INVESTIGATING THE GLIAL CONTRIBUTION TO PERSISTENT NEUROPATHIC PAIN

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ABSTRACT

SARAH TAVES WHITLEY: Investigating the Glial Contribution to Persistent Neuropathic Pain
(Under the guidance of Ken McCarthy)

Persistent neuropathic pain is the coordinated activation and sensitization of glial and neuronal elements both peripherally and centrally. Here, we have investigated the role of glial fibrillary acidic protein (GFAP)-positive glial cells including astrocytes in the central nervous system and non-myelinating Schwann cells in the peripheral nervous system and their individual contributions to persistent neuropathic pain.

We used three-dimensional reconstruction of ultrastructural data to establish the morphological relationship between astrocyte processes and incoming C and A-delta fiber synapses with second-order pain neurons. We found that not only do astrocytes contact 100% of the C and A-delta fiber glomerular synapses, but they also provide a high degree of ensheathment of each glomerulus. This encapsulation of the glomerular synapses puts astrocytes in a position to potentially modulate neuronal activity and synapse structure.

Next, we used transgenic and knockout mouse models to interfere vesicular gliotransmitter release: a glial-specific IP_3R2 conditional knockout mouse and a transgenic mouse expressing a dominant-negative SNARE protein. However, neither blocking IP_3 dependent Ca^{2+} release or SNARE-dependent vesicle release have any effect on basal nociception or on the development or maintenance of mechanical sensitivity following nerve injury. We then used two mouse models to interfere with distinct elements of intracellular

inflammatory pathways known to be active in persistent pain conditions: a transgenic mouse with suppressed NFκB activity and a COX2 conditional knockout mouse, both of which were selectively expressed in GFAP-positive cells. In both lines of mice, we observed a robust yet temporary alleviation of pain behavior from one to five weeks post-nerve injury. This finding indicates that the NFκB-COX2 signaling pathway in GFAP-positive glia is critical to the maintenance of a specific phase of persistent neuropathic pain.

GFAP-positive glia include peripheral non-myelinating Schwann cells, which ensheath unmyelinated nociceptive neurons, as well as central astrocytes, which ensheath neuronal synapses throughout the brain. We used the tet-Off transgenic mouse expression system in a novel manner to tease apart the peripheral vs central roles of GFAP-positive cells. The administration of oxytetracycline, a blood-brain barrier impermeable analog of doxycycline, was capable of turning off transgene expression in the peripheral nervous system while keeping transgene expression intact in the central nervous system. The blockade of peripheral transgene expression reversed the alleviation of pain behavior post-nerve injury in mice with suppressed NFκB activity. Thus, the central suppression of NFκB is insufficient to relieve mechanical sensitization following nerve injury. The implicates non-myelinating Schwann cells in an important role in the maintenance of a specific phase of persistent neuropathic pain from one to five weeks post-injury.

This work is dedicated to my husband, Jeremy Whitley, who has pushed me to keep my scientific passion alive while becoming a wife and a mother.

This work is also dedicated to my children, Gianna and (soon to be) Logan, who remind me of what is really important at the end of the day.

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ACSF	artificial cerebral-spinal fluid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATP	adenosine triphosphate
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
CaMK	calmodulin-dependent kinases
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
CCL2	chemokine C-C motif ligand 2 (MCP-1)
CCR2	chemokine C-C motif receptor 2
CD11B	cluster of differentiation 11B
CGRP	calcitonin gene related peptide
cKO	conditional knockout
CNS	central nervous system
COX2	cyclooxygenase 2
DAG	diacylglycerol
DHPG	(S)-3,5-Dihydroxyphenylglycine
dnSNARE	dominant negative N-ethylamaleimide-sensitive factor adaptor protein receptor
DRG	dorsal root ganglion

eGFP	enhanced green fluorescent protein
EM	electron microscopy
EPSC	excitatory postsynaptic current
ERK	extracellular signal-regulated kinases
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter
Glt-1	glutamate transporter 1
GluR1	glutamate receptor 1
hGFAP	human glial fibrillary acidic protein
Iba-1	ionized calcium binding adaptor molecule 1
IGF-1	insulin-like growth factor 1
IKKdn	dominant negative IκB kinase
IKKβ	IκB kinase beta
IL-1β	interleukin -1 beta
iNOS	inducible nitric oxide synthase
IP_3	inositol-1,4,5-trisphospahate
IP_3R2	inositol-1,4,5-trisphospahate receptor 2
JNK	c-Jun N-terminal kinase

KCC2	K^+ Cl^- co-transporter
KO	knockout
LTP	long term potentiation
MAPK	mitogen activated protein kinase
MCH	major histocompatability complex
MCP-1	monocyte chemoattractant protein -1
MEK	mitogen activated protein kinase kinase
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
NFkB	nuclear factor kappa B
NGF	nerve growth factor
NK-1	neurokinin receptor - 1
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NRG-1	neuregulin-1
Oxy	oxytetracycline
PDGF	platlet-derived growth factor
PGE2	prostaglandin E 2
PI3K	phosphatidylinositol 3-kinase

PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLCg1	phospholipase C gamma 1
pNFkB	phospho-nuclear factor kappa B
PNS	peripheral nervous system
p-p38	phospho- p38
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
SNI	spared nerve injury
Tam	tamoxifen
tetO	tet operon
TIMP-2	tissue inhibitor of metalloproteinases 2
TNFR	tumor necrosis factor receptor
TNFα	tumor necrosis factor alpha
tTA	tetracycline transactivator
TTX	tetrodotoxin

TTX-R	tetrodotoxin resistant
WT	wild-type

CHAPTER 1.

INTRODUCTION TO NEUROPATHIC PAIN

Chronic pain conditions affect 1.5 billion people worldwide (Global Industry Analysts 2011). In the United States alone, chronic pain affects 116 million people annually, which is more than heart disease, cancer and diabetes combined (Committee on Advancing Pain Research, Education et al. 2011). Severe and constant chronic pain affects 9% of the adult US population (Worldwide 1999). One in three sufferers of chronic pain is less able to maintain an independent lifestyle (Division 2001). Additionally, one-third of chronic pain sufferers describe their pain as being almost the worst pain they can possibly imagine. Rather than experiencing frequent flare-ups, their pain is more likely to be constant, and two-thirds of chronic pain sufferers have been living with their pain for over 5 years.

Pain can arise from discrete causes such as trauma or disease, or its cause can be unknown. Regardless of the causative agent, persistent pain can lead to sleep disturbances, depression, anxiety, fatigue, reduced quality of life and an inability to work or socialize (Moore, Straube et al. 2010). As a public health problem, it costs our society between $261 to $300 billion in increased health care and $297-$336 billion due to lost productivity (based on days of work missed, hours lost and lower wages) (Committee on Advancing Pain Research, Education et al. 2011). These costs total $635 billion annually. Furthermore, the incidence of chronic pain is projected to rise due to the increasing size of the elderly population. Pain is the most frequently reported symptom in greater than 50% of community-dwelling

individuals and in greater than 80% of nursing home residents (Worldwide 1999). From the year 2000 to 2050, the world's over-80 population is projected to triple (Division 2001).

Pain can be mild and easily handled with over-the-counter medications, it can be acute and resolve with treatment, it can be recurrent over months or years, or it can be chronic and debilitating, requiring almost constant attention and accommodation. Current data on the incidence, prevalence and consequences of pain are not consistent or complete because there is a lack of consensus on the terminology and categorization of these types of pain. This makes obtaining a definitive picture of the extent and significance of pain difficult. In the clinical setting "chronic pain" most often denotes long-term or persistent pain regardless of its origin. While pain serves a vital function as a warning sign of injury or infection, once this role has been exhausted, continued pain is maladaptive.

Maladaptive pain can arise from musculoskeletal disorders, principally fibromyalgia, low back pain and osteoarthritis, cancer pain, headache, visceral pain and neuropathic pain. Neuropathic pain, on which this dissertation is focused, is characterized by continuous or intermittent spontaneous pain, typically described as burning, aching or shooting (Kroenke, Krebs et al. 2009). To a lesser degree, patients also report mechanical pain, thermal hypersensitivity and cold sensitivity. Neuropathic pain is most commonly associated with painful diabetic neuropathy or lumbar nerve root compression but can also begin with cancer-related pain, spinal cord injury, post-stroke pain, HIV-associated neuropathy, phantom limb pain and trigeminal neuralgia. "Neuropathic pain differs from normal nociception in that the experience is not the direct outcome of the signaling of injury of peripheral tissue or organs" (Perl 2011). Of the global population, 3-4.5% suffers from

neuropathic pain, with the incidence rate increasing with age (Global Industry Analysts 2011).

1.1. TREATMENT

The mainstay of treatment for pain of moderate intensity has been non-selective and cyclooxygenase-2 (COX2)-selective non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are anti-inflammatory, analgesic and antipyretic and all seem to have similar efficacy in the treatment of pain disorders (Kroenke, Krebs et al. 2009). Rofecoxib (marketed under the name Vioxx) was linked to an increased risk of myocardial infarction and stroke. At first, the increased cardiovascular risk was thought to be a class effect of the COX2 inhibitors; however, in 2005, the US Food and Drug Administration announced that a greater risk for cardiovascular events may be a class effect for all NSAIDs. These agents are also associated with gastrointestinal and renal toxicity (Whelton 2000; Dieppe, Bartlett et al. 2004); furthermore, there is also an acknowledged ceiling effect of NSAIDs.

After NSAIDs and COX2 inhibitors, the first line of defense in the pharmacological management of neuropathic pain includes tricyclic antidepressants, selective norepinephrine and serotonin reuptake inhibitors, calcium channel blockers, and topical lidocaine. A large number of clinical trials have found tricyclic antidepressants are effective in treating several types of neuropathic pain. They are also efficacious for the treatment of depression, a commonly comorbid condition in patients with chronic pain, but have also been proven efficacious in non-depressed patients (Max, Culnane et al. 1987). It generally takes 6 to 8 weeks for an adequate trial of a tricyclic antidepressant medication to be assessed in a patient. Tricyclic antidepressants can cause dry mouth, orthostatic hypotension, constipation and urinary retention, most likely through their anti-cholinergic activity. More serious complications can arise with cardiac toxicity in patients with ischemic cardiac disease or

ventricular conduction abnormalities. Duloxetine and venlafaxine are selective serotonin norepinephrine reuptake inhibitors that have been proven effective in peripheral neuropathic pain (duloxetine only in diabetic peripheral neuropathy) (O'Connor and Dworkin 2009). Cardiac abnormalities have been reported in a small number of patients; however, they are generally well-tolerated. The calcium channel α_2-δ ligands gabapentin and pregabalin bind voltage-gated calcium channels at the α_2-δ subunit and inhibit neurotransmitter release. Gabapentin has a well-documented moderate effect on pain, mood and sleep disturbances in several large clinical trials in a number of neuropathic pain conditions (Finnerup, Otto et al. 2005). Pregabalin has not undergone the same degree of testing but has shown effects comparable to gabapentin, albeit with higher trial withdrawal rates (presumably due to the side effects of treatment) (Finnerup, Otto et al. 2005). Calcium channel blockers can both produce dose-dependent dizziness and sedation. Gabapentin must be titrated slowly and takes a 3- to 8-week trial period, while pregabalin can be titrated more quickly and only requires a 4-week trial period. A 5% topical lidocaine patch has shown good efficacy and is easily tolerated with only mild local skin reactions and no systemic side effects (Finnerup, Otto et al. 2005). However, this patch is most appropriate in localized neuropathic pain and is unlikely to benefit those with central neuropathic pain.

Second-line medications are reserved for patients who do not respond to first-line medications due to concerns regarding their long-term safety and potential for abuse. Tramadol is a weak μ-opioid receptor agonist that also inhibits the reuptake of serotonin and norepinephrine. Though less effective than strong μ-opioid agonists such as morphine and oxycodone, it provides relatively rapid pain relief with less potential for abuse (Dworkin, O'Connor et al. 2007). Tramadol binds to the μ-opioid receptor (its M1 metabolite to an even

greater extent) (Grond and Sablotzki 2004), which produces some of its analgesic effects. Around 40% of tramadol analgesia can be reversed by opioid antagonists (Raffa, Friderichs et al. 1992; Raffa and Friderichs 1996). In addition, tramadol inhibits the reuptake of noradrenalin and serotonin, the primary neurotransmitters involved in descending pain inhibition from the brain, accounting for approximately 40% and 20% of its analgesic effect, respectively (Collart, Luthy et al. 1993; Desmeules, Piguet et al. 1996; Raffa and Friderichs 1996). The adverse effects of this drug are similar to those of other opioids; however, tramadol can also interact with selective serotonin reuptake inhibitors and selective serotonin and norepinephrine reuptake inhibitors to cause serotonin syndrome, a potentially fatal reaction. Opioid analgesics, such as oxycodone and morphine, have been shown to provide as great of pain relief as tricyclic antidepressants and gabapentin (Raja, Haythornthwaite et al. 2002) and are proven in a variety of neuropathic pain conditions (Finnerup, Otto et al. 2005). However, they have more adverse events than other treatments and are generally recommended only for patients in whom first-line medications are ineffective or insufficient. The most common adverse events include constipation, nausea and sedation. More seriously, all patients undergoing long-term opioid therapy will develop physical dependence. Patients with histories of drug abuse or a family history of drug abuse are considered risk factors for the misuse or abuse of opioid analgesics and must be monitored closely. Despite these factors, opioid use for chronic pain has risen dramatically over the last 20 years, in which time a new phenomenon has been described: opioid induced hyperalgesia. The molecular mechanisms are still under debate; however, recently, opioids have been shown to induce long-term potentiation in neurons via both presynaptic and postsynaptic mechanisms (Drdla, Gassner et al. 2009; Zhou, Chen et al. 2010). This excitatory effect may counteract the initial

inhibitory effect resulting in increased synaptic activity and the opioid induced hyperalgesia being seen clinically.

Third-line medications are reserved for patients who do not tolerate or find first- and second-line medications ineffective for the management of their pain. These include medications that are typically given as antidepressants or anticonvulsants, as well as dextromethorphan, memantine, mexiletine and topical capsaicin. Antidepressants such as bupropion, citalopram, and paroxetine have been used with some success. Bupropion is a noradrenaline and dopamine reuptake inhibitor, and in a small trial, relieved neuropathic pain of multiple etiologies (Finnerup, Otto et al. 2005). Citalopram and paroxetine are both selective serotonin reuptake inhibitors. Both have demonstrated modest efficacy in neuropathic pain trials (Jackson and St Onge 2003). Anticonvulsants include carbamazepine, lamotrigine, oxcabazepine, topiramate, and valproate (valproric acid). Lamotrigine has a complex trail of clinical trial data in which lamotrigine seemed to be efficacious in very specific types of pain including painful diabetic neuropathy and central post-stroke pain (Finnerup, Otto et al. 2005). Topiramate failed to relieve pain in three large clinical trials totaling 1259 patients, while another trial found significant effects (Finnerup, Otto et al. 2005). All studies have had high withdrawal rates due to side effects. Similarly, valproate shows conflicting results from different clinical trials. Its primary mechanism of action is thought to be the inhibition of GABA transaminase to increase levels of the inhibitory neurotransmitter GABA. It may also block voltage-gated sodium channels and T-type calcium channels. Carbamazepine is efficacious in trigeminal neuralgia but inconsistent in other types of neuropathic pain (Dworkin, O'Connor et al. 2007). Oxcarbazepine is an anticholinergic anticonvulsant with mood elevating properties typically used for the

treatment of epilepsy. It has been shown to be effective in clinical trials in relieving the pain associated with trigeminal neuralgia (Nasreddine and Beydoun 2007). Its efficacy in treating painful diabetic neuropathy is less clear. Dextromethorphan and memantine are orally administered NMDA receptor antagonists. Clinical trials have shown conflicting results regarding their efficacy (Dworkin, O'Connor et al. 2007). Mexiletine is an orally administered lidocaine analogue with modest to no difference compared to a placebo in a controlled trial (Finnerup, Otto et al. 2005). Benefits were only commonly observed at doses with possible proarrhythmic side effects. Capsaicin is derived from chili peppers and is thought to deplete substance P from primary afferent neurons (Yaksh, Farb et al. 1979; Carpenter and Lynn 1981; Bernstein, Bickers et al. 1987). Capsaicin needs to be applied several times daily for 6-8 weeks for pain relief over the entire affected area. Capsaicin's main disadvantage is that each application elicits a burning sensation, which can persist for days. However, clinical trials have shown that 57% of neuropathic pain patients achieved at least a 50% reduction in pain levels (Mason, Moore et al. 2004). One-third of patients also experienced local adverse events. Overall, third-line medications often have conflicting clinical trial results, may only be efficacious in treating a small subset of patients and often have serious side effects.

Neuropathic pain treatments often only treat a subset of patients. This results in patients who live in pain while trying a litany of different treatments, each of which can have serious side effects, including nausea, dizziness, sedation and physical addiction. A panel from the International Association for the Study of Pain noted, "Although few clinical trials have been conducted, no medications have demonstrated efficacy in patients with lumbrosacral radiculopathy, which is probably the most common type of neuropathic pain."

They also noted that little is known regarding the treatment of mild to moderate neuropathic pain because clinical trials enroll subjects with severe neuropathic pain. Long-term effectiveness is also unknown because most clinical trials are less than three months in length. Lastly, even current pain treatments deemed efficacious, meaning that clinical trials have shown that the treatment is more effective than the placebo, often do not control pain in patients to the level that they can resume routine activities and/or occupations.

1.2. SOMATOSENSORY NEUROTRANSMISSION

1.2.1. Peripheral Nervous System

Our bodies play host to a wide variety of sensory information that is detected every moment by the peripheral nervous system. In the skin, primary afferent fibers are responsible for the detection and transmission of somatosensory information to the central nervous system. These sensory neurons can detect vibration, light touch, pressure, stretch, heating, cooling, noxious chemicals, and nociceptive information through a wide variety of receptors. Sensory neurons are pseudo-unipolar cells with one terminus innervating epithelia, muscles, tendons or other organelles and the other terminus entering the central nervous system. Their cell bodies rest in the dorsal root ganglia or in the trigeminal ganglia, in the case of sensory information from the head. These neurons are either specialized to detect a single type of stimuli or are polymodal and capable of detecting multiple types of stimuli (Julius and Basbaum 2001; Myers, Campana et al. 2006). Neurons that detect noxious cold, noxious heat, noxious chemicals and hard pressure, which are somatosensory stimuli that identify danger to the organism, are called nociceptors (Sherrington 1906).

There are two groups of nociceptive sensory neurons that are classified based on the type of nerve fiber: lightly myelinated, medium diameter, A-delta fibers convey the initial sensation of nociception and non-myelinated, small diameter, C fibers convey a secondary

but prolonged sense of burning pain (Julius and Basbaum 2001). These two sensations are familiar to anyone who has stubbed his/her toe. A third type of fiber exists, a thickly myelinated, large diameter and fast conduction fiber, the A-beta fiber, which transmits information regarding non-noxious sensory stimuli. These fibers do not participate in acute nociception; however, they may play a role in the development of allodynia (Zimmermann 2001).

