to pro-inflammatory/pro-pain molecules (Wei et al., 2015), and release of immune-related molecules by cells of the immune system (Nance and Sanders, 2007). Indeed, there is a rich body of literature, spanning from experiments conducted by Wall and colleagues in the 1970s up to now that has found that sympathetic input can affect pain *via* somatic control (vasoconstriction, immune cell activation) as well as by direct effects on sympathetic fiber activation (Levine et al., 1986c). These studies linked sympathetic activity with pain, but the underlying causes leading to altered sympathetic tone remained unknown.

3.4.2 The Sympathetic Nervous System & Pain Sensitivity: The Role of COMT

One way in which sympathetic tone may be altered is through changes in the metabolism of sympathetically-linked neurotransmitters, epinephrine and norepinephrine. A growing body of evidence demonstrates that catecholamines and pathways that regulate their bioavailability are altered in patients with fibromyalgia and TMD. These patients exhibit increased levels of the catecholamines epinephrine and norepinephrine (Light et al., 2009a) and decreased levels of the enzyme catechol-O-methyltransferase (COMT) (Smith et al., 2014b), one of two enzymes responsible for metabolizing epinephrine and norepinephrine to their inactive derivatives (the other related enzyme being monoamine oxidase) (Mannisto and Kaakkola, 1999a).

COMT is ubiquitously expressed in a variety of peripheral and central nervous system tissues (Tunbridge et al., 2006). The gene encoding COMT includes two different promoter sites, P1 and P2. The P1 promoter leads to the transcription of mRNA transcript associated with the shorter COMT isoform, S-COMT. The P2 promoter leads to the transcription of a longer mRNA transcript, which can be translated to encode the S-COMT isoform as well as the MB-COMT isoform. (Tunbridge et al., 2006) Transcript and protein expression levels of S- and MB-COMT isoforms vary

across tissue types and within brain regions. Generally speaking, S-COMT is more commonly expressed in peripheral tissues and organs and MB-COMT is more abundant in central nervous system tissues. While the area of COMT protein regulation remains uncharted territory, current research suggests that these ratios of MB- and S-COMT protein expression is likely a result of differential binding of transcription factors to the P1 or P2 promoter sites (Tunbridge et al., 2006).

In humans, single nucleotide polymorphisms in the COMT gene are linked to decreases in mRNA stability (Nackley et al., 2006), lower transcript and protein expression levels (Nackley et al., 2006), and reductions in enzymatic activity (Smith et al., 2014b) which are associated with experimental (Diatchenko et al., 2005) and clinical pain (Diatchenko et al., 2005, McLean et al., 2011, Orrey et al., 2012). Based on the frequency of allele variation, it is estimated that up to two-thirds of chronic pain patients may carry those genetic variations that alter COMT activity (Diatchenko et al., 2005). The first study to link COMT gene variants with pain was published by Zubieta and colleagues in 2003, which found that a genetic polymorphisms in the COMT gene was linked with reported prolonged experimental pain and elevations in sensory and affective measures of pain (Zubieta et al., 2003). Subsequent findings mirror these data, showing a negative correlation between COMT enzymatic activity and pain sensitivity. Diatchenko and colleagues reported that particular COMT gene variants were linked to a higher incidence of temporomandibular joint pain. Also, the presence of certain SNPs increased risk of developing temporomandibular joint disorder by a factor of 2.3 (Diatchenko et al., 2005). Similar to TMD findings, researchers found that in certain populations of patients with fibromyalgia, including those from Spain and Korea, fibromyalgia incidence was linked to COMT gene variants and high pain sensitivity (Vargas-Alarcon et al., 2007, Park et al., 2016). These variations in the COMT gene are important because they alter the expression and activity of COMT, thereby altering the metabolism of substrates of COMT, including catecholamines (dopamine, epinephrine, and norepinephrine) and estrogen (Nackley et al.,

2006).

Animal studies reflect findings in the clinic: decreased COMT activity as a result of either pharmacological inhibition or genetic knockout results in increased mechanical and thermal pain sensitivity. Given that decreased COMT activity should result in elevated levels of catecholamines, Nackley and colleagues looked at different catecholamine receptors that could be involved in nociception, including dopamine, α -adrenergic and β -adrenergic receptors. They concluded that the development of COMT-dependent pain is mediated through the activation of β_2 - and β_3 -adrenergic receptors (Nackley et al., 2007). However, the mechanisms downstream of β_2 and β_3 -adrenergic receptor activation remained unclear. Furthermore, while research pointed to the importance of these receptors in the development of COMT-dependent pain, it is unknown whether these receptors are required for the maintenance of pain.

3.4.3 The Sympathetic Nervous System & Pain Sensitivity: Beta-Adrenergic Receptors

An emerging body of evidence now links β_2 -adrenergic receptors (β_2 ARs) and β_3 -adrenergic receptors (β_3 ARs) with pain. β_2 ARs are located on keratinocytes, macrophages, and peripheral afferent neurons in the periphery as well as on microglia in the central nervous system. Activation of these receptors results in increased release of pro-inflammatory molecules including cytokines and nitric oxide as well as increased pain sensitivity. β_3 ARs are expressed on adipocytes where it acts as the prime producer of the cytokine interleukin-6 in the periphery (Tchivileva et al., 2009b) and on dorsal root ganglion, where it appears to play an important role in neuropathic pain development and maintenance (Kanno et al., 2010). Furthermore, β ARs are expressed on microglia and astrocytes in the central nervous system, where they can exert pro-inflammatory effects downstream of β AR stimulation (Gharami and Das, 2004, Wohleb et al., 2011, Johnson et al., 2013).

One way in which β_2 - and β_3 ARs may increase pain is through hyperalgesic priming. During hyperalgesic priming, epinephrine acts on small-fiber nociceptors to reduce nociceptive thresholds by increasing the number of action potentials elicited and reducing the latency for the first spike *via* β ARs (Khasar et al., 1999b). In this sense, β AR agonists can independently increase pain by action on the periphery. Downstream of β AR activation, the kinases phosphokinase c (PKC), phosphokinase A (PKA), and extracellular-regulated kinase 1/2 may help mediate the activation of nociceptors through their ability to modify TTX-sensitive nociceptors (Khasar et al., 1999b, Aley et al., 2001).

β -Adrenergic receptor signaling is involved in acute settings and may be important for the initiation of a nociceptive event (Khasar et al., 1999b). Subsequent studies have shown that β ARs residing on nociceptors play an important role in pain. These include C-fiber afferent nociceptors located in the meninges (Wei et al., 2015) and keratinocytes located at the site of the nociceptor terminal (Li et al., 2013). Therefore, assessing the role of β AR activation on peripheral nociceptors may play an important role in the potentiation of pain signaling.

3.4.4 The Sympathetic Nervous System & Pain Sensitivity: Epinephrine and Norepinephrine

Although a body of evidence suggests that epinephrine and norepinephrine act on β ARs residing in the periphery contribute to pain hypersensitivity, these neurotransmitters may act oppositely, in an anti-nociceptive manner, in the spinal cord. Descending input from α_1 - and α_2 -adrenergic receptors reduces the transmission of nociceptive signals through three main ways: (i) reducing the release of excitatory neurotransmitters from peripheral nociceptors at the central terminal, (ii) initiating inhibitory K^+ currents in postsynaptic secondary afferents, or (iii) activation of inhibitory interneurons residing in the dorsal spinal horn (Pertovaara, 2013).

Because adrenergic receptors are located on a variety of cell types that elicit different intracellular responses, it is not surprising that their activity in the transmission of pain is different at peripheral and spinal sites. A number of authors have concluded similarly. In a comprehensive review of catecholamine metabolism and signaling, Segall and colleagues found that epinephrine and norepinephrine act oppositely in the peripheral and central nervous system (Segall et al., 2012). Further, activity of the enzyme responsible for metabolizing these catecholamines, catechol-O-methyltransferase (COMT), follows the same nociception pattern: intraperitoneal administration of a COMT inhibitor, which should presumably increase epinephrine and norepinephrine, leads to increased mechanical and thermal sensitivity (Nackley et al., 2007, Kambur et al., 2010); intrathecal administration of a COMT inhibitor does not alter nociception (Kambur, 2013). While these studies are highly specific to bodily site and receptor-subtype activation, their findings underline the importance of discriminating for site-appropriate targets that effectively alter pain sensitivity and their downstream targets along the pain transmission pathway. While these studies have outlined how COMT may play an important role in the onset of pain, the role of COMT in the maintenance of pain remains unknown.

3.5 Immune System Involvement in Pain

3.5.1 Cytokines and Chemokines: General Overview

Cells involved in the innate and adaptive immune response must release chemical signals that indicate the need to mount an immune response. Cytokines and chemokines are small signaling proteins that serve a variety of roles in the immune system, including recruitment of immune cells, activation of intracellular pathways, and trafficking of receptors on/off the membrane (Grace et al., 2014). Considered a specific type of cytokine, chemokines are responsible for chemotaxis, or the

movement of immune cells. Both chemokines and cytokines fall into the category of either pro- or anti-inflammatory. Pro-inflammatory cytokines ramp up an innate and/or adaptive immune response. Cytokines involved in the innate immune response include tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). These molecules are the first to be released by macrophages after an inflammatory event or damage, signaling for the recruitment of immune cells and associated molecules to the site of infection. Furthermore, innate immune cytokines are important because they initiate an immune response: when pro-inflammatory cytokine levels remain high, this may activate lymphoid-derived cells to trigger an antigen-specific response by the adaptive immune system. Pro-inflammatory cytokines involved in the adaptive immune response include interleukin-2 (IL-2), interleukin-8, interleukin-18, and interferon- γ (IFN γ). These molecules play an important role in the activation of B- and T-cells, which drive an antigen-specific response to an infection. In addition, pro-inflammatory cytokines are able to upregulate particular genes involved in inflammation, thus creating a positive feedback loop to potentiate immune signaling/response (Grace et al., 2014).

Opposite to their pro-inflammatory counterparts, anti-inflammatory cytokines act to reduce inflammation, which returns the expression of pro-inflammatory cytokines to baseline levels. Anti-inflammatory cytokines include interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13). Accumulating pre-clinical and clinical evidence suggests that impaired anti-inflammatory cytokine response may extend an inflammatory event and thereby potentiate pain (Milligan et al., 2005, Grace et al., 2014, Ji et al., 2014).

3.5.2 Cytokines and Chemokines: Their Potential Role in Pain

Cytokines can potentiate pain through their actions in both peripheral and central nervous systems. In the periphery, for example, tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β)

can bind to receptors expressed on peripheral nociceptor terminals to directly reduce their activation threshold (Binshtok et al., 2008a, Gudes et al., 2014). Furthermore, interleukin-6 (IL-6) has been found to be a key inducer of hyperalgesic priming, which leads to hyperactivation of nociceptors following an inflammatory insult and predisposes neurons to become active with prostaglandin E2 (PGE2). Cytokines can also indirectly increase pain sensitivity. For example, TNF α can promote the release of a cascade of pro-inflammatory cytokines, including IL-1 β , IL-6 and chemokine (C-C motif) ligand 2 (CCL2), which can thereby influence pain states.(Cunha et al., 2005) Furthermore, TNF α binds to the TNF receptor to activate intracellular cascades that upregulate pain-relevant genes (Grace et al., 2014).

In the central nervous system, pain and inflammation are potentiated by microglia and astrocytes that synthesize, release, and bind cytokines. Microglia express a variety of receptors that sense cytokines and other pro-inflammatory signals (e.g. LPS, ATP) including TLR2/4, IL-1R, CCR2, P2X4R, P2X13R, P2Y12R and P2X7R. TLR2/4 can stimulate p38 and extracellular regulated kinases (ERK). The purinergic receptors (P2X₄R, P2X₁₃R, P2Y₁₂R and P2X₇R) respond to ATP release following cell damage. (Grace et al., 2014) Through these receptors, then, microglia may act as important first mediators of central nervous system inflammation. Downstream of P2 receptor activation, microglia have been found to release IL-1 β and initiate the intracellular p38 pathway. Because microglia express this wide variety of immune related molecules, it is thought that they are the first to respond to an inflammatory event and their activity downstream of activation results in the initiation of a neuroinflammatory event. After their activation, microglia are capable of releasing TNF α , which can bind to TNFR residing on astrocytes, leading to activation of p38 (Ji and Suter, 2007).

Astrocytes also express tumor necrosis factor receptor 1 (whose ligand is TNF α),

interleukin-18 receptor (whose ligand is IL-18), and chemokine receptor 2 (whose ligand is CCL2). Research on the intracellular pathways downstream of these cytokines is more limited; however, it is thought that activation of these astrocytically-located receptors leads to increased activation of mitogen activated protein kinases (MAP kinases) such as p38 and ERK1/2 as well as the intracellular nuclear factor- κ B (NF- κ B) pathway (Grace et al., 2014). Astrocytic p38 and ERK1/2 activation can result in TNF α production (Lee et al., 2000), as well as NF- κ B activation. Given that many intracellular events result in their activation, p38 and ERK1/2 play an important role as key central intracellular cascades that can control neuroinflammation and/or centrally-derived chronic pain states.

In addition, previous studies in astrocyte cell lines *in vitro* have demonstrated that the cytokine TNF α can bind directly to the P2 promoter of the *Comt* gene and inhibit its transcription through activation of NF- κ B. (Tchivileva et al., 2009a) Because TNF α is produced as a result of an inflammatory insult that can activate the NF- κ B pathway, we hypothesized that inhibition of NF- κ B decreases inflammatory pain and prevents downregulation of COMT expression while constitutive activation of NF- κ B increases inflammatory pain and increases downregulation of COMT expression.

Pro-inflammatory molecules can also act on neurons such that they stimulate pain and/or produce pro-inflammatory molecules. For example, TNFRs residing on GABAergic neurons in the spinal dorsal horn can stimulate p38, leading to attenuated activity of GABAergic neurons and disinhibition of pain transmission (Zhang et al., 2010). Neurons located in the DRG also express toll-like receptors (TLRs), which following activation by DAMPs, can sensitize nociceptors and lead to local synthesis of pro-inflammatory molecules including IL-1 β , CCL5, and prostaglandin E2 (PGE2) (Grace et al., 2014). DRG neurons can be activated by cytokines, as well: TNF α and IL-1 β

have been found to act on TNFR and IL-1R, respectively, to initiate the p38 pathway and altering the activity of TTX-r sodium channels (Gudes et al., 2015).

While more limited research exists linking anti-inflammatory signaling molecules and their role in pain, emerging evidence has shown that IL-10 can act as a powerful anti-nociceptive molecule when administered intrathecally (Milligan et al., 2005). Furthermore, those molecules that promote the resolution of injury, including poly-unsaturated fatty acid derivatives such as resolvins, protectins and lipoxins. For example, ResolvinE1 has been shown to act through chemerin chemokine-like receptor 1 to inhibit activation of transient potential vanilloid receptor 1 (TRPV1), ERK1/2, and TNF α (Xu et al., 2013).

3.5.3 Nitric Oxide

Nitric oxide (NO) is a small molecule that is involved in the transmission of pain and has been found to be released downstream of IL-1 and the activated nuclear factor-kappa B (NF- κ B) inflammatory pathway (McMahon et al., 2005). NO has traditionally been viewed as a molecule responsible for blood vessel dilation. In the presence of NO, endothelial cells lining the blood vessels “relax,” thereby allowing for greater blood flow to the localized site in order to effectively deliver molecules responsible for injury resolution (Schmidtko et al., 2009).

NO and nitric oxide synthases can increase pain sensitivity and initiate pro-inflammatory pathways. Nitric oxide is produced by nitric oxide synthases, which include three different isoforms: inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS). While expression of eNOS and nNOS are largely constitutive, expression of iNOS can be upregulated by activation of NF- κ B (Aktan, 2004). Spinal nNOS has been implicated as a key mediator of inflammatory and neuropathic pain, while iNOS may contribute to inflammatory pain (Schmidtko et al., 2009). Pain sensitivity can be heightened through NO –

mediated activation of the enzyme guanylate cyclase which has been shown to increase neuronal excitability and decrease inhibition of inhibitory neurons in the central nervous system (Latremoliere and Woolf, 2009). NO and cytokines influence one another's release in a feed-forward mechanism, such that NO stimulates cytokine release and vice versa (Holguin et al., 2004). Furthermore, because NO has a great propensity to bind with the free-radical superoxide (O_2^-) and form the molecule peroxynitrite (ONOO⁻), prolonged elevations in NO levels may exacerbate inflammatory states and increase pain (Schmidtko, 2015).

4. Summary and Aims

Previous work by our lab demonstrated that sympathetic dysregulation following altered expression/activity of COMT drives pain through the activation of β_2 - and β_3 ARs. However, the downstream mechanisms through which β_2 - and β_3 AR activation led to increased pain remained unknown. Thus, the present studies sought to reveal the downstream molecular mechanisms required for the onset and maintenance of pain in peripheral and central sites linked to catecholamine dysregulation. These studies employed the COMT inhibitor dinitrocatechol or OR486, which is a second-generation inhibitor similar in structure to the backbone of catechols (Mannisto and Kaakkola, 1999a). Because systemically administered OR486 is capable of penetrating the blood brain barrier, the effects of OR486 are not only in the periphery but also in the central nervous system. Therefore, this COMT inhibitor provided a means to mimic observations in clinical studies that show universal decreases in COMT expression and activity (Nackley et al., 2006, Nackley et al., 2007).

The following dissertation will attempt to describe (i) the short term effects of COMT inhibition on pain sensitivity and pro-inflammatory molecule release *via* β_2 - and β_3 ARs and (ii) the long effects during and following chronic COMT inhibition on pain behaviors, circulating and

central cytokine release, and activation of glia and neurons. Here, we demonstrate that there are distinct mechanisms that underlie onset and maintenance of pain. Specifically, we will show that other molecules, independent of β_2 - and β_3 -adrenergic receptor activation play an important underlying role after COMT-dependent pain has been established. In addition to studying the downstream effects of decreased COMT inhibition, this body of work will also focus on the means by which COMT protein expression is decreased as a result of inflammatory insults. We report that NF- κ B activated by an inflammatory insult results in decreased COMT protein expression in central nervous system tissues. Results gathered from this research is important because it demonstrates that genetic factors as well as environmental events predispose an individual to decreased COMT expression that can result in increased pain.

Chapter 2: The Onset of COMT-dependent Pain

1. Introduction

A growing body of literature demonstrates that catecholamines and pathways regulating their bioavailability influence pain. Patients with chronic pain conditions, such as fibromyalgia and temporomandibular disorders (TMD), exhibit increased levels of the catecholamines epinephrine and norepinephrine (Evaskus and Laskin, 1972, Torpy et al., 2000, Staud, 2004, Light et al., 2009a) as well as decreased levels of the enzyme catechol-O-methyltransferase (COMT) (Gursoy et al., 2003, Diatchenko et al., 2005, Vargas-Alarcon et al., 2007), which metabolizes epinephrine and norepinephrine to their inactive derivatives (Mannisto and Kaakkola, 1999a). Consistent with these findings, animal studies show that epinephrine administration (Coderre et al., 1990, Khasar et al., 1999a, Khasar et al., 1999b) or COMT inhibition (Nackley et al., 2007, Kambur et al., 2010) increases mechanical and thermal hyperalgesia. Pharmacologic studies reveal that COMT-dependent pain, decreased COMT resulting in enhanced sensitivity, is mediated *via* both β_2 - and β_3 -adrenergic receptors (β_2 ARs β_3 ARs). While antagonism of either β_2 - or β_3 ARs block acute COMT-dependent pain, this enhanced sensitivity was completely blocked by antagonizing both β_2 - and β_3 ARs (Nackley et al., 2007).

β_2 ARs and β_3 ARs are G-protein coupled receptors expressed in peripheral, spinal, and supraspinal sites involved in pain transmission. Stimulation of β_2 - or β_3 ARs on peripheral afferents directly sensitize nociceptors (Khasar et al., 1999a, Sun et al., 2006) and produce allodynia (Kanno et al., 2010) through activation of intracellular kinases. Additionally, stimulation of β_2 - or β_3 ARs indirectly enhance pain transmission through the release of pro-inflammatory molecules including

nitric oxide and cytokines (Maimone et al., 1993, Akimoto et al., 2002, Frost et al., 2004, Furlan et al., 2005, Canova et al., 2006, Tan et al., 2007, Hodges et al., 2008, Figueroa et al., 2009, Tchivileva et al., 2009b).

Nitric oxide (NO) is a gaseous molecule whose production by NO synthases can be induced by stimulation of β_2 ARs on endothelial cells, smooth muscle, sympathetic afferent neurons, and macrophages (Akimoto et al., 2002, Hodges et al., 2008, Figueroa et al., 2009) or stimulation of β_3 ARs on adipocytes and fibroblasts (Furlan et al., 2005, Canova et al., 2006). Following release, NO lowers the firing threshold of nociceptors (Aley et al., 1998, Boehning and Snyder, 2003) to enhance experimental inflammatory and neuropathic pain (Omote et al., 2001, Holguin et al., 2004, Kuboyama et al., 2011). Furthermore, NO can stimulate release of additional molecules involved in nociception, including pro-inflammatory cytokines (Holguin et al., 2004, Chen et al., 2010).

Pro-inflammatory cytokines linked to pain include tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and chemokine (C-C motif) ligand 2 (CCL2, MCP-1). β_2 - and β_3 AR stimulation promotes the production and release of TNF α , IL-1 β , IL-6, and CCL2 (Maimone et al., 1993, Frost et al., 2004, Tan et al., 2007, Tchivileva et al., 2009b, Roth Flach et al., 2013). Signaling by TNF α , IL-1 β , and IL-6 lead to lower nociceptor firing thresholds and enhanced pain sensitivity (Obreja et al., 2002, Obreja et al., 2005, Binshtok et al., 2008b, Czeschik et al., 2008). Similarly, CCL2 enhances nociceptor firing in models of neuropathic pain (Sun et al., 2006, Jung et al., 2008).

Of note, NO and cytokines influence one another's release. NO drives the production and release of cytokines such as TNF α and IL-1 β (Jongeneel, 1995, Watkins et al., 1999, Chen et al., 2010, Cury et al., 2011), while cytokines can upregulate NO synthase expression and promote NO release (Lamas et al., 1991, Teixeira et al., 1993, Grabowski et al., 1996, Sung et al., 2005). This

positive feedback loop may contribute to the development and/or maintenance of pain (Cury et al., 2011). While NO and cytokines are released following β_2 - and β_3 AR stimulation and are linked with pain, their role in COMT-dependent pain has not been established.

To investigate the role of NO and cytokines in COMT-dependent pain mediated by β_2 - and β_3 ARs, we measured plasma NO and cytokines following administration of a COMT inhibitor in the presence or absence of β_2 - and β_3 AR antagonists. Additionally, we measured mechanical and thermal pain sensitivity following COMT inhibition in the presence or absence of a NO synthase inhibitor or TNF α , IL-1 β , IL-6, or CCL2 neutralizing antibodies. Our results demonstrate that (1) COMT-dependent pain is accompanied by increases in peripheral NO derivatives and cytokines mediated by β_2 - and β_3 ARs, (2) inhibition of NO synthesis and neutralization of the innate immunity cytokines TNF α , IL-1 β , IL-6 block COMT-dependent pain, and (3) NO and cytokines potentiate one another's biosynthesis, such that NO promotes the release of TNF α , IL-1 β , IL-6, and CCL2 while TNF α and IL-6 promote NO release.

2. Materials and Methods:

2.1 Subjects

Adult male Sprague Dawley rats (Charles River Laboratories, Raleigh, NC) were used in all experiments. Rats weighed between 215-265 g for β_2 - and β_3 AR antagonism and NO synthase inhibition experiments and between 315-360 g for cytokine neutralization experiments.

2.2 Drugs and chemicals

As described in Nackley et al., 2007 (Nackley et al., 2007), OR486 was dissolved in DMSO and diluted in 0.9% saline (3:2). ICI18551, SR59230A, and L-NAME were dissolved in DMSO and 0.9% saline (1:4). Functional grade antibodies against tumor necrosis factor α (α -TNF α), interleukin-1 β (α -IL-1 β), interleukin-6 (α -IL-6), chemokine (C-C motif) ligand 2 (α -CCL2) or IgG control were dissolved in 0.9% saline. OR486, ICI118,551, and SR59230A were purchased from Tocris (Ellisville, MO). L-NAME was purchased from Sigma-Aldrich (St. Louis, MO). Neutralizing antibodies against TNF α , IL-1 β , CCL2 and Armenian hamster IgG controls were purchased from eBiosciences (San Diego, CA), while the antibody against IL-6 (polyclonal goat IgG) was purchased from R&D Systems (Minneapolis, MN).

2.3 General Experimental Conditions

Animals were handled and habituated for 4 days prior to testing day. On testing day, animals were habituated to the environment for 10-15 minutes and then stable baseline responses to mechanical or thermal stimuli were established in separate groups of rats. Following baseline testing, animals received drug treatment and behavior was reassessed. Responses to mechanical stimuli were reassessed at 30, 75 and 120 minutes following OR486 and responses to thermal heat were reassessed at 120 minutes following OR486.

Experimenter was blinded to first and second experimental treatments. Animals were grouped separately based upon the three types of treatments used: β_2 - and β_3 AR antagonists, NOS inhibitors, and neutralizing antibodies.

We first sought to determine if COMT-dependent pain is accompanied by increases in NO and cytokines and if this was mediated by β_2 - and β_3 ARs. Separate groups of animals received

intraperitoneal (i.p.) ICI118,551 (0.5mg/kg) together with SR59230A (5.0mg/kg) or vehicle 30 minutes before i.p. OR486 (30 mg/kg) or vehicle.

We then sought to elucidate the role of NO and cytokines in driving COMT-dependent pain. To determine if NO production was required for the development of COMT-dependent pain, separate groups of animals received i.p L-NAME (30 mg/kg) or vehicle 30 min before i.p. OR486 (30 mg/kg) or vehicle. L-NAME dosage was based on that used in Kuboyama et al., 2011 (Kuboyama et al., 2011). To determine if cytokine action was required for the development of COMT-dependent pain, separate groups of animals received intravenous (i.v.) α -TNF α (75 ug), α -IL-1 β (75 ug), α -IL-6 (75 ug), α -CCL2 (75 ug) or IgG control (75 ug) dissolved in 250 μ L 0.9% saline 2h prior to i.p. OR486 (30 mg/kg) or vehicle. Dosages of neutralizing antibody were determined by two sources: previous reports using neutralizing antibodies and the effective neutralizing dose that would neutralize cytokines at the average dosages we observed at 180 minutes following OR486 administration (Lindenlaub et al., 2000, Tan et al., 2007). We chose to administer the antibodies by i.v. injection to optimize the circulation of the antibody in a relatively short amount of time.

Finally, we sought to establish if NO and cytokines influenced one another's biosynthesis. To determine if NO synthesis was required for cytokine release, plasma collected from animals in the L-NAME experiments was measured for levels of TNF α , IL-1 β , IL-6 and CCL2. To determine if cytokine action was required for NO release, plasma from animals receiving neutralizing antibodies against TNF α , IL-1 β , IL-6, and CCL2 was measured for levels of total nitrite (nitrite and nitrate).

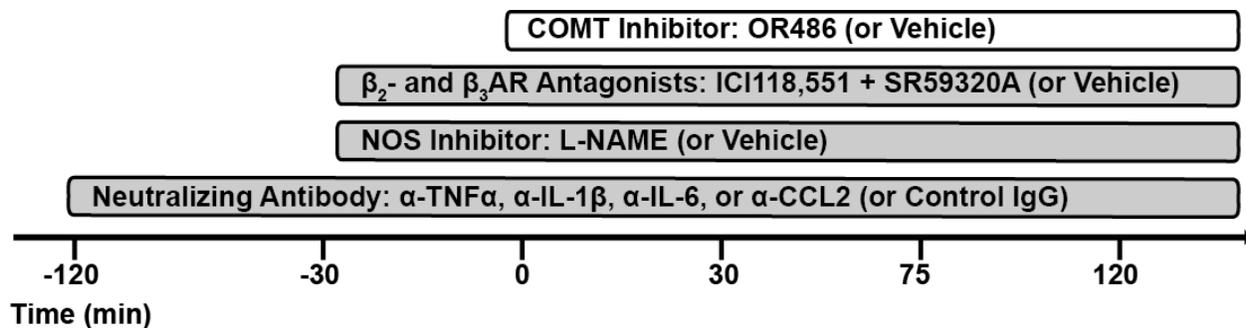


Figure 2.1. Timeline of administered treatments used in this study.

The COMT inhibitor OR486 or vehicle were administered in the presence or absence of the β_2 - and β_3 -adrenergic receptor antagonists ICI118,551 and SR59230A, the NO synthase inhibitor L-NAME, or neutralizing antibodies against TNF α , IL-1 β , IL-6, or CCL2.

2.4 Assessment of Mechanical Allodynia and Mechanical Hyperalgesia

Paw withdrawal threshold was measured using the von Frey up-down method, as described in Nackley et al., 2007 and below. Nine calibrated von Frey monofilaments (bending forces of 0.40, 0.68, 1.1, 2.1, 3.4, 5.7, 8.4, 13.2, and 25.0 g; Stoelting) with equal logarithmic spacing between filaments were applied to the plantar surface of the hind paw. A series of six applications of monofilaments with varying gram forces was applied for 3 s to the plantar surface of the hindpaw. Testing began with the middle filament in the series (3.4 g). If the response included the withdrawal of the hindpaw, an incrementally lower filament was applied. In the absence of a paw withdrawal, an incrementally higher filament was applied. These data were entered into Paw Flick module within the National Instruments LabVIEW 2.0 (Austin, TX) software. A logarithmic algorithm accounted for the order and number of withdrawal responses as well as the gram force of the final filament to calculate mechanical threshold, the gram force that would elicit paw withdrawal in 50% of trials ($10^{[X_f+k\delta]}$ / 10,000, where X_f = value (in log units) of the final von Frey hair used; k = tabular value of positive and negative responses, and δ = mean difference (in log units) between stimuli). Mechanical

allodynia was defined as a heightened response to a normally innocuous stimulus and was determined as a significant decrease in paw withdrawal threshold from baseline.

After determining paw withdrawal threshold, paw withdrawal frequency to a noxious von Frey monofilament was assessed. The highest gram force filament (25.0 g) was applied to the hind paw 10 times. Stimulus was applied for 1s followed by a 1s interval without a stimulus. The number of paw withdrawals was recorded for each hindpaw. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals to a noxious mechanical stimulus from baseline.

2.5 Assessment of Thermal Hyperalgesia

Thermal hyperalgesia was measured using the radiant method by applying radiant heat to the hind paw as described in Hargreaves et al., 1988 (Hargreaves et al., 1988). Animals were placed in individual Plexiglass chambers and habituated for approximately 10 minutes. Following habituation, a radiant beam of light was applied to the plantar surface of the rat hind paw through a glass floor heated to 30°C. Latencies of paw withdrawal from the heat stimulus were recorded in duplicate. If the second paw withdrawal latency was not within ± 4 seconds of the first withdrawal latency, then a third measure was recorded. The two latencies closest in value were averaged and included in the analysis. Thermal hyperalgesia was defined as a decrease in paw withdrawal latency to a noxious thermal stimulus compared to baseline.

2.6 Tissue Collection

Following behavioral testing, animals were euthanized by injection of 0.5 mL Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI). Arterial blood was collected and placed in EDTA plasma tubes, then centrifuged for 15 minutes at 15,000 x g. Following collection, plasma was stored at -80°C.

2.7 Measurement of NO Derivatives

To measure nitrite, NO in blood plasma was assessed using the Griess Reaction (Promega, Madison, WI). To measure total nitrite (nitrite and nitrate), NO in blood plasma was assessed by kit from R&D Systems (Minneapolis, MN).

2.8 Measurement of Cytokines

To determine if COMT inhibition raised TNF α plasma levels downstream of β_2 - and β_3 AR stimulation, plasma TNF α was measured by the UNC Proteomics/Immunotechnologies Core using ELISA kits from Biosource (Camarillo, CA). To determine if COMT inhibition raised TNF α plasma levels downstream of NO production, plasma TNF α was measured by chemiluminescent ELISA (Life Technologies Carlsbad, CA) due to discontinuation of aforementioned Biosource kit. IL-1 β was measured by the UNC Cytokine Analysis Facility using the Luminex Rat Cytokine Multiplex Array from R&D Systems (Minneapolis, MN). IL-6 and CCL2 were measured by ELISA (eBioscience, San Diego, CA; R&D Systems, Minneapolis, MN, respectively). Selected ELISAs and multiplex were based upon minimum assay range and analyte sensitivity. All plasma samples were diluted at 2x.

2.9 Statistical Analysis

All behavioral data were analyzed using a t-test to verify that there were no significant differences in baseline values. Baseline mechanical allodynia values did differ in two groups and were normalized by adding the difference between the average baseline threshold values and the average baseline threshold value for all groups to each animal at each time point. Mechanical allodynia and hyperalgesia data were analyzed by two-way analysis of variance (ANOVA). Thermal hyperalgesia

and molecular data were analyzed using a one-way ANOVA. Post-hoc comparisons were performed using the Bonferroni test and were corrected for multiple testing. $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1 COMT inhibition results in increased pain sensitivity and production of pro-inflammatory mediators via β_2 - and β_3 ARs

To recapitulate our lab's previous results demonstrating that acute COMT-dependent pain is mediated by both β_2 - and β_3 ARs, we measured pain behavior in animals receiving the β_2 AR antagonist ICI118,551 together with the β_3 AR antagonist SR59230A prior to the COMT inhibitor OR486. As expected, animals receiving OR486 exhibited mechanical allodynia ($F_{3,137}=9.223$, $P < 0.0001$; Fig. 2A), mechanical hyperalgesia ($F_{3,139}= 11.45$, $P < 0.0001$; Fig. 2B) and thermal hyperalgesia ($F_{3, 54}=5.336$, $P < 0.003$; Fig. 2C) compared to those receiving vehicle. COMT-dependent increases in pain sensitivity were observed 30 to 120 min following drug administration and were completely blocked by co-administration of β_2 - and β_3 AR antagonists.

Following the conclusion of behavioral experiments, blood plasma was collected to measure circulating levels of NO derivatives, TNF α , IL-1 β , IL-6, and CCL2. Animals receiving OR486 exhibited increased levels of nitrite ($F_{3,23}= 3.929$, $P < 0.03$; Fig. 2D), TNF α ($F_{2,18}=5.663$, $P < 0.02$; Fig. 2E), IL-1 β ($F_{3,27}=3.428$, $P < 0.04$; Fig. 2F), IL-6 ($F_{3,19}=1.354$, $P=0.2$; Fig. 2G), and CCL2 ($F_{3,27}=3.569$, $P < 0.03$; Fig. 2H). COMT-dependent increases in nitrite and cytokines were completely blocked by co-administration of ICI118,551 and SR59230A.

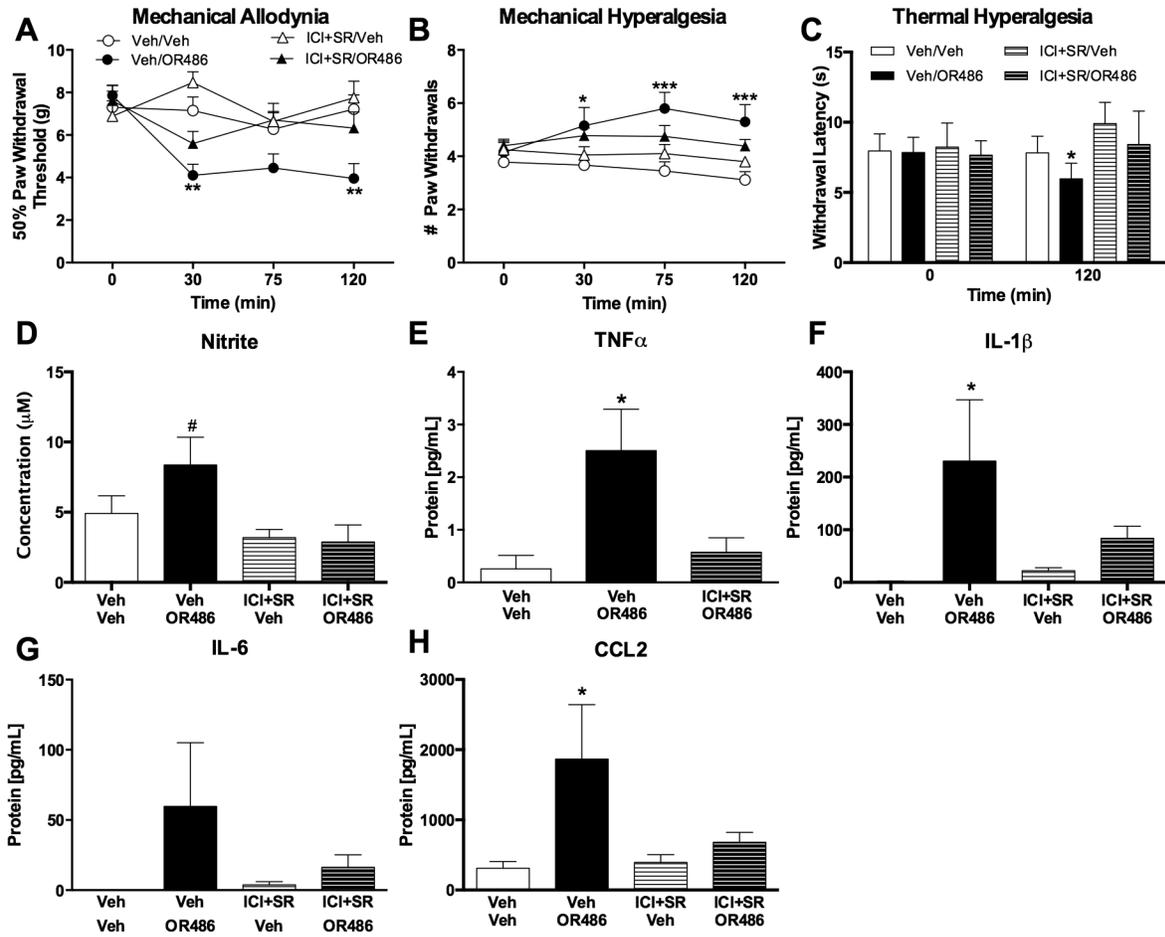


Figure 2.2. COMT inhibition increases pain, NO derivatives, and cytokines *via* $\beta_{2,3}$ ARs.

Animals receiving OR486 (30 mg/kg) exhibit **(A)** mechanical allodynia, **(B)** mechanical hyperalgesia, and **(C)** thermal hyperalgesia, as well as increased circulating levels of **(D)** nitrite, **(E)** TNF α , **(F)** IL-1 β , **(G)** IL-6, and **(H)** CCL2. COMT-dependent increases in pain, nitrite, and cytokines were completely blocked by co-administration of ICI118,551 (0.5 mg/kg) and SR59230A (5.0mg/kg). N=6-10 per group. Data are mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 different from Veh/Veh, # P <0.05 different ICI+SR/Veh and ICI+SR/OR486.

3.2 NO synthase inhibition and cytokine neutralization prevent COMT-dependent pain

As NO and cytokines are released following stimulation of β_2 - and β_3 ARs and have been implicated in the development of pain in other models, we sought to determine their role in the development of acute COMT-dependent pain. To first evaluate the contribution of NO synthesis, we measured pain behavior in separate groups of animals that received the NO synthase inhibitor L-NAME or vehicle 30 min prior to OR486. Administration of L-NAME prior to OR486 blocked the development of mechanical allodynia ($F_{3,138}=5.195$, $P<0.003$; Fig. 3A), mechanical hyperalgesia ($F_{3,138}=5.195$, $P<0.003$; Fig. 3B), and thermal hyperalgesia ($F_{3,54}=6.337$, $P<0.001$; Fig. 3C). Therefore, NO production by NO synthases is required for the development of COMT-dependent increases in mechanical and thermal pain.

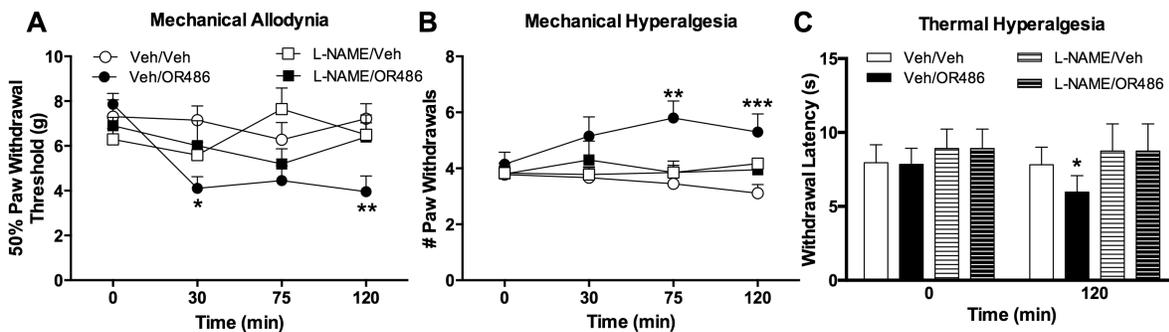


Figure 2.3. Inhibition of NO synthesis prevents COMT-dependent pain.

Administration of the universal nitric oxide synthase inhibitor L-NAME (30 mg/kg) prior to OR486 (30 mg/kg) normalized (A) mechanical allodynia, (B) mechanical hyperalgesia, and (C) thermal hyperalgesia. N=8-10 per group. Data are mean \pm SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ different from Veh/Veh.

To next evaluate the individual contributions of TNF α , IL-1 β , IL-6, and CCL2 to acute COMT-dependent pain, we measured pain behavior in separate groups of animals receiving neutralizing

antibodies against TNF α , IL-1 β , IL-6, and CCL2 or control IgG prior to OR486. Results show that neutralization of the innate immunity cytokines (TNF α , IL-1 β , and IL-6), but not CCL2, prevented OR486-dependent increases in mechanical and thermal pain. Administration of α -TNF α ($F_{3,84}=10.71$, $P<0.0001$; Fig. 4A), α -IL-1 β ($F_{3,83}=19.34$, $P<0.0001$; Fig. 4D), and α -IL-6 ($F_{3,87}=10.96$, $P<0.0001$; Fig. 4G) blocked mechanical allodynia. Additionally, pretreatment with α -TNF α ($F_{3,89}=30.95$, $P<0.0001$; Fig. 3B), α -IL-1 β ($F_{3,89}=29.72$, $P<0.0001$; Fig. 4E), and α -IL-6 ($F_{3,93}=23.33$, $P<0.0001$; Fig. 4H) blocked mechanical hyperalgesia. Finally, α -TNF α ($F_{3,47}=5.312$, $P<0.004$; Fig. 4C), α -IL-1 β (α -IL-1 β : $F_{3,49}=5.639$, $P<0.002$; Fig. 4F), and α -IL-6 ($F_{3,48}=3.339$, $P<0.003$; Fig. 4I) blocked thermal hyperalgesia at 120 min. However, α -CCL2 was not effective at blocking mechanical allodynia (Fig. 4J), mechanical hyperalgesia (Fig. 4K) or thermal hyperalgesia (Fig. 4L). Therefore, the innate immunity cytokines TNF α , IL-1 β , and IL-6 are required for the development of COMT-dependent pain.

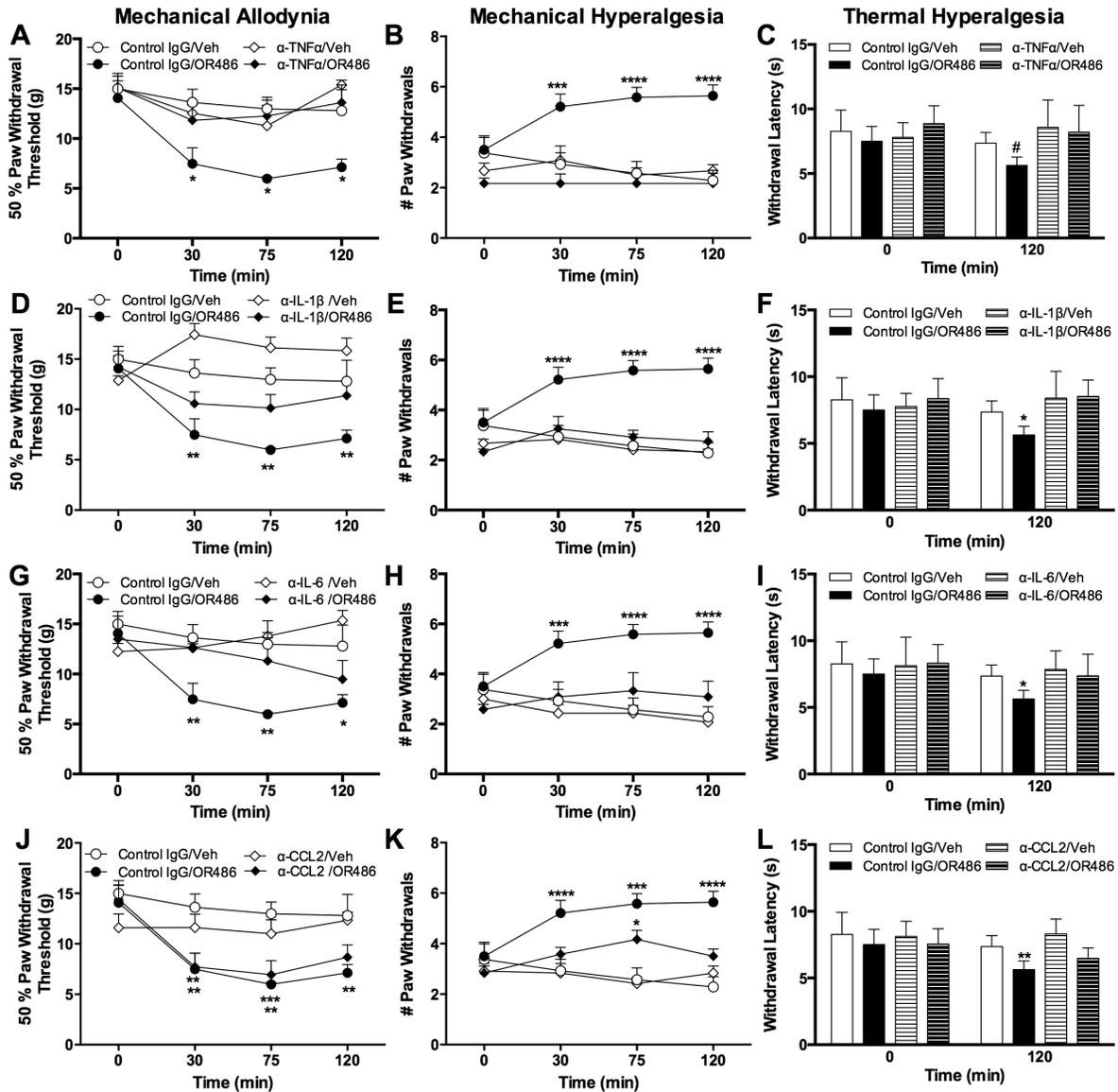


Figure 2.4. Neutralization of TNF α , IL-1 β , and IL-6, but not CCL2, blocks COMT-dependent pain. Administration of α -TNF α (75 μ g), α -IL-1 β (75 μ g), or α -IL-6 (75 μ g) prior to OR486 (30 mg/kg) normalized (A, D, G) mechanical allodynia, (B, E, H) mechanical hyperalgesia, and (C, F, I) thermal hyperalgesia. (J-L) Administration of α -CCL2 failed to block OR486-induced increases in mechanical and thermal pain. N=6-8 per group. Data are mean \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 different from Control IgG/Veh. # P <0.05 different from α -TNF α /Veh and α -TNF α /OR486.