Electrophysiologically nociceptive afferent fibers can be distinguished by their differing conduction velocities at physiological temperatures; moderate conduction A-delta fibers range from 2 to 30 meters/second and slowly conducting C fibers conduct at less than 2 meters/second with a mean of only 0.7 meters/second (Light 1992). C-fibers travel in groups called Remak bundles, in which individual fibers are not separated by myelin, as in A-beta and A delta fibers, but by the cytosol of non-myelinating Schwann cells. Unlike other sensory neurons, action potential conduction along C-fibers also differs in that they express a tetrodotoxin (TTX)-resistant sodium channel in addition to the normal TTX-sensitive channel. These fibers selectively respond to noxious stimuli but not to innocuous stimuli such as light touch (Burgess and Perl 1967). C-fibers can be further subdivided into two groups based on their expression of chemical markers (Snider and McMahon 1998; Julius and Basbaum 2001). One group, known as peptidergic neurons due to their release of the neuropeptides substance P, calcitonin gene-related peptide (CGRP) and somatostatin, expresses nerve growth factor receptor TrkA and terminates in lamina I and in the outer layer of lamina II (Molliver and Snider 1997; Zylka, Rice et al. 2005) (Priestley, Michael et al. 2002). However, 20% of all A-delta fibers also peptidergic (McCarthy and Lawson 1989). The second group, known as nonpetidergic neurons for their lack of neuropeptide expression,

expresses glial derived neurotrophic factor receptor c-ret, binds isolectin B4 (IB4), expresses the P2X3 receptor, expresses the Mrgprd group of orphan G protein-coupled receptors (GPCR) and terminates in inner lamina II (Silverman and Kruger 1988; Molliver, Wright et al. 1997; Bradbury, Burnstock et al. 1998; Snider and McMahon 1998; Priestley, Michael et al. 2002; Zylka, Rice et al. 2005; Myers, Campana et al. 2006).While their termination zones in the dorsal horn are quite distinct, most molecular markers for the peptidergic and nonpeptidergic groups do have some overlap. At the ultrastructural level, peptidergic neurons form non-glomerular synapses in lamina I, nonpeptidergic neurons form C1 type glomerular synapses in outer lamina II, and A-delta fibers form C2 type glomerular synapses in inner lamina II (Ribeiro-da-Silva and Coimbra 1982; Bernardi, Valtschanoff et al. 1995; Bailey and Ribeiro-da-Silva 2006). Using electron microscopic postembedding immunohistochemistry and electrophysiology, all three types of fibers have been shown to be glutamatergic (Schneider and Perl 1985; Valtschanoff, Phend et al. 1994).

The functional relevance of these distinctions between peptidergic and nonpeptidergic primary afferents is not entirely clear. Both peptidergic and nonpeptidergic afferents project to pathways leading to the brain. Through neurons in lamina II, the nonpeptidergic circuit projects to affective areas of the brain such as the amygdala, ventromedial nucleus of the hypothalamus and the bed nucleus of the stria terminalis (Braz, Nassar et al. 2005). The peptidergic circuit projects to these same areas as well; however, the neurons of this circuit project via lamina I and the parabrachial nucleus rather than through lamina II (Gauriau and Bernard 2002). Only the peptidergic circuits are known to project to areas thought to be important for the sensory discriminative aspects of pain, such as the thalamus.

Ablation studies may also provide clues regarding the functional aspects of these circuits. The intrathecal delivery of saporin (a ribosomal toxin) bound to substance P resulted in the destruction of substance P receptor (NK-1)-possessing cells in lamina I. While this did not affect acute thermal or mechanical nociceptive assays, the hypersensitivity following inflammatory pain, using the capsaicin model, was nearly abolished (Khasabov, Rogers et al. 2002). The genetic elimination of Mrgprd expressing cells, which accounts for ~75% of the IB4-positive population, results in specific deficits in noxious mechanosensation, but not in thermal or cold nociception (Cavanaugh, Lee et al. 2009).

The cell bodies of the primary afferents lie in the dorsal root ganglion or trigeminal nucleus, in the case of those afferents innervating the head. Primary afferent cell bodies can be distinguished by the same set of molecular markers. In addition, the size of the neuronal cell bodies is a clear marker of the afferent fiber type. The large cell bodies correspond to those of the A-beta fibers; A-delta and C-fibers possess much smaller cell bodies. Local satellite glial cells form rings around the neuronal cell bodies.

1.2.2. Central Nervous System

The circuitry in the dorsal horn connects incoming primary afferents to outgoing projection neurons and is likely an important player in the development of central sensitization. The simplest nociceptive pathway is that of a primary afferent entering the dorsal horn and synapsing to a projection neuron. Projection neurons then pass contralaterally in the spinal cord and ascend via the spinothalamic track to the thalamus, which relays information to the somatosensory cortex and higher centers of cognitive and emotional experience. However, the dorsal horn is not a simple relay station but contains circuitry that can greatly modulate nociceptive information before relaying it to higher order brain centers. It is also a site of integration for descending input from the brain. The

complexity of the dorsal horn has been a major inhibitor of its study in nociception and maladaptive pain.

Innervation of the dorsal horn is organized along the rostral-caudal and mediolateral axes (Swett and Woolf 1985; Woolf and Fitzgerald 1986). Along the dorsal-ventral axis, the dorsal horn is organized into laminae with lamina I being the most superficial, IX being the most ventral and X wrapping around the central canal of the spinal cord (Rexed 1952). The secondary neurons of the superficial laminae can be differentiated into several populations according to their morphology and electrophysiological properties. Lamina I contains both projection and non-projection neurons, with most projection neurons displaying a transient firing pattern and local neurons showing a sustained firing pattern (Grudt and Perl 2002). Lamina I projection neurons target the thalamus, caudal ventrolateral medulla, lateral parabracial area, nucleus of the solitary tract, and the periaqueductal gray area (Gauriau and Bernard 2004; Gauriau and Bernard 2004). There is extensive collateralization, as some lamina I projection neurons extend processes to three of these areas (Al-Khater and Todd 2009). Lamina II contains five major types of neurons, as defined by electrophysiology and morphology (Grudt and Perl 2002). The majority of synaptic connections in lamina II are GABAergic between islet cells and transiently-firing central cells, which both receive monosynaptic input from C-fibers (Lu and Perl 2003). Central neurons of lamina II then excite outer lamina II vertical neurons receiving A-delta input, which can in turn excite lamina I neurons (Lu and Perl 2005). The wide dynamic range (WDR) neurons of lamina V receive convergent input from nociceptive and innocuous sources. They project to the thalamus and seem to provide information about sensory-motor integration (Craig 2004).

Under physiological conditions, the dorsal horn is responsible for maintaining a delicate balance of inhibitory and excitatory tone, and this balance is upset during pathology.

Nociceptive information is processed in a myriad of areas in the brain. PET scans and fMRI studies have shown activation of the thalamus, periaqueductal gray, secondary somatic and insular regions, anterior cingulate cortex, and variably in the primary somatosensory cortex (Peyron, Laurent et al. 2000). These hemodynamic responses to pain reflect the sensory, cognitive and emotional aspects of pain. However, how each of these areas contributes to the perception and control of pain remains to be determined.

There are three major pathways of descending inhibition from the brain that project to the dorsal horn. A serotonergic pathway projects from the nucleus raphe magnus and a noradrenergic pathway from the locus coeruleus. Both pathways terminate diffusely throughout lamina II and are thought to communicate via volume transmission (Zoli, Jansson et al. 1999). The third pathway projects from the rostral ventral medulla and makes GABAergic connections to lamina II neurons. Opioids are also involved in both the ascending and descending nociceptive pathways. Antinociception is thought to occur via the inhibition of ascending nociceptive transmission and the modulation of the descending pathways from the locus coeruleus and dorsal raphe nucleus (Basbaum and Fields 1984; Mansour, Fox et al. 1995; Porreca, Burgess et al. 2001). These serotonin, noradrenergic and opioid pathways are the major targets of most pain treatments and ongoing clinical trials.

Nociception is of vital importance for survival; thus, it has become a highly regulated pathway within the nervous system of humans. Nociceptive input elicits pain as well as emotional, neuroendocrine, and autonomic responses. The activity of this system results in activity-dependent plasticity or a progressive increase in the response of the system to

repeated stimuli (Woolf and Salter 2000). Long-term maladaptive pain has its roots in this activity-dependent plasticity through peripheral and central mechanisms of sensitization, though the degree of peripheral versus central contribution remains to be determined.

1.3. SENSITIZATION

1.3.1. Peripheral Sensitization

After peripheral nerve injury, extensive changes in the state of these primary afferents occur. Nerve injury results in the interruption of trophic factors from the innervated tissue and Wallerian degeneration. These activating signals result in lowered activation thresholds, spontaneous activity, and changes in gene expression in dorsal root ganglion (DRG) neurons and can lead to fundamental changes in physiology that underlie the pathology of maladaptive pain.

Peripheral nerve injury results in a major interruption of retrograde signals, particularly trophic signals from the tissue being innervated. For example, nerve growth factor (NGF) typically activates three signaling pathways: extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) (Kaplan and Miller 2000). In the DRG, NGF also activates the MAPK family members p38 and JNK (Ji, Samad et al. 2002; Mamet, Lazdunski et al. 2003). Peripheral nerve injury increases the phosphorylation of ERK, p38 and JNK, not only in the neurons of the DRG but also in their associated satellite cells, causing a local increase in inflammatory mediators (Kenney and Kocsis 1998; Jin, Zhuang et al. 2003; Obata, Yamanaka et al. 2003). Peripheral blockade of nerve growth factor (NGF) using anti-NGF antibodies elicits changes in neuropeptide expression in DRG neurons similar to that of neurons undergoing axonal injury (Shadiack, Sun et al. 2001). PI3K and ERK are required for NGF- induced hyperalgesia (Zhuang, Xu et al. 2004); however, the roles of p38 and JNK in NGF-induced hyperalgesia remain to be

investigated. NGF's effects are mainly effected through its receptor, TrkA, which is only expressed on the peptidergic population of C-fibers.

In 1850, Augustus Waller described the process following nerve transection involving an initial reaction at the site of injury followed by progressive degeneration and phagocytosis of the axons distal to the injury site (Stoll, Jander et al. 2002). This process is now known as Wallerian degeneration. Ongoing Wallerian degeneration distal to the site of the lesion involves the activation of Schwann cells and their release of cytokines to recruit nonresident macrophages. One of the best understood cytokines in this process is tumor necrosis factor α (TNFα). TNFα is released immediately following nerve injury by Schwann cells, mast cells, endothelial cells and fibroblasts. By three days post insult, macrophages invade the nerve injury releasing additional cytokines, chemokines and proteases, and clear the debris through phagocytosis (Stoll, Jander et al. 2002). This process of degeneration and clearing is closely related to the peak periods of hyperalgesia (Myers, Yamamoto et al. 1993). Blocking TNFα upregulation or the recruitment of macrophages can interfere with the rate and magnitude of Wallerian degeneration and the duration of the associated pain state (Myers, Campana et al. 2006). Activated Schwann cells can also phagocytose debris, but not with the same efficiency as macrophages. Tissue damage leads to the release of multiple chemical messengers that sensitize nociceptors, including tumor necrosis factor α, prostaglandins, protease-activated receptor 2, protons, histamine, adenosine triphosphate (ATP), bradykinin, nerve growth factor, serotonin and nitric oxide (NO) (Abbott, Hong et al. 1996; Bullock and Johnson 1996; England, Bevan et al. 1996; Koda, Minagawa et al. 1996; Handy and Moore 1998; Cesare, Dekker et al. 1999; Cook and McCleskey 2002; Kawabata, Kawao et al. 2002; Kawabata, Kawao et al. 2002; Tsuda, Shigemoto-Mogami et al. 2003; Baumann, Chaudhary

et al. 2004; Calixto, Medeiros et al. 2004). For instance, exposure to prostaglandin E2 (PGE2) activates PKA, leading to sensitization through an increased probability of TTX-R Na+ channel opening ($Na_v1.8$ and $Na_v1.9$) and a corresponding reduction in action potential threshold (Gold, Levine et al. 1998; Lai, Porreca et al. 2004). Even intact, non-injured nerves exposed to this milieu of sensitization factors can become hyperactive. This is well demonstrated by ventral rhizotomy, in which the only the ventral root, or motor component, of the spinal nerve is ligated while leaving sensory neurons intact. Sensory neurons contact the degenerating motor neurons but not the injury site; nonetheless, this model leads to neuropathic pain behavior in rats (Sheth, Dorsi et al. 2002). In the chronic constriction, partial sciatic nerve ligation and spinal nerve ligation injury models, the intact fibers are directly opposed to fibers undergoing Wallerian degeneration.

In the spared nerve injury model, the majority of degeneration occurs in anatomically separate compartments with two exceptions. The spared nerve injury model, as developed by Deocostard and Woolf in 2000 and adapted for mouse by Basbaum's group in 2003 (Decosterd and Woolf 2000; Shields, Eckert et al. 2003), entails exposing the sciatic nerve at the level of the thigh. The common peroneal and sural divisions of the sciatic nerve are tightly ligated. Then, a 5-mm section of nerve is removed below the ligature. In this case, the intact tibial nerve is not exposed to the Wallerian degeneration of the distal segments of the common peroneal and sural nerves; however, it is exposed to the inflammatory reaction occurring at the proximal nerve stump because all three nerves lie in close apposition at the level of the thigh.

Additionally, reinnervation of the deinnervated areas occurs over time. Within the first five days following nerve lesion, there is extensive degeneration until two weeks post-

injury. Two weeks to four months following lesion, regeneration and the remyelination of intact nerves occurs, though these nerves never remyelinate to their full extent (Guilbaud, Gautron et al. 1993). Growth into these territories could expose the tibial nerve to this same degenerative and sensitizing chemical milieu depending on the timing of degeneration and reinnervation. Although it has not been studied in the spared nerve injury model, using the chronic constriction injury model in the rat, distal skin reinnervation to pre-injury levels by peptidergic neurons has been shown to occur by four to eight weeks post lesion, and in non-peptidergic neurons, it occurs between 16 weeks and 1.5 years post lesion. Further, in these neurons, myelinated fibers never fully recover (Peleshok and Ribeiro-da-Silva 2011).

Neuropathic lesion leads to changes in chemical markers in the DRG, innervation patterns of peripheral tissue and electrical activity. In a model of spinal nerve injury in rats, the proportion of spontaneously active C and A-delta neurons increases, their activation thresholds to both thermal and mechanical stimuli are lowered, and they exhibit an enhanced response magnitude to suprathreshold thermal and mechanical stimuli (Shim, Kim et al. 2005). In some cases, primary afferents send action potentials traveling in the reverse direction back into the peripheral tissue, resulting in the local release of neuropeptides like substance P and neurokinin A (Cao, Mantyh et al. 1998). These molecules produce vasodilation, venule permeability, plasma extravasation, edema, and leukocyte influx. This process, known as neurogenic inflammation, only further excites local primary afferents. While many peripheral mechanisms may play a part in the generation and or early maintenance of neuropathic pain, long-term changes also occur in the central nervous system.

1.3.2. Central Sensitization

Nerve lesion is known to induce changes in both the peripheral and central nervous system. Increased long-lasting discharge of peripheral nociceptors induces plasticity and

changes in the spinal cord and brain, leading to increased responsiveness. This is referred to as central sensitization. Central sensitization probably developed as a defense mechanism specific to nociception that contributes maintaining the integrity of the organism by inducing hypersensitivity following frequent nociceptive afferent discharge. It results in an increase in spontaneous activity, a reduced threshold for activation of nociceptors, and an increase in the receptive field.

Described more than 30 years ago, dorsal horn plasticity was first demonstrated as wind-up; where repetitive stimulation of peripheral C-fibers produced increased magnitude and duration of action potentials in the dorsal horn (Mendell and Wall 1965). However, the phenomenon only lasts for seconds. Long-term potentiation (LTP), similar to that seen in hippocampus, was first demonstrated using high frequency stimulation (Liu and Sandkuhler 1995). Later, LTP was demonstrated using low-frequency stimulation of dorsal root at C-fiber intensity, which is akin to nociceptive input (Ikeda, Stark et al. 2006). Although the circuitry of the hippocampus and dorsal horn are quite different, glutamate is the predominant neurotransmitter in both, and LTP involves the activation of the NMDA receptor, trafficking of AMPA receptors, and the activation of similar kinases (Grosshans, Clayton et al. 2002; Ji, Kohno et al. 2003; Ikeda, Stark et al. 2006; Latremoliere and Woolf 2009). However, late-phase transcription-dependent LTP has not been demonstrated in the dorsal horn.

Central sensitization first occurs with changes in the threshold and activation kinetics of NMDA and AMPA receptors and changes in receptor trafficking. Increased primary afferent activity and the release of glutamate activates AMPA receptors depolarizing the cell. Depolarization removes the magnesium blockade, keeping the NMDA receptor inactive.

NMDA receptor activation then allows the influx of Ca^{2+}, activating signaling pathways including PKC, CaMK, PKA and PI3K. These signaling pathways can result in changes in receptor phosphorylation and trafficking. In the absence of further input, these changes are brief and will fade. However, the long-term, pathological activation of primary afferents results in central sensitization associated with decreases in descending inhibition, loss of injured neuronal connections, integration of A-beta fiber input into the nociceptive circuit, decreases in inhibitory neurotransmission, and glial activation.

After peripheral nerve injury, spontaneous activity from injured and non-injured primary afferents can initiate and maintain activity-dependent central sensitization. Alterations in gene expression of the primary afferents, including ion channels, receptors, and neurotransmitters, all produce increased excitatory neurotransmission. After peripheral nerve injury, primary afferents release glutamate as well as substance P and BDNF. On postsynaptic neurons, the activation of the NK-1 receptor by substance P and the Trk B receptor BDNF both lead to the activation of ERK. At the same time as excitatory drive increases from the periphery, higher brain centers increase descending excitatory drive from the rostral ventral medulla and decrease descending inhibition to the dorsal horn, resulting in further excitation of dorsal horn nociceptive neurons.

Structural changes also contribute to alterations in the synaptic function. Nerve injury leads to the loss of central terminals in the dorsal horn. The loss of synaptic input along with an increase in neurotrophic chemicals may allow sprouting of A-beta fibers, or excitatory interneurons with monosynaptic A-beta fiber input, from lamina III-V into lamina I and II to contact nociceptive neurons (Schoffnegger, Ruscheweyh et al. 2008). Immunostaining for c-fos and electrophysiological recordings show that post-nerve lesion A-beta fiber activation

can activate lamina I and II nociceptive neurons. Furthermore, while substance P expression is usually restricted to nociceptors, large diameter neurons in the DRG, usually A-beta afferents, have been reported to undergo a 'phenotypic switch' and begin to express substance P and BDNF, which further implicates their role in the maintenance of central sensitization (Todd 2010).