3.3 Interplay between NO and cytokine protein expression in COMT-dependent pain

We then sought to determine if these pro-inflammatory molecules could influence the synthesis and release of one another downstream of β_2 - and β_3 AR stimulation. Blood plasma was collected from animals that received L-NAME or cytokine neutralizing antibodies prior to OR486 and peripheral levels of NO derivatives and cytokines were measured. In NO inhibition experiments, levels of TNF α , IL-1 β , IL-6, and CCL2 were elevated in animals receiving vehicle prior to OR486. Pre-administration of L-NAME blocked OR486-mediated increases in TNF α ($F_{3,39}=0.2989$, $P<0.83$; Fig. 5A), IL-1 β ($F_{3,27}=3.255$, $P<0.04$; Fig. 5B), IL-6 ($F_{3,18}=1.354$, $P<0.3$; Fig. 5C), and CCL2 ($F_{3,27}=2.761$, $P=0.06$; Fig. 5D).

In cytokine neutralization experiments, total nitrite (nitrite + nitrate) concentrations in blood plasma were elevated in animals receiving control IgG prior to OR486. Pre-administration of α -TNF α ($F_{3,21}=3.230$, $P<0.05$; Fig. 6A) or α -IL-6 ($F_{3,22}=3.772$, $P<0.03$; Fig. 6C) prior to OR486 blocked elevations in total nitrite. However, pre-administration of α -IL-1 β (Fig. 6B) or α -CCL2 (Fig. 6D) failed to block OR486-mediated increases in total nitrite levels. Thus, NO and cytokines drive one another's biosynthesis.

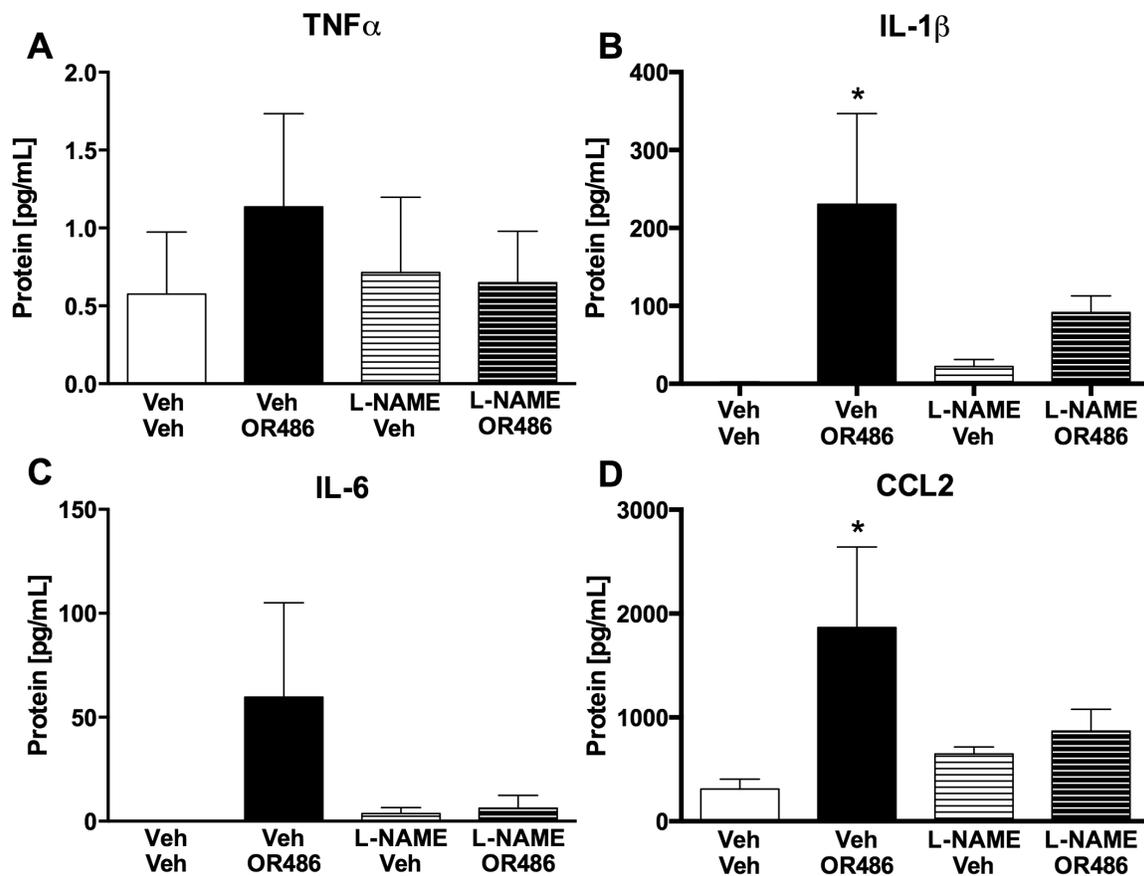


Figure 2.5. Inhibition of NO synthesis prevents COMT-dependent increases in cytokines.

Administration of the nitric oxide synthase inhibitor L-NAME (30 mg/kg) prior to OR486 (30 mg/kg) blocked increases in circulating levels of (A) TNF α , (B) IL-1 β , (C) IL-6, and (D) CCL2.

N=6-10 per group. * P <0.05 different from Veh/Veh.

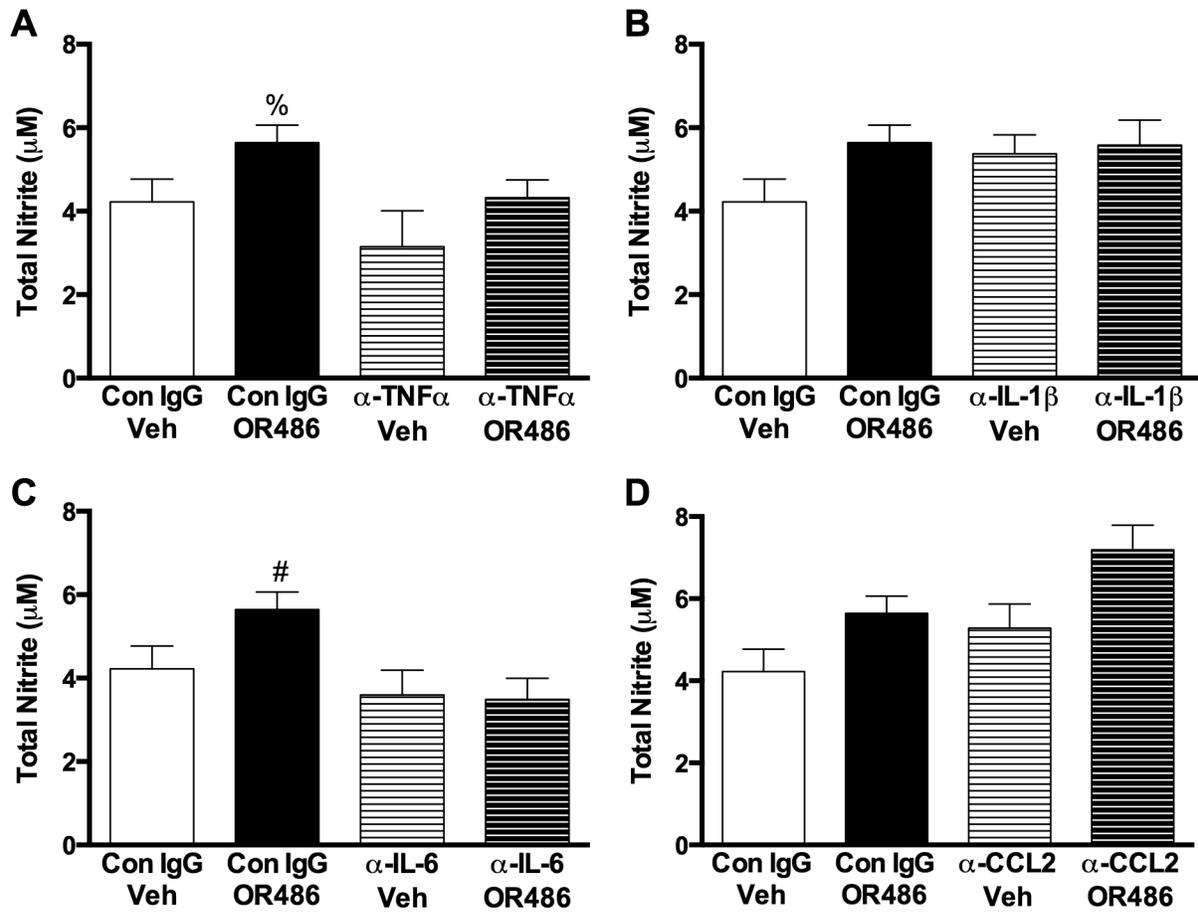


Figure 2.6. Neutralization of TNF α and IL-6 prevents COMT- dependent increases in NO.

OR486-induced increases in total nitrite (nitrite and nitrate) were blocked by pretreatment with **(A)** α -TNF α (75 μ g) or **(C)** α -IL-6 (75 μ g), but not **(B)** α -IL-1 β (75 μ g) or **(D)** α -CCL2 (75 μ g). N=6-8 per group. Data are mean \pm SEM. % P <0.05 different from α -TNF α /Veh, # P < 0.05 different from α -IL-6/Veh and α -IL-6/OR486.

4. Discussion

Our laboratory previously demonstrated that COMT inhibition produces remarkable increases in mechanical and thermal pain sensitivity through stimulation of both β_2 - and β_3 ARs (Nackley et al., 2007). However, the molecular mechanisms whereby these receptors drive COMT-dependent pain have remained unknown. Here, we identify NO, TNF α , IL-1 β , and IL-6 as molecules downstream of β_2 - and β_3 AR stimulation that are critical for the development of pain associated with decreased COMT activity. Furthermore, we demonstrate that NO and cytokines act in a positive feedback loop to induce one another's biosynthesis.

4.1 Role of Nitric Oxide in COMT-dependent Pain

NO is a paracrine signaling molecule produced by three different nitric oxide synthase isoforms: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). While previous studies have linked NO to inflammatory and neuropathic pain, here we provide the first demonstration that NO contributes to COMT-dependent pain. Specifically, we found that stimulation of β_2 - and β_3 ARs following COMT inhibition resulted in increased levels of NO derivatives and that inhibition of NO synthesis with L-NAME prevented the development of COMT-dependent mechanical allodynia, mechanical hyperalgesia, and thermal hyperalgesia. These findings are in line with results from clinical and animal studies showing NO is upregulated following injury and inflammation (Omote et al., 2001, Holguin et al., 2004, McMahon et al., 2005, Schmidtko et al., 2009, Chen et al., 2010, Cury et al., 2011, Kuboyama et al., 2011) and that genetic or pharmacologic blockade of NO can suppress pain in these models (Omote et al., 2001, Holguin et al., 2004, Marchand et al., 2005, Chen et al., 2010, Ramachandran et al., 2010, Kuboyama et al., 2011).

NO is able to produce pain through several mechanisms, including the canonical stimulation of cyclic guanylyl monophosphate (cGMP), which can enhance activity of Ca²⁺-activated K⁺

channels and, thus, the firing rate of nociceptors. NO can also stimulate cyclic adenosine monophosphate (cAMP)-mediated production of pro-pain prostaglandins (PGE₂) that sensitize primary afferents (Aley et al., 1998, Boehning and Snyder, 2003). Furthermore, NO can stimulate cAMP production through S-nitrosylation of adenylyl cyclase and the phosphorylation of cAMP response element binding (CREB) protein by cGMP. Activation of CREB leads to enhanced expression of cytokines such as IL-1 β and TNF α (Jongeneel, 1995, Watkins et al., 1999, Chen et al., 2010). While others have linked NO production with β_2 - and β_3 AR stimulation in the context of inflammation (Akimoto et al., 2002, Tan et al., 2007, Hodges et al., 2008, Figueroa et al., 2009), this is the first demonstration that NO synthesis is critical for COMT-dependent pain and cytokine production.

4.2 Role of Pro-Inflammatory Cytokines in COMT-dependent Pain

TNF α , IL-1 β , and IL-6 are innate immunity cytokines, considered to be the first-responders to injury or pro-inflammatory events. In an acute setting, these cytokines convey a protective advantage by promoting wound healing (Dinarello et al., 1990). However, sustained elevations of these cytokines can promote tissue damage and pain. Here, we found that COMT inhibition led to the release of TNF α , IL-1 β , IL-6, and CCL2 mediated by β_2 - and β_3 ARs. We also found that neutralization of the innate immunity cytokines TNF α , IL-1 β and IL-6, but not CCL2, prevented COMT-dependent mechanical and thermal sensitivity.

Stimulation of β_2 - and β_3 ARs located on cells in the periphery and central nervous system can enhance production of TNF α , IL-1 β , IL-6, and CCL2 (Nance and Sanders, 2007, Tan et al., 2007, Johnson et al., 2008, Tchivileva et al., 2009b, Wang et al., 2010, Wohleb et al., 2011, Johnson et al., 2013, Li et al., 2013), which can then enhance pain sensitivity. Elevations in these cytokines have been found in local synovial joint fluid from patients with TMD (Kubota et al., 1998) and in

blood from patients with fibromyalgia and migraine (Sarchielli et al., 2006a, Slade et al., 2011, Uzar et al., 2011). Neutralization of TNF α , IL-1 β , and IL-6 reduces the development of allodynia and hyperalgesia in models of neuropathic pain (Lindenlaub et al., 2000, Sommer et al., 2001, Obreja et al., 2005, Binshtok et al., 2008b), suggesting that these cytokines are critical for pain.

Cytokines downstream of β_2 - and β_3 AR stimulation likely drive COMT-dependent pain through direct and indirect mechanisms. Previous studies have demonstrated that TNF α , IL-1 β and IL-6 can bind to their respective receptors on nerve terminals to directly sensitize peripheral nociceptors (Obreja et al., 2002, Obreja et al., 2005, Binshtok et al., 2008b, Czeschik et al., 2008). TNF α can also drive sensitization of nociceptors through receptor-independent increases in the production of other pro-inflammatory cytokines. Cunha and colleagues found that α -TNF α blocked CFA-induced increases in pain and IL-1 β production (Cunha et al., 2005). They speculated that TNF α acts as the first cytokine in the cascade to stimulate the sequential release of IL-6, IL-1 β , and PGE $_2$.

In contrast to the innate immunity cytokines, administration of α -CCL2 did not prevent the development of COMT-dependent pain. This may be due to one of two possibilities: (1) that higher dosages of α -CCL2 may reduce COMT-dependent pain and NO release or (2) that CCL2 is critical for the maintenance versus the development of pain. Previous studies have shown that CCL2 recruitment of monocytes and neutrophils to the site of injury occurs at later time points after 2 hours (Pflucke et al., 2013). Furthermore, CCL2 is released from spinal dorsal horn astrocytes, which are glial cells involved in the maintenance of pain states (Gao and Ji, 2010b).

4.3 Interplay between NO and Cytokines in COMT-dependent pain

Mounting evidence suggests that a positive feedback loop exists between NO and cytokines, such that they can induce one another's biosynthesis. Here, we found that inhibition of NO

synthesis effectively blocked COMT-dependent increases in TNF α , IL-1 β , IL-6 and CCL2, while neutralization of TNF α and IL-6 blocked COMT-dependent increases in the production of NO derivatives. Disruption of NO, TNF α or IL-6 signaling reduces the pro-inflammatory feedback mechanism important for COMT-dependent pain. This synergistic relationship between NO and cytokines has been observed as a key characteristic of inflammation. NO has long been known to act as a putative molecule dictating macrophage trafficking (Boehning and Snyder, 2003) and cytokine production and release (Holguin et al., 2004, Chen et al., 2010, Kuboyama et al., 2011). Furthermore, NO can influence the transcription of cytokines such as TNF α (Jongeneel, 1995) and IL-1 β (Watkins et al., 1999). Cytokines can also influence NO synthesis, as TNF α , IL-1 β and IL-6 have been found to increase NOS transcription by directly binding to the promoter or by stimulating p38-MAPK (Lamas et al., 1991, Lowenstein et al., 1993, Sung et al., 2005). The collective work from our lab and others demonstrates that NO and cytokines influence one another's biosynthesis and suggest that it is the 'net effect' of these molecules that ultimately influences pain.

4.4 Greater Implications and Clinical Relevance

As observed here, decreased COMT activity enhances pain by increasing the production of NO and cytokines *via* β_2 - and β_3 ARs. Evidence suggests that peripherally delivered neutralizing antibodies do not cross the blood brain barrier (Wittrup and Verdine, 2012), though evidence for blood-brain trafficking is less clear for L-NAME. While we observed that the release of pro-inflammatory molecules contribute to acute COMT-dependent pain, it is possible that there are also mechanisms of pain transmission and transduction occurring in spinal and supraspinal sites. Taken together, we believe that acute COMT-dependent pain is driven largely in the periphery.

Genetic variants resulting in decreased COMT activity have been associated with chronic pain conditions such as fibromyalgia [24] and TMD (Diatchenko et al., 2005), which are linked to

increased levels of catecholamines (Evaskus and Laskin, 1972, Torpy et al., 2000) and production of pro-inflammatory molecules (Larson et al., 2000, Di Franco et al., 2010, Bote et al., 2012). Specifically, patients with fibromyalgia (Larson et al., 2000, Bote et al., 2012) and TMD (Shafer et al., 1994, Kubota et al., 1998, Slade et al., 2011, Fan et al., 2012) exhibit higher levels of NO derivatives (e.g. nitrite and nitrate) and cytokines such as $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , and CCL2 . Recent reports suggest that β -adrenergic mechanisms involved in COMT-dependent pain may overlap with those observed in complex regional pain syndrome (Li et al., 2013), which is also linked to stimulation of β ARs and increased production of pro-inflammatory cytokines. While this study identifies the molecules contributing to the development of COMT-dependent pain by pre-emptively blocking the action of β_2 - and β_3 ARs, NO synthases, and cytokines, future studies will identify processes required for the maintenance of COMT-dependent pain following its induction. Therefore, we may more closely mimic the treatment chronic pain conditions following their development. Based on these and future studies, β AR antagonist therapy used to mitigate catecholamine signaling and alleviate pain in patients with fibromyalgia and TMD (Wood et al., 2005, Light et al., 2009a, Tchivileva et al., 2010) may benefit other patient populations suffering from pain conditions of shared etiology.

5. Conclusions.

In conclusion, these findings elucidate the molecules downstream of β_2 - and β_3 ARs that drive acute COMT-dependent pain. Elevated levels of norepinephrine/epinephrine, resulting from decreased COMT activity, stimulate β_2 - and β_3 ARs to promote the release of NO and the innate immunity cytokines $\text{TNF}\alpha$, $\text{IL-1}\beta$, and IL-6 , which in turn produce heightened pain sensitivity. The chemokine CCL2 was elevated in COMT-deficient animals, but its blockade did not prevent the development of acute COMT-dependent pain. Additionally, we found that NO and innate immunity

cytokines function in a positive feedback loop to strengthen their own biosynthesis. This amplification mechanism may form the basis for the development of prolonged hypersensitive pain states. Finally, these data suggest that patients suffering from pain conditions associated with abnormalities in catecholamine signaling may benefit from therapeutics that selectively regulate the activity of β_2 - and β_3 ARs and downstream effectors.

Chapter 3: The Maintenance of COMT-dependent Pain

1. Introduction

Chronic pain disorders such as fibromyalgia and temporomandibular disorders (TMD) negatively impact the lives of millions of Americans and cost \$635 billion in treatment and lost wages (Gaskin and Richard, 2012). In line with an established literature linking sympathetic nervous system activity and chronic pain (McMahon, 1991, Baron et al., 1999, Torpy et al., 2000, Martinez-Lavin et al., 2002, Finan et al., 2011), a growing body of evidence demonstrates that catecholamines and pathways that regulate their bioavailability are altered in patients with fibromyalgia and TMD. These patients exhibit increased levels of the catecholamines epinephrine and norepinephrine (Baron et al., 1999, Mannisto and Kaakkola, 1999b, Torpy et al., 2000, Martinez-Lavin et al., 2002), and decreased levels of the enzyme catechol-O-methyltransferase (COMT), which metabolizes epinephrine and norepinephrine to their inactive derivatives (Mannisto and Kaakkola, 1999a). Based on the frequency of allele variation, it is estimated that up to two-thirds of chronic pain patients may carry genetic variations that alter COMT activity (Barbosa et al., 2012a, Desmeules et al., 2014). Furthermore, TMD patients display increased mechanical pain coupled with genetic variations that alter COMT expression or activity (Smith et al., 2014a). Consistent with these findings, humans using COMT inhibitors for the treatment of Parkinson's disease report heightened visceral and generalized pain (Gordin et al., 2004) and animal studies show that inhibition of COMT (Nackley et al., 2007, Kambur et al., 2010) produces increased mechanical pain, which we define as COMT-dependent pain.

Despite years of clinical and animal research that focus on the role of sympathetic activity and catecholamines in chronic pain conditions, there are few studies that assess specific molecules and mechanisms that act downstream from catecholamines and β ARs to maintain these chronic pain conditions. Previously, we found that the *onset* of COMT-dependent pain is blocked by antagonists for β_2 - and β_3 -adrenergic receptors (β_2 ARs and β_3 ARs) and their downstream effectors nitric oxide, tumor necrosis factor- α (TNF α), interleukin-1 β , and interleukin-6 (Nackley et al., 2007, Hartung et al., 2014). In this study, we seek to explore if β ARs downstream effectors are responsible for the maintenance of pain following COMT inhibition.

It is well established that epinephrine and norepinephrine and cytokine release are tightly interwoven. β_2 ARs are expressed on macrophages, B cells, and T cells (Szelényi and Vizi, 2007). In general, activation of β ARs on these cells leads to an immunosuppressive effect, decreasing the transcription of cytokines (Szelényi and Vizi, 2007, Padro and Sanders, 2014). However, in the central nervous system, local epinephrine and norepinephrine has been found to mediate the production of pro-inflammatory molecules such as IL-1 β (Johnson et al., 2008, Johnson et al., 2013). Therefore, catecholamine dysregulation can have a divergent effect in the periphery and in central nervous system tissues. Importantly, the release of pro-inflammatory molecules in the central nervous system has been associated with increased pain and central sensitization (Marchand et al., 2005).

Sustained increases in catecholamines and activation of β ARs can lead to activation of the MAPK p38, a known molecule that is activated by phosphorylation following a neuropathic or inflammatory insult. Stimulation of β ARs on microglia (Wang et al., Johnson et al., 2013), macrophages (Szelenyi et al., 2006), and astrocytes (Gharami and Das, 2004) can lead to the activation of p38. P38 has been found to be a key modulator of neuropathic and inflammatory pain

by activating glia, modulating the transmission of pain signals, and leading to the transcription of pro-pain or pro-inflammatory molecules through cAMP response element binding protein (CREB) and nuclear factor- κ B (NF- κ B) activation (Ji et al., 2009). In healthy tissues, glia are relatively quiescent, but in the presence of an insult, they can be activated, thereby locally releasing pro-inflammatory molecules, altering the local environment to encourage synaptic plasticity, and upregulating the transcription of pro-pain molecules (McMahon et al., 2005).

Similar to p38, extracellular regulated kinase 1/2 (ERK1/2) has been found to be activated in the dorsal root ganglion and lamina I of the dorsal spinal horn following a variety of painful stimuli, including capsaicin (Wei et al., 2006), light touch (Gao and Ji, 2010c), neuropathic pain (Obata and Noguchi, 2004, Song et al., 2005), inflammatory pain (Ji et al., 2002), NMDA (Cheng et al., 2008) or AMPA receptors (Ji et al., 2009). In addition, ERK1/2 can be phosphorylated *via* β ARs in microglia (Wang et al., 2010), macrophages (Tan et al., 2007), astrocytes (Gharami and Das, 2004), and A β fibers (Khasar et al., 1999b). Activation of ERK1/2 results in upregulation of CREB-mediated transcription (Song et al., 2005), plasticity, and glial production of pro-inflammatory molecules (Ji et al., 2009). Studies have found that microglia express activated ERK1/2 at early time points, while astrocytes express activated ERK1/2 at later time points (Zhuang et al., 2007). Thus, cell-specific ERK1/2 signaling may be an indicator of pain progression (McMahon et al., 2005, Ji et al., 2009). Furthermore, ERK1/2 leads to NF- κ B-dependent transcription of genes (Grace et al., 2014).

Interestingly, one particular molecule that can activate cells or MAP kinases is TNF α , a pro-inflammatory cytokine that regulates immune system activation and pro-inflammatory cytokine release (Cunha et al., 2005). Previously, we found that circulating levels of TNF α were elevated just hours following COMT inhibition (Hartung et al., 2014). TNF α can directly activate nociceptors

and increase their firing through its binding to its receptor, TNFR1. TNF α can also increase pain in the central nervous system through its ability to activate p38 in astrocytes, microglia (Ji et al., 2009) and neurons (Zhang et al., 2010, Gudes et al., 2014). TNF α can more directly act in the spinal cord and the dorsal root ganglia (DRG) to elicit pain. In the spinal cord, transplantation of TNF α -activated astrocytes results in mechanical allodynia (Gao et al., 2010). In DRG, TNF α can increase synaptic transmission as well as decrease the activation threshold of neurons. TNF α can also dictate pain states by increasing glial activation in the spinal cord and DRG *via* phosphorylation of p38 or ERK1/2 (Ji et al., 2009). For example, bath application of TNF α on peripheral nociceptors results in increased nociceptor excitability by altering sodium channel activity *via* the MAP kinase p38 (Gudes et al., 2014). Further, TNF α can increase the activation of p38 in microglia or satellite cells located in the spinal cord or DRG, and lead to local release of cytokines (Grace et al., 2014) that act on local neurons to enhance spontaneous neuronal firing, increase neuronal excitability and reduce inhibitory activity (Gao and Ji, 2010b). Conversely, intrathecal TNF α inhibition leads to decreased allodynia and reduced spinal p-p38 (Svensson et al., 2003, Svensson et al., 2005). In sum, TNF α may represent an important target for patients with fibromyalgia or TMD, particularly because it is effective for patients with other chronic pain conditions such as rheumatoid arthritis (Kumar et al., 2003).

The MAP kinases p38 and ERK1/2 play key roles in a variety of pain models. For example, in neuropathic pain models, p-p38 is upregulated in DRG (Jin et al., 2003) neurons and in spinal microglia (Tsuda et al., 2004, Zhuang et al., 2007). As a result, p-p38 translocates to the nucleus and subsequently triggers the transcription of pro-inflammatory cytokines including TNF α (Kumar et al., 2003). When p38 is inhibited in either neuropathic or inflammatory pain, this treatment reduces cytokine production and microglial marker expression.(McMahon et al., 2005) Another MAP kinase,

ERK1/2, is required for sensitization. In sum, downstream activation of either neuronal p38 or ERK1/2 can contribute to nociceptor sensitization (Xiachun et al., 2006, Gudes et al., 2014). Thus, both p38 and ERK1/2 activation, either by TNF α or independent of TNF α , could play important roles in altering pain behaviors and cellular (glial or neuronal) activation.

Taken together, we sought to understand if pain resulting from COMT inhibition would require β_2 - and β_3 -ARs and/or pro-pain molecules including cytokines as well as the MAPKs p38 and ERK1/2. We find that the mechanisms required for the onset and maintenance of pain are distinct: while β_2 - and β_3 ARs are required for the onset of pain resulting from catecholamine dysregulation, systemic blockade of β_2 - and β_3 ARs does not reverse COMT-dependent pain. Furthermore, COMT inhibition results in increased levels of TNF α , p38, and ERK1/2 in the central and peripheral nervous system and intrathecal inhibition of either p38 or ERK1/2 reverses COMT-dependent pain.

2. Methods

2.1 Animals

A total of 138 male Sprague Dawley rats initially weighing between 200-350 g were used.

2.2 General Experimental Conditions

For β_2 - and β_3 AR antagonism experiments, all animals underwent surgery to receive two osmotic mini-pumps (Alzet): one pump to subcutaneously deliver the COMT inhibitor OR486 or vehicle (5:3:2, DMSO: Ethanol: 0.9% Saline) and another pump to subcutaneously deliver a cocktail containing the β_2 AR-specific antagonist ICI118,551 (15 mg/kg/day) and the β_3 AR-specific

antagonist SR59230A (1.5 mg/kg/day) or vehicle (4:1, 0.9% Saline: DMSO). Dosages were chosen based on the efficacy of dosages outlined in Ciszek et al., 2016.

For p38 and ERK inhibitor experiments, all animals underwent surgery to receive two osmotic mini-pumps (Alzet): one pump to subcutaneously deliver the COMT inhibitor OR486 or vehicle beginning on day 0 and the other pump to intrathecally (i.t.) deliver the p38 inhibitor SB203580 (12 µg/day), the ERK1/2 inhibitor U0126 (12 µg/day) or vehicle (1:1, DMSO: 0.9% Saline) beginning after behavioral testing on day 14.

2.3 Surgical Procedures

For all surgeries, animals were anesthetized using isoflurane (5% induction, 1.5-5% maintenance). The incision sites were shaved, disinfected with ethanol and betadine, and sterile surgical technique was implemented for all surgical procedures. Wound clips were used to close incisions.

To deliver peripherally-administered drugs, a pump containing OR486 and either a pump with ICI118,551 and SR59230A beginning on day 0 a pump with PE-50 catheter to delay delivery of ICI118,551 and SR59230A beginning on day 7, a 2-2.5 cm incision was made between the shoulder blades and hemostats were used to make a small pocket to place the Alzet pumps.

To delay delivery of ICI118,551 and SR59230A until day 7, we used the Lynch method (Lynch et al., 1980), in which we placed coiled polyethylene tubing (PE-50) onto the stainless steel flow moderator, which delayed drug delivery according to the length and volume of the tubing.

To deliver intrathecally-administered drugs SB203580 or U0126, we used the same method as was used in Ciszek et. al, 2016. Here, polyurethane rat intrathecal short catheters (Durect Corporation, Cupertino, CA) were cut on the distal end to 12.5 cm to reach the approximate site of

the lumbar spinal cord. Six animals did not awaken after surgeries and these animals were replaced in subsequent groups.

2.4 Assessment of Mechanical Allodynia and Mechanical Hyperalgesia.

Paw withdrawal threshold was measured using the von Frey up-down method, as described in Nackley et al., 2007. Nine calibrated von Frey monofilaments (bending forces of 0.40, 0.68, 1.1, 2.1, 3.4, 5.7, 8.4, 13.2, and 25.0 g; Stoelting, Wood Dale, IL) with equal logarithmic spacing between filaments were applied to the plantar surface of the hind paw. A series of 6 applications of monofilaments with varying gram forces was applied for 3 seconds to the plantar surface of the hindpaw. Testing began with the middle filament in the series (3.4 g). If the response included the withdrawal of the hindpaw, an incrementally lower filament was applied. In the absence of a paw withdrawal, an incrementally higher filament was applied. These data were entered into Paw Flick module within the National Instruments LabVIEW 2.0 (Austin, TX) software. A logarithmic algorithm accounted for the order and number of withdrawal responses as well as the gram force of the final filament to calculate mechanical threshold, the gram force that would elicit paw withdrawal in 50% of trials ($10^{[Xf+kd]}/10,000$, where Xf = value [in log units] of the final von Frey hair used; k = tabular value of positive and negative responses, and d = mean difference [in log units] between stimuli). Mechanical allodynia was defined as a heightened response to a normally innocuous stimulus and was determined as a significant decrease in paw withdrawal threshold from baseline.

After determining paw withdrawal threshold, paw withdrawal frequency to a noxious von Frey monofilament was assessed. The highest gram force filament (25.0 g) was applied to the hind paw 10 times. Stimulus was applied for 1 second followed by a 1-second interval without a stimulus. The number of paw withdrawals was recorded for each hindpaw. Mechanical hyperalgesia was defined as an increase in the number of paw withdrawals to a noxious mechanical stimulus from

baseline.

We assessed baseline mechanical sensitivity on day 0. Following surgery, mechanical sensitivity was reassessed on days 1, 3, 7, 14, 21, 28 and 35. In all behavioral experiments, the experimenter was blinded to treatment.

2.5 Collection of CSF and blood plasma for cytokine analysis

After behavioral testing, animals were euthanized by injection of 1.0 - 1.5 mL Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI). For plasma collection, arterial blood was collected and placed in EDTA plasma tubes, then centrifuged for 10 minutes at 15,000 g. For cerebrospinal fluid collection, fluid was collected from the cisterna magna. After collection, plasma and CSF were flash frozen and stored at -80°C.

2.6 Collection of tissues for Western Blotting

Brain regions (including locus coeruleus, periaqueductal gray, nucleus accumbens, hypothalamus, thalamus, and somatosensory cortex), DRG, spinal were collected from animals on day 14 during COMT inhibitor administration and on days 21 and 35 after COMT inhibition. First, we assessed differences in cellular activation, including neurons, glial cells, and MAPKs using Western blotting to serve as a screen to inform IHC experiments. Time points, structures and protein markers with the most significant alterations in cellular activation and/or MAPK activation were assessed with IHC. Collected DRG and spinal cord were flash frozen and stored until further use. Tissue samples were homogenized in a Precellys tissue homogenizer with tissue protein extraction reagent (TPER, ThermoFisher, Carlsbad, CA) and Halt protease and phosphatase inhibitor (ThermoFisher, Carlsbad, CA).

2.7 Western Blotting

To accommodate for variation in tissue homogenates, protein concentrations were normalized following BCA measurement of protein content to evenly load protein into wells of SDS-PAGE gels. Gels were using standard SDS-PAGE methods and transferred onto PVDF membrane (Life Technologies, Carlsbad, CA). Membranes were then be blocked, probed with primary antibody, and subsequently probed with a corresponding secondary antibody. Following addition of ECL Prime (GE Biosciences, Marlborough, MA), blots were be developed using chemiluminescence on a GE ImageQuant and assessed for intensity of target signal relative to a loading control, β actin. To assess cellular activation, blots were be probed for expression of the cellular markers Iba1 (Wako, 1:1000), GFAP (Cell Signaling, 1:300), p-ERK1/2 (Cell Signaling, 1:1000) and p-p38 (Cell Signaling, 1:500) primary antibodies.

2.8 Collection of tissues for immunohistochemistry

Rats were deeply anesthetized with Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI) and when no longer responsive to paw- or tail-pinch, they were perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS, collected DRG and spinal cord were placed in 4% PFA postfixative overnight. Spinal cord and DRG were cryoprotected in 30% sucrose in 0.01M PBS. Tissues were then embedded in Optimal Cutting Temperature (OCT) cryomatrix and sectioned by cryostat. Free-floating spinal cord sections were sliced to 30 μ m-thick sections, placed in chilled 0.01M PBS containing 0.1% sodium azide, and stored until use. Dorsal root ganglia were sliced to 12 μ m-thick sections on Superfrost Plus (ThermoFisher, Carlsbad, CA) microscope slides. Slides were dried for at least 30 min before storage in -80°C freezer.

2.9 Measurement of cytokines in CSF and blood plasma

To determine whether COMT inhibition altered cytokine levels in circulating and central tissues both during and after COMT inhibition, plasma and CSF were collected at baseline, 14d, 21d and 35d for measurement of pro- and anti-inflammatory cytokines. Cytokines levels in plasma and CSF were measured by the UNC Cytokine Analysis Facility using the Pro Rat Cytokine 24-plex Array (Bio-Plex, Hercules, CA). To confirm results from multiplex, CCL2 levels in plasma were measured using the CCL2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN). All plasma and CSF samples were diluted at 2x.

2.10 Immunohistochemistry

A combination of antibodies was used to determine p-MAP kinase expression in microglia, astrocytes and neurons in spinal cord as well as satellite cells and neurons in dorsal root ganglia. For spinal cord staining, anti-p-p38 (1:500, Cell Signaling Cat. No. 9211) or anti-p-p42/44 (1:300, Cell Signaling Cat No. 9101, Danvers, MA) in combination with either anti-CD11b (1:500, AbD Serotec), anti-S100 (1:500, Abcam), or anti-NeuN (1:500, Millipore, Billerica, MA) were used. For DRG staining, the Molecular Probes Neurotrace 530/615 Red Fluorescent Nissl Stain (1:300, Molecular Probes, Eugene, OR) was used to stain neuron cell bodies. Secondary antibodies were either anti-rabbit or anti-mouse FITC or Alexa Fluor 594 (Jackson Immuno, West Grove, PA). Stained tissues were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were collected on an Olympus IX51 widefield fluorescent microscope. Quantification of immunofluorescence for spinal cord was conducted using the Fluorescent Intensity Function available on Olympus CellSens software. Quantification of immunopositive cells for dorsal root ganglia was performed manually.

2.11 Statistical Analysis

Group differences in behavior were analyzed by two-way ANOVA and corrected for repeated measures. Post hoc comparisons were made using the Bonferroni test. $P \leq 0.05$ were considered significant. For i.t. experiments, behavior was analyzed by performing a two-way ANOVA on days 0-14 and days 21-35. Differences in mechanical allodynia or hyperalgesia were analyzed by averaging data from days 21-35, performing a one-way ANOVA, and correcting for repeated measures. Post hoc comparisons were made with the Holm-Sidak test. For multiplex cytokine experiments, data was analyzed for each cytokine using a one-way anova and treatment differences over all time points were analyzed. For immunohistochemistry experiments, following image acquisition, protein expression was measured by relative fluorescence or by cell counting. Cell counters were blinded to treatment. Group differences in p-p38 protein expression were analyzed by one-way ANOVA and corrected for repeated measures. Post hoc comparisons were made using the Bonferroni test. $P \leq 0.05$ were considered significant. Group differences in pERK1/2 protein expression were analyzed by t-test.

3. Results

3.1 β_2 - and β_3 AR antagonism prevents the onset of COMT-dependent pain

To confirm previous experiments from our lab demonstrating that sustained COMT inhibition results in increased mechanical pain, we administered OR486 for 14d by implantation of a subcutaneous Alzet mini-pump. As sustained COMT inhibition resulted in increased mechanical sensitivity while the COMT inhibitor OR486 was on board and 7-21d after OR486 administration had ceased. As expected, we found that sustained OR486 resulted in mechanical allodynia ($F_{1,128}=199.7$, $P<0.0001$) and mechanical hyperalgesia ($F_{1,128}=415.7$, $P<0.0001$) beginning on day 1

and lasted while OR486 was on board. Notably, we also found that mechanical allodynia and mechanical hyperalgesia lasted for up to 21d following the cessation of OR486 administration.

We then sought to determine if β_2 - and β_3 ARs were required for the initiation of COMT-dependent pain. Previously, we found that co-administration of the β_2 AR antagonist ICI118,551 together with the β_3 AR antagonist SR59230A was capable of preventing the onset of COMT-dependent pain. Therefore, here we co-administered two specific pharmacological antagonists together with OR486. We found that administration of the β_2 - and β_3 AR antagonists together prevented the development of OR486-dependent mechanical allodynia ($F_{3,208}=64.19$, $P<0.0001$) and hyperalgesia ($F_{3,208}=194.2$, $P<0.0001$) beginning on day 1 and lasting through day 35 of the behavioral paradigm (Figure 1A,B).

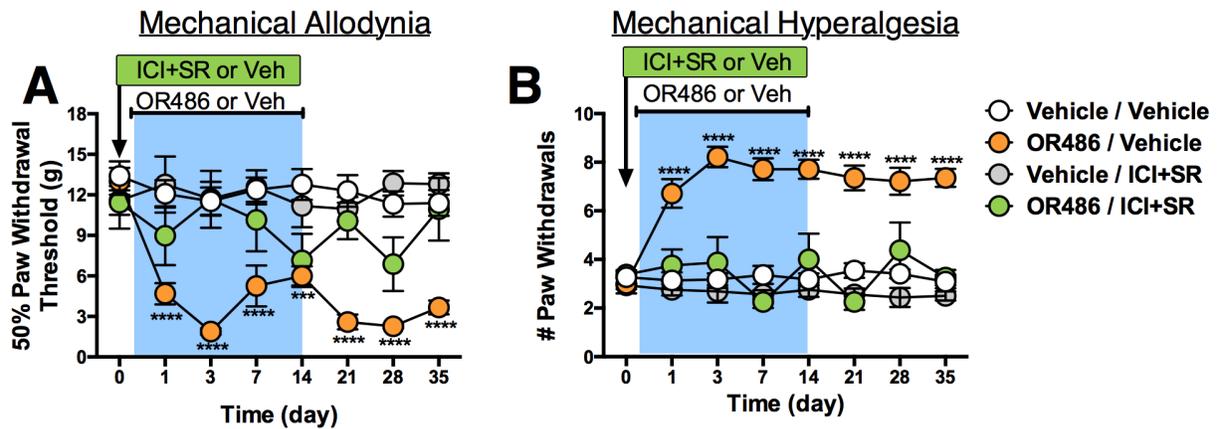


Figure 3.1. β_2 - and β_3 ARS are required for the onset of COMT-dependent pain.

Animals receiving OR486 for 14d exhibit (A) mechanical allodynia and (B) mechanical hyperalgesia, which are blocked by co-administration with ICI118,551 (β_2 AR antagonist) and SR59230A (β_3 AR antagonist). N=4-8 per group. Data are mean \pm SEM. ****p<0.0001.

3.2 β_2 - and β_3 AR antagonism does not reverse COMT-dependent pain

To next evaluate if β_2 - and β_3 ARs were important in the maintenance of COMT dependent pain, animals underwent surgery as discussed above. However, here, we wanted to determine if delayed delivery of drug would reverse COMT-dependent pain. Therefore, on surgery day, we implanted a mini-osmotic pump that delayed delivery of the β_2 - and β_3 AR antagonists until day 7. We found that blockade of β_2 - and β_3 ARs did not reverse COMT-dependent pain. Rather, these animals exhibited mechanical hyperalgesia and allodynia similar to that of the animals receiving only OR486 (Figure 2A,B). Therefore, β_2 - and β_3 ARs are required for the onset, but not the maintenance of COMT-dependent pain.

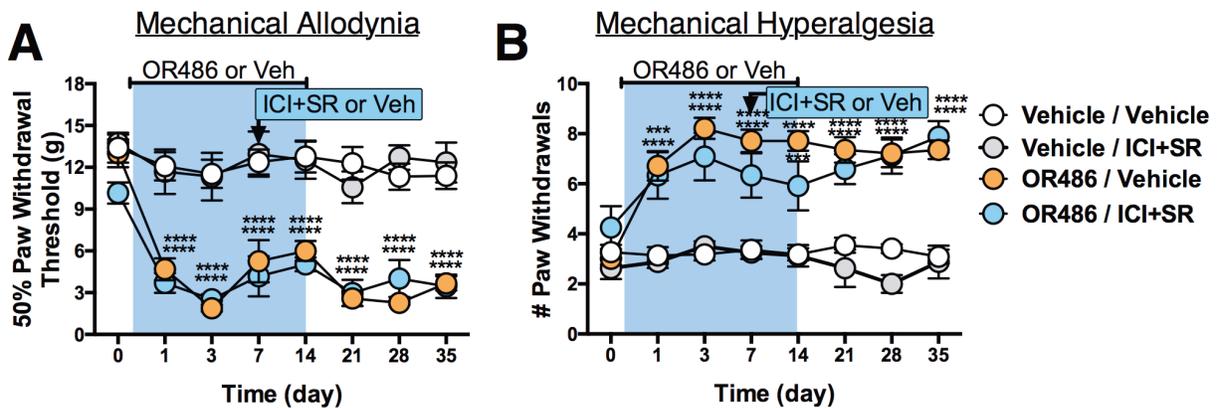


Figure 3.2. β_2 - and β_3 ARS are not required for the maintenance of COMT-dependent pain.

Animals receiving OR486 for 14d exhibit (A) mechanical allodynia and (B) mechanical hyperalgesia, which is not blocked by co-administration with ICI118,551 (β_2 AR antagonist) and SR59230A (β_3 AR antagonist). N=4-8 per group. Data are mean \pm SEM. ****p<0.0001.

3.3 Catecholamine dysregulation results in altered in circulating and central cytokine release

Because we found that β_2 - and β_3 ARs were not required for the onset of COMT-dependent pain, we sought to determine the changes taking place in the periphery and the CNS before, during,

and after sustained OR486 administration. Previously, we found that intraperitoneal administration of OR486 led to increases in pro-inflammatory molecules (TNF α , IL-1 β , IL-6, and CCL2) 4h after OR486 administration (Hartung et al., 2014). We collected blood plasma and CSF from animals at baseline, 14d, 21d and 35d and measured circulating and central cytokines using a cytokine multiplex that measures pro- and anti-inflammatory cytokines.

In blood plasma, we did detect significant group differences between animals receiving vehicle compared to those receiving OR486. However, while we did not observe individual differences in cytokines, there was a noticeable trend towards universal decreases in both pro-and anti-inflammatory cytokines in animals receiving OR486 compared to controls (Figure 3A-X).