After partial nerve injury, there is a selective loss of GABAergic and glycinergic inhibition in lamina II (Moore, Kohno et al. 2002). Interestingly these same changes did not occur with complete nerve transection. There are conflicting results regarding whether this is due to the downregulation of neurotransmission or a loss of GABAergic neurons; however, these discrepancies may be due to technical differences (Polgar, Hughes et al. 2005; Polgar and Todd 2008). GABAergic transmission may also become less inhibiting during neuropathic pain states. Local increases in BDNF downregulate the K^+ Cl^- exporter 2 channels (KCC2) that help to maintain the neurons' low intracellular Cl^- concentration. A decrease in the number of KCC2 channels increases the intracellular Cl^- concentration. Consequently, the activation of $GABA_A$ receptors produces a diminished Cl^- current. Activated microglial cells seem to be the main producers of BDNF.

Nerve injury produces massive activation of both microglia and astrocytes in lamina I and lamina II, as well as infiltration by macrophages and T-cells. Microglia are the innate immune cell of the CNS and comprise 10-20% of its cell population (Farber and Kettenmann 2005). Microglia are highly dynamic and monitor their surroundings by continuously sampling the extracellular milieu (Hanisch and Kettenmann 2007). In addition to monitoring for cellular debris or pathogens, the typical role of innate immune cells, microglia are also capable of responding to neuro- or glial transmission through a diverse array of

neurotransmitter receptors including opioid, purine, glutamate, dopamine, acetylcholine, serotonin, adrenergic and GABA receptors (Pocock and Kettenmann 2007). Activation of microglia results in process retraction, from its previously ramified state, and hypertrophy (Streit, Walter et al. 1999). Functional changes associated with activation include increases in major histocompatibility complex (MHC) I and II, enhanced cytoplasmic ionoized calcium-binding adaptor molecule 1 (Iba1), increased surface expression of cluster of differentiation 11b (CD11B), activation of PI3K/Akt and MAPK, increased release of cytokines and chemokines, proliferation and migration (Nakajima and Kohsaka 2001). Separate from its antimicrobial activity, minocycline is microglial inhibitor. When given either before or after peripheral nerve injury, administration of minocycline inhibits the development of microgliosis and microglial p38 mitogen-activated protein kinase (MAPK) (Ledeboer, Sloane et al. 2005; Piao, Cho et al. 2006). However, only when given prior to, or within one day of, nerve injury does it prevent the development of allodynia (Raghavendra, Tanga et al. 2003; Ledeboer, Sloane et al. 2005). This implicates microglia in a role of development, but not the maintenance of, neuropathic pain.

Following peripheral nerve injury, astrocytes become reactive and hypertrophy as early as seven days post-neuropathic injury and remain in this state for the duration of behavioral allodynia, which is tested through four weeks post-injury (Tanga, Raghavendra et al. 2004). Blockade of astrocyte metabolism using fluorocitrate prevents astrocytes from becoming reactive and blocks pain behavior (Hassel, Paulsen et al. 1992; Milligan, Twining et al. 2003). Astrogliosis may result in the perturbation of the existing roles of astrocytes within a neuronal network (this is the topic of the following chapter) or in additional roles

specific to reactive astrocytes, such as the release of cytokines and chemokines, which can further sensitize surrounding neurons (this is the topic of chapter 4).

CHAPTER 2.

ASTROCYTE MODULATION OF EXCITATORY NEURONAL ACTIVITY IN THE CENTRAL NERVOUS SYSTEM

As animal systems became more complex, the nervous system evolved from providing simple reflexive behavior to being capable of elaborate cognitive tasks. As organisms increase in complexity, not only does the number of neurons increase, but there is also an explosive increase in the number of glia, as well as an increase in their morphological complexity. *Caenorhabditis elegans* consists of 302 neurons and 50 glial cells (Oikonomou and Shaham 2011). Only four of these glia mimic astrocyte morphology in that they extend sheet-like processes that surround the nerve ring and ensheathe a small number of synapses (Oikonomou and Shaham 2011). Reticular or "astrocyte-like glia" in *Drosophila* form a network of glial tissue that surrounds terminal axons, dendrites and synapses (Pereanu, Shy et al. 2005; Awasaki, Lai et al. 2008; Doherty, Logan et al. 2009; Spindler, Ortiz et al. 2009). The ratio of glial cells to neurons increases with increasing nervous system complexity; this ratio is approximately 10:90 in invertebrates, whereas, in vertebrates, this ratio is approximately 50:50 (Hartline 2011). The volume of a cortical rodent astrocyte ranges from 14,700 and 22,906 μm^3 (Bushong, Martone et al. 2002; Ogata and Kosaka 2002; Chvatal, Anderova et al. 2007; Halassa, Fellin et al. 2007). In human brain samples, protoplasmic astrocytes of the neocortex are approximately 2.6 times larger and extend 10 times more processes than those in the rodent brain (Oberheim, Takano et al. 2009). While the cortical synaptic density between rodents and humans is similar—1397 versus approximately 1100

synapses/mm^3 respectively (DeFelipe, Alonso-Nanclares et al. 2002), the domain range of astrocytes has increased dramatically. The domain of a rodent astrocyte covers 20,000 to 120,000 synapses, and human astrocytes can cover from 270,000 to 2 million synapses (Bushong, Martone et al. 2002; Oberheim, Takano et al. 2009).

For many years, astrocytes were relegated to the role of supportive cells in the central nervous system, providing structural and metabolic support, removal of neurotransmitters from the synapse and buffering extracellular potassium levels. However, over the last 30 years, their role has developed from that of a silent supportive cell to that of an active partner at the synapse, with important roles in both physiology and pathophysiology. Astrocytes provide energy to surrounding neurons and modulate the formation and efficiency of synapses (Pfrieger and Barres 1996; Pfrieger and Barres 1997; Smith 1998). They regulate extracellular glutamate concentrations through glutamate transporters (Rothstein, Martin et al. 1994; Chaudhry, Lehre et al. 1995). Astrocytes likely communicate with one another through intercellular calcium signaling via gap junctions (Parpura, Basarsky et al. 1994; Porter and McCarthy 1996; Verkhratsky and Kettenmann 1996; Vernadakis 1996) and may release gliotransmitters, such as glutamate, D-serine and ATP, in a calcium-dependent manner. They are an integral part of the central nervous system, and their positioning is essential for their function.

2.1. TRIPARTITE SYNAPSE STRUCTURE.

Astrocytes have intricate spongiform morphology with fine terminal processes that interact with nearby synapses, vasculature and neighboring astrocytes. While the cell body is roughly 600 μm^3 of an astrocyte's domain, the area that its processes spread through the neuropil, is much larger, 66,000 μm^3 (Bushong, Martone et al. 2002). The location and distribution of astrocyte processes is important for regulating the extracellular milieu and

limiting neurotransmitter diffusion. On average, a single cortical astrocyte domain surrounds four neuronal cell bodies and hundreds of dendrites (Halassa, Fellin et al. 2007). In the CA1 region of the hippocampus, it has been estimated that a single astrocyte domain engulfs 140,000 synapses (Bushong, Martone et al. 2002). Thus, an astrocyte has the potential to interact with many nearby neurons. This architecture has led to the development of the tripartite synapse model. Structurally, it is composed of the presynaptic terminal, the postsynaptic bouton and the ensheathing astrocyte process. The percentage of contacted synapses as well as the degree of ensheathment varies dramatically from brain region to brain region. In the cerebellum, nearly all of the parallel and climbing fiber synapses are contacted by astrocyte processes (Spacek 1985). Of contacted climbing fiber synapses the median ensheathment by astrocytes is 94% of the synapse perimeter. In the parallel fiber synapses population, the median ensheathment dropped to 67% (Xu-Friedman, Harris et al. 2001). In the hippocampus, only 57% of synapses are contacted by astrocytes. Of these synapses, the astrocyte processes only ensheathed an average of 43% of the synaptic interface (Ventura and Harris 1999). At the synaptic glomeruli of the olfactory bulb and thalamus, astrocytes processes form multi-lamellar sheets surrounding the entire poly-synaptic unit (Spacek and Lieberman 1974; Chao, Kasa et al. 1997). This nonuniform distribution of astrocyte processes raises the question of whether the distribution is random or whether processes are growing toward specific synapses.

In cell culture models, astrocytes extend processes toward synapse related molecules, including glutamate (Hatten 1985; Cornell-Bell, Thomas et al. 1990; Matsutani and Yamamoto 1997). Using organotypic slice culture, astrocyte process interaction with dendritic spines has been shown to be highly dynamic, with processes rapidly extending and

retracting (Haber and Murai 2006). This movement is neuronal activity-dependent, where neuronal activation encourages astrocyte encroachment of the synaptic zone (Genoud, Quairiaux et al. 2006). Ultrastructural studies in the mouse whisker barrel cortex have shown that 24 hours of whisker stimulation causes a significant increase in the astrocytic envelopment of excitatory synapses on dendritic spines (Genoud, Quairiaux et al. 2006). This work suggests that the amount of glutamate in the synapse is directly correlated to the degree of astrocyte coverage. The fine astrocytic processes covering a synapse can be very narrow, less than 50 nm wide, and below the resolution of light microscopy. The determination of the degree of synaptic ensheathment by an astrocytes process requires serial section electron microscopy (EM). Release probability at individual synapses is largely regulated by the size of the readily releasable vesicle pool, which can be determined at the EM level (Harris and Sultan 1995; Murthy, Schikorski et al. 2001; Dobrunz 2002; Branco, Marra et al. 2010). Though no published work has directly correlated the number of docked synaptic vesicles to the degree of astrocyte ensheathment at individual synapses, analysis of the average percentage of astrocyte process coverage at specific varieties of synapses is correlated to the relative number of docked vesicles or the known release probability of a given variety of synapse.

Astrocyte processes are now considered an integral third component of synapses along with the pre- and postsynaptic terminals. The positioning of astrocyte processes is key in determining the degree to which astrocytes can participate in signaling via the tripartite synapse model. Contact between astrocyte processes and synaptic elements have been shown to modify the structural and physiological properties of synapses. Proximity to presynaptic release sites greatly affects the astrocyte's ability to remove glutamate or other

neurotransmitters from the synaptic cleft. Furthermore, astrocytes may release gliotransmitters including glutamate, D-serine and ATP to directly participate in neuronal signaling. The molecular pathways that allow astrocytes to communicate with synaptic compartments are only beginning to be understood.

Table 1. The relationship between release probability and astrocyte synapse coverage.

Synapse	Release Probability	Average % Astrocyte Coverage	Reference
Cerebellar Climbing Fibers	High	87%	Xu-Friedman, 2001
Cerebellar Parallel Fibers	Low	67%	Xu-Friedman, 2001
Hippocampal Perforated Synapses	Many docked vesicles	80%	Ventura & Harris, 1999
Macular Synapses	Few docked vesicles	50%	Ventura & Harris, 1999

2.2. SYNAPSE FORMATION AND STRENGTH

Astrocytes are involved in the development, maturation and stabilization of synapses. During synaptogenesis, astrocytes can release cholesterol and thrombospondins to increase synapse number and promote synapse maturation (Ullian, Sapperstein et al. 2001; Christopherson, Ullian et al. 2005). Conversely, the interaction of astrocytic ephrin A3 with EphA4 receptors on dendritic spines leads to spine retraction, reducing spine number and size (Murai, Nguyen et al. 2003; Bourgin, Murai et al. 2007; Fu, Chen et al. 2007) (Zhou, Martinez et al. 2007). Synaptic strength can be modulated both by ephrin A3-induced modifications of glutamate transport and TNFα induced changes in AMPA receptor trafficking on the post-synaptic membrane.

2.2.1. Thrombospondins

Thrombospondins are a family of five large extracellular matrix proteins that can mediate cell-cell and cell-matrix interactions through communication with membrane receptors, other extracellular matrix proteins and cytokines (Adams and Lawler 2004). Thrombospondins 1 and 2 are the most studied members of this family. They are secreted by astrocytes during early postnatal ages when the majority of excitatory synapses are forming (Christopherson, Ullian et al. 2005), as well as after ischemic injury (Lin, Kim et al. 2003; Liauw, Hoang et al. 2008) and after spinal cord injury (Wang, Chen et al. 2009). After ischemic stroke, thrombospondin-1/2-deficient mice show deficits in synaptic plasticity and functional recovery (Liauw, Hoang et al. 2008). In culture, thrombospondin-induced synapse formation produces ultrastructurally normal synapses; however, these synapses are postsynaptically silent due to a lack of surface AMPA receptors (Christopherson, Ullian et al. 2005). Both in early post-natal stages and after injury, astrocytes appear to take on an immature form. Thrombospondins are thought to work together with another yet unidentified molecule secreted from astrocytes in order to produce functional synapses.

α2δ-1 is a neuronal thrombospondin receptor involved in central nervous system synaptogenesis (Eroglu, Allen et al. 2009). It is a subunit of the voltage gated L-type calcium channel and is thought to play a role in channel regulation and trafficking. It is also a high-affinity receptor for gabapentin and pregabalin, which are anticonvulsant drugs often prescribed for neuropathic pain conditions (Marais, Klugbauer et al. 2001; Field, Cox et al. 2006). α2δ-1 is expressed both presynaptically and postsynaptically in the dorsal horn (Li, Song et al. 2004). After spinal nerve injury, presynaptic α2δ-1 subunit expression increases and is temporally correlated with the development of neuropathic allodynia (Luo, Chaplan et al. 2001; Luo, Calcutt et al. 2002; Li, Song et al. 2004). The administration of α2δ-1

antisense oligonucleotides blocks the upregulation of α2δ-1 and decreases mechanical allodynia (Li, Song et al. 2004; Li, Zhang et al. 2006). Post neuropathic injury, thrombospondin 4 is upregulated in dorsal root ganglion neurons (Valder, Liu et al. 2003). Thrombospondin 4 can also bind α2δ-1 through its EGF-like domain and is secreted by astrocytes; however, its role in the pathophysiology of pain has yet to be studied.

2.2.2. Ephrins

Binding of the Eph receptor and ephrin from distinct cell surface membranes induces a bidirectional signaling cascade that alters cell adhesion and provokes actin cytoskeletal rearrangement. Ephrins A and B, as well as Eph receptors, are broadly expressed by neurons in the developing and mature central nervous system (Murai and Pasquale 2011). Ephrin-A3 becomes enriched in astrocyte processes within the first few weeks of post-natal development (Stein, Savaskan et al. 1999; Carmona, Murai et al. 2009; Galimberti, Bednarek et al. 2010). Ephrin-A3 activates EphA4 on dendritic spines and regulates the functional properties of astrocytes. The activation of EphA4 leads to retraction of the dendritic spine and to a reduction in their number and size (Murai, Nguyen et al. 2003; Bourgin, Murai et al. 2007; Fu, Chen et al. 2007; Zhou, Martinez et al. 2007). While other ephrin and Eph receptor signaling pairs may be involved, most research has focused on ephrin-A3 and EphA4 pairing.

In ephrin-A3 knockout mice, EphA4 tyrosine phosphorylation (an indication of its level of activity) is greatly reduced in the hippocampus and elevated in transgenic mice overexpressing ephrin-A3 in astrocytes (Filosa, Paixao et al. 2009). Upon activation, EphA4 phosphorylates and complexes with Cdk5 to bind and activate the guanine nucleotide exchange factor Ephexin1. Ephexin1 activates RhoA, which is required to decrease spine size and synapse number. EphA4 tyrosine phosphorylation also signals through phospholipase Cγ1 to activate the phosphatidylinositol 4,5 bisphosphate (PIP_2) pathway to

produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. These mobilize calcium signaling and promote protein kinase C (PKC) activation. EphA4 signaling reduces the membrane association of F-actin severing and depolymerization factor cofilin (Zhou, Martinez et al. 2007). Coflin is downstream of both RhoA signaling (Bernstein and Bamburg 2010) and the cdk5-Ephexin1 PLCγ1 pathways. These pathways may concomitantly regulate the activity of cofilin to modify the architecture of actin filaments in spines (Murai and Pasquale 2011).

EphA4 signaling also regulates adhesion receptors critical for dendritic spine maintenance. This EphA4 signaling pathway disrupts β1 integrin function in neurons, promoting spine retraction and loss (Bourgin, Murai et al. 2007). This cell contact-dependent communication between astrocyte processes and neurons may maintain the architecture of the synapse by restricting the dendritic spine's ability to change shape (Murai, Nguyen et al. 2003).

Forward EphA4 signaling in dendritic spines activates pathways that promote the modification of the actin cytoskeleton and adhesion molecules important for the maintenance of spine density and morphology. Additionally, forward EphA4 signaling could modulate synaptic strength. In cultured neurons, EphA4 modulates the cell surface expression of GluR1-containing AMPA receptors (Fu, Hung et al. 2011). Stimulation of EphA4 promotes its interaction with the anaphase-promoting complex (APC), enabling Cdh1 to bind and polyubiquinate GluR1. Polyubiquitination and degradation of GluR1 decreases synaptic efficacy.

The binding of ephrins to their Eph receptor not only promotes forward signaling downstream of EphA4 but also reverse signaling in the Ephrin-A3 expressing cell. The

importance of Ephrin-A3 reverse signaling in astrocytes has been demonstrated *in vivo.* Transgenic mice overexpressing ephrin A3 under the astrocytic promoter GFAP have reduced GLT-1 and GLAST immunolabeling (Filosa, Paixao et al. 2009). Ephrin A3 knockout mice have increased immunolabeling for glial glutamate transporters GLT1 (EAAT2) and GLAST (EAAT1) in the hippocampus and cortex and an increase in glial glutamate transporter currents measured by patch-clamp in acute hippocampal slices (Carmona, Murai et al. 2009; Filosa, Paixao et al. 2009). Treatment of wild-type, but not ephrin-A3 knockout, slices with EphA2 FC fusion protein to promote reverse ephrinA-A3 signaling also reduces glutamate uptake. Interestingly, both ephrin A3 and EphA4 knockout mice exhibit a reduction in long-term potentiation (LTP) following theta-burst stimulation but not tetanic stimulation (Filosa, Paixao et al. 2009). The reduction of LTP following theta-burst stimulation can be rescued by the pharmacological blockade of glutamate transporters, arguing that the increased levels of glutamate transporters observed in ephrin A3 and EphA4 knockout mice are clearing glutamate too efficiently from the synapse to allow for the development of LTP. Tetanic stimulation, which results in higher levels of glutamate release, may overwhelm even the increased population of glutamate transporters. However, rhythmic bursting, similar to theta bursting, of CA1 neurons is observed when animals explore novel environments (Winson 1978; Otto, Eichenbaum et al. 1991; O'Keefe 1993).