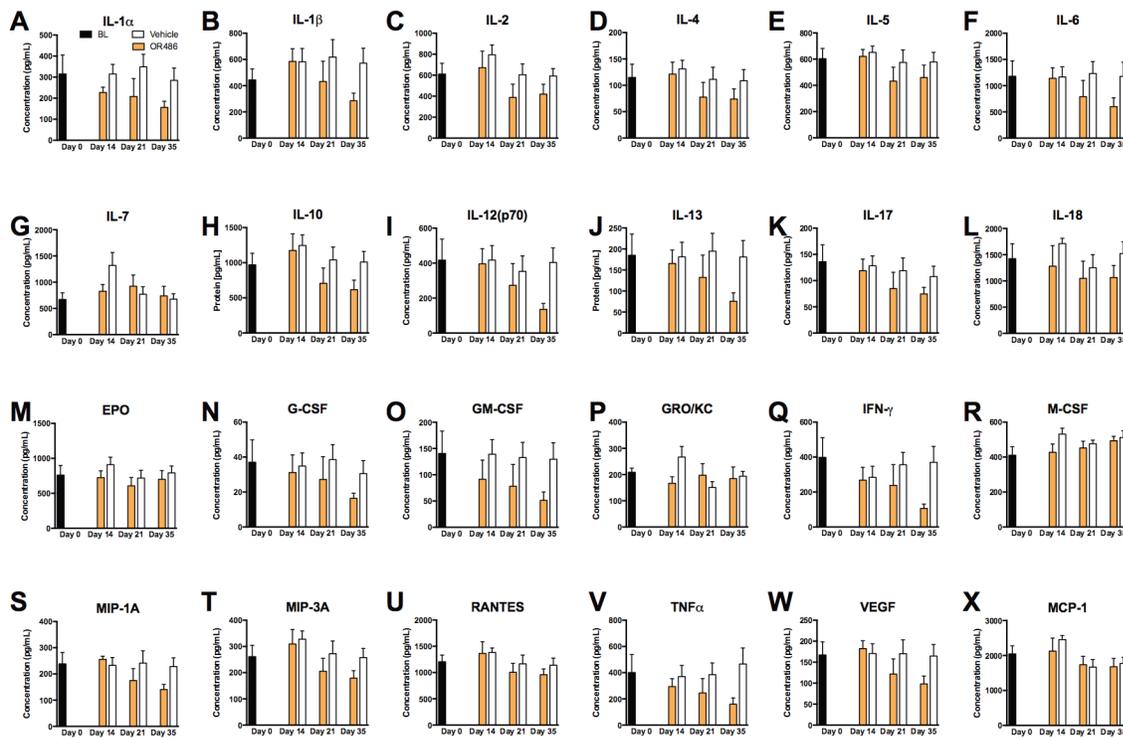


Figure 3.3. Sustained COMT inhibition results in decreases in pro- and anti-inflammatory cytokines. Animals receiving OR486 for 14d exhibited lower levels of cytokines in blood plasma. N=4-6 per group. Data are expressed as mean \pm SEM.

In CSF, we did find that there were increases in some pro-inflammatory markers. While there were trends towards a pro-inflammatory state in the CSF (Figure 4A-N), we observed group differences in CSF levels in TNF α that trended toward significance ($p < 0.076$, Figure 5).

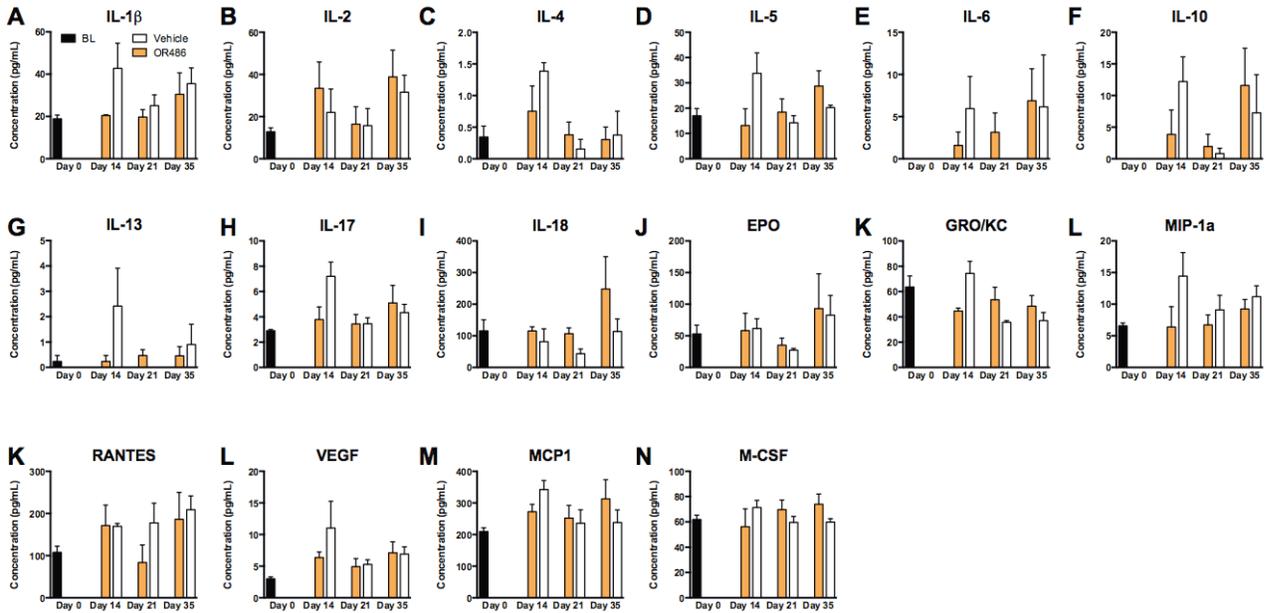


Figure 3.4. Sustained COMT inhibition results in no differences in pro- and anti-inflammatory cytokines in cerebrospinal fluid.

Animals receiving OR486 for 14d exhibited lower levels of cytokines in blood plasma. N=3 per group. Data are expressed as mean \pm SEM.

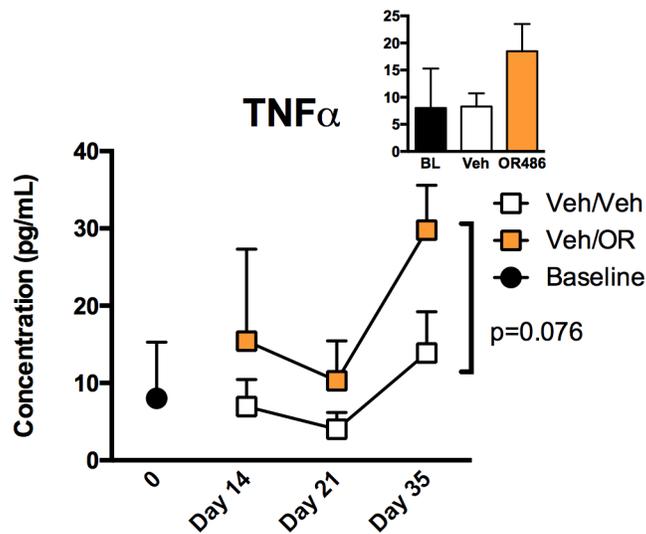


Figure 3.5. Sustained COMT inhibition results in elevated levels of central $\text{TNF}\alpha$.

Animals receiving OR486 for 14d exhibited elevated levels of $\text{TNF}\alpha$ in cerebrospinal fluid. Inset is average of baseline or $\text{TNF}\alpha$ concentration over days 14-35. Group differences between groups were $P=0.076$. $N=3$ per group. Data are expressed as mean \pm SEM.

3.4 Catecholamine dysregulation results in elevated levels of p-p38 and p-ERK1/2

Because of their implications with pain and βAR activation, we sought to understand if MAP kinases were altered in animals receiving OR486 in both dorsal root ganglia and the dorsal horn of the spinal cord. We found that COMT inhibition resulted in a significant increase in p-p38 in the dorsal spinal horn on days 14, 21 and 35 (Figure 6A-I). We observed significant differences in the relative fluorescence of p-p38 in lamina I/II on days 14 and 21 (Figure 6G,H). P-p38 co-localized with neurons and astrocytes in spinal dorsal horn (Figure 6J-L).

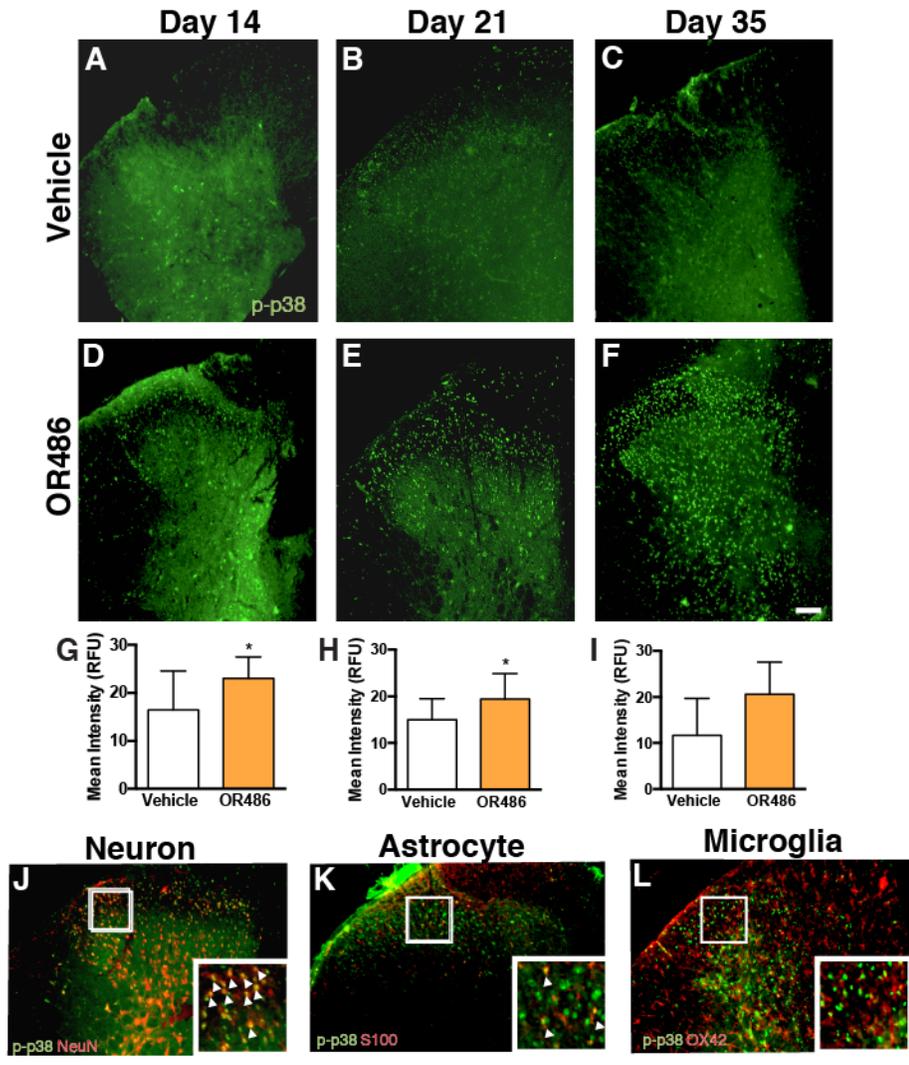


Figure 3.6. P-p38 levels are elevated in dorsal spinal horn.

Animal receiving OR486 exhibited elevated levels of activated, phospho-p38 in the spinal dorsal horn on (A-I) Days 14-35. N=3-4 per group. P-p38 is expressed in (J-L) neurons and astrocytes, but not microglia, in the dorsal horn. Scale bar = 100 μ m. Data are expressed as mean \pm SEM.

*p<0.05

While we found differences in p-p38 in the dorsal spinal horn, we did not observe differences in levels of ERK1/2 in the dorsal spinal horn. However, we did see increased that there

were observable increases in the number of cells expression p-ERK1/2 in nociceptors in the dorsal root ganglia at day 14 (Figure 7).

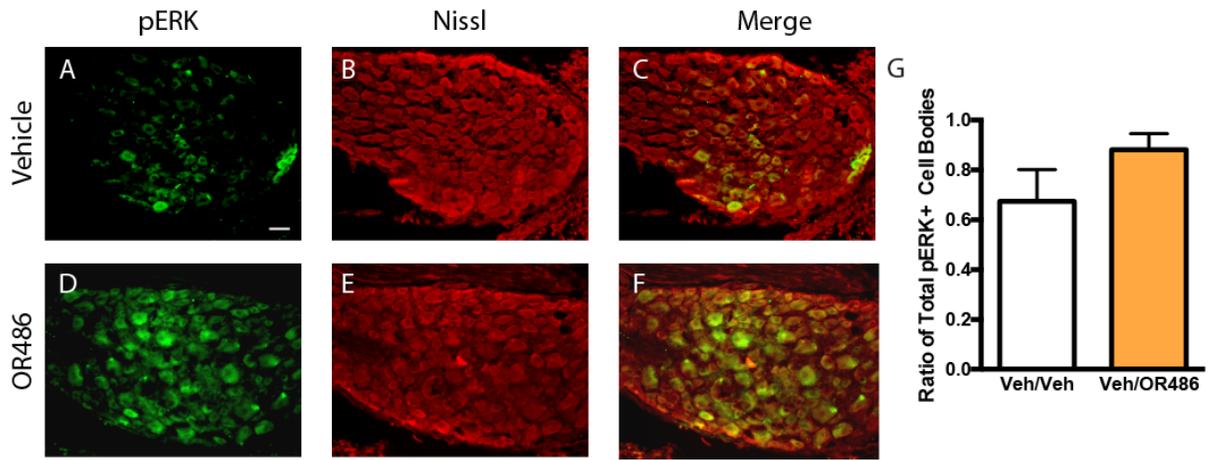


Figure 3.7. Phospho-ERK1/2 levels are elevated in dorsal root ganglion on Day 14.

Animal receiving OR486 exhibited (G) elevated, albeit not significant, levels of activated, p-ERK in dorsal root ganglia. Scale bar = 50 μ m. N=3-4 per group. Data are expressed as mean \pm SEM. P=0.067

We report changes in p-p38 and ERK1/2 in the spinal cord and DRG respectively, but we also assessed expression of the phosphorylated versions of these molecules in supraspinal sites. Using a combination of Western blotting and immunohistochemistry, we measured expression of phosphorylated p38 and ERK1/2 as well as the markers for astrocyte (GFAP), and microglial (Iba1) activation (Supplemental Figure 1). We did not detect significant differences in phosphorylated MAPKs nor in cellular activation. These data suggest that the most dynamic changes were occurring not in the brain, but rather in spinal cord and DRG.

3.5 Blockade of p38 or ERK1/2 reverses COMT-dependent pain

Because we observed that OR486 administration resulted in elevated levels of activated p38 and ERK1/2 in the spinal cord and DRG, we then sought to determine their role in the maintenance of COMT-dependent pain. To first evaluate the role of spinal p38 in COMT-dependent pain, we implanted an osmotic mini-pump to intrathecally deliver the p38 inhibitor SB203580 or vehicle on day 14.5 (after measuring mechanical pain). As expected, animals receiving OR486 developed mechanical allodynia ($F_{3,105}=13.43$, $P<0.0001$) and hyperalgesia ($F_{3,102}=13.35$, $P<0.0001$). Administration of SB203580 reversed both mechanical allodynia ($F_{3,63}=16.96$, $P<0.0001$) and hyperalgesia ($F_{3,63}=16.89$, $P<0.0001$). Therefore, p38 is required for the maintenance of COMT-dependent pain (Figure 8A,B).

We then sought to determine if ERK1/2 played a role in the maintenance of COMT-dependent pain. We measured pain behavior in animals receiving OR486 for 14d and performed surgery to deliver an intrathecal dose of either the ERK1/2 inhibitor U0126 or vehicle. Animals receiving OR486 developed mechanical allodynia ($F_{3,90}=9.707$, $P<0.0001$) and hyperalgesia ($F_{3,88}=12.97$, $P<0.0001$). Results show that ERK1/2 inhibition reversed mechanical allodynia ($F_{3,54}=15.05$, $P<0.0001$), but did not reverse mechanical hyperalgesia (Figure 8C,D).

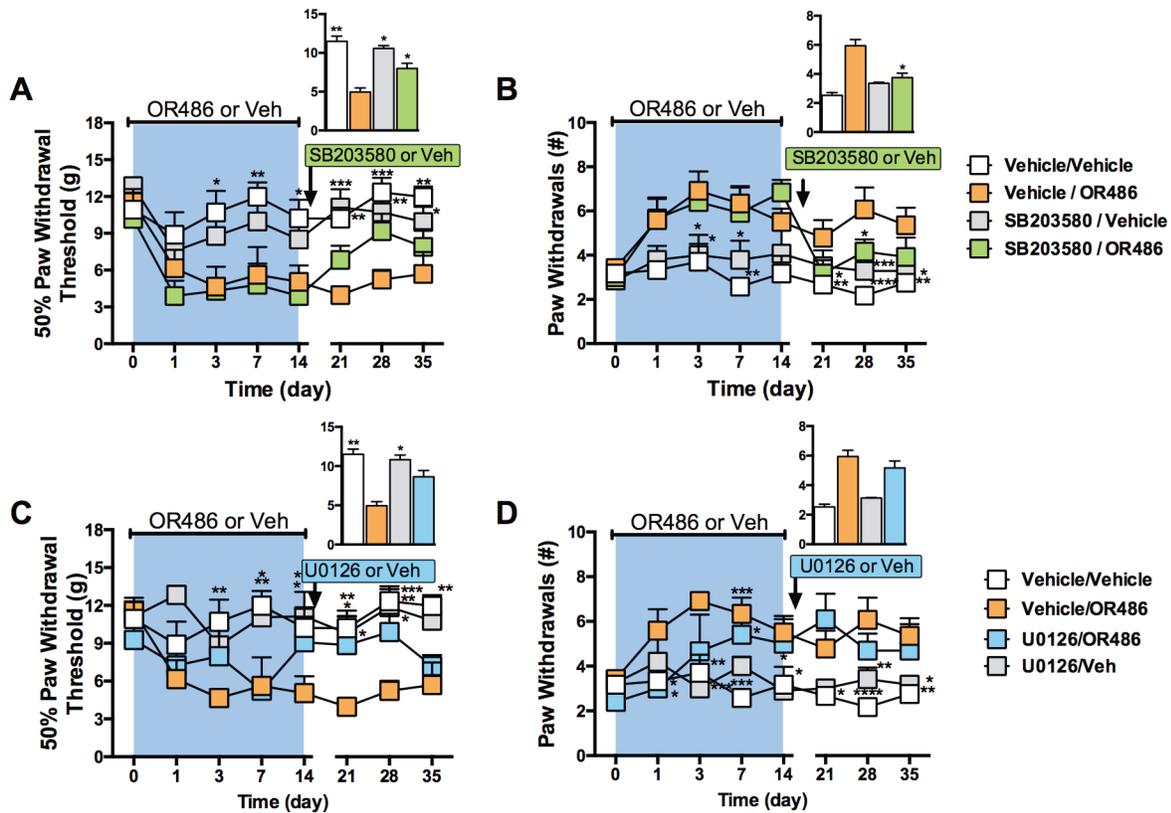


Figure 3.8. p38 and ERK1/2 contribute to the maintenance of COMT-dependent pain.

Animals receiving OR486 for 14d exhibit (A) mechanical allodynia and (B) mechanical hyperalgesia, which is reversed by intrathecal administration of SB203580 (p38 inhibitor).

Administration of intrathecal U0126 (ERK1/2 inhibitor) reversed (C) mechanical allodynia but

not (D) mechanical hyperalgesia. Insets are average of days 21-35. N=6-8 per group. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Vehicle/OR486.

4. Discussion

4.1 β_2 - and β_3 ARs are required for the onset, but not the maintenance, of COMT-dependent pain

Previously, our group found that blockade of β_2 - and β_3 ARs was capable of preventing the onset of COMT-dependent pain that lasted less than 4.5h (Hartung et al., 2014). Since then, we have found that the onset of sustained COMT inhibition results in pain through peripheral, but not spinal or supraspinal, β_2 - and β_3 ARs (Ciszek et al., 2016). These data are important because they suggest a peripheral site of action for the onset of COMT-dependent pain. Together with data demonstrating that β_2 - and β_3 AR antagonism does not reverse COMT-dependent pain, these findings suggest that peripheral β ARs are important for the onset of COMT-dependent pain, but they do not contribute to the maintenance of COMT-dependent pain.

One way in which β_2 - and/or β_3 ARs may drive the onset, but not the maintenance, of pain is through changes in the second messenger cascade associated with β AR activation. In a report by Khasar and colleagues, researchers found that epinephrine-induced hyperalgesia led to a priming event such that subsequent administration of epinephrine resulted in β ARs decoupling from the canonical G_s downstream pathway and switching to $G_{i/o}$. This switch caused an increase in the firing of IB4+ nociceptors as well as a shift from PKA and PKC second messenger pathways to ERK1/2 activation (Khasar et al., 1999b). Because we too observed modest increases in ERK1/2 in DRG, this may be the means by which β AR activation no longer plays a decreased role in pain. Another way in which β AR may play a lesser role is through action by β -arrestin, which can bind to β ARs to either desensitize or internalize the receptor (DeWire et al., 2007). β -arrestin binds to β ARs that are phosphorylated by the β AR kinase known as G-protein receptor kinase 2 (GRK2)(Krasel et al., 2005). Furthermore, GRK2 is involved in the switch intracellular pathways within sensory neurons

to prolong epinephrine-induced pain (Wang et al., 2011). Therefore, GRK2 and β -arrestin may play an important role in β AR desensitization and/or intracellular signaling during the transition from the onset to the maintenance of COMT-dependent pain.

4.2 p38 and ERK1/2 involvement in maintenance of COMT-dependent pain

Because of their close links to β AR signaling and chronic pain states, we next sought to understand what molecules may be important for the maintenance of pain. We chose to study MAPKs for two main reasons: (i) their activation is considered to be a correlate of elevated nociception in various pain models and (ii) they are activated by pro-inflammatory molecules (Ji et al., 2009). We found that p-p38 levels were elevated primarily in neurons, but also in astrocytes, following 14d of COMT-dependent pain. Furthermore, we saw that these changes were significantly different through day 21. Together with our data demonstrating that p38 inhibition reverses COMT-dependent pain, these data point to persistent activation of p38 as a key contributor to COMT-dependent pain.

While previous reports for neuropathic and inflammatory pain find that p-p38 co-expresses with microglia (Boyle et al., 2006, Ji and Suter, 2007), here we found that p-p38 was expressed in neurons and astrocytes. One way in which p-p38 may act in spinal neurons is through its ability to trigger inhibitory synaptic transmission of GABAergic interneurons in the spinal cord. Furthermore, researchers found that p38 is activated downstream of TNF α and its receptor tumor necrosis factor receptor 1 (TNFR1)(Zhang et al., 2010). While our data does not directly assess which type of neurons co-express p-p38, many of the p-p38-positive neurons were located in lamina I/II, where a number of glutamic acid decarboxylase 65 (GAD65) GABAergic interneurons are located (Lorenzo et al., 2014). Similar to results in neurons, p-p38 activation in astrocytes can be driven through

TNFR1 activation, leading to the transcription of inducible nitric oxide synthase (iNOS) *in vitro* (Da Silva et al., 1997). Given that we observed elevated levels of central TNF α throughout the paradigm, this may be one possible mechanism by which p-p38 is activated *via* TNFR1 residing on neurons and astrocytes located in the dorsal spinal horn.

We also observed that there were elevated levels of ERK1/2 in the dorsal root ganglion, but not in the spinal cord. One reason why ERK1/2 may not be activated in the spinal cord is because observable differences in ERK1/2 in the spinal cord are generally limited to high-frequency stimuli that activate A δ -fibers. Our data are in line with those of Khasar and colleagues, who found that epinephrine treated nociceptors led to elevated levels of ERK1/2 downstream of the β_2 AR-G $_{i/o}$ pathway. This pathway represents one of three possible pathways that could be activated: the other two being the PKC and PKA pathways (Khasar et al., 1999b). While we did not distinguish between ERK1 and ERK2 here, researchers have found that ERK2 may play a more central role in mechanical sensitization, particularly as a result of inflammatory pain (Alter et al., 2010, O'Brien et al., 2015). Active ERK1/2 may lead to a number of different effects, including phosphorylation of the NR1 subunit of NMDA receptors as well as induction of the transcription factor cAMP-response element binding protein, which is important for up regulation of genes involved in long-term potentiation as well as other pro-pain genes like Cox2, TrkB, and BDNF (Ji et al., 2009).

4.3 Catecholamine dysregulation and pain

Our data also show that there is a general trend towards decreased cytokine levels in blood plasma and increased cytokine levels in CSF. Clinically, it is well established that elevated levels of norepinephrine may play a role in the pathology of immune mediated diseases (Cohen et al., 2007). In particular, sustained norepinephrine elevations have been linked to immunosuppression in peripheral tissues and immune cells (Padro and Sanders, 2014). β ARs are expressed on a variety of

different immune cells including macrophages, lymphocytes and mast cells (Szelenyi et al., 2006). Research indicates that while β AR stimulation in already activated immune cells (e.g. by LPS or a pathogen) may decrease the production of cytokines, AR stimulation on quiescent immune cells may result in the production of cytokines (Szelenyi et al., 2006, Szelenyi and Vizi, 2007). Experimental data support this hypothesis: increased activity of the sympathetic nervous system results in decreased release of Th1-type cytokines (e.g. $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6). This downregulation in cytokine release may be a result of a macrophage specific G_s - to G_i switch, as Magocsi and colleagues found that $\text{TNF}\alpha$ levels were elevated in cultured macrophages following β AR stimulation *via* G_i -protein coupling to macrophage specific β ARs (Magocsi et al., 2007). These data may describe why we observed universal decreases in circulating cytokines throughout our paradigm.

At present, there is not a consensus on whether circulating pro- or anti-inflammatory cytokine levels are altered in patients with chronic pain disorders. In a study conducted in female patients with fibromyalgia, researchers found that macrophage release of pro-inflammatory cytokines $\text{IL-1}\beta$, $\text{TNF}\alpha$, IL-6 , IL-10 , IL-8 , IL-18 , MCP-1 was higher compared to healthy controls (Bote et al., 2012). Given that our study looked only at whole blood, not at the individual cell level, it is possible that we were not able to detect these more subtle, cell-specific changes in cytokine release, particularly in macrophages. However, it is also possible that NE release has an opposite effect on myeloid- and lymphoid-derived cells (Szelenyi and Vizi, 2007). In addition, due to the circadian nature of the release of both norepinephrine/epinephrine and cytokines (Padro and Sanders, 2014), it may be more difficult to correlate circulating levels of these molecules in a clinical study.

In a study that assessed levels of cytokines from patients with fibromyalgia, Kosek and colleagues found that levels of the cytokines IL-4 , IL-8 , IL-10 were upregulated in the CSF of fibromyalgia patients compared to healthy controls (Kadetoff et al., 2012, Kosek et al., 2015), similar

to results from our data. These data support more basic science studies that have found that β AR activation in the central nervous system can result in neuroinflammation (Johnson et al., 2008). Given that the sympathetic nervous system may be involved in these chronic pain conditions and that central β AR activation increases production of inflammatory cytokines, assessing cytokine levels in the CSF may be an informative measure to assess disease progression in pain patients.

4.4 Onset and maintenance: a changing site of action

Previously, our group found that COMT-dependent pain was primarily driven by mechanisms in the peripheral nervous system. Of note, blockade of either β_2 - and β_3 ARs or pro-inflammatory molecules in the periphery was able to prevent the onset of acute (<4.5h) COMT-dependent pain (Hartung et al., 2014). Furthermore, blockade of β_2 - and β_3 ARs at the onset of sustained COMT-dependent pain prevented the development of mechanical and thermal pain (Ciszek et al., 2016). Here, though, we find that COMT-dependent pain outlasts its initiating stimulus, COMT inhibition, by some three weeks and is not maintained by β_2 - and β_3 ARs. We also see that these animals do not have heightened inflammation in the periphery. Taken these lines of evidence together, it is clear that the maintenance of COMT-dependent pain is not driven by peripheral mechanisms. Rather, our findings suggest that COMT-dependent pain is maintained by a central mechanism. This evidence includes the upregulation of cytokines in CSF, together with increased p-p38 expression in spinal neurons and astrocytes, increased pERK1/2 in sensory neuron cell bodies in DRG, and the ability for spinally delivered p38 or ERK1/2 inhibitors to reverse mechanical hyperalgesia and/or allodynia.

Indeed, it is well known that recurring high-frequency stimulation of nociceptors results in a pain-facilitatory state in which central synapses are altered at the neuronal and biochemical level to

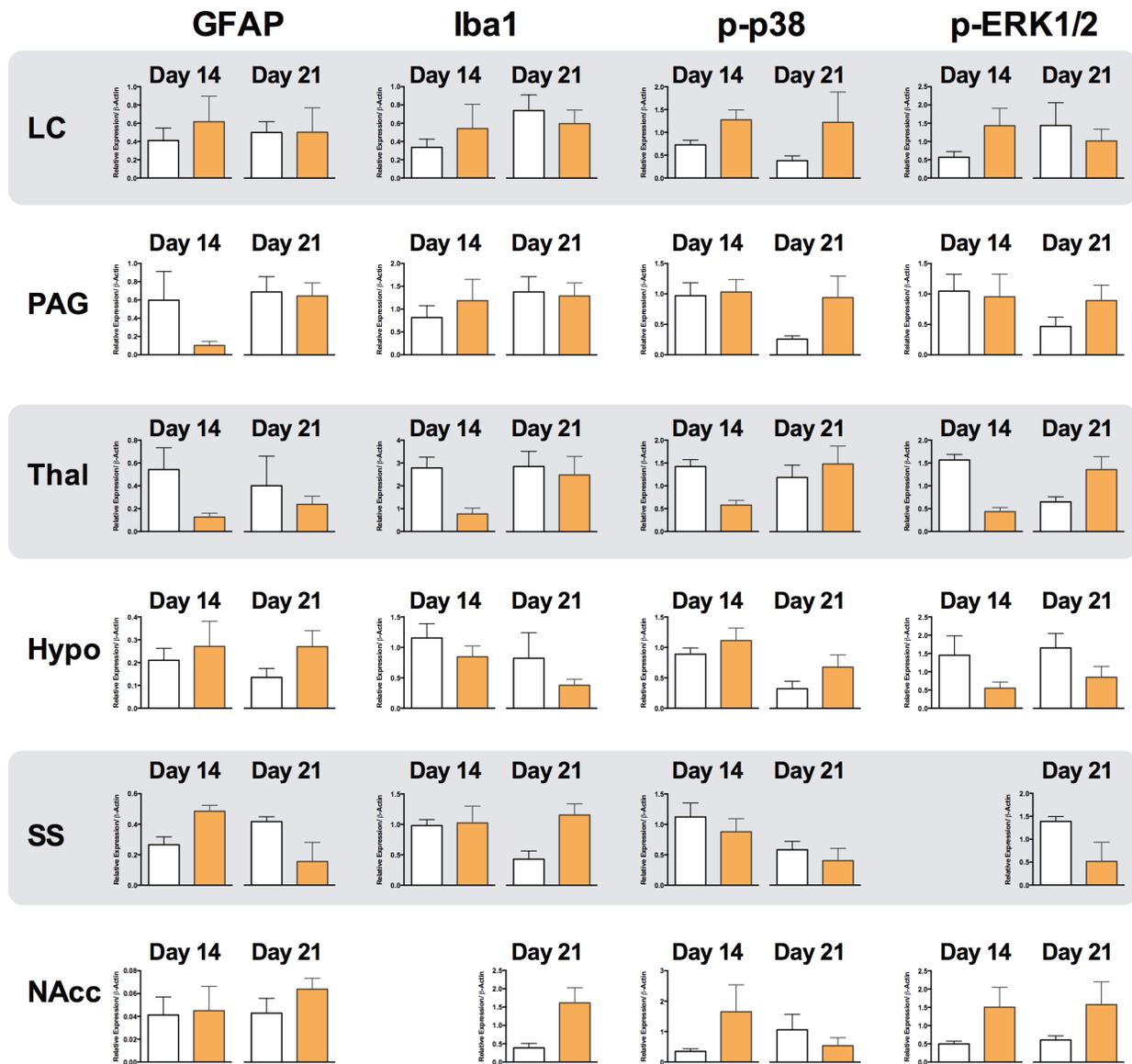
thereby heighten pain transmission (Grace et al., 2014). While we did not directly measure activation of nociceptor activity, we do see that in the absence of a known stimulus (e.g. COMT inhibitor), animals that received 14d of the COMT inhibitor continued to exhibit mechanical allodynia and hyperalgesia for weeks following the cessation of the drug. Furthermore, p-p38 has been attributed to be activated in the spinal dorsal horn of rodents with that have elevated activation of spinal nerve ligation (Jin et al., 2003), intraplantar CFA (Boyle et al., 2006), and peripheral nerve injury (Tsuda et al., 2004). Therefore, heightened nociceptive activity may have resulted in this pain facilitory response of p38 activation.

4.5 Clinical implications and greater observations

Here, we observed that sustained COMT inhibition results in increased pain through activation of peripherally located β_2 - and β_3 ARs during the onset phase and centrally-located MAPKs during the maintenance phase. While β AR antagonists have been used for some chronic pain conditions such as TMD and migraine, they are only effective for a subset of patients or require patients to take a beta-blocker as a preventative measure against future pain, and only reduce the frequency of migraine, but do not relieve their intensity or prevent them completely (Silberstein et al., 2012). However, there is limited evidence that β AR-antagonism acts as an effective treatment for patients with fibromyalgia, as most studies are conducted with small sizes (Light et al., 2009b) or without the use of a placebo in the study (Wood et al., 2005). Therefore, novel therapeutics that incorporate effectors downstream catecholamine dysregulation may be more successful at relieving pain experienced by chronic pain patients. However, it may also be important to assess some of the compensatory mechanisms that may occur as a result of decreased COMT activation, such as decreased release of catecholamines in the central nervous system. This, counterintuitively, may be why drugs such as duloxetine, a dual reuptake inhibitor that increases levels of serotonin and

norepinephrine in the central nervous system is efficacious for patients with fibromyalgia (Arnold et al., 2004): observed increases in pain following COMT inhibition may result in decreased release of norepinephrine in the central nervous system, thereby attenuating the effects of descending inhibition by α ARs. Therefore, future studies will look at the role of descending inputs on the facilitation of pain in our model.

At present, there are few studies that have assessed the efficacy of p38 inhibitors in populations of patients with idiopathic pain conditions such as TMD and fibromyalgia. However, there are a number of promising Phase II trials for patients with osteoarthritis or neuropathic pain (Hayes et al., 2014). As drug development for these p38 inhibitors continues, our data suggest that future work should incorporate patient populations with one or more idiopathic pain condition. Given that there is a direct link between $\text{TNF}\alpha$ and p38 activation in basic science data, use of $\text{TNF}\alpha$ inhibitors may also be an alternative mechanism to treating patients with fibromyalgia. As such, use of a spinally-delivered inhibitor of $\text{TNF}\alpha$ activity, etanercept, has been found effective in patients with fibromyalgia (Tobinick, 2010). Taken together, these data suggest that while β AR antagonists may be more effective for patients that have recently developed idiopathic pain disorders, while drugs that target specific downstream effectors such as p38 or $\text{TNF}\alpha$ in the CNS may be more effective for those patients with more advanced stages (e.g. were diagnosed earlier) of idiopathic pain. Future studies will assess the role of β ARs in the ability to induce changes in p38 and $\text{TNF}\alpha$ activation.



Supplemental Figure 3.1 COMT Inhibition does not result in significant differences in cell activation in discrete brain regions.

No differences were detected in locus coeruleus (LC), periaqueductal gray (PAG), thalamus (Thal), hypothalamus (Hypo), somatosensory cortex (SS), or nucleus accumbens (NAcc) for astrocyte activation (GFAP), microglial activation (Iba1), p-p38, or pERK1/2. Protein expression relative to β-actin. Data expressed as mean \pm SEM.

Chapter 4: Nuclear Factor- κ B Regulates Pain and COMT Expression

1. Introduction

The signaling complex nuclear factor- κ B (NF- κ B) is a key regulator of molecules and pathways important for inflammation and pain. NF- κ B can exist as homo- or heterodimers composed of the Rel family proteins including p65, p50, p52, RelB, and c-Rel, with the p65/p50 heterodimer being the canonical form.(Kaltschmidt and Kaltschmidt, 2009) In its inactive state, NF- κ B is bound in the cytoplasm bound to inhibitors of κ B (I κ B α). Upon activation by pro-inflammatory cytokines (e.g., interleukin-1 β ; IL-1 β and tumor necrosis factor α ; TNF α), growth factors (e.g., epidermal growth factor; EGF) (Kaltschmidt et al., 2005), or adjuvants that stimulate toll-like receptors 2 and 4 (e.g., complete Freund's adjuvant; CFA)(Lee et al., 2004), the I κ B kinase (IKK) phosphorylates I κ B α , tagging it for degradation. As a result, I κ B α releases the p65/p50 complex, which subsequently translocates to the nucleus and initiates transcription of κ B-associated inflammatory genes such as TNF α , IL-1 β , and cyclooxygenase-2 (COX-2) that directly or indirectly influence pain.(Scholz and Woolf, 2002, Kaltschmidt and Kaltschmidt, 2009).

Activation of NF- κ B in humans is implicated in a variety of pain conditions such as rheumatoid arthritis, migraine, and nerve injury (Niederberger and Geisslinger, 2008). In patients with rheumatoid arthritis, NF- κ B-linked proteins are upregulated in synovial tissues (Marok et al., 1996). In addition, patients that experience chronic migraines express increased I κ B α in blood monocytes concurrent with increased migraine intensity (Sarchielli et al., 2006b). Accordingly, NF- κ B is upregulated in the dorsal root ganglia and spinal cord of animals experiencing inflammatory

and neuropathic pain (Ma and Bisby, 1998, Ledeboer et al., 2005, Wu et al., 2006, Lee et al., 2011). Inflammation-evoked pain is attenuated by knockdown of p50 (Niederberger et al., 2007), gene silencing of p65 (Luo et al., 2014), or by administration of a proteasome inhibitor that prevents I κ B α dissociation from p50 and p65 (Ahmed et al., 2014). Similarly, pain following nerve injury is reversed by administration of an NF- κ B decoy that prevents activated NF- κ B from binding to a consensus DNA fragment and initiating transcription (Sakaue et al., 2001, Inoue et al., 2006).

While NF- κ B is ubiquitously expressed in a variety of cell types,(Lawrence, 2009) its contribution to pain is driven in large part by its signaling in astrocytes. Astrocytes are glial cells located in the central nervous system that participate in the transmission of neuronal signaling and provide neuroprotection during synaptic remodeling or neurodegeneration (Kaltschmidt et al., 2005). In animal studies, astrocytic NF- κ B is upregulated following viral coat protein-induced inflammation (e.g. gp120) (Ledeboer et al., 2005), CFA-induced inflammation (Ledeboer et al., 2005, Luo et al., 2014), and nerve injury (Lee et al., 2011). Likewise, loss of astrocytic NF- κ B signaling attenuates pain following formalin administration (Fu et al., 2008) or spinal nerve injury (Ma and Bisby, 1998). While these studies point to the contribution of astrocytic NF- κ B activity in pain, more research is required to identify downstream pain-relevant molecules.

Recent work by our group suggests that activation of NF- κ B in astrocytes may produce pain by regulating the expression of catechol-o-methyltransferase (COMT), an enzyme that inactivates catecholamines and modulates pain (Tchivileva et al., 2009a). Specifically, we found *in vitro* evidence demonstrating that TNF α -induced activation of NF- κ B in a human astrocyte (H4) cell line led to binding of p65 to the P2 promoter region of the *Comt* gene, thereby reducing COMT mRNA and protein expression. Reduced COMT expression and activity is associated with heightened pain sensitivity in patients with chronic pain conditions (Diatchenko et al., 2005, Smith et al., 2014b).

Furthermore, pharmacological inhibition of COMT increases pain in animals (Nackley et al., 2007, Hartung et al., 2014). Therefore, COMT may represent a novel molecular target for NF- κ B that dictates pain behaviors.

Building on *in vitro* work demonstrating that NF- κ B downregulates COMT expression together with *in vivo* work demonstrating the role of COMT in pain, the purpose of the present study was to examine the relationship between systemic and astrocyte-specific NF- κ B activity, pain, and COMT expression in an animal model of inflammation. Specifically, we evaluated the effects of NF- κ B inhibition in rats or overexpression in mice on pain behavior and COMT expression following local administration of CFA. We hypothesized that induction of NF- κ B by the pro-inflammatory stimulant CFA would result in enhanced pain and decreased COMT protein expression.

2. Materials and Methods

2.1 Animals

Seventy-eight adult male Sprague-Dawley rats (250-320g; Charles River Laboratories, Raleigh, NC) were used in behavior and molecular experiments. Eighty-eight male C57Bl6 expressing constitutively active form of I κ B kinase (IKK) in GFAP+ cells (IKK constitutive activity, hence “IKKca”) and littermate control (Co) mice (25-35 g; a gift from the lab of Dr. Ken McCarthy) were used in behavior and molecular experiments. All procedures were approved by the University of North Carolina Animal Care and Use Committee and adhered to the guidelines of the Committee for Research and Ethical Issues of the IASP (Zimmermann).

2.2 General Experimental Methods

To evaluate the role of NF- κ B in regulating inflammatory pain and COMT expression in rats, separate groups received an intraperitoneal (i.p.) injection of the NF- κ B inhibitor MG132 (10 mg/kg) or vehicle (10% DMSO in 0.9% saline) one hour prior to a unilateral intraplantar (i.pl.) injection of CFA (200 μ l) or saline (200 μ L). In mice, separate groups of IKKca or Co mice received a unilateral i.pl. injection of CFA (20 μ L) or incomplete Freund's adjuvant (IFA; 20 μ L). IFA is identical in its chemical composition to CFA, but does not contain *Mycobacterium tuberculosis*, which is responsible for the induction of inflammation. Behavioral responses to mechanical and thermal stimuli were reassessed at acute (1h, 6h, 1d), subchronic (3d, 5d, 7d), and chronic (9d, 11d, and 13d) phases of inflammation, as outlined by Raghavendra et al., 2004 (Raghavendra et al., 2004). In all studies, the experimenter was blinded to the experimental conditions.

2.3 Assessment of Mechanical and Thermal Pain Sensitivity

Rats and mice were handled and habituated to the testing environment for 4d prior to establishing baseline responsiveness to mechanical and thermal stimuli. First, animals were placed in plexiglass cages positioned over an elevated mesh stainless steel platform and habituated to the environment for 20 min. Mechanical allodynia and hyperalgesia were assessed using repetitive presentation of either an innocuous or noxious stimulus, as used in Ringkamp et al., 1999 and Fecho et al., 2005. Mechanical allodynia was assessed by placing a normally innocuous von Frey filament (3.632g for rat; 0.166g for mouse) onto the plantar surface of the hindpaw ten times for 1s with an inter-stimulus interval of 1s. Mechanical hyperalgesia was assessed by placing a normally noxious von Frey filament (15g for rat; 1.494g for mouse) onto the plantar surface of the hindpaw ten times for 1s with an inter-stimulus interval of 1s. Mechanical allodynia or hyperalgesia was defined as an

increase in the frequency ($[\text{number of paw withdrawals}/10] \times 100\%$) of paw withdrawals evoked by stimulation with the von Frey monofilaments compared to baseline.

Thermal hyperalgesia was assessed using the Hargreaves method as described previously.(Nackley et al., 2007) Briefly, animals were placed on an elevated heated glass platform maintained at 30°C and habituated to the environment for 20 min. A radiant beam of light was applied to the plantar surface of the hindpaw and the latency to withdrawal for a maximum of 20s was recorded. Withdrawal latency was measured in duplicate for each paw, with an inter-stimulus interval of 5 min. If the difference between the duplicate measures was >4s, a third measurement was taken. The two most consistent measurements were included in the analysis. Thermal hyperalgesia was defined as a decrease in paw withdrawal latency compared to baseline.

2.4 Assessment of COMT Protein Expression in Nervous System Tissues

COMT expression was measured in tissues collected from separate groups of rats sacrificed at 6h, 1d, or 5d following experimental treatment and from mice sacrificed at 1h, 6h, 1d, 3d, 5d, or 11d following experimental treatment. The specific tissues collected include the dorsal root ganglia (DRG), spinal cord, and brain regions (hindbrain [locus coeruleus and periaqueductal gray], midbrain [thalamus and hypothalamus] or forebrain [primary somatosensory cortex and nucleus accumbens]).

Total protein from each discrete tissue region was diluted in tissue protein extraction reagent (TPER; Thermo Scientific; Rockford, IL) with protease inhibitor (Pierce; Rockford, IL) and homogenized using the Precellys Homogenizer system (MD, USA). Soluble protein concentrations were assessed and normalized using a BCA Protein Assay Kit (Pierce; Rockford, IL), run at 10-50 µg on precast 12% Tris-Glycine gels (Bio-Rad; Hercules, CA), and transferred onto PVDF membranes using the iBlot Dry Blotting system (Invitrogen; Waltham, MA). Following transfer, blots were

blocked using 5% w/v bovine serum albumin diluted in TBST for 1h and incubated in either anti-COMT primary antibody (BD Biosciences; San Diego, CA) diluted at 1:7,500 in blocking buffer solution or anti- β -actin primary antibody (Cell Signaling; Danvers, MA) diluted at 1:3,000 at 4°C overnight. Chemiluminescence was detected on a ImageQuant TL system and analyzed using ImageQuant TL 3000 software (GE Healthcare; Piscataway, NJ). COMT is expressed as one of two isoforms: a membrane-bound isoform (~30 kDa) and soluble isoform (~25 kDa). Densitometry volume was analyzed as the sum of both COMT isoforms normalized to β -actin densitometry values.

2.5 Statistical Analysis

Behavioral data were analyzed by one-way analysis of variance (ANOVA) for repeated measures. Post hoc comparisons were performed using the Bonferroni's test. Normalized protein data were analyzed by unpaired t-test with post-hoc tests. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 NF- κ B inhibition attenuates inflammation-evoked increases in pain sensitivity in rats

To evaluate the role of NF- κ B in regulating pain sensitivity in the CFA model of inflammation, separate groups of rats received i.p. administration of the NF- κ B inhibitor MG132 or vehicle 1h prior to i.pl. administration of CFA or saline. Behavioral responses to mechanical and thermal stimuli were then assessed during acute, subchronic, and chronic phases of inflammation.