The mechanisms of ephrinA3-mediated down-regulation of glutamate transporters are unknown. Ephrin-A3 and EphA4 knockout animals have similar levels of GLT1 and GLAST mRNA; thus, the effects are most likely post-translational. GLT1 and GLAST colocalize with ephrin-A3 on astrocytic processes, so perhaps their interaction depends on their proximity (Carmona, Murai et al. 2009).

Synaptic activity may promote the trafficking of EphA4 to the membrane, allowing EphA4 activation and the downregulation of synaptic GluR1, as well as the mobilization of the cytoskeleton and adhesion molecules, which acts as a homeostatic mechanism to stabilize activity following bursts of neuronal network activity. Alternatively, synaptic activity also drives the cleavage of EphA4, which could lead to an increase in the number of dendritic spines. While the efficacy of the extracellular fragment of EphA4 in binding to ephrin-A3 has not been determined, it would be expected to inhibit ephrin reverse signaling, thereby enhancing glutamate transport via GLT1 and GLAST transporters and decreasing synaptic activity (Murai and Pasquale 2011). *In vivo*, prolonged synaptic activity has been shown to increase glial glutamate transporter levels as well as synaptic coverage by astrocyte processes (Genoud, Quairiaux et al. 2006).

Ephrin A3 signaling has not been studied in the dorsal horn in the context of chronic pain; however, spinal cord injury, which is also associated with chronic pain, causes an increase in EphA receptor expression on both oligodendrocytes and astrocytes (Willson, Irizarry-Ramirez et al. 2002). The remodeling of synapses in the dorsal horn post injury is likely to require astrocyte process movement and dendritic spine remodeling, making ephrins another good target for study using neuropathic pain models.

2.2.3. TNFα

Long-term changes in a cell's synaptic activity lead to adjustments in the strength of all the synapses on that cell. This form of synaptic plasticity is known as synaptic scaling and is thought to be crucial for preventing neural networks from becoming unstable (Buckby, Jensen et al. 2006). Synaptic scaling used to be thought of as a cell autonomous process because it could be induced by the hyperpolarization of individual neurons and independent of glutamate receptor signaling (Turrigiano 2006). However, Stellwagen and Malenka found

that a drop in neuronal activity, as induced by a prolonged activity blockade by TTX, triggers the release of TNFα from glial cells. TNFα signaling released from glia can induce the trafficking of AMPA receptors to postsynaptic sites to increase excitatory synaptic strength (Beattie 2002). Conversely, blocking TNFα has the opposite effect (Beattie 2002). Soluble TNFα acts to compensate for the decrease in activity by increasing the level of cell-surface AMPA receptors (Stellwagen and Malenka 2006). Glial-derived TNFα containing media was prepared using mixed wild-type glial and TNFα KO neuronal cell cultures in which activity was blocked using TTX. This media was then applied to hippocampal slices prepared for electrophysiological recording. Interestingly, medium from cultures treated with TTX for 24 hours did not increase synaptic strength; it required 48 hours of conditioning before the effect was produced. This finding raises the possibility that TNFα is a factor in long-term synaptic scaling and that there may be other factors involved at shorter time-scales.

Several lines of evidence suggest a role for TNFα in the production of central sensitization in the dorsal horn as well. TNFα is induced in spinal cord glial cells after neuropathic lesions (DeLeo, Colburn et al. 1997; Xu, Xin et al. 2006; Hao, Mata et al. 2007). Intrathecal injection of TNFα induces thermal hyperalgesia and mechanical allodynia (Kwon, Shim et al. 2005; Kawasaki, Zhang et al. 2008; Gao, Zhang et al. 2009). Intrathecal injection of a TNFα inhibitor inhibits behavioral sensitivity following peripheral nerve injury (Sommer, Schafers et al. 2001; Schafers, Svensson et al. 2003; Marchand, Tsantoulas et al. 2009). Additionally, the application of TNFα to spinal cord slices increases spontaneous EPSC frequency but not EPSC amplitude (Kawasaki, Zhang et al. 2008; Youn, Wang et al. 2008; Li, Xie et al. 2009; Zhang, Berta et al. 2011). While this may argue for a presynaptic mechanism, the circuitry of the dorsal horn is complex, and the effect may not be mediated

by the cells being recorded from. More detailed studies will be needed to determine the role of TNFα in the dorsal horn, particularly after neuropathic lesions.

2.3. NEUROTRANSMITTER UPTAKE

In vitro and in situ astrocytes have been shown to express a wide variety of neurotransmitter transporters including glutamate, GABA, adenosine and dopamine. Glutamate transporters, or excitatory amino acid transporters (EAATs), are among the most studied family of neurotransmitter uptake transporters. Glia express EAAT1 and EAAT2, neurons express EAAT 3 and EAAT4, and EAAT5 is found exclusively in the retina. In rodents, the orthologs for EAAT1-3 are named glutamate aspartate transporter (GLAST), glutamate transporter 1 (Glt-1) and excitatory amino acid carrier 1 , whereas the titles of EAAT4 and 5 are conserved. While both neurons and astrocytes express glutamate transporters, the astrocytic glutamate transporters, particularly Glt-1, are the predominant transporters in most brain areas (Danbolt 2001).

The role of neurotransmitter uptake has been found to be much more complex than that of a simple drainage system and plays a role in the modulation of synaptic transmission. The kinetics of excitatory postsynaptic currents (EPSCs) are dependent on the time course of glutamate in the synaptic cleft (Trussell 1998; Jonas 2000). Factors that influence synaptic glutamate concentrations include the amount and rate of glutamate release from presynaptic sites, the release location, the glutamate diffusion coefficient, the microstructure of the synapse, the affinity of glutamate binding to receptors and glutamate uptake by transporters (Clements and Rose 1996; Anderson and Swanson 2000; Danbolt 2001). The microstructure of the synapse is, in part, determined by the astrocyte envelopment of the synaptic cleft, which differs among CNS regions. Synapses between Schaffer collaterals and hippocampal pyramidal cells have little glial covering, and the inhibition of glutamate transporters has

little effect on the decay of evoked EPSCs (Isaacson and Nicoll 1993; Ventura and Harris 1999). In contrast, in the synapses between the parallel and climbing fibers on Purkinje cells in the cerebellum, glutamate diffusion is limited by astrocyte ensheathment, and the inhibition of glutamate transport increases the duration of glutamatergic transmission to the postsynaptic neuron (Overstreet, Kinney et al. 1999).

Examination of BAC-generated Glt-1-eGFP/GLAST-DsRed double transgenic mice shows that in most areas of the brain, Glt-1 and GLAST are coexpressed in astrocytes, whereas the spinal cord has largely segregated populations of Glt-1- and GLAST-positive astrocytes (Regan, Huang et al. 2007). In neurons of the dorsal horn, glutamate uptake by Glt-1 contributes to the termination of EPSCs (Weng, Chen et al. 2007; Xin, Weng et al. 2009; Zhang, Xin et al. 2009). Immunohistochemical and western blot studies have shown an initial increase followed by a long-term decrease in glutamate transporters after nerve injury (Xin, Weng et al. 2009). This timeline correlates with astrocyte hypertrophy and the chronic phase of pain observed in neuropathic pain models (Vega-Avelaira, Moss et al. 2007). However, the decrease in transporter protein may be associated with the decrease in primary afferent innervations and may not generate a lack of synaptic glutamate clearance at intact primary afferent fibers.

2.4. CALCIUM MEDIATED RELEASE OF GLIOTRANSMITTERS FROM ASTROCYTES

Unlike neurons, astrocytes do not respond to stimuli with action potentials, leading earlier researchers to believe that astrocytes were “silent cells”. However, this viewpoint shifted dramatically when astrocytes in culture and *in situ* were shown to have a wide variety of G protein-coupled receptors (GPCRs) (McCarthy and de Vellis 1978; van Calker, Muller et al. 1978; Porter and McCarthy 1997). The most widely studied astrocytic GPCR pathway

is the canonical G alpha q-coupled pathway that elicits increases in intracellular calcium. Upon activation, Gαq activates phospholipase C (PLC). PLC then hydrolyzes the membrane lipid phosphatidylinositol 4,5 bisphosphate, cleaving it into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG remains bound to the membrane, whereas IP_3 is released into the cytosol where it can activate the IP_3 receptor on the endoplasmic reticulum. Activation of IP_3 receptors causes the release of calcium from intracellular stores. Of the three subtypes of IP_3 receptors known, astrocytes are only known to possess IP_3 receptor subtype 2 (IP_3R2). Conditional knockout of IP_3R2 under the GFAP promoter abolished astrocyte calcium oscillations and increases in response to Gq GPCR agonists or neuronal stimulation (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010).

The Gq-IP3 receptor pathway elicitation of calcium increases is thought the most prevalent physiological method of increasing intracellular calcium in astrocytes. Cultured astrocytes express voltage-gated calcium channels and P2X7 receptors (Panenka, Jijon et al. 2001; Latour, Hamid et al. 2003; D'Ascenzo, Vairano et al. 2004); however, there is little functional evidence of these channels *in situ*. Specific subpopulations of astrocytes, such as Bergmann glia and complex cells, have been found to display voltage-gated calcium channels as well as AMPA receptors; however, these subpopulations seem to be the exception rather than the rule (Burnashev, Khodorova et al. 1992; Hamilton, Vayro et al. 2010). *In vitro*, *in situ* and *in vivo* astrocytes respond to neuronal stimulation with calcium elevations attributed to the activation of their wide range of Gq-coupled GPCRs (Agulhon, Petravicz et al. 2008).

Increases in intracellular calcium can elicit many changes in cells, some permissive and others active. In the astrocyte field, research has focused on the calcium-mediated release

of gliotransmitters. In neuronal cells, voltage-gated calcium channels at the presynaptic membrane open in response to voltage changes, triggering an influx of calcium over a relatively small microdomain. This calcium elevation causes the release of neurotransmitter-filled vesicles into the synaptic cleft via the soluble NSF attachment protein receptor (SNARE) complex. In ultrastructural studies, astrocytes have been found to contain sparsely distributed vesicles complete with vesicular glutamate transporters 1 or 2 as well as the vesicular SNARE proteins cellubrevin, SNAP-25 and syntaxin (Bezzi, Gundersen et al. 2004; Wilhelm, Volknandt et al. 2004; Zhang, Pangrsic et al. 2004; Montana, Malarkey et al. 2006). However, much of this work was completed in cultured astrocytes. The presence of vesicular machinery provides the basis for many studies supporting the role of astrocytes in the active modulation of neuronal transmission via gliotransmission.

Astrocytes are thought to listen to neuronal activity via their GPCRs, to respond via calcium signaling and to in turn affect neuronal transmission via the vesicular release of gliotransmitters, including glutamate, adenosine triphosphate (ATP) and D-serine (Halassa, Fellin et al. 2007). Gq GPCR stimulation triggers the release of glutamate (Parpura, Basarsky et al. 1994; Pasti, Volterra et al. 1997; Araque, Parpura et al. 1998), and this glutamate can activate presynaptic mGluR receptors (Fiacco and McCarthy 2004; Andersson, Blomstrand et al. 2007) or postsynaptic NMDA receptors (Pasti, Zonta et al. 2001; Angulo, Kozlov et al. 2004; Fellin, Pascual et al. 2004; Jourdain, Bergersen et al. 2007). ATP may also be released from astrocytes in a calcium- and SNARE-dependent manner. Cultured astrocytes release ATP, which is rapidly converted to adenosine, in response to Gq GPCR-mediated calcium increases (Pangrsic, Potokar et al. 2007). Interfering with the vesicular release of ATP through the transgenic expression of dominant-negative SNARE protein (dnSNARE)

selectively in astrocytes reduced long-term potentiation in the hippocampus and interfered with the formation of heterosynaptic depression (Pascual, Casper et al. 2005; Serrano, Haddjeri et al. 2006). Astrocytes release D-serine in culture (Schell, Molliver et al. 1995; Mothet, Pollegioni et al. 2005) and are thought to be the sole source of D-serine in the brain (Schell, Brady et al. 1997; Oliet and Mothet 2006). In the supraoptic nucleus, D-serine levels change as a function of the astrocytic coverage of neuronal synapses and affect both LTP and LTD (Panatier, Theodosis et al. 2006).

Astrocytes display the same receptor milieu of Gq GPCRs as neurons; therefore, the selective stimulation of astrocyte signaling, while not perturbing neuronal function, is a major hurdle for all studies on gliotransmission. The selective stimulation of astrocyte signaling has been achieved using methods as crude as mechanical stimulation of the cell, which has a high potential of damaging the cell membrane, to the more commonly used photolysis of caged calcium or IP_3 molecules delivered by patch pipette. Although these techniques have been used in numerous studies and increase calcium selectively in astrocytes, the calcium response is spatially and temporally very distinct from endogenous responses. Fiacco et al. (2007) used a transgenic mouse model in which the expression of the MrgA1 receptor, a Gq-coupled GPCR normally found only in a specific subset of primary nociceptive neurons and not in the brain, was driven by an inducible astrocyte-specific promoter. Selective activation of astrocyte Gq GPCR signaling was achieved in acute hippocampal slices through the bath application of the MrgA1 ligand. Additionally, the calcium activation spatially and temporally matched that of endogenous ligands. This highly selective activation of astrocyte intracellular calcium was unable to reproduce the effects

observed with other methods, such as calcium or IP_3 uncaging (Fiacco, Agulhon et al. 2007). This study therefore does not support the current model of gliotransmission.

The Gq GPCR-mediated release of gliotransmitters relies on the activation of IP_3R2 to release calcium from internal stores. IP_3R2 knockout mice show abolished intrinsic calcium oscillations as well as agonist- or neuronal stimulation-evoked calcium increases in astrocytes, while leaving neuronal calcium responses intact (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010). Contrary to the current model of gliotransmission, the lack of calcium signaling in astrocytes had no effect on basal excitatory neurotransmission or on short- or long-term potentiation in the hippocampus (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010). This series of papers does not support the current model of gliotransmission. More work will need to be done in the field in order to resolve these differences.

2.5. THE IMPACT OF THE PHYSIOLOGICAL ROLES ASTROCYTES ON NEUROPATHIC PAIN

Astrogliosis has been considered a marker for stress in the central nervous system since the early 1900s (Ramon y Cajal 1928). Astrocyte activation, or astrogliosis, is seen visualized by hypertrophy of the cell body and initial large processes using immunohistochemical staining for GFAP and S100beta. These morphological changes have been linked to numerous diseases and insults, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, alcoholism and physical trauma. Astrocyte hypertrophy is accompanied by increased release of cytokines and chemokines and changes in glutamate and glycine transporter levels (Cavaliere, Cirillo et al. 2007). There is no single molecular profile of a reactive astrocyte that is uniform through the conditions that produce astrogliosis.

Under conditions of chronic neuropathic pain, astrogliosis begins as early as 7 days post-neuropathic injury and remains for the duration of behavioral allodynia, through 4 weeks (Tanga, Raghavendra et al. 2004). Astrogliosis may result in added roles of astrocytes, such as the release of cytokines and chemokines, which can further sensitize surrounding neurons. Alternately, astrogliosis may perturb of the existing roles of astrocytes within a neuronal network. Studies have shown that the inhibition of these changes alleviates the behavioral sensitization that occurs in chronic pain (Sweitzer, Colburn et al. 1999). Transgenic inhibition of inflammatory pathways selectively in astrocytes lessens behavioral sensitization following formalin administration and chronic constriction injury (Fu, Zhang et al. 2007; Fu, Zhang et al. 2010). Decreases in glutamate transporter expression following the development of astrogliosis (Sung, Lim et al. 2003; Binns, Huang et al. 2005) may account for the increase in glutamate levels in the spinal cord during chronic pain and may produce the AMPA and NMDA receptor activation underlying the hyperexcitability of secondary pain transmission neurons (Cavaliere, Cirillo et al. 2007). Furthermore, the administration of glial metabolic inhibitors decreases behavioral hypersensitivity after the induction of neuropathic pain (Tawfik, Nutile-McMenemy et al. 2007).

First, we examined the tripartite synapse structure of primary afferents in the dorsal horn to determine whether astrocytes were in a position to regulate excitatory neuronal transmission in the dorsal horn. Astrocyte interactions with synapses can affect their formation, stability and strength through cell contact and secreted factors. Astrocytes can also modulate neuronal signaling through neurotransmitter transporters and the release of gliotransmitters. However, their active participation in neurotransmission is dependent upon their ensheathment of neuronal synapses. Determining astrocytic ensheathment around

nociceptive synapses is an important first step for the field in determining the possible astrocyte contribution to pain processing. There is considerable variability between brain regions in synaptic coverage by astrocyte processes, and this variability even extends to the synapse type within a single region. As blockade of the astrocyte glutamate transporter Glt-1 affects dorsal horn EPSCs, I hypothesize that astrocyte processes will contact synapses in the dorsal horn.

Next, we chose to investigate the role of gliotransmission in the dorsal horn through the use of transgenic mouse models blocking the calcium-dependent and/or SNARE-dependent release of gliotransmitters from astrocytes in both naïve and nerve lesioned animals. Using these transgenic mouse models, our laboratory has shown a lack of evidence for gliotransmission in the hippocampus. However, after nerve lesion, astrocytes become reactive and hypertrophy. This regression to a more immature or reactive state may be similar to the state of cultured astrocytes. In culture, there is a great deal of evidence, from our laboratory as well as others, supporting the release of gliotransmitters and their effect on nearby neurons. We hypothesize that blocking the calcium- and/or SNARE-dependent release of gliotransmitters from astrocytes will alter the behavioral response of animals to nerve lesion.

CHAPTER 3.

TRIPARTITE SYNAPSE STRUCTURE SURROUNDING GLOMERULAR SYNAPSES OF INCOMING PRIMARY AFFERENTS

3.1. OVERVIEW

Astrocytes interact with synapses in every studied area of the central nervous system. Their roles include shaping the microstructure of the synapse, physically insulating the synapse, glutamate transport, potassium clearance, metabolic support and possibly gliotransmission. Key to their involvement in neurotransmission is their position near the synaptic cleft. Increasingly, tripartite synapse structure is being seen as the new functional unit of neurotransmission in the brain. In the dorsal horn of the spinal cord, nociceptive primary afferents form glomerular synapses with multiple pre- and postsynaptic cells. We used serial-section block-face tomography to reconstruct 3-dimensional (3-D) images of C1- and C2-type glomerular synapses and their associated astrocyte processes. We found that one hundred percent of the glomerular synapses in the dorsal horn were contacted by astrocyte processes. Furthermore, the astrocytes provided a high degree of ensheathment of these synapses, surrounding, on average, 87 ± 3.16% of each C1-type synapse and 77.20 ± 6.98% of each C2-type synapse.

3.2. INTRODUCTION

Astrocytes have intricate spongiform morphology with fine terminal processes that interact with nearby synapses, vasculature and neighboring astrocytes. While the astrocyte cell body comprises approximately 600 μm^3 of an astrocyte's domain, the area covered by its

processes, which spread through the neuropil, is much larger (66,000 μm^3) (Bushong, Martone et al. 2002). On average, a single cortical astrocyte domain surrounds four neuronal cell bodies and hundreds of dendrites (Halassa, Fellin et al. 2007). In the CA1 region of the hippocampus, it has been estimated that a single astrocyte domain contacts 140,000 synapses (Bushong, Martone et al. 2002). In humans, a single astrocyte may contact more than one million synapses (Oberheim, Wang et al. 2006). Thus, an astrocyte has the potential to interact with many nearby neurons.

The dorsal horn of the spinal cord is the first site in the central nervous system where nociceptive information from the periphery is processed. Incoming nociceptive primary afferents are divided into three types of fibers—peptidergic and nonpeptidergic unmyelinated C fibers and lightly myelinated A-delta fibers—each of which has its own termination zone. The peptidergic C fibers are known for their release of the neuropeptides substance P, calcitonin gene-related peptide (CGRP) and somatostatin and form non-glomerular type synapses in lamina I (Zylka, Rice et al. 2005; Cavanaugh, Lee et al. 2009). The nonpeptidergic C fibers are IB4 positive and teminate in outer lamina II (Woodbury, Ritter et al. 2000; Cavanaugh, Lee et al. 2009). A-delta fibers, 20% of which are also peptidergic, project to inner lamina II (McCarthy and Lawson 1989). At the ultrastructural level, peptidergic neurons form non-glomerular synapses, while the nonpeptidergic and A-delta neurons form glomerular synapses (Ribeiro-da-Silva and Coimbra 1982; Bernardi, Valtschanoff et al. 1995; Bailey and Ribeiro-da-Silva 2006). Using electron microscopic postembedding immunohistochemistry and electrophysiology, all three types of fibers have been shown to be glutamatergic (Schneider and Perl 1985; Valtschanoff, Phend et al. 1994).

Glomerular-type synapses are composed of a large primary afferent synapsing with multiple post-synaptic cells and are observed in several areas of the brain, including the somatosensory thalamus, olfactory bulb and cerebellum. The architecture of the astrocytes processes around each of these multi-synaptic units is unique. In the dorsal horn, the incoming primary afferents associated with glomeruli are quite large, often exceeding 900 nm in width. Previous studies using ultrathin sections have found that astrocyte processes may exceed 1 μm in diameter; however, they are typically smaller than 200 nm and frequently narrow to only 50 nm, which is well below the resolution of light microscopy. To capture the large size of the primary afferent and glomerulus while retaining the delicate architecture of the astrocyte processes, we utilized block-face serial-section electron tomography to make three-dimensional reconstructions of the entire synaptic unit. Here, we show that astrocytes are an integral part of the architecture of glomerular synapses in the dorsal horn.

3.3. MATERIALS & METHODS

Adult C57BL/6 mice were used between postnatal days 60 and 80. All mice were housed in the animal facilities at the University of North Carolina, Chapel Hill, in accordance with Institutional Animal Care and Use Committee guidelines.

3.3.1. Spinal cord tissue procurement for fixed tissue preparations

The mice were anesthetized by an intraperitoneal injection of urethane (2 mg/kg) and perfused with 2% formaldehyde / 2.5% glutaraldehyde in 0.15 M cacodylate buffer. The lumbar section of the spinal cord was removed and post-fixed in the perfusion solution for an additional 2 hours. One hundred micron-thick transverse sections were cut on a vibratome (Leica) and post-fixed overnight.

3.3.2. Electron Microscopy Processing

After washing thoroughly in PBS on ice, the slices were placed in 0.5% osmium tetroxide in PBS on ice for 1 hour. The slices were washed 3 times for 2 minutes each in ice-cold distilled deionized water. The slices were dehydrated with series of ethanol and acetone solutions. All the solutions were ice cold, except the final acetone solution, which was room temperature. Each step lasted 10 minutes. The slices were placed in 1:1 Durcupan:acetone overnight and subsequently transferred to 3:1 Durcupan:acetone for 6-8 hours. The slices were then transferred to 100% Durcupan for 24 hours and incubated overnight in fresh 100% Durcupan. The slices were flat-embedded between glass slides and placed in 50-60ºC oven for 3 days.

For electron tomography, several serial, 500 nm thick, semithin sections were imaged using a 400 keV TEM. The sections were rotated from -60 to +60 degrees while collecting images every 2 degrees. A back projection algorithm was then used to reconstruct a computed volume based on the series of tilt images. This technique allowed us to reconstruct entire synapses.

3.3.3. Stereology

Non-biased volume estimates were made using point-counting stereology, as previously described (Lehre and Rusakov 2002; Vanhecke, Studer et al. 2007). A uniform grid of points was applied over the image. Point spacing of 470 nm in the x and y planes generated grids of approximately 3000 points. Each image covered 650-670 μm^2 of tissue. The points were assigned to the following categories: neuropil, astrocyte process or blood vessel/cell body. The number points in each compartment was totaled and multiplied by the area of the tissue represented.

3.3.4. Synapse Reconstruction

Synapse reconstruction, modeling and measurements were performed using IMOD (Copyright (c) 1994-2011 by the Boulder Laboratory for 3-Dimensional Electron Microscopy of Cells and the Regents of the University of Colorado).

3.4. RESULTS

3.4.1. Astrocyte content of lamina II in the dorsal horn.

Astrocytes were identified by their irregular, tortuous shape and by the presence of glycogen granules in a relatively clear cytoplasm. Intermediate filaments could be found in the larger processes, but not in the fine processes nearing synapses. The astrocytic content of the neuropil was estimated from three randomly selected large sections of 668 μm^2 each (volumes 1-3 in Table 2). Non-biased point-counting stereology was used to assign the neuropil into the following categories: astrocyte process, other neuropil and cell body/vasculature. Due to the large size of the cell bodies and vasculature in comparison to the neuropil and their variable distribution between the images, they were not included when determining the astrocyte fraction of the local neuropil. Astrocytic processes occupied 17.6 ± 0.8% of the local neuropil.

Table 2. Stereological measurements of the astrocytic, neuropil and cell body/vasculature content of lamina II in the dorsal horn.

	Volume 1	Volume 2	Volume 3	Avg Volume (um^2)
Astrocyte Processes	454	347	402	188.47 (± 14.52)
Other Neuropil	2406	1944	2492	1071.91 (± 79.97)
Cell Bodies / Vasculature	165	625	22	127.21 (± 95.50)
Astrocyte Fraction of Neuropil	**18.87%**	**17.85%**	**16.13%**	**17.6% (± 0.80)**

Values are mean ±SEM.

3.4.2. Tripartite Synapse Structure

In the dorsal horn, non-peptidergic fibers are thought to form C1-type terminals, whereas C2-type terminals arise from A-delta fibers. Both C1- and C2-type terminals are large and form glomerular-type synapses with multiple postsynaptic and sometimes presynaptic contacts. At the ultrastructural level, C1-type terminals are identified by their highly scalloped shape and dark cytoplasm. They contain densely packed round vesicles and can contain dense-core vesicles. C2-type terminals are distinguished by their lighter cytoplasm and somewhat less scalloped shape in comparison to C1-type terminals. Additionally, C2 type terminals do not contain dense core vesicles.

We prepared two series, one of 302 sections and another of 50 sections, for the examination of C1- and C2-type glomerular synapses in lamina II of the dorsal horn. These volumes covered 14,126.22 μm^3 and 2,338.78 μm^3 of tissue, respectively. In each series, five C1-type and five C2-type primary afferents were identified, with the C1-type generally located more dorsally, though the populations of these two types of synapses intermingled to a small degree. At every synaptic unit, we traced the glomerular boundary through 45 to 85 serial sections until the glomerulus terminated on both ends. Only terminals that made synaptic contact with either the primary afferent, or other terminals synapsing with the primary afferent were included within the glomerular boundary. Generally, all the postsynaptic terminals within a glomerulus made synaptic contact with the primary afferent. However, there were many more presynaptic terminals within the glomerulus than made direct synaptic contact with the primary afferent. Of the 6-9 presynaptic terminals within a glomerulus, only 1-3 of these terminals made direct synaptic contact with the primary afferent (see Table 3). The rest made contact with second order neurons that were in synaptic

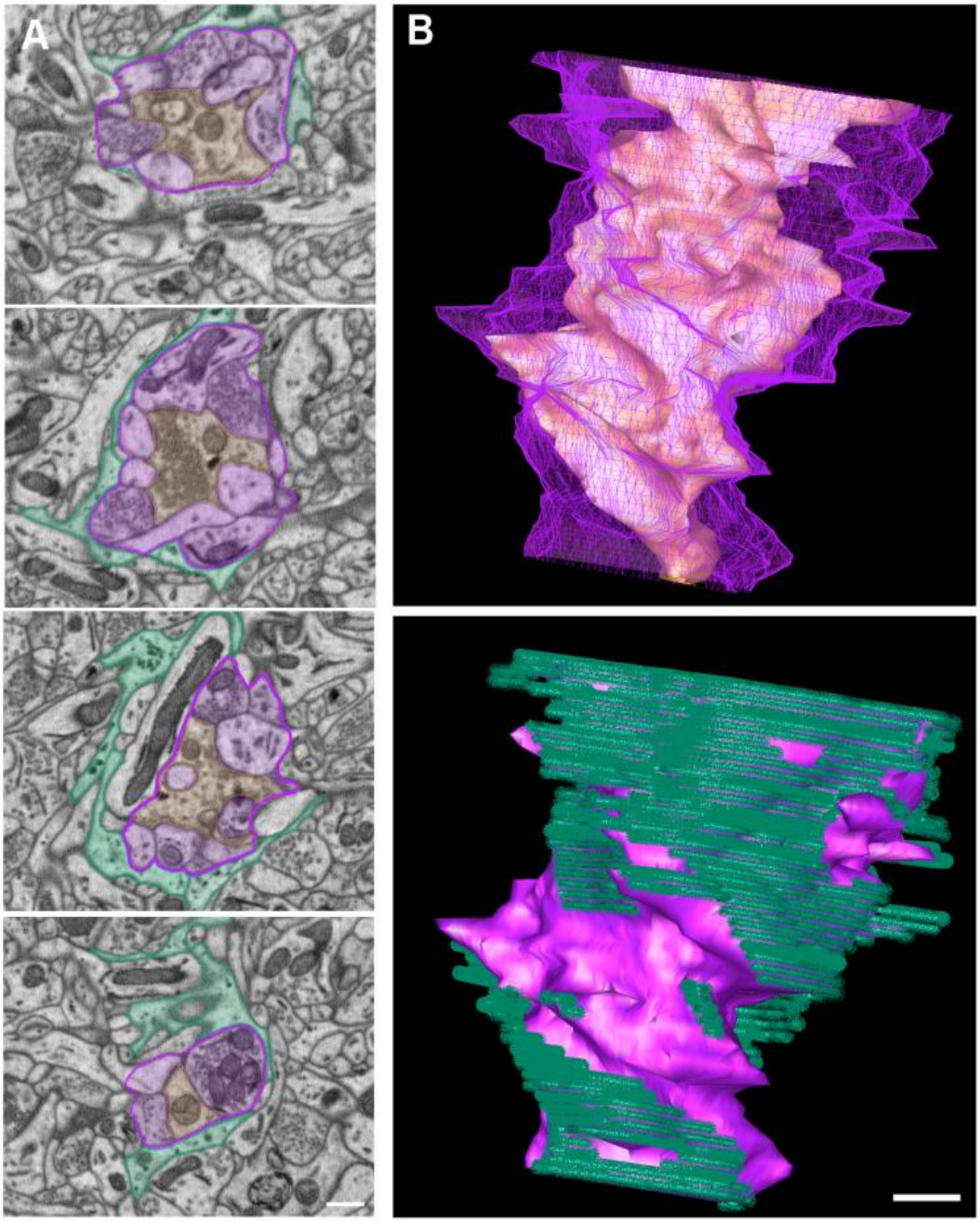

Figure 1. An example of a C1-type terminal forming a glomerular synapse. The images were pseudocolored to identify the primary afferent (*orange*), the glomerular boundary (*purple*) and the astrocytic processes that make contact with the glomerulus (*green*). In **A**, four representative sections spaced evenly through the glomerulus are shown. The scale bar is 500 nm. In **B**, a 3-D reconstruction based on serial section traces is shown. The top image shows the large C1-type primary afferent (*orange*) surrounded by a translucent outline of the glomerulus (*purple*). The space between the primary afferent and the exterior border of the glomerulus is filled with pre- and postsynaptic terminals as seen in A. The bottom image shows a solid glomerular sheath (*purple*) and the area contacted by astrocyte processes (*green*). The scale bar is 500nm.

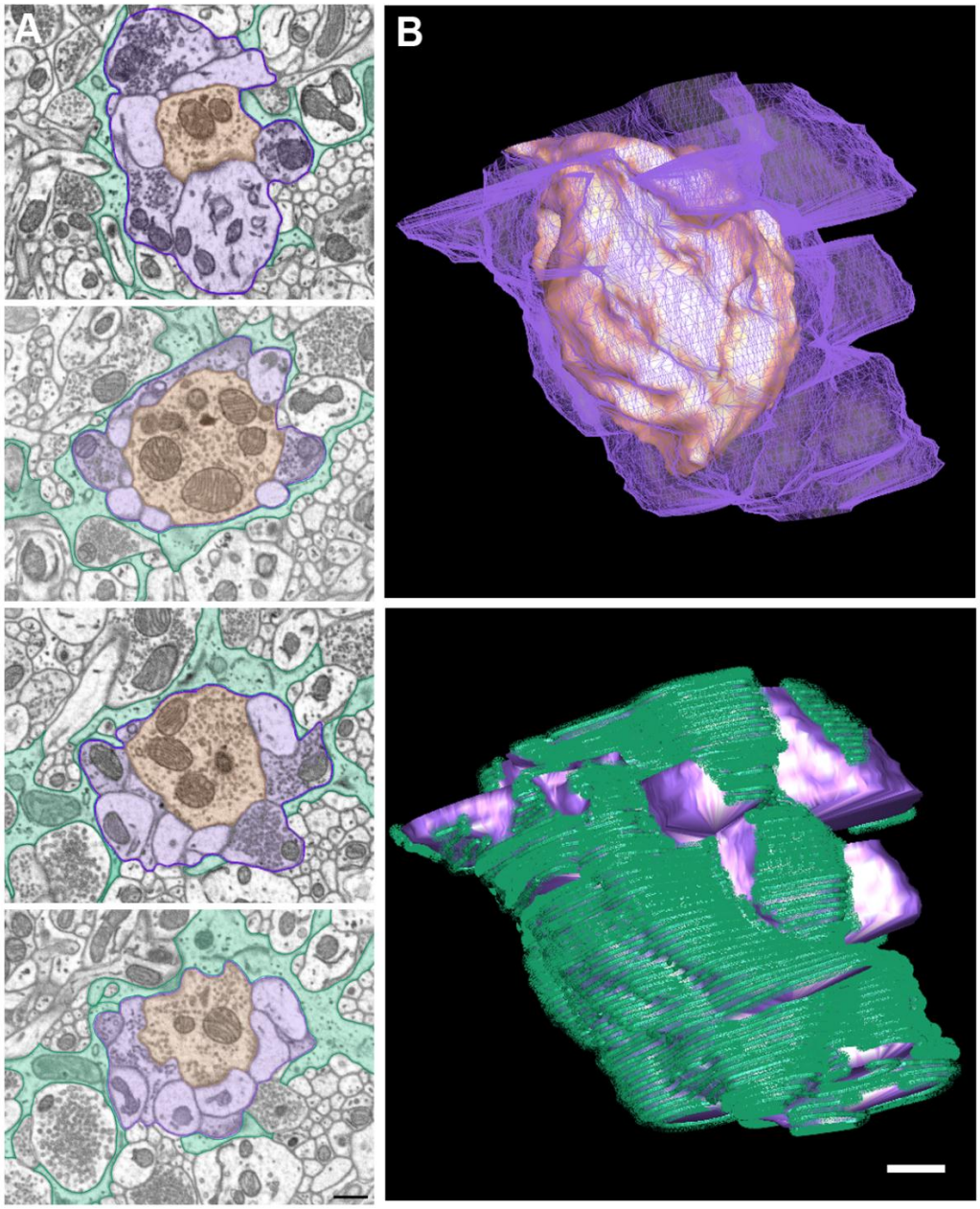

Figure 2. An example of a C2-type terminal forming a glomerular synapse. The images were pseudocolored to identify the primary afferent (*orange*), the glomerular boundary (*purple*) and the astrocytic processes that make contact with the glomerulus (*green*). In **A**, four representative sections spaced evenly through the glomerulus are shown. The scale bar is 500 nm. In **B**, a 3-D reconstruction based on serial section traces is shown. The top image shows the large C1-type primary afferent (*orange*) surrounded by a translucent outline of the glomerulus (*purple*). The space between the primary afferent and the exterior border of the glomerulus is filled with pre- and postsynaptic terminals as seen in A. The bottom image shows a solid glomerular sheath (*purple*) and the area contacted by astrocyte processes (*green*). The scale bar is 500nm.

contact with the primary afferent. Both C1- and C2-type glomerular synapses displayed this characteristic.

Figures 1 and 2 show examples of C1- and C2-type terminals forming glomerular synapses. In these figures, we have identified and traced the primary afferent (*orange*), the outline of the glomerulus (*purple*) and the astrocytic processes that contact the glomerulus (*green*). The highly scalloped shape of the C1-type terminal compared to the C2-type terminal (Fig 2) is evident in the individual slices as well as in the 3-D reconstruction. The space between the primary afferent and the glomerular sheath contains the presynaptic and postsynaptic terminals within the glomerulus. At reconstructed synapses, we quantified the number of synapses between the primary afferent and presynaptic and postsynaptic terminals (Table 3). The C1-type terminals had fewer postsynaptic contacts (mean 7.4) than the C2-type terminals (mean 12.4); both types of terminals had the same mean number of presynaptic synapses (mean 1.8).

Every C1- and C2-type glomerular synapse was contacted by an astrocyte process. Furthermore, the ensheathment of a glomerulus was never below 55.5%. Both C1- and C2-type glomeruli were highly ensheathed by astrocyte processes, with the C1-type being more highly ensheathed than the C2-type synapse (87% and 77%, respectively). However, there was no significant difference in the astrocytic ensheathment of C1- and C2-type glomeruli.

Table 3. Quantitative comparison of C1-type and C2-type glomerular synapses.

Characteristic	**C1 Type**	**C2 Type**
Total Synapses with Postsynaptic Contacts	7.4 ± .75	12.4 ± 1.43
Total Synapses with Presynaptic Contacts	1.8 ± 0.58	1.8 ± 0.20
Mean Astrocytic Ensheathment	**87.59 ± 3.16%**	**77.20 ± 6.98%**

Values are mean ± SEM.

3.5. DISCUSSION

The astrocytic content of the neuropil varies by brain region. In the stratum radiatum of the CA1 region of the hippocampus, these measurements vary between 4 and 10% (Ventura and Harris 1999; Lehre and Rusakov 2002). In the cerebellar cortex, the Bergmann glial content is much greater, with 33% astrocyte content (Lehre and Rusakov 2002). We found that, in lamina II of the dorsal horn, astrocyte processes occupy 17.6 ± 0.8% of the local neuropil.

Serial reconstructions of C1- and C2-type glomerular synapses in the dorsal horn reveal that, despite belonging to different populations of primary afferents, these glomerular synapses are very similar at the ultrastructural level. C1-type primary afferents, corresponding to the non-peptidergic C fiber population, have a slightly lower number of postsynaptic contacts as well as a somewhat greater degree of astrocytic ensheathment. C2-type primary afferents, corresponding to the A-delta fiber population, have a slightly higher number of postsynaptic contacts and a greater variability of the degree of astrocyte ensheathment.

Astrocyte ensheathment of synapses varies greatly between brain regions and between types of synapses within a given brain region. In the hippocampus, 57-62% of synapses are contacted by astrocytes (Ventura and Harris 1999; Witcher, Kirov et al. 2007). Of the contacted synapse population, larger perforated synapses are approximately 80% ensheathed; in contrast, smaller macular synapses are only 50% ensheathed. In the cerebellum, nearly all the climbing fiber and parallel fiber synapses are contacted by astrocytes; however, the degree of ensheathment also varies by synapse type. Climbing fiber synapses are 87% ensheathed, whereas parallel fibers are 67% ensheathed. These differences in synapse ensheathment contribute to the kinetics of excitatory postsynaptic currents in two

major ways. The microstructure of the synapse is, in part, determined by the astrocyte envelopment of the synaptic cleft. This shaping of the synapse has also been observed using electron microscopy, where a preference for post- versus presynaptic contact is observed in hippocampal astrocytes (Lehre and Rusakov 2002). Second, the proximity of glutamate transporters to the site of release can limit the duration of glutamatergic transmission to the postsynaptic neuron (Overstreet, Kinney et al. 1999). In neurons of the dorsal horn, glutamate uptake by Glt-1 contributes to the termination of EPSCs (Weng, Chen et al. 2007; Xin, Weng et al. 2009; Zhang, Xin et al. 2009).

In addition to the dorsal horn, glomerular-type synapses can also be found in the sensory thalamus, olfactory bulb, trigeminal nucleus, lateral geniculate or as rosettes in the cerebellar cortex. In the sensory thalamus, astrocyte processes completely ensheathe the glomerulus, occasionally with multiple lamellae (Spacek and Lieberman 1974). Olfactory glomeruli are highly organized units in which an individual astrocyte is confined to a single glomerulus (Bailey and Shipley 1993; Roux, Benchenane et al. 2011). Within each glomerulus, astrocyte processes are excluded from the "sensory-synaptic subcompartment" and only comingle with the neuropil components of the "central-synaptic compartment" (Chao, Kasa et al. 1997). In this case, it appears that astrocytes only interact with the brain-derived components of the glomerulus rather than the olfactory input. In the trigeminal and lateral geniculate nuclei, astrocytic processes are largely restricted to the periphery of a synaptic glomerulus (Peters and Palay 1966; Ide and Killackey 1985). In the cerebellum, Bergmann glia form specialized appendages to interact with discrete units of cells (Grosche, Matyash et al. 1999; Grosche, Kettenmann et al. 2002). Due to the difficulty of serial section reconstruction of these large glomerular-type synapses, quantitative measurements of the

degree of ensheathment have not been made. We have found that 100% of C1- and C2-type synapses in the dorsal horn are contacted by astrocytes. C1-type glomerular synapses are ensheathed to a somewhat greater degree than C2-type synapses (87% compared to 77%). This high degree of ensheathment may serve multiple purposes key to maintaining the high-fidelity processing of nociceptive signaling in the dorsal horn.

Astrocyte processes provide a structure that may shield the synapse from interference from nearby synapses and extrasynaptic signaling. Astrocytic sheaths around glomeruli provide a degree of physical separation between synapses. Perhaps more importantly, astrocyte membranes also possess glutamate transporters that can act as dynamic functional barriers to actively remove transmitters from the synaptic cleft, thereby reducing spillover and maintaining high-fidelity, discrete signaling events between synapses. This active glial barrier could ensure spatial and temporal precision in synaptic transmission.

In addition to the reuptake of neurotransmitters, astrocytes may also release "gliotransmitters". The stimulation of Gq-coupled G protein-coupled receptors results in increases in intracellular calcium levels and may promote the release of gliotransmitters such as ATP, D-serine and glutamate (Halassa, Fellin et al. 2007). These gliotransmitters can have various effects on the pre- or postsynaptic cells depending upon their complement of neuronal receptors.

Astrocytes are also responsible for the removal of evoked increases in extracellular K^+ concentration through reuptake by Na^+/K^+ ATPase. The extracellular K^+ level is a key determinant of neuronal membrane potential. Minor decreases in extracellular K^+ concentration may suppress excitability by increasing the gap between the resting membrane

potential and the threshold for the activation of voltage-gated channels (Nedergaard and Verkhratsky 2012).

Lastly, astrocytes are also responsible for the lactate production necessary to fuel active synapses. Extracellular increases in the K^+ level, associated with increased neuronal activity, lead to a rise in lactate production (Rose and Ransom 1996; Ransom, Ransom et al. 2000). The activation of astrocytic Na^+/K^+ ATPase triggers the release of lactate, providing metabolic support of synaptic transmission (Adachi, Cruz et al. 1995). Astrocytic envelopment of glomerular synapses may serve to isolate them from spillover and extrasynaptic transmission, help to maintain high-fidelity signaling through the uptake of neurotransmitters, play a role in synaptic transmission through the release of gliotransmitters, control extracellular K^+ levels and locally deliver energy to active synapses.

It is interesting to note that astrocyte processes are also highly mobile. In the hippocampus, long-term potentiation is accompanied by astroglial remodeling, including the extension and retraction of processes (Hirrlinger, Hulsmann et al. 2004; Haber, Zhou et al. 2006). Ultrastructural studies in the barrel cortex have shown that 24 hours of whisker stimulation can induce increases in astrocyte coverage of synapses (Genoud, Quairiaux et al. 2006). This finding implies that stimulation encourages an astrocytic encroachment of the bouton-spine interface. Under conditions of persistent pain, nociceptive primary afferents become hypersensitized, with increased firing to normally non-noxious stimuli and sometimes spontaneous firing. On a large scale, hypertrophy develops in the large processes of dorsal horn astrocytes in the context of chronic pain (Garrison, Dougherty et al. 1991). However, what happens at the scale of a single synapse and its surrounding fine astrocytic processes is still largely unknown, yet could have profound effects on neuronal signaling.

Astrocyte domain structure in the dorsal horn has never been studied in normal mice or under the conditions of neuropathic pain; however, the loss of domain structure in reactive astrocytes has been investigated following epilepsy and stab wound conditions. During epilepsy, a state of increased glutamatergic activity and dendritic sprouting, astrocytes lost domain regulation and process overlap increased 10-fold (Oberheim, Tian et al. 2008). However, using a model of unilateral entorhinal cortex lesion, which promotes a state of neuronal hypoactivity, no changes in domain structure were observed (Wilhelmsson, Bushong et al. 2006). The effect of these conditions on tripartite synapse structure was not examined in either study. It remains to be determined how domain structure or tripartite synapse structure in the dorsal horn might be affected in chronic pain conditions.

The fundamental disorganization of tripartite synapse structure after hypertrophy or an inability to regain organization could have a significant impact on dorsal horn neuronal networks. Changes in astrocyte ultrastructure around the synapse caused by astrocyte hypertrophy could affect the functioning of the tripartite synapse. For instance, retraction of the astrocyte process from the synapse would result in the withdrawal of glutamate transporters from the synaptic cleft and potentially decrease glutamate clearance. Retraction of astrocyte synaptic ensheathment would also expose the synapse to neurotransmitter spillover from nearby synapses and extrasynaptic transmission from descending inputs from the brain. Lastly, retraction of astrocyte processes may also open the synapse to new and detrimental synaptic connections. The extension of astrocyte processes could protect the synapses from these factors, but it could also be detrimental to necessary remodeling following the disruption of primary afferents.

CHAPTER 4.

TRANSGENIC MANIPULATION OF GLIOTRANSMISSION DURING PERSISTENT NEUROPATHIC PAIN HAS NO EFFECT ON MECHANICAL SENSITIVITY

4.1. OVERVIEW

The activation of astrocytes, or astrogliosis, has been shown to coincide with the long-term maintenance phase of persistent neuropathic pain. Blockade of this activation through glial metabolic inhibitors inhibits astrogliosis and reduces pain behaviors. However, the astrocyte-specific contribution to neuropathic pain has yet to be identified. Astrocytes may play a role in physiology and pathophysiology through the release of gliotransmitters, glutamate, ATP and D-serine. Determining the astrocytic pathways involved is complicated by their use of similar cellular machinery, as nearby microglia and neurons, to accomplish very different tasks. This makes selective pharmacological manipulation difficult.

Here, we have taken advantage of the astrocyte-specific glial fibrillary acidic protein promoter to drive transgenes that will interfere with the intracellular pathways leading to gliotransmitter release. We found that neither reduction of intracellular calcium release through removal of the IP_3R2 receptor nor obstruction of vesicular release through the expression of a dominant negative SNARE protein had any effect on basal mechanical sensitivity or pain sensitization following a neuropathic lesion.

4.2. INTRODUCTION

Under conditions of persistent neuropathic pain, astrogliosis begins as early as seven days post-neuropathic injury and remains for the duration of behavioral allodynia, up to four weeks after the injury (Tanga, Raghavendra et al. 2004). Blockade of gliosis through the administration of glial metabolic inhibitors decreases behavioral hypersensitivity after the induction of neuropathic pain (Tawfik, Nutile-McMenemy et al. 2007). These observations suggest that astrocytes may play an important role in the maintenance of persistent neuropathic pain. However, because neurons use the same host of cellular machinery as astrocytes, the pharmacological agents often used to study astrocytes lack cellular specificity. We have taken advantage of the astrocyte-specific promoter glial fibrillary acidic protein (GFAP) to drive transgenes, or remove floxed genes, specifically in astrocytes. This technique allows us to tease apart the roles that neurons and astrocytes play in physiology and pathophysiology.

The fine processes of astrocytes wrap the synaptic cleft, putting them in a key position to respond to presynaptic transmitter release and to actively participate in regulating neuronal excitability. Studies have reported that through their spatial and temporal coding of calcium increases, astrocytes participate in the modulation of neuronal networks through gliotransmitter release (Araque, Parpura et al. 1999; Haydon and Carmignoto 2006; Montana, Malarkey et al. 2006), such as glutamate (Parpura, Basarsky et al. 1994), D-serine (Schell, Molliver et al. 1995) and ATP (Guthrie, Knappenberger et al. 1999). Astrocytes release calcium, their primary intracellular signaling molecule, from internal stores both spontaneously (Parri, Gould et al. 2001; Nett, Oloff et al. 2002; Hirase, Qian et al. 2004) and through the activation of Gq-linked G protein coupled receptors (GPCRs) in response to neuronal stimulation (Porter and McCarthy 1996; Aguado, Espinosa-Parrilla et al. 2002;

Perea and Araque 2005). Gq-linked GPCRs trigger calcium release through the production of IP_3, which activates IP_3 receptors on the endoplasmic reticulum. There are three subtypes of IP_3 receptors; astrocytes only possess subtype 2 (IP_3R2) (Hertle and Yeckel 2007; Petravicz, Fiacco et al. 2008). We used IP_3R2 conditional knockout (cKO) mice to selectively inhibit astrocyte calcium signaling. These mice were created by crossing two single-transgenic lines: one in which the human GFAP (hGFAP) promoter drives the expression of Cre recombinase and the other expressing a floxed IP_3R2 gene.

In IP_3R2 full knockout (KO) mice, there is no calcium release from internal stores in astrocytes of the brain, whereas neuronal calcium signaling remains intact (Petravicz, Fiacco et al. 2008). In the spinal cord, the cKO transgenic mice had greatly attenuated spontaneous and Gq GPCR-mediated increases in astrocyte calcium and would therefore be expected to have decreased calcium-dependent gliotransmitter release. We challenged these mice with spared nerve injury to produce neuropathic pain. Although astrogliosis developed, there were no differences in basal mechanical sensitivity or pain sensitization following the neuropathic lesion between transgenic and littermate control animals.

The vesicular release of neuroactive molecules also depends on soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE) machinery, which mediates vesicle fusion with the plasma membrane. We used a line of transgenic mice in which the expression of a dominant negative SNARE domain was driven selectively to astrocytes. Mice were obtained by crossing mice from two different lines: one with the GFAP promoter driving expression of the tet-off tetracycline transactivator (tTA) and another in which the cytosolic domain of the vesicle protein synaptobrevin II, which acts as a dominant negative repressor, as well as the reporter eGFP, are coexpressed under the tetO

promoter. Mice are maintained on doxycycline, to suppress gene expression through development, until they are taken off doxycycline at weaning. Pascual et al. first showed that vesicular release of ATP from astrocytes is inhibited in these animals (Pascual, Casper et al. 2005). Following release, ATP is converted to adenosine by extracellular ectonucleotidases. Ambient levels of adenosine provide an inhibitory tone for local neurons by activating adenosine 1 receptors on presynaptic terminals, which decreases release probability. In the dorsal horn, A1 receptor activation decreases glutamate release from the majority of A delta and C fibers (Lao, Kawasaki et al. 2004; Choi, Cho et al. 2011). Adenosine 1 receptor agonists have potent antinociceptive effects in several pain models and have demonstrated efficacy in human clinical trials in reducing allodynia and hyperalgesia (Zylka 2011). However, we found that dnSNARE animals had no differences in basal mechanical sensitivity or pain sensitization following neuropathic lesion in comparison to littermate control animals.

4.3. MATERIALS & METHODS:

4.3.1. Animals

IP_3R2 KO mice were generated as described previously (Li, Zima et al. 2005; Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010). Briefly, a 539 bp fragment of exon 3 of IP_3R2 (116 bp) was inserted into a targeting vector between two loxP sites. Mice were bred to heterozygosity for the floxed allele (IP_3R2+/flox) and crossed to Pro-Cre mice. Pro-Cre mice were crossed to IP_3R2+/flox mice to generate germline heterozygous null mutant offspring (IP_3R2+/–), which were interbred to generate homozygous full mutant mice (IP3R2–/–) and littermate controls (IP_3R2+/+). IP_3R2 floxed mice were then bred to GFAP-Cre mice to produce astrocyte-specific IP_3R2 cKO mice. Littermate floxed, Cre-negative mice were used as wild-type control mice. All mice were backcrossed onto C57BL/6 for nine

generations. All mice were housed in the animal facilities at the University of North Carolina at Chapel Hill in accordance with Institutional Animal Care and Use Committee guidelines.

Dominant negative SNARE mice were generated as described previously (Pascual, Casper et al. 2005). Briefly, we expressed the cytosolic portion of the SNARE domain of synaptobrevin 2 (amino acids 1 to 96), as well as the lacZ and eGFP reporters. These mice were bred to a GFAP-tTA line containing a 2.2 kb fragment of the human GFAP promoter driving the expression of the tTA. To prevent potential developmental influences of the transgenes, the animals were maintained on 25 mg/mL doxycycline in their drinking water until weaning.

4.3.2. Spinal cord tissue preparation for calcium imaging

Young mice (postnatal days 15-21) were anesthetized by an intraperitoneal injection of urethane (2 mg/kg). Once deeply anesthetized, as monitored by the loss of the toe-pinch reflex, the mice were cooled briefly at 4˚C and transcardially perfused with ice-cold oxygenated slicing buffer containing the following (in mM): 75 sucrose, 2.5 KCl, 80 NaCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 2.5 $NaHCO_3$, 1.3 ascorbate, 3 pyruvic acid and 0.1 kynurenic acid (290 mOsM). The lumbar section of the spinal cord was removed, and 500-µm thick transverse sections were cut on a vibratory microtome (Vibratome 300, St. Louis, MO) in ice-cold oxygenated slicing buffer solution.

4.3.3. Calcium Imaging

Slices for calcium imaging were incubated for 30 minutes with 11 µM fluo-4, 0.07% pluronic acid and 0.4% DMSA in oxygenated slicing buffer at 35 degrees Celsius. The slices were then transferred to normal slicing buffer and allowed to cool to room temperature for at least 30 minutes prior to calcium imaging experiments, which were performed in artificial cerebral spinal fluid (ACSF). The ACSF contained (in mM) 125 NaCl, 25 dextrose, 1.25

NaH_2PO_4, 2.5 KCl, 26 $NaHCO_3$, 1.3 $MgCl_2$ and 0.1 Trolox-C. Regions of interest were placed over the cell bodies of astrocytes. Increases in average fluorescence in regions of interest indicate an increase in Ca^{2+} concentration. Fold increase over baseline was calculated for each trace and reported as $\Delta F/F_0$.

TTX, histamine, carbachol and (RS)-3,5-dihydroxyphenylglycine (DHPG) were obtained from Tocris Bioscience (Bristo, UK). Fluo-4 was obtained from Invitrogen (Carlsbad, CA).

4.3.4. Spared Nerve Injury Surgery

Using isoflurane, mice at least 50 days old were deeply anesthetized, as monitored by toe-pinch and eye-blink reflexes. The left hind leg was secured with tape, and the leg hair was removed with Nair and cleaned with iodine and alcohol. The three divisions of the sciatic nerve were exposed at thigh level. The common peroneal and sural nerves were ligated, and a 1-3 mm portion of the nerve was removed below the ligature to prevent regrowth. The musculature was replaced in its original position, and the skin was closed with wound clips. The area was again cleaned with iodine and alcohol, and an anti-bacterial anti-fungal agent applied. The animals were housed in cages with soft bedding. Sham surgeries entailed exposure of the nerves without disturbing them.

4.3.5. Behavioral testing

Animals were singly placed in a Plexiglas enclosure on top of a metal grate. To determine paw withdrawal frequency, a von Frey hair (IITC Life Sciences, Woodland Hills, CA) calibrated to 3.61 log force (to monitor allodynia) or 4.31 log force (to monitor hyperalgesia) was applied ten times to the center of the foot pad at an interval of at least one minute between applications. The number of times the paw was briskly withdrawn due to the stimulus was recorded. To determine the paw withdrawal threshold, the up-down paradigm

(Chaplan, Bach et al. 1994) was used with a series of von Frey hairs calibrated between 2.36 and 4.56 log force.

4.3.6. Immunohistochemistry

Animals were deeply anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Once deeply anesthetized, as monitored by the toe-pinch and eye-blink reflexes, the animals were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) solution. Lumbar segments 4-6 of the spinal cord were removed and post-fixed for two hours. The tissue was cryoprotected with 30% sucrose in PBS and subsequently frozen in OCT. Fourteen micrometer-thick sections were made on a cryostat (Leica, Wetzlar, Germany). Immunohistochemistry was performed with primary antibodies against mouse GFAP (1:500; Dako), rabbit Iba-1 (1:500; Wako), mouse NeuN (1:500; Millipore) and biotinylated IB4 lectin (1:100; Vector). Secondary antibodies were either anti-mouse or anti-rabbit Alexa 568 or Alexa 488 or avidin-conjugated Alexa 568. The slides were coverslipped with Vectashield with DAPI.

4.4. RESULTS

4.4.1. Dorsal horn astrocytes from IP_3R2 KO lack spontaneous and Gq-coupled GPCR Ca^{2+} increases, whereas cKO reduced the number of responding cells

Full knockout of the IP_3R2 gene has been reported to obliterate astrocyte calcium responses in the cortex and hippocampus without affecting the neuronal calcium response (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010). We generated cKO mice to restrict the IP_3R2 knockout to GFAP-positive cells. Then, to address the functional role of IP_3R2 in dorsal horn astrocytes, we performed Ca^{2+} imaging experiments on bulk-loaded slices from littermate control, IP_3R2 full KO and IP_3R2 cKO mice (Fig. 3). Bath application of a Gq-coupled GPCR agonist cocktail (10 μM histamine, 10 μM carbachol and 10 μM

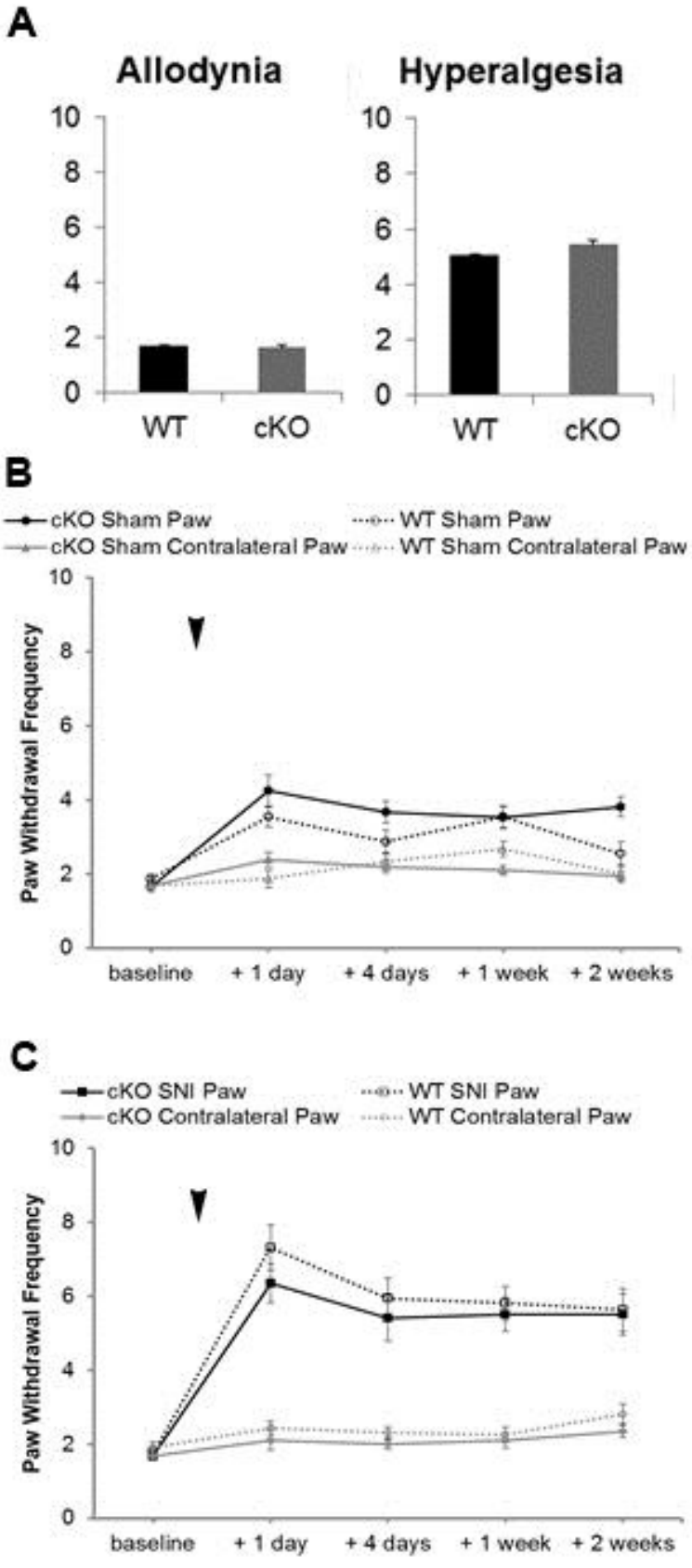
A
Allodynia
Hyperalgesia
10
8
6
4
2
0
WT
cKO
B
cKO Sham Paw
WT Sham Paw
cKO Sham Contralateral Paw
WT Sham Contralateral Paw
Paw Withdrawal Frequency
baseline
+ 1 day
+ 4 days
+ 1 week
+ 2 weeks
C
cKO SNI Paw
WT SNI Paw
cKO Contralateral Paw
WT Contralateral Paw
Paw Withdrawal Frequency
baseline
+ 1 day
+ 4 days
+ 1 week
+ 2 weeks

Figure 3. IP_3R2 conditional knockout mice show no difference in the development of mechanical allodynia post-spared nerve injury. Mechanical allodynia was monitored by the repeated application of a 3.61 log force von Frey hair to the plantar surface of the ipsilateral and contralateral hind paws. Hyperalgesia was monitored in the same manner with a 4.31 log force von Frey hair. The paw withdrawal frequency was recorded as the number of brisk withdrawals from ten repeated applications. A. There was no difference in mechanical allodynia or hyperalgesia between naïve IP_3R2 cKO (n=24) and littermate control (n=12) animals. B. There was no difference in mechanical allodynia between genotypes at any time point following spared nerve injury in the ipsilateral or contralateral hind paws. (cKO n=20; WT n=16) C. There was no difference mechanical allodynia between IP_3R2 cKO (n=21) and littermate control (n=15) animals following sham operation in the ipsilateral or contralateral hind paws. The arrow marks the date of spared nerve injury surgery.

DHPG) to littermate control slices elicited robust increases in astrocytic intracellular calcium levels (91.9% of 37 cells from 3 animals total). In contrast, the GPCR cocktail failed to elicit Ca^{2+} increases in slices from IP_3R2 KO mice (0% of 27 cells from 3 animals total). Furthermore, no astrocytes from IP_3R2 KO slices displayed spontaneous calcium oscillations.

To assure slice health, 100 µm ATP was applied to the slices, which elicited a robust intracellular Ca^{2+} increase in astrocytes, presumably through P2X receptors (data not shown). When applied to IP_3R2 cKO slices, the Gq GPCR agonist elicited calcium responses in a smaller population of astrocytes than in the littermate controls but did not abolish the Ca^{2+} responses (42.5% of 61 cells responded from 3 animals). Similar to littermate control slices, some astrocytes from IP_3R2 cKO slices also displayed spontaneous intracellular oscillations.

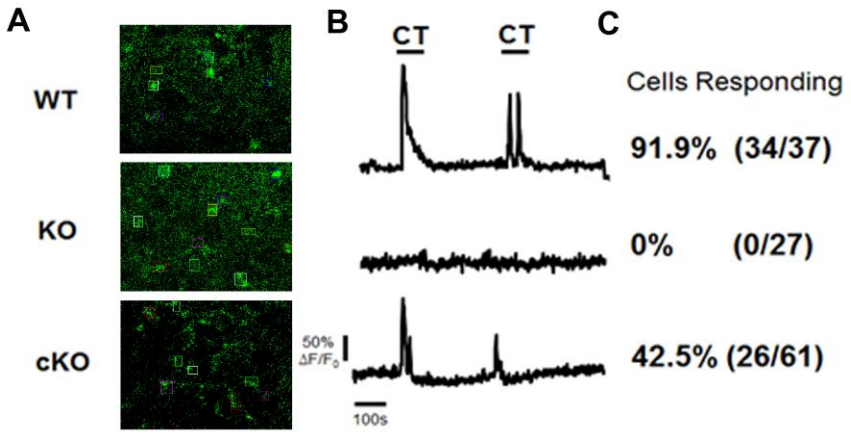

Figure 4. Knockout of IP_3R2 obliterates astrocyte calcium responses to a Gq-coupled GPCR agonist cocktail; however, the conditional knockout only reduced the number of responding cells. Three hundred micrometer-thick transverse spinal cord slices were prepared from 25-day-old wild-type, IP_3R2 KO and IP_3R2cKO mice. **A**. Representative slices in which astrocytes were loaded with fluo-4 calcium indicator dye, which increases in fluorescence with calcium increases. Regions of interest were placed over the cell bodies of bulk-loaded astrocytes in lamina II of the dorsal horn to record their responses to agonist application. **B**. Representative calcium traces to bath application of an agonist cocktail (histamine, carbachol and DHPG; 10 µm each). **C.** The cocktail elicited Ca^{2+} responses in astrocytes from wild-type littermate control slices but not in IP_3R2 KO slices. In cKO slices, 42.5% of the dorsal horn astrocytes still responded to the Gq cocktail.

Taken together, these observations show that the activation of Gq-linked GPCRs on dorsal horn astrocytes evokes intracellular Ca^{2+} increases through IP_3R2-mediated mechanisms. While a full KO of the IP_3R2 gene obliterates astrocytic intracellular Ca^{2+} responses, it may also affect cell populations that we are not monitoring. Specifically knocking out IP_3R2 in GFAP cells significantly decreased the population of astrocytes that responded to a Gq-linked GPCR agonist cocktail; less than half of the astrocytes responded to this cocktail. Although recombination in the IP_3R2 cKO was not complete, we chose to take a conservative approach and utilize the cKO animal for further pain behavioral testing to avoid the potential confounds of the full KO animal.

4.4.2. Reduction in IP_3R2-dependent Ca^{2+} increases does not affect the development of mechanical allodynia following spared nerve injury

Spontaneous and evoked astrocytic Ca^{2+} elevations have been shown to cause gliotransmitter release, which can modulate neuronal synaptic activity (Hassinger, Atkinson et al. 1995; Araque, Sanzgiri et al. 1998; Kang, Jiang et al. 1998; Parri, Gould et al. 2001; Fiacco and McCarthy 2004; Liu, Xu et al. 2004; Liu, Xu et al. 2004). To address the role of astrocytic IP_3R2 in nociception, we monitored the sensitivity of the hind paw to normally non-noxious stimulation and normally noxious stimulation by application of low or high force Von Frey hairs to the plantar surface of the paw (log force 3.61 or 4.31, respectively). There was no difference in mechanical allodynia or hyperalgesia between naïve IP_3R2 cKO and littermate control mice (Figure 4A).

Under neuropathic pain conditions, astrocytes are known to hypertrophy (Tanga, Raghavendra et al. 2004). This hypertrophy lasts for the duration of pain behavior and is thought to contribute to the longevity of the pain behavior. Glial metabolic inhibitors block the development of hypertrophy and reduce allodynia following the induction of neuropathic

pain (Tawfik, Nutile-McMenemy et al. 2007). Much of the data showing the release of gliotransmitter from astrocytes has been collected from cultured astrocytes; however, several publications have debated whether gliotransmitter release occurs *in situ* and *in vivo* (Fiacco, Agulhon et al. 2007; Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010; Nedergaard and Verkhratsky 2012). The hypertrophic state of astrocytes under neuropathic pain conditions may more closely mimic their semi-reactive state in culture.

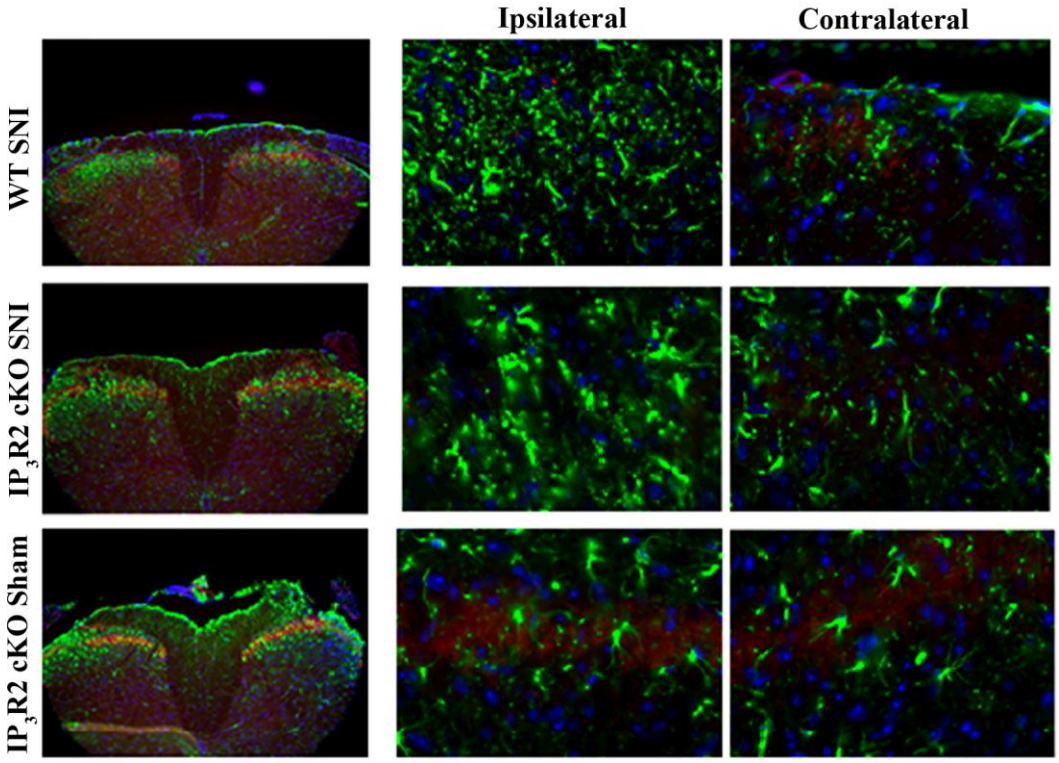

Figure 5. Astrogliosis develops normally following SNI in IP_3R2 knockouts and littermate controls. Immunohistochemistry was performed on mice two weeks following spared nerve injury or sham surgery. IB4-positive (*red*) terminals mark primary afferent projections into the spinal cord. Loss of IB4 staining confirmed the loss of peripheral inputs into the superficial laminae of the dorsal horn of the spinal cord following nerve lesion. No loss of IB4 staining was observed in the sham-operated animals. Astrogliosis was monitored using GFAP (*green*) and was evident only in the ipsilateral side of the dorsal horn in SNI animals and not in the contralateral side or in sham animals.

Therefore, we performed spared nerve injury, a model of persistent neuropathic pain, and monitored mice for the development of mechanical allodynia using Von Frey hair stimulation of the hind paws (log force 3.61). No difference between IP_3R2 cKO (n=20) and littermate control (n=16) mice was detected in the development of mechanical allodynia on the ipsilateral or contralateral paws over two weeks (Figure 4A). One group of mice was monitored until four weeks post-SNI to observe the effects of neuropathic pain on the longevity of mechanical sensitivity. No significant difference was found between IP_3R2 cKO (n=12) and WT (n=6) up to four weeks post-surgery (data not shown). Lastly, we also performed sham surgeries on a subset of animals to detect whether astrocytes from IP_3R2 cKO mice might become hyperreactive to sensitizing stimulation (an effect that might not be evident with spared nerve injury due to a ceiling effect on pain behavior post-injury). However, no difference was observed between IP_3R2 cKO (n=21) and littermate control (n=15) animals post-sham surgery (Fig. 4B).

4.4.3. The development of astrocyte hypertrophy is unaltered in IP_3R2 cKO mice following spared nerve injury

To determine whether astrocytes become activated following SNI surgery, we performed immunohistochemistry two weeks post-SNI, which is when reactive astrogliosis occurs in the superficial laminae of the dorsal horn. As shown in figure 5, IB4 staining (*red*) showed a clear loss of nerve terminals on the ipsilateral side of the injury in SNI animals but not in sham-operated animals. Loss of nerve terminals coincided with areas of astrocyte hypertrophy (*green*), evidence of reactive astrogliosis. Astrogliosis occurred ipsilateral to the injury site in IP_3R2 cKO and littermate control animals, but not in sham-operated animals.

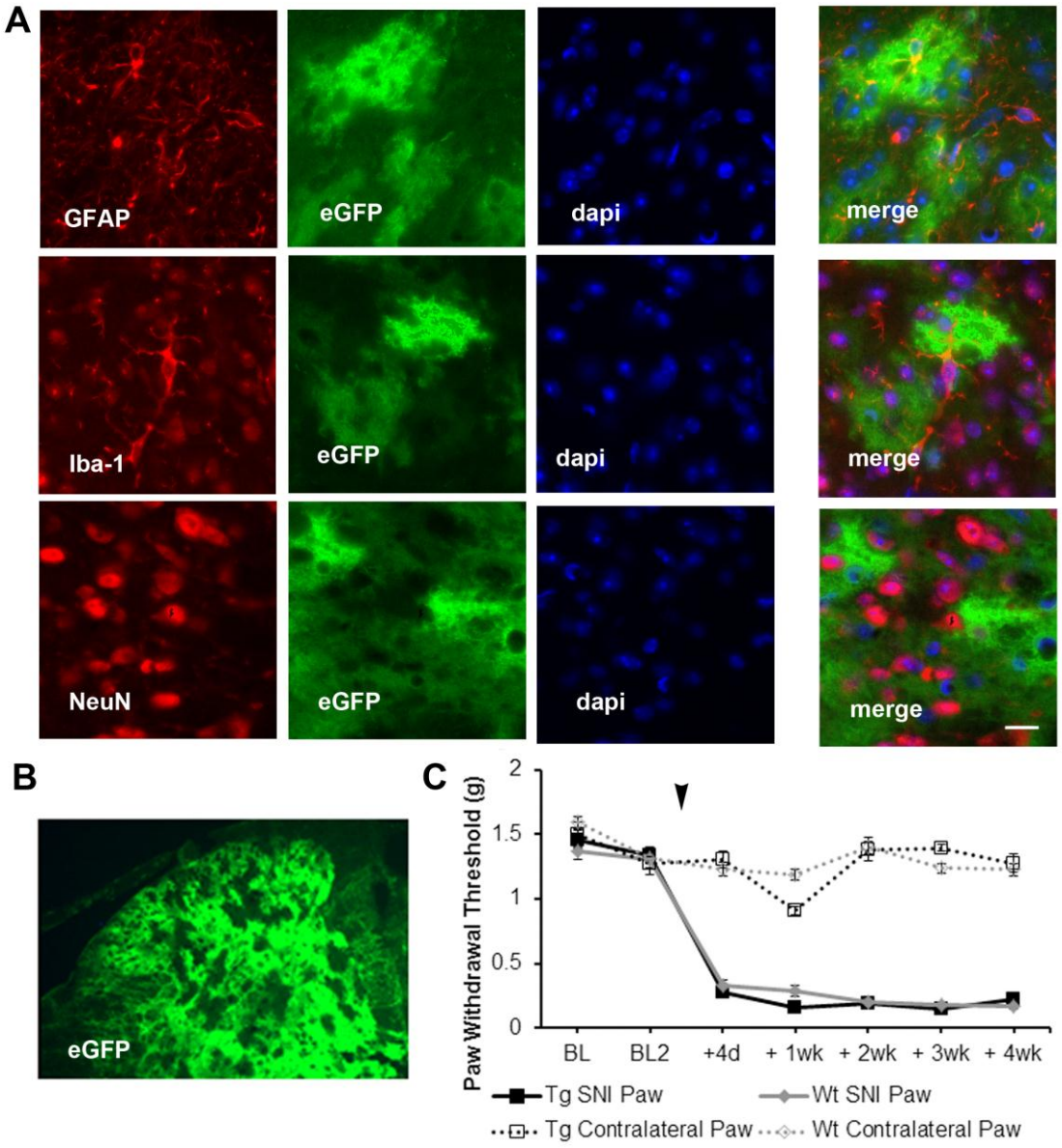

Figure 6. In the dorsal horn of the spinal cord, transgenic dnSNARE was expressed specifically astrocytes but did not affect mechanical sensitivity in naïve or nerve-lesioned animals. A. Immunohistochemistry shows that eGFP colocalized with the astrocytic marker GFAP (*red*) and not with the microglial marker Iba-1 (*red*) or the neuronal marker NeuN (*red*) in the dorsal horn of the spinal cord. **B.** In dnSNARE transgenic mice, native eGFP expression was robust throughout the gray matter of the spinal cord. **C**. Paw withdrawal threshold showed no difference between transgenic and littermate control mice in mechanical sensitivity either in naïve mice (repeated measures on two baseline days) or at any time up to four weeks post-neuropathic lesion. The arrow marks the date of spared nerve injury surgery.

4.4.4. Transgenic dominant negative SNARE protein is expressed in astrocytes of the superficial laminae of the dorsal horn

Gliotransmission is thought to depend upon Gq-coupled GPCR-activated calcium release from internal stores to promote vesicle release. Vesicular release of neuroactive molecules also depends SNARE machinery, which mediates vesicle fusion with the plasma membrane. We used a second transgenic approach to block gliotransmitter release through the utilization of a dominant negative SNARE (dnSNARE) mouse. Figure 6A shows robust eGFP expression in the dorsal horn of the spinal cord. We used immunohistochemistry to show that eGFP colocalized with GFAP, an astrocyte marker, but not with markers for other cell types, such as microglia (Iba-1) or neurons (NeuN) (Fig. 6A).

4.4.5. Dominant negative SNARE does not affect the development of mechanical allodynia following spared nerve injury

To determine whether vesicular release from astrocytes plays a role in the development or maintenance of mechanical allodynia following neuropathic injury, we performed spared nerve injury on dnSNARE mice and littermate controls. We observed typical development of astrogliosis in the dorsal horn ipsilateral, but not contralateral, to the site of nerve lesion by 14 days post-injury (data not shown). We monitored the paw withdrawal threshold using von Frey hair stimulation of the hind paws. We saw no difference in withdrawal threshold between dnSNARE (n=10) and littermate controls (n=9) in naïve mice or following spared nerve injury up to four weeks post-injury (Fig. 6C).

4.5. DISCUSSION

Astrocytes have been implicated in the maintenance of persistent pain through their release of cytokines and chemokines and concurrent hypertrophy. With the chronic constriction injury and spared nerve injury neuropathic pain models, astrogliosis begins as early as seven days post-injury and remains for the duration of monitored behavioral

allodynia (Tanga, Raghavendra et al. 2004). Astrogliosis may cause astrocytes to take on new non-physiological roles, such as the release of cytokines and chemokines, which can further sensitize surrounding neurons. Studies have shown that the inhibition of these changes alleviates the behavioral sensitization that occurs in persistent pain (Sweitzer, Colburn et al. 1999). Transgenic inhibition of inflammatory pathways selectively in astrocytes lessens behavioral sensitization following formalin administration and chronic constriction injury (Fu, Zhang et al. 2007; Fu, Zhang et al. 2010). Decreases in glutamate transporter expression following the development of astrogliosis (Sung, Lim et al. 2003; Binns, Huang et al. 2005) may account for the increase in glutamate levels in the spinal cord during persistent pain and may produce the AMPA and NMDA receptor activation underlying the hyperexcitability of secondary pain transmission neurons (Cavaliere, Cirillo et al. 2007). Furthermore, the administration of glial metabolic inhibitors decreases behavioral hypersensitivity after the induction of neuropathic pain (Tawfik, Nutile-McMenemy et al. 2007). The development of gliosis may not only result in new "reactive" roles for astrocytes but may also perturb their existing functions within a neuronal network.

In the present study, we investigated the role of gliotransmission in the dorsal horn through the use of transgenic mouse models blocking the calcium-dependent or SNARE-dependent release of gliotransmitters from astrocytes. In agreement with previous publications examining multiple areas of the brain, including the hippocampus and cortex, we found that full KO of the IP_3R2 gene abolished spontaneous and Gq-linked GPCR calcium responses in dorsal horn astrocytes. In the hippocampus, full KO of the IP_3R2 gene had no effect on neuronal calcium dynamics, AMPA or NMDA receptor activation or LTP (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010). However, full KO of IP_3R2

affects other organ systems, such as the heart and olfactory system (Li, Zima et al. 2005; Fukuda, Shirasu et al. 2008). Therefore, we generated a conditional knockout of the IP_3R2 gene under the control of the astrocyte-specific GFAP promoter. Calcium imaging in the cKO animals revealed that 42.5% of dorsal horn astrocytes were still able to respond to a Gq-GPCR agonist cocktail. This is a reduction of 50% from wild-type slices in which 91.9% of astrocytes responded. We took a conservative approach and continued to utilize the cKO animals for further behavioral assays.

In naïve animals, neither cKO of astrocyte intracellular calcium mobilization nor expression of the dnSNARE protein had any effect on mechanical sensitivity. This is supported by other studies from our laboratory that show that transgenic blockade of intracellular calcium release (Petravicz, Fiacco et al. 2008; Agulhon, Fiacco et al. 2010) or SNARE machinery (unpublished observations) specifically in astrocytes has no effect on neuronal responses. However, others have found that dnSNARE mice exhibit a small reduction in mechanical threshold, suggesting that they have increased basal sensitivity to pain (Foley, McIver et al. 2011). dnSNARE mice have also been shown to exhibit reduced release of adenosine triphosphate and reduced adenosine 1 receptor (A1R) activation (Halassa, Florian et al. 2009). ATP is converted to adenosine through extracellular ectonucleotidases, which is known to be anti-nociceptive in both naïve and neuropathic pain conditions (Zylka, Sowa et al. 2008; Gong, Li et al. 2010). However, in our study, as well as in that of Foley et al., dnSNARE mice exhibited no difference in the development of mechanical sensitivity following spared nerve injury (Foley, McIver et al. 2011).

After nerve lesion, astrocytes become reactive and hypertrophy. This regression to a more immature or reactive state may activate new processes or interfere with normal

astrocytic signaling. We investigated the role of gliotransmission in the development of neuropathic pain. We found that transgenic blockade of calcium- or SNARE-dependent release of gliotransmitters from astrocytes did not alter the development of astrogliosis or the behavioral responses of animals to nerve lesion. Both IP_3R2 cKO and dnSNARE animals developed mechanical allodynia with the same time course and to the same degree as littermate controls following spared nerve injury. Previous studies have shown that astrocytes participate in the nerve injury response through their hypertrophy, inflammatory response and possibly through changes in their glutamate transporter regulation; however, they do not participate in the nerve injury response through intracellular calcium- or vesicular-dependent release of gliotransmitters.

CHAPTER 5.

NEUROINFLAMMATORY INTERACTIONS BETWEEN NEURONS AND GLIA DURING PERSISTENT NEUROPATHIC PAIN

5.1. OVERVIEW

Persistent pain manifests itself as an expression of neuronal plasticity first characterized by the peripheral sensitization of primary sensory neurons in the dorsal root ganglion and later by the central sensitization of spinal dorsal horn and cortical neurons (Woolf and Costigan 1999; Woolf and Salter 2000; Julius and Basbaum 2001; Ji, Kohno et al. 2003; Bhave and Gereau 2004; Kuner 2010). The pathogenic role of neuroinflammation in the development of persistent pain has gained attention as it has become clear that proinflammatory cytokines are key modulators of pathophysiology and communication between immune cells, neurons and glia during the processes of degeneration, regeneration and pain (Myers, Campana et al. 2006). A temporally phasic pattern is emerging in many of the key inflammatory signaling pathways during the generation of neuropathic pain. These patterns may be indicative of a change in the underlying mechanisms of pain sensitization over time, as an acute injury progresses toward either resolution or persistent maladaptive pain. Identification of the markers and mechanisms of specific phases of pain could lead us closer to controlling the outcome of acute injury or relieving persistent maladaptive pain.

In this chapter, I will examine the neuroinflammatory environment of injured peripheral nerves, the associated DRG and the dorsal horn, from the acute onset of injury through the available data, which often only extends until three weeks post-injury. First, I

will examine the order of cellular activation in the peripheral and central nervous systems. Then, I will emphasize several molecules to highlight the phasic patterns involved in the nerve injury response. I have incorporated data from several models of neuropathic pain, including spared nerve injury (SNI), chronic constriction injury (CCI) and crush injury. Although using several models may produce variances, it is impossible to produce a coherent timeline without pooling the data from several models. This is not an exhaustive list; instead, this description provides examples of molecules that may be prototypical of other others in their class. There are no doubt other inflammatory mediators that mimic these temporally phasic patterns following nerve injury.

5.2. CELLULAR ACTIVATION FOLLOWING NERVE INJURY

5.2.1. Peripheral Cellular Activation Following Nerve Injury

Acute nerve injury immediately results in the release of large amounts of ATP, calcium and chloride from transected neurons. These molecules promote a brief phase of vasoconstriction and platelet aggregation and activate resident macrophages and Schwann cells. Within minutes to hours, other inflammatory mediators, including bradykinin, prostaglandins, H+, nerve growth factors, ATP and proinflammatory cytokines, are released from injured tissues and activated resident macrophages and Schwann cells (Oh, Tran et al. 2001; Cook and McCleskey 2002; Sommer and Kress 2004; Stein, Clark et al. 2009). These inflammatory mediators promote vasodilation and increase vascular permeability to allow the influx of leukocytes and subsequent macrophage recruitment. Importantly, the peripheral terminals of nociceptive neurons express the receptors for many of these inflammatory mediators, many of which promote sensitization (Basbaum, Bautista et al. 2009).

At any stage post-injury, the activation of nociceptors by inflammatory mediators causes hyperactivity of key transduction molecules, such as transient receptor potential

subtype V1 (TRPV1) and A1 (TRPA1), as well as longer-term changes such as the upregulation of the sodium channels Nav 1.7, 1.8 and 1.9 (Myers and Shubayev 2011). For instance, intraplantar, perineurial, DRG or spinal cord exposure to TNFα or IL-1β, two of the most studied cytokines involved in neuropathic pain, directly sensitize C and A fibers, reducing mechanical activation thresholds and sometimes evoking spontaneous discharge and increasing the spontaneous and evoked firing of deep dorsal horn neurons (Fukuoka, Kawatani et al. 1994; Sorkin, Xiao et al. 1997; Junger and Sorkin 2000; Reeve, Patel et al. 2000; Onda, Hamba et al. 2002; Schafers, Svensson et al. 2003; Ozaktay, Kallakuri et al. 2006). These electrophysiological changes facilitate neuronal sensitization, in which smaller signals generated by changes in the axonal environment are recognized and amplified in the ascending pain pathway (Myers and Shubayev 2011). It is the ongoing axonal environment that primes the nerve for regeneration following injury is also responsible for the development of peripheral sensitization.

The first responders to peripheral nerve injury are resident macrophages, mast cells and Schwann cells. Resident macrophages comprise approximately 4% of the endoneurial cavity (Mueller, Wacker et al. 2001). Their reaction to nerve injury is immediate; however, their early role is limited due to their small resident population. Activated macrophages take on an amorphous shape, start expressing MHC class I and II markers and begin to phagocytose nearby debris. Mast cells secrete vasoactive agents partially responsible for the increased permeability of the blood-nerve barrier and thus facilitate the recruitment of non-resident, blood-born macrophages (Omura, Ohbayashi et al. 2004). Schwann cells become activated immediately following injury. Similar to resident macrophages, they are also

capable of phagocytosing myelin debris (Reichert, Saada et al. 1994); however, their main role is the recruitment of macrophages to the injury site.

The axons of the peripheral nervous system are in close contact with Schwann cells. Myelinated fibers are larger in diameter and consist of A-beta fibers originating from spinal cord motor neurons and A-delta fibers originating from large primary sensory neurons in the DRG. Each myelinating Schwann cell wraps itself around a single axon to produce the myelin sheath critical for rapid saltatory conduction. In contrast, small diameter C-fibers, which include nociceptive neurons and all postganglionic sympathetic neurons, are arranged into groups called Remark bundles in which a single non-myelinating Schwann cell wraps around several axons using its processes to keep the axons separate from one another. Both myelinating and non-myelinating Schwann cells are S100B positive, but only non-myelinating Schwann cells are GFAP positive (Wewetzer, Grothe et al. 1997).

Within two days of nerve injury, Schwann cells begin to dedifferentiate and downregulate their expression of genes encoding myelin-associated proteins, such as myelin basic protein, myelin protein zero and peripheral myelin protein 22 (Trapp, Hauer et al. 1988; Mitchell, Griffiths et al. 1990). Dedifferentiation may occur due to a reduction of cAMP signaling through JNK (Monje, Soto et al. 2010). Perhaps due to their dedifferentiation, the populations of myelinating and non-myelinating Schwann cells are no longer distinguishable post-injury. Within three days, Schwann cells upregulate MAC-2, a galactose-specific lectin, making them capable of myelin-phagocytosis (Reichert, Saada et al. 1994).

Within four days of nerve injury, Schwann cells undergo massive proliferation controlled by neuregulin-1 (NRG-1), which is expressed on neurons and interacts with the tyrosine kinase receptors erbB2 and erbB3 on Schwann cells (Corfas, Velardez et al. 2004;

Jessen and Mirsky 2005). Transgenic disruption of NRG1-erbB signaling by the transgenic expression of dominant negative erbB in non-myelinating Schwann cells leads to the extensive proliferation and death of non-myelinating Schwann cells, changes similar to those produced by nerve lesion (Chen, Rio et al. 2003). This death is followed by C-fiber apoptosis and progressive heat and cold pain sensory loss, possibly due to a loss of trophic support from Schwann cells. NRG-1-erbB signaling can also induce the Ras/Raf/MEK extracellular signal-related kinase (ERK) pathway, which induces cell cycle arrest (Marshall 1995; Lloyd, Obermuller et al. 1997). Trophic signals activate ERK to both initiate and terminate cell mitosis; therefore, identifying their upstream modulators is critical to elucidating the mechanisms of Schwann cell survival after nerve injury.

Recruited macrophages are attracted by locally produced chemokines including MCP-1 expressed by denervated Schwann cells (Toews, Barrett et al. 1998; Siebert, Sachse et al. 2000). The phagocytic process is then amplified by the activated macrophages, which may, in turn, attack Schwann cells. Activated macrophages secrete components of the complement cascade as well as coagulation factors, proteases, hydrolases, interferons, TNFα and other cytokines that facilitate the degeneration and phagocytosis of the nerve fiber (Shubayev, Kato et al. 2010). This process in the distal nerve is referred to as Wallerian degeneration. The time course and magnitude of Wallerian degeneration through the alteration of macrophage activity and TNFα expression can modulate the pain experience (Myers, Heckman et al. 1996; Liefner, Siebert et al. 2000; Sommer, Lindenlaub et al. 2001; Shubayev, Angert et al. 2006). However, altering the clearance of debris may also impact nerve regeneration.

Glial cells of the DRG also have late-phase reactions to peripheral nerve injury. Satellite glial cells display an increase in GFAP staining three days through at least six weeks post-injury (Woodham, Anderson et al. 1989). Resident macrophages are also normally present in the DRG; however, by one week post-nerve lesion, non-resident macrophages have infiltrated the DRG, where they remain elevated for at least three months (Hu and McLachlan 2003; Tandrup et al 2000). Proinflammatory cytokines released from macrophages can then directly elicit ectopic action potentials, alter the phenotype of sensory neurons and promote neuropathic activity (Scholz and Woolf 2007).

While data have been pooled between neuropathic pain models, including the crush, CCI and SNI models, in order to examine the timelines of individual inflammatory mediators, there are distinct differences regarding the underlying pathologies and recoveries between the different models. Crush lesions of the sciatic nerve leave the Schwann cell basal lamina intact, allowing for more efficient nerve regeneration along the basal lamina. One month after crush injury, the majority of both small myelinated fibers and unmyelinated fibers are in the process of regenerating, and the inflammatory cells are restricted to areas around blood vessels. One month following CCI, the nerve consists of patches of degenerating, regenerating and preserved neural tissue. One month following SNI, the distal nerve stumps have undergone Wallerian degeneration. For three weeks post-SNI, the proximal stumps undergo retrograde degeneration. Finally, at one month following SNI, there is evidence of regeneration, with abundant small myelinated and unmyelinated axons and proliferating Schwann cells. In the dorsal horn, there was a persistent loss of IB4 and SP-positive terminals in the crush and SNI models, while in the CCI model, nerve terminal density returned to baseline levels (Casals-Diaz, Vivo et al. 2009).

Several months post-nerve injury, many chronically denervated Schwann cells apoptose, either due to loss of neurotrophic support or as a result of endogenously produced NGF (Grinspan, Marchionni et al. 1996). Those Schwann cells that survive chronic denervation are morphologically and functionally different from acutely denervated Schwann cells and are no longer capable of supporting regenerating axons (Li, Terenghi et al. 1997; You, Petrov et al. 1997; Terenghi, Calder et al. 