Prior to the induction of inflammation, responsiveness to mechanical and thermal stimuli did not differ between groups. Following administration of i.pl. CFA, rats exhibited mechanical

allodynia, mechanical hyperalgesia, and thermal hyperalgesia in the ipsilateral paws. CFA-induced increases in pain sensitivity were observed 1h following administration and lasted throughout the 13-day testing period. Administration of MG132 prior to CFA attenuated inflammation-evoked mechanical allodynia and thermal hyperalgesia during acute, subchronic, and chronic phases ($F_{9,112} = 6.06$, $P < 0.0001$; Figure 1A and $F_{9,112} = 6.027$, $P < 0.0001$; Figure 1C, respectively). Pre-emptive administration of MG132 also attenuated inflammation-evoked mechanical hyperalgesia during subchronic and chronic phases ($F_{9,112} = 13.19$, $P < 0.0001$; Figure 1B). No differences in behavioral responsiveness to mechanical and thermal stimuli were observed following MG132 administration in the absence of CFA or in the contralateral non-injected paws.

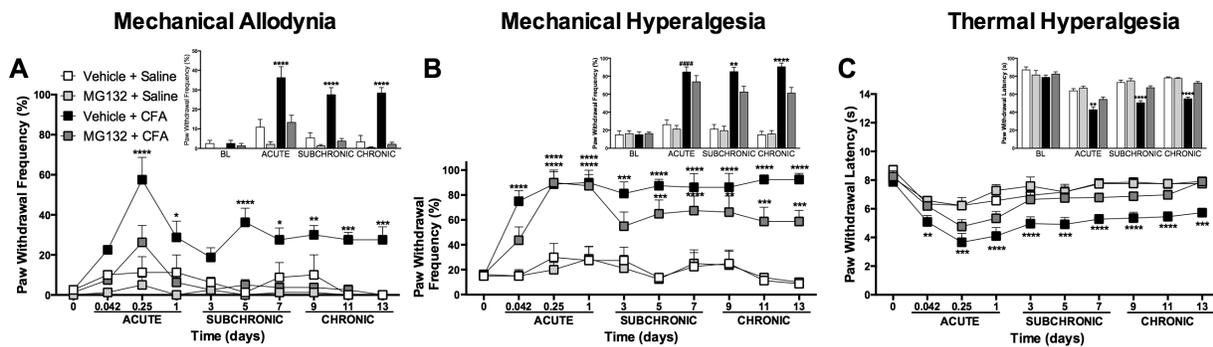


Figure 4.1. Blockade of NF- κ B reduces CFA-induced pain in rats.

Intraplantar administration of CFA (200 μ L) increases (A) mechanical allodynia, (B) mechanical hyperalgesia, and (C) thermal hyperalgesia. Pretreatment with MG132 (20 mg/kg dosage) attenuated mechanical allodynia and hyperalgesia compared to Vehicle/CFA. N=8 per group. Data are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ different from Vehicle + Saline for larger graphs and different from MG132+CFA for inset graphs.

3.2 Constitutive activity of astrocytic NF- κ B prolongs CFA-induced pain in mice

We next sought to determine if constitutive activity of the astrocytic NF- κ B pathway would alter inflammatory pain behaviors. Separate groups of IKKca and Co mice received i.pl. CFA or IFA. Behavioral responses to mechanical and thermal stimuli were then assessed during acute, subchronic and chronic phases of inflammation.

Compared to their Co littermates, IKKca mice displayed prolonged increases in mechanical allodynia ($F_{27,457} = 2.130$, $P < 0.001$; Figure 2A) and mechanical hyperalgesia ($F_{27,456} = 3.247$, $P < 0.0001$; Figure 2B) in the affected ipsilateral paw beginning on day 3, which lasted throughout the 13-day testing period (Figure 2A-C). Furthermore, while we observed no changes in mechanical or thermal pain in the unaffected contralateral paw of Co mice, we did observe increased mechanical allodynia and hyperalgesia in the contralateral paw of IKKca mice beginning on day 3, during the subchronic phase of inflammatory pain, and lasting until the end of the behavioral paradigm. We did not observe significant differences in thermal hyperalgesia in IKKca and Co mice. As expected, no differences in pain behaviors were observed between IKKca and Co mice following IFA administration for mechanical allodynia ($F_{9,162} = 0.270$, $P = 0.982$), mechanical hyperalgesia ($F_{9,162} = 0.926$, $P = 0.504$), or thermal hyperalgesia ($F_{9,178} = 0.496$, $P = 0.876$) (Supplementary Figure 1A-C).

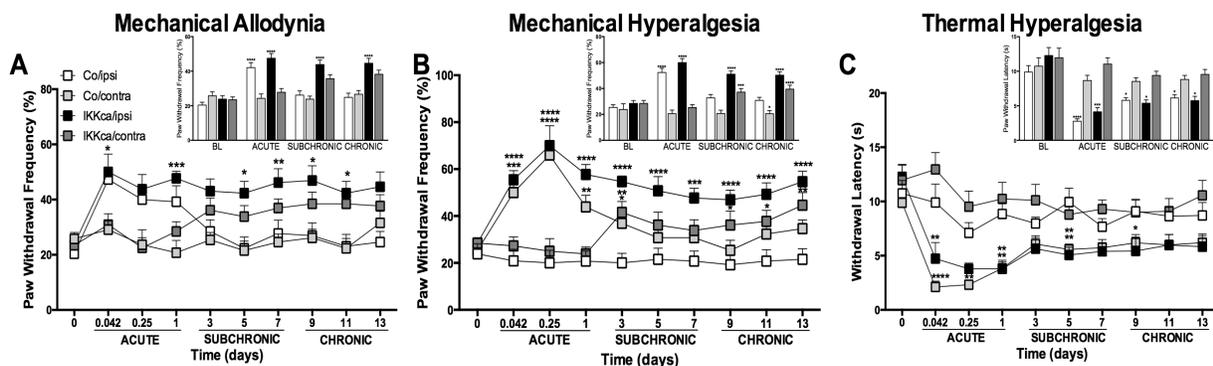


Figure 4.2. Mice expressing constitutively active IKK exhibit enduring pain following CFA treatment compared to Co.

Intraplantar administration of CFA (20 μ L) increases (A) mechanical allodynia, (B) mechanical hyperalgesia, and (C) thermal hyperalgesia. While CFA-mediated (A) mechanical allodynia and (B) mechanical hyperalgesia subside in Co mice, IKKca mice exhibit prolonged mechanical allodynia and hyperalgesia compared to Co contra. No differences were observed in thermal hypersensitivity following CFA administration between IKKca and Co mice. N=10-13 per group. Data are mean \pm SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 different from Co/ contra.

3.3 NF- κ B inhibition blocks inflammation-evoked reductions in COMT protein expression in rats

After establishing that NF- κ B activity mediated pain behavior in the CFA model of inflammation, we sought to assess the role of NF- κ B in regulating the expression of COMT. Separate groups of rats received an i.p. dose of the NF- κ B inhibitor MG132 or vehicle 1h prior to i.pl. administration of CFA or saline and were sacrificed at 6h, 1d, and 5d after CFA injection. Tissues from forebrain (nucleus accumbens, primary somatosensory cortex) and midbrain (thalamus and hypothalamus), hindbrain, and lumbar spinal cord were collected for analysis of COMT protein levels. CFA administration produced noticeable reductions in midbrain for both membrane-bound and soluble isoforms of COMT protein expression at 6h and 1d (Figure 3). Administration of MG132 prior to CFA normalized reductions in COMT expression in midbrain. No differences in COMT expression were observed in forebrain, hindbrain or spinal cord tissue samples (data not shown).

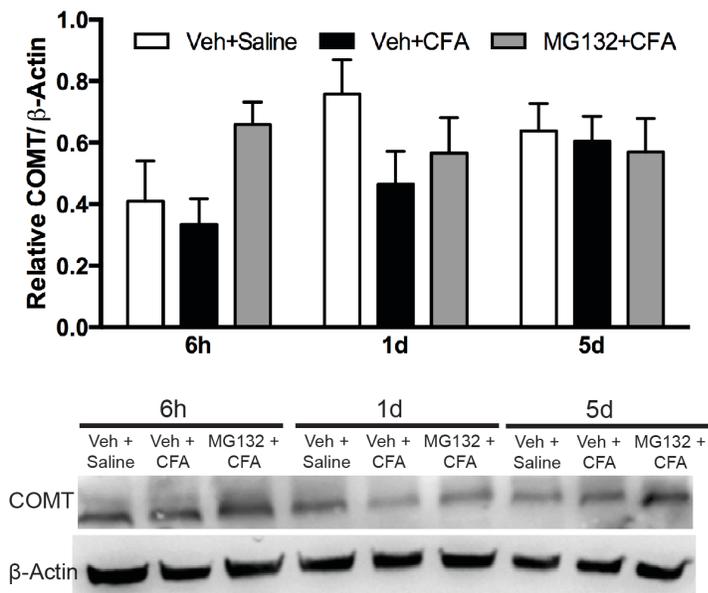


Figure 4.3. CFA inflammation reduces COMT expression in rat midbrain *via* an NF- κ B mechanism. Western blot analysis of COMT expression of both membrane-bound and soluble isoforms in rat midbrain lysates after intraplantar administration of CFA and MG132 prior to CFA. N=3/group. Data are mean + SEM.

3.4 Constitutive activity of astrocytic NF- κ B reduces COMT protein expression in mice

Finally, we sought to determine if animals expressing astrocyte-specific IKKca exhibited altered COMT expression following CFA. Separate groups of IKKca and Co mice were administered CFA and tissue was collected at acute, subchronic and chronic time points from discrete pain-associated brain regions, spinal cord and dorsal root ganglia for COMT protein expression analysis. As the trends in COMT expression levels (for both membrane-bound and soluble isoforms) were similar according to brain region (e.g. hindbrain, midbrain and forebrain), the data were averaged in this manner. Analyses showed significant group differences in COMT expression between IKKca and Co mice in forebrain ($t_{12}=4.014$, $P<0.002$) and midbrain ($t_{12}=3.676$,

$P < 0.01$). Specifically, similar to the results obtained in rats receiving the NF- κ B inhibitor, IKKca mice exhibited significant decreases in COMT expression in midbrain (thalamus and hypothalamus) as well as in forebrain (nucleus accumbens and somatosensory cortex). We then assessed if there were significant differences at specific timepoints. Compared to Co mice, IKKca mice expressed significantly less COMT in forebrain at 1h ($t_{12}=3.10$, $P < 0.003$) (Figure 4A,B) and in midbrain at 1h ($t_{11}=3.04$, $P < 0.01$) and 1d ($t_{12}=3.14$, $P < 0.008$) post-CFA (Figure 4C,D).

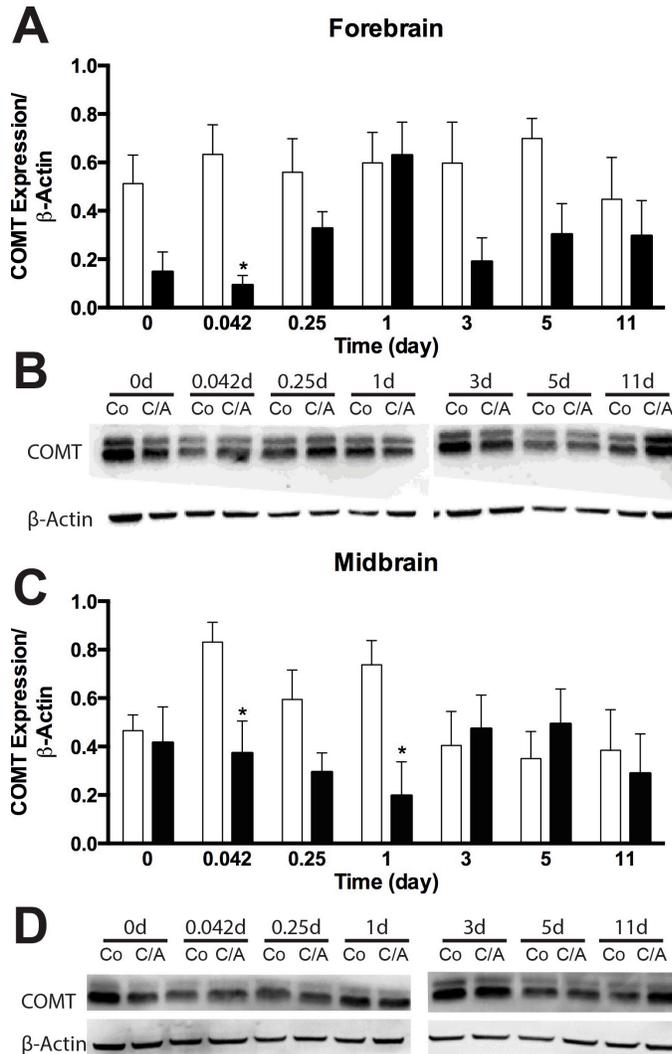


Figure 4.4. CFA inflammation reduces COMT expression in forebrain and midbrain of *IKKca* mice. Western blot analysis and representative Western blots of COMT expression in mouse (A) forebrain and (B) midbrain tissues of *IKKca* and Co mice receiving CFA. N=3-4/group. Data are mean \pm SEM. *P<0.05.

4. Discussion

The present study employed pharmacologic and genetic approaches in an animal model of inflammation to examine the relationship between NF- κ B, pain, and COMT expression. We found

that animals with inhibited NF- κ B activity exhibited decreased CFA-induced pain, while those with enhanced astrocytic NF- κ B activity exhibited increased CFA-induced pain. Furthermore, we provide the first systems-level demonstration showing that CFA-induced inflammation leads to reductions in COMT protein expression *via* an NF- κ B mechanism.

NF- κ B is widely recognized as a master switch that is essential for immune responses. An emerging literature shows that NF- κ B plays an important role in the central nervous system, where it can drive pain associated with injury and inflammation. For example, animals with p50 knockout (Niederberger et al., 2007) or p65 knockdown (Luo et al., 2014) show reduced pain following administration of intraplantar formalin or intradermal CFA, respectively. CFA contains *Mycobacterium tuberculosis*, which can directly stimulate toll-like receptors-2 and -4, which in turn signal through MyD88 to stimulate various inflammatory cascades, including the NF- κ B pathway. Administration of MG132, which induces upstream I κ B α nuclear translocation to more fully block NF- κ B-mediated transcription, (Vu et al., 2008) reduces pain following systemically-administered CFA. The present study extended these findings by testing the ability of MG132 to prevent the development of mechanical and thermal hypersensitivity following intraplantar CFA. Accordingly, we found that rats receiving a pre-emptive dose of MG132 exhibited reduced hypersensitivity to mechanical and thermal stimuli for the duration of the two-week testing paradigm.

Based on evidence demonstrating that nociceptive stimuli induce NF- κ B activity in astrocytes, we were particularly interested in understanding if astrocytic NF- κ B signaling regulated CFA-induced pain. Astrocytic NF- κ B regulates the expression of genes linked to immune responses, triggering the transcription of TNF- α , IL-1 β and COX-2 (Niederberger et al., 2007). In turn, TNF α or IL-1 β can increase the transmission of pain signals by directly lowering the activation threshold for nociceptors (Binshtok et al., 2008a, Gudes et al., 2015). Astrocytic NF- κ B can also strengthen

plasticity-related events that increase the transmission of pain. For example, in a study assessing memory performance, mice expressing a dominant negative isoform of $\text{I}\kappa\text{B}\alpha$ in astrocytes, thereby inhibiting astrocytic $\text{NF-}\kappa\text{B}$ activity, showed deficits in memory tasks that correlated with decreased expression of postsynaptic density protein 95 (PSD95) (Bracchi-Ricard et al., 2008). In the present study, we found that mice expressing a constitutively active form of IKK in astrocytes displayed increased pain in the CFA-injected ipsilateral paw that lasted throughout the duration of the two-week testing paradigm. Interestingly, the IKKca mice also displayed increased mechanical allodynia and hyperalgesia in the unaffected contralateral paw beginning during the subchronic phase of injury. This may be a result of a phenomenon known as spreading (Radhakrishnan et al., 2003). It should also be noted, though, that repetitive mechanical stimuli, as used in this study, may result in increased activation of spinally-located second-order sensory neurons, possibly a result of temporal summation (Herrero and Cervero, 1996, Ringkamp et al., 1999). Given that IKKca and Co mice did not show significant differences in mechanical or thermal sensitivity at baseline or after IFA injection, a pro-inflammatory event, such as CFA administration, may be required for the emergence of a behavioral phenotype, in this case prolonged mechanical hypersensitivity. To our knowledge, this is the first gain-of-function study to demonstrate that enhanced astrocytic $\text{NF-}\kappa\text{B}$ activity prolongs the duration of inflammatory pain as well as its ability to spread contralaterally.

While $\text{NF-}\kappa\text{B}$ can influence the transcription of genes involved in inflammation and plasticity, another gene that contains a known κB binding site is *COMT*. Following $\text{NF-}\kappa\text{B}$ induction by $\text{TNF}\alpha$, binding of p65 to the known P2-*COMT* promoter in a human astrocyte cell line (H4 cells) *in vitro* dampens *COMT* mRNA and protein expression (Tchivileva et al., 2009a). In line with these findings, we observed $\text{NF-}\kappa\text{B}$ -linked reductions in *COMT* expression at 6h and 1d following CFA in midbrain in rats and 1h and 1d following CFA in forebrain and midbrain in mice (Figure 5).

COMT expression likely was not downregulated during the entirety of the inflammation period because NF- κ B activity is known to fluctuate during an inflammatory event (Gilmore, 2006). COMT expression in brain normalized at later stages of inflammation. Furthermore, inflammation-linked COMT expression in mice and rats is tissue- and, in this case, brain region-specific (Nissinen, 2010). At present, few studies have assessed how COMT expression is regulated, particularly in the brain. Genetic studies have predicted that promoters for the COMT gene contain binding motifs for transcription factors including Sp1, AP-2 and NF-D. Furthermore, altered COMT expression in brain has been associated with administration of mood stabilizing drugs, local infusions of inflammatory agents, as well as developmental vitamin D deficiencies (Nissinen, 2010).

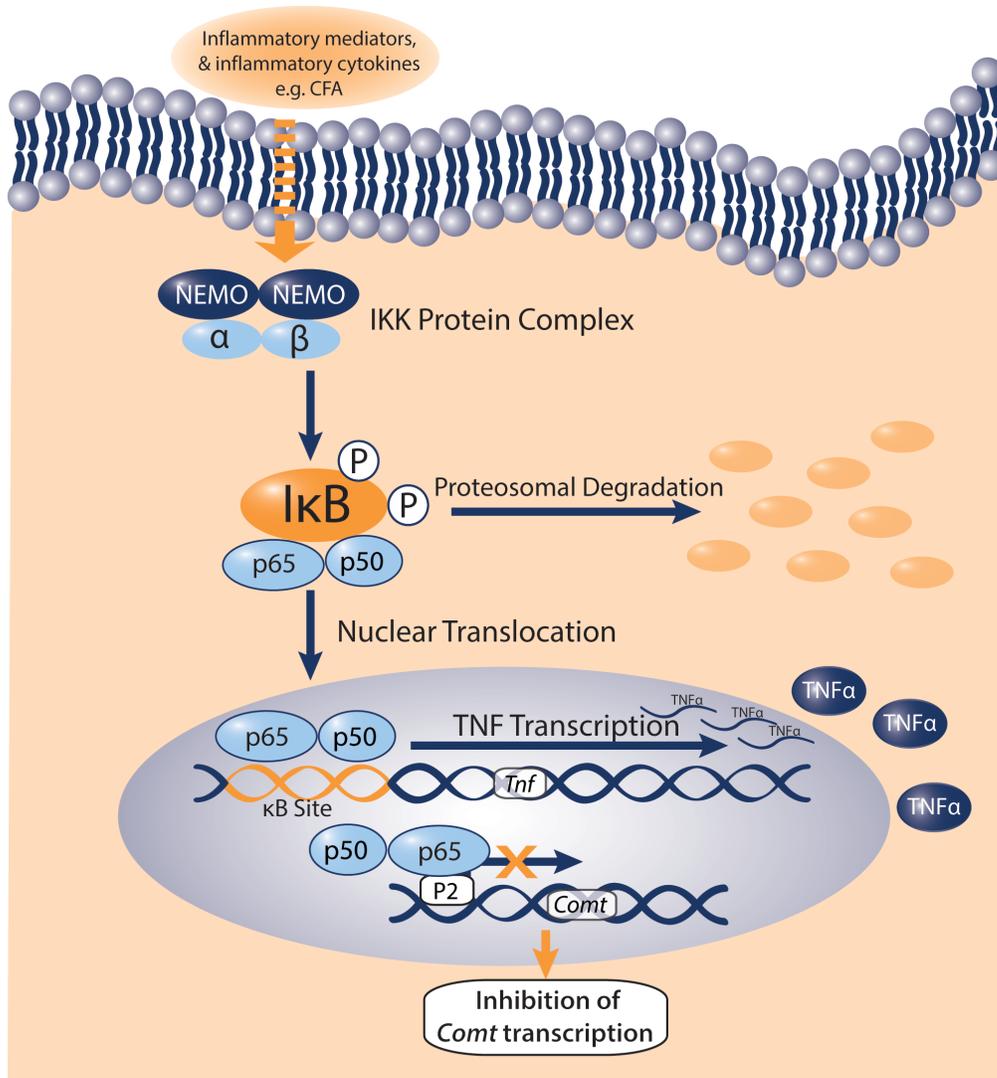


Figure 4.5. Schematic depicting how NF-κB mediated transcription of TNFα may regulate COMT expression.

Our *in vivo* data, together with *in vitro* findings from Tchivileva et al., 2009, suggest an NF-κB-dependent mechanism in which a pro-inflammatory stimulus such as TNFα or CFA can initiate NF-κB, thereby permitting nuclear translocation of p65, which subsequently binds to the P2 promoter of COMT and prevents its transcription.

Reductions in COMT expression can increase pain sensitivity in both humans and animals. In humans, decreased COMT expression and/or activity as a result of genetic variance have been linked to increased susceptibility to temporomandibular joint disorders (Diatchenko et al., 2005) and fibromyalgia (Gursoy et al., 2003, Barbosa et al., 2012b). In animals, decreased COMT activity, as a result of pharmacological inhibition, increases pain sensitivity (Nackley et al., 2007, Kambur et al., 2010). Heightened pain sensitivity persists after the cessation of COMT inhibitor administration, suggesting that even transient reductions in COMT expression, such as those observed in brain in this study, may result in heightened pain states (Hartung and Nackley, 2014). In addition to increasing pain sensitivity, decreased COMT activity also results in the release of pro-inflammatory molecules including TNF α , which is required for the development of COMT-dependent pain (Hartung et al., 2014). As discussed earlier, TNF α may also contribute to NF- κ B activation and downstream alterations in COMT expression, thereby driving COMT-dependent pain. Indeed, these studies highlight the complexity of overlapping regulatory pathways that may be involved in the potentiation of pain and/or COMT expression.

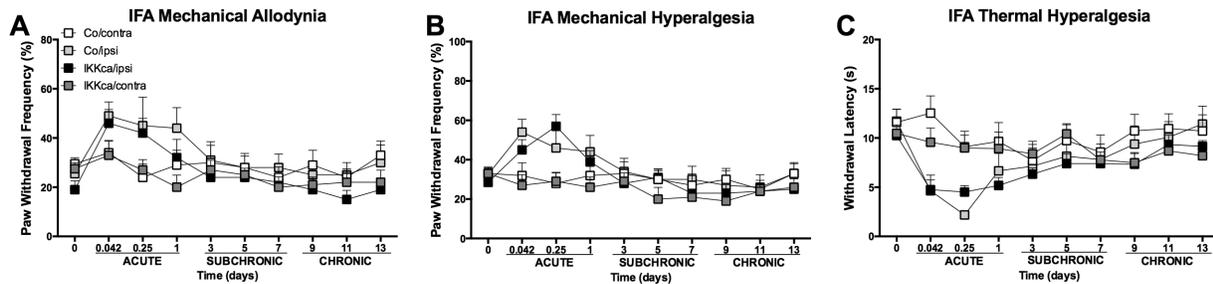
Here, we demonstrate that COMT protein expression is altered as a result of NF- κ B-mediated inflammation. At present, drugs that block NF- κ B activity, such as I κ B inhibitors (Niederberger and Geisslinger, 2008), or indirectly block the initiation of NF- κ B, such as the TNF α antagonist etanercept, are currently in use for individuals experiencing chronic pain conditions such as fibromyalgia and rheumatoid arthritis. Thus, future studies will assess how NF- κ B and/or COMT play a role in mediating pain behaviors in other models of inflammatory and neuropathic pain. Given that sex differences appear to affect the etiology of inflammatory pain, it will also be important to test this relationship in females.

5. Conclusion

This study highlights the role of NF- κ B in regulating inflammatory pain and COMT expression in the CNS. Pharmacological inhibition (which quiets NF- κ B activity) attenuates inflammatory pain, while constitutive activation of IKK in astrocytes (which enhances NF- κ B activity) leads to lasting inflammatory pain. Negatively correlated with pain phenotypes, COMT expression was decreased in animals with greater NF- κ B activity. This is the first demonstration that inflammation can influence COMT expression in the brain in an NF- κ B-dependent method.

6. Acknowledgments

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Supplementary Figure 4.1.

Co and IKKca mice exhibit heightened (A) mechanical allodynia, (B) mechanical hyperalgesia, and (C) thermal hyperalgesia behaviors at 1h and 6h following IFA. There are no group differences in IFA-induced pain behavior, which resolves by day 1 or 3. Data are mean \pm SEM.

Chapter 5. Discussion

5.1 Summary

5.1.1 Impact of Catecholamine Dysregulation on the Onset and Maintenance of Pain

Here, we report catecholamine dysregulation through COMT inhibition results in increased pain. This is driven through β_2 - and β_3 AR stimulation and downstream release of circulating pro-inflammatory molecules including nitric oxide (NO) and innate immune cytokines (TNF α , IL-1 β , IL-6 and CCL2). Blockade of either NO or TNF α , IL-1 β , or IL-6 was able to prevent COMT-dependent pain. Furthermore, nitric oxide and pro-inflammatory cytokines act in a feed-forward manner, such that increased NO results in increased cytokines and vice-versa. These studies represent the molecules and events involved in the onset of COMT-dependent pain.

While this work proposes a possible mechanism by which pain is initiated, we also sought to understand the mechanisms involved in the maintenance of pain. In order to assess this, we pharmacologically blocked COMT for 14 days and assessed pain and molecule release during and after COMT blockade. As our group has reported previously, we found that blockade of COMT for 14 d led to increased mechanical allodynia and hyperalgesia, which lasted for 21 days following cessation of COMT antagonist administration. Notably, blockade of β_2 - and β_3 AR prevented the onset of pain, but did not reverse pain. These data suggest that while β_2 - and β_3 ARs are required for the onset of pain, they do not contribute to the maintenance of COMT-dependent pain.

Our group then sought to uncover the β AR-independent mechanisms of COMT-dependent pain. To do so, we chose to assess levels of various correlates of pain, including cytokines, cellular activation and mitogen activated protein kinases (MAPKs). We found that increases in pain are associated with increased TNF α in CSF as well as increased phosphorylated p38 in the spinal dorsal horn. We also observed that ERK1/2 was increased on day 14 in DRG. In order to understand if MAPKs were required for the maintenance of pain, we administered a continuous intrathecal dose of either a p38 inhibitor or an ERK1/2 inhibitor beginning on day 14. We found that intrathecal administration of a p38 inhibitor on day 14 reversed COMT-dependent mechanical allodynia and mechanical hyperalgesia. While intrathecal administration of an ERK1/2 inhibitor reversed mechanical allodynia, it did not reverse mechanical hyperalgesia. Taken together, these data suggest that while the onset of pain may be regulated by previously identified β_2 - and β_3 ARs, extended dysfunction of catecholamine regulation may result in MAP kinase activation that drive pain (Figure 5.1)

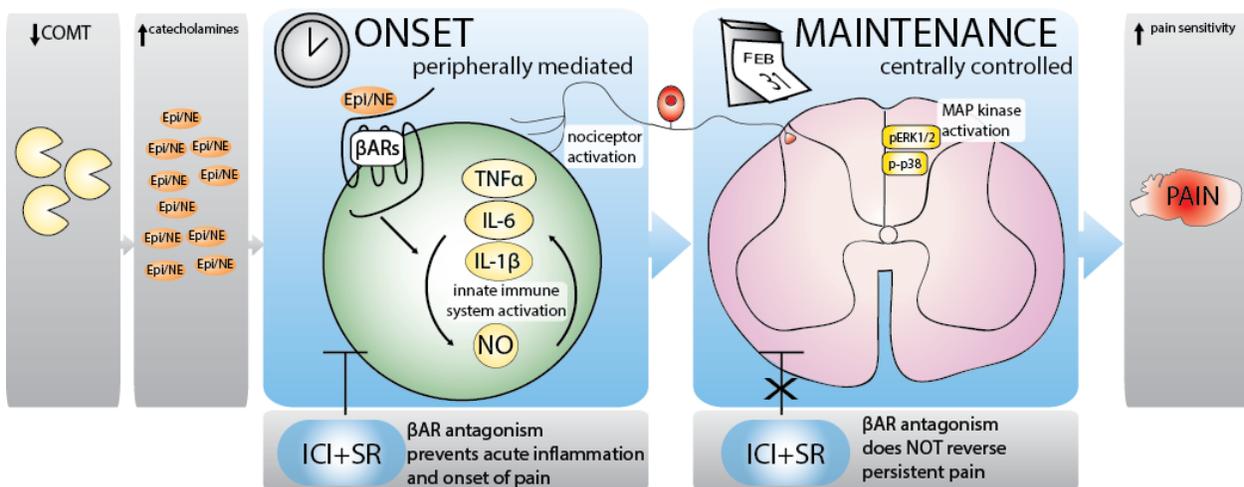


Figure 5.1 Proposed model for the onset and maintenance of pain.

Distinct mechanisms underlie the onset of pain linked to catecholamine dysregulation, such that β_2 - and β_3 ARs are required for the onset of pain, while p38 contributes to the maintenance of pain.

5.1.2 Role of the Immune System on Inflammatory Pain and COMT Protein Regulation

While we looked specifically at the downstream effects of COMT inhibition on pain, we were also interested in understanding how COMT protein expression could be altered. Previous data conducted *in vitro* indicated that the expression of COMT could be altered based on TNF α -induced stimulation of NF- κ B in astrocytes (Tchivileva et al., 2009a). We were interested if a similar phenomenon occurred *in vivo*, such that inflammatory stimuli would alter COMT protein expression *via* NF- κ B. Therefore, we assessed if NF- κ B activity stimulated by CFA could either increase or decrease COMT expression. We administered an intraplantar dose of CFA to the rat hindpaw in rats with NF- κ B inhibition or in mice with NF- κ B constitutive activity. In rats, we found that inhibition of NF- κ B prevented CFA-induced pain as well as decreases in COMT expression in the forebrain. Oppositely, in mice, we found that constitutive activity of astrocytic NF- κ B prolonged mechanical sensitivity and reduced expression of COMT protein expression compared to wildtype mice. Taken together, these data support the hypothesis that decreases in COMT expression elicited by inflammation are correlated with increased pain. Indeed, a wide variety of environmental factors can contribute to the differential expression of COMT (Tunbridge et al., 2006). As in our acute model, even transient decreases in COMT expression can result in increased pain and release of pro-inflammatory molecules. This may be just one of many mechanisms by which COMT expression is altered downstream of an injury, infection, or after psychological distress.

Results indicate that there are COMT reductions in response to CFA and NF- κ B constitutive activity in astrocytes. These results are interesting for a few reasons. First, these data suggest that NF- κ B activity in astrocytes plays an important role in the prolongation of pain. At present, there is very little research assessing astrocyte-derived NF- κ B in various pain modalities or in particular pain regions. Furthermore, there are few research groups that are actively assessing the roles of NF- κ B in the central nervous system in neurological-related disorders. Some of these studies include those conducted by Kleinschmidt and colleagues, who suggest that NF- κ B in neurons, particularly neurons in the forebrain (e.g. nucleus accumbens) may play an important role in depression and anxiety. These studies have been followed-up by Scott Russo's group, showing that the I κ B kinase can regulate social defeat stress (Christoffel et al., 2011). Indeed, social defeat stress has been shown to be regulated, at least in part, by stimulation of beta-adrenergic receptors (Wohleb et al., 2011). Given that inhibition of either NF- κ B or β ARs can give way to a reduction in stress, it would be worthwhile to assess if β AR stimulation prolongs the activation of NF- κ B, and if this has a measurable behavioral effect.

5.1.3 Integration of Observations

Here, we find that the mechanisms and sites of action contributing to the onset and maintenance of COMT-dependent pain are distinct from one another. Taking these data together with recent findings in our group showing that COMT-dependent pain mechanisms originate in the periphery (Ciszek et al., 2016), a period of sustained COMT inhibition results in a shift to the central nervous system, whereby COMT-dependent pain is maintained. At present, we do not know the particular mechanisms that induce this shift from the periphery to the central nervous system. One possible mechanism is neurogenic neuroinflammation. In neurogenic neuroinflammation, a

hypernociceptive response paired with increased inflammation can trigger elevated release of pro-inflammatory cytokines, activation of MAP kinases such as p38, and long term potentiation between peripheral nociceptor terminals and secondary afferents (Xanthos and Sandkuhler, 2014). Similar events can occur without inflammation: high levels of nociceptive activity in the periphery, such as through intraplantar capsaicin administration or neuropathic pain, can result in increased activation of ERK1/2 or p38, respectively (Ji et al., 2009), in the dorsal spinal horn.

These increases in MAPKs, which can induce mechanisms related to sensitization (Ji et al., 2009, Zhang et al., 2010), may prolong states of pain in the absence of injury or inflammation. This may explain why we observed sustained mechanical allodynia and hyperalgesia throughout the behavioral paradigm, even after the COMT inhibitor is no longer on board. Furthermore, because we did see that p38 inhibition was able to reverse COMT-dependent pain, this suggests that p38 drives pain, while β AR signaling plays a lesser role in the maintenance phase. Data from these studies underscore the importance of peripheral inputs to the central nervous system that then affect the activity and expression of proteins in the central nervous system.

Finally, we found that alterations in COMT expression can result in an increase in inflammation, and that similarly, inflammation may result in altered COMT expression. Given that there are known triggers that are linked to chronic pain conditions, initiation of this possible positive feedback loop may result in increased pain. While COMT is just one enzyme of many different molecules and processes that can alter sympathetic tone, it is clear that COMT plays a key role in driving pain while increasing acute inflammation and long-term sensitization. These data follow a line of evidence that shows that short term catecholamine dysregulation, such as an increase in epinephrine and norepinephrine, results in release of circulating inflammatory mediators if there is no pre-existing inflammation in that system (Szelenyi et al., 2006). Furthermore, because $\text{TNF}\alpha$ acts as a key regulator of COMT transcription (Tchivileva et al., 2009a), observed elevations in $\text{TNF}\alpha$

following COMT inhibition may trigger a feed-forward mechanism such that TNF α acts to decrease local expression of COMT and thereby potentiate COMT-dependent pain.

5.2 Future Directions

Data collected from these studies have revealed a number of questions that necessitate study. This is by no means an exhaustive list of possible experiments, but an outline of those studies that are most important and/or interesting that should be conducted to better understand the mechanisms of pain elicited by catecholamine dysregulation.

5.2.1 Determining the Complete Duration of COMT-Dependent Pain

While I followed rats with chronic COMT inhibition for 21 days following a 14 day administration of COMT inhibitor, we continued to observe that elevated mechanical pain throughout the behavioral paradigm. Future studies should follow rats given 14d of COMT inhibitor until pain ceases, if it does at all. By determining the length of time it takes for pain to resolve following COMT-inhibition, we may begin to assess if those mechanisms that delay resolution. In addition we can assess if animals in this latent pain stage respond differently (e.g. intensified and/or prolonged pain) or if they are more pain sensitive when given a stress challenge.

5.2.2 Measuring the Excitability of COMT-Dependent Pain Nociceptors

One of the most important questions that our lab must answer is if peripheral nociceptor firing is increased downstream as a result of COMT-dependent pain. It is possible that nociceptors elicit action potentials at lower thresholds as a result of COMT-dependent pain, which was seen in a form of sensitization elicited by β AR activation (Khasar et al., 1999b). Work from these experiments

may reveal that peripheral nociceptors are more sensitized in the early stages of COMT-dependent pain, but that nociceptor sensitization subsides and gives way to more spinally located central sensitization at later stages. By determining whether or not nociceptor activation is altered, we can more easily understand the peripheral component of COMT-dependent pain. Furthermore, if we observe an initial difference in nociceptor excitability followed by no difference in COMT-dependent pain, these observations may indicate a central sensitization mechanism.

5.2.3 Locating Beta-Adrenergic Receptors on Peripheral Neurons

We find that β ARs contribute to the onset, but do not play a role in the maintenance of COMT-dependent pain. However, it is unclear how β AR dynamics are altered as a result of COMT inhibition. The most straightforward hypotheses are that peripheral β_2 - and β_3 ARs are downregulated on pain relevant molecules or that the G-proteins associated with β_2 - and β_3 AR change during the transition from the onset to the maintenance of COMT-dependent pain. To address the first hypothesis, that the abundance of β_2 - and β_3 ARs are reduced after 7d of COMT inhibition, it would be important to measure the expression of β_2 - and β_3 ARs in pain relevant tissues. Because COMT-dependent pain appears to be maintained peripherally, it would be worthwhile to measure expression of β_2 - and β_3 ARs in dorsal root ganglia as well as nerve terminals using immunohistochemistry. In using immunohistochemistry in particular, we would be able to see where these receptors are going to be expressed on both cell bodies as well as nerve terminals. At present, there are few well-characterized antibodies that target either β_2 - or β_3 ARs, so alternatively, receptors could be labeled using an *in situ* hybridization probe specific for either the β_2 - or the β_3 AR transcript.

To address the second hypothesis, that β_2 - and β_3 ARs switch their second messenger cascade downstream of pain, it may be important to assess β AR coupling using a co-

immunoprecipitation to study the coupling of either β_2 - or β_3 ARs to the various $G\alpha$ subunits in pain. Khasar and colleagues find that while β ARs generally couple with $G\alpha_s$, which increases cAMP production, chronic β AR activation results in $G\alpha_i$ coupling, which decreases cAMP production (Khasar et al., 1999b).

5.2.4 Assessing Tissue-Specific Dynamics of Cytokine Release and Immune Cell Abundance in Peripheral and Central Tissues in COMT-Dependent Pain Animals

Previously, we found that COMT inhibition led to increases in pain and pro-inflammatory cytokines *via* β_2 - and β_3 AR activation. While cytokine levels were elevated at 3h, we did not observe differences in circulating cytokines prior to this timepoint. This could be a result of a delay following the activation of immune cells or accumulation of released cytokines reaching detectable levels only as early as 3 h. In our studies, we observed increased levels of $TNF\alpha$ in cerebrospinal fluid at 14d, 21d and 35d of our timeline. However, measuring cytokine release in either blood or cerebrospinal fluid does not account for tissue-specific release of cytokines, particularly to pain-relevant regions of the central nervous system. Therefore, it would be valuable to assess if there are changes in local cytokine secretion at the tissue level in DRG and spinal cord tissues from animals receiving the COMT inhibitor over time.

Others have reported that elevated adrenergic signaling leads to increases in immune system derived cells that express transcripts encoding for pro-inflammatory cytokines (McIlvried et al., 2016). Therefore, future work can determine if there are changes the abundance of cells and the proteins they secrete in peripheral and central nervous system tissue following COMT inhibition. One method in which this could be conducted is through use of a combination of flow cytometry and cell sorting. As such, immune-related cells would be sorted through from nervous system tissue

(DRG and spinal cord) and from blood. Here we would assess the abundance of immune cells located in nervous system tissue and blood during short-term and long-term OR486 administration. Given that we have observed increases in levels of cytokines in blood during the short term, we would predict to see increased levels of activated immune cells in blood during an acute timepoint (defined previously as <4h), while we would predict that the levels of myeloid cells in nervous system tissues would be elevated during longer time periods (>7d). Furthermore, once the subtypes of cells have been identified in these tissues, assessing cell-type specific mRNA transcript expression may reveal the mechanisms by which these immune cells directly or indirectly influence peripheral afferent activation or nociceptive processing. If indeed COMT inhibition alters the abundance and types of immune cells located in the periphery, then it may also be worthwhile to understand how the immune system responds to extended catecholamine dysregulation.

5.2.5 Assessing the Role of Environmental Triggers on Genetically-Predisposed Animals

One important feature that has yet to be explored is why COMT polymorphisms affect some individuals, leading to increased experimental and clinical pain (Diatchenko et al., 2005), while others remain unaffected. Based on Hardy-Weinberg ratios, it is estimated that approximately one-third of individuals have the SNPs related to lower COMT expression and activity. However, not nearly as many individuals have one or more chronic pain conditions. One possible explanation for this discrepancy between the number of individuals with gene variants and those individuals with chronic pain is that the effects of genetic polymorphisms require one or more environmental triggers, such as injury or a repeated stressor (Diatchenko et al., 2006, McLean et al., 2011).

In a laboratory setting, we see a similar phenomenon in mice as observed in humans. While mice that have complete knockout of COMT have significantly higher levels of mechanical and thermal hyperalgesia compared to their wildtype littermates, those animals that are heterozygous for

the COMT knockout gene (and that presumably express half the COMT compared to wildtype animals) do not display mechanical and thermal hyperalgesia, similar to their wildtype littermates (unpublished data from B.P Ciszek). The amount of COMT expressed by heterozygous mice is sufficient such that the animals do not have altered mechanical or thermal sensitivity. However, in a study conducted years earlier focusing on stress and anxiety, these COMT +/- heterozygotes display increased anxiety as measured by elevated internal temperature after handling, decreased time spent in the open arms of an elevated plus maze, and decreased time exploring novel objects (Papaleo et al., 2008). Given their altered responses to stressors, can stress alter COMT +/- mice pain phenotypes? For these experiments, we would measure baseline levels of pain sensitivity in COMT +/+, +/-, and -/- mice, expose these animals to a stressor such as swim stress or a three day paradigm of chronic variable stress, and measure pain behaviors 24 h following, thereby reducing the effects of stress analgesia. I would hypothesize that while all animals, regardless of genotype, would have an increase in pain sensitivity, those with the +/- genotype would have similar increases in pain sensitivity to the -/- mice, thereby uncovering the initially dormant phenotype.

5.2.6 Assessing the Role of Sex in Mechanisms of COMT-dependent Pain

Because chronic pain conditions generally affect women with much greater frequency than men, it is necessary to begin assessing how chronic pain is driven in females. Up to this point, while behavioral assessments with the COMT-dependent pain model show no sex-dependent differences in the initiation of pain (Ciszek et al., 2016), molecular studies have been limited exclusively to males. However, recent evidence from the Mogil lab suggests an alternative mechanism of neuroinflammation: in female rodents, chronic neuropathic pain is not driven by microglia but by T-cells (Sorge et al., 2015). While our pain model is different from neuropathic pain, this finding highlights the importance of conducting studies that incorporate both males and females, as we may

observe similar differences in cellular activation in our own COMT-dependent pain model. Therefore, replicating these COMT-dependent studies in female rodents may be important to revealing the unique mechanisms that may trigger and potentiate pain more commonly in females.

One way in which we may see differences in males and females is through activation of p38. Evidence presented by the Ji group demonstrates that there are sex-specific differences in the role of spinal p-p38 in some types of inflammatory and neuropathic pain. In this study, Taves and colleagues report that p38 phosphorylation was more pronounced the spinal dorsal horn downstream of neuropathic pain in males, but not in females. Furthermore, intrathecal administration of p38 inhibitor prevented formalin-induced pain and reversed neuropathic constriction injury pain in male mice and rats, but was not effective for female rodents (Taves et al., 2015). While we have observed increases in spinal p-p38 in males as well as reversal of COMT-dependent pain in males, we have not yet looked to see if these findings are the same in females. Therefore, future work should assess pain behaviors and molecular changes, including p38 activation, occurring in both females and males.

5.2.7 Overexpressing COMT in Neuropathic and Inflammatory Pain Models

Data from our NF- κ B studies suggest that reductions in COMT are associated with increases in mechanical pain sensitivity. These data indicate that COMT may play an important role in the induction of inflammatory pain, such that decreases in its expression may be important in the chronicity of pain. Based on these studies, it may be useful to see if overexpression of COMT in the forebrain, where we found the greatest reductions in COMT expression following CFA injection(Hartung et al., 2015)(Hartung et al., 2015) (Hartung et al., 2015), can prevent or reduce some of the effects of inflammatory or neuropathic pain. COMT

overexpression will be driven with a Camk2a promoter, leading to the greatest increases in COMT expression in the forebrain. These CaMK2a-COMT mice have previously been used in the context of learning and memory (Simpson et al., 2014), but given their direct links to our lab's work with COMT, may be an important tool to demonstrate a converse concept: that overexpression of COMT prevents increases in pain. Future studies using a mouse that overexpresses COMT and administering various types of well-known pain inflammatory and neuropathic pain models may reveal how COMT expression plays a key role in either the induction or the prolongation of pain. Results from these experiments could have interesting results, since overexpression of COMT would lower the availability of norepinephrine and thereby reduce the effects of possible descending input from α -adrenergic receptors. Indeed these experiments would require the need to measure levels of local catecholamines in the forebrain, measure mechanical and thermal pain in COMT overexpressers compared to their wildtype littermates, assess if the COMT overexpressers display altered pain behavior responses (either increased or decreased) in different models of inflammatory and/or neuropathic pain, and ultimately determine if α - and/or β -adrenergic receptors play an important role in the inhibition or facilitation of pain signals.

5.3 Conclusions

The aims of this dissertation were developed as a means to better understand how long-term catecholamine dysregulation leads to pain that last weeks following its onset. These studies indicate that distinct mechanisms underlie both the onset and maintenance of COMT-dependent pain, adding to a growing body of evidence now indicating that the transition to chronic pain includes a sequence of events in which the maintenance of pain is nearly independent of what is required its induction. These studies suggest that catecholamine dysregulation is sufficient to induce long-term changes in pain, as observed in patients suffering from post-traumatic stress disorder or stress-

related trauma, two patient groups that are often diagnosed with increased pain sensitivity, either following an injury or without any visible tissue damage. Third, given that these studies more closely mimic what is observed in patient populations (e.g. decreased COMT expression), research using this model may be useful in the development of either future clinical studies looking to find differences in particular molecular targets in chronic pain patients or to develop pharmaceuticals that target particular molecules depending on the patients chronic pain “phase,” the duration of time that the patient has exhibited heightened pain. As the field of pain research continues to grow, it will certainly be important to better characterize and address a number of issues that contribute to chronic pain: the different categories of pain, the length of time an individual has experienced symptoms, their genetic and experiential background, and their sex. Taking these factors wholly, we may find treatments that are more effective for patients that suffer from chronic pain.

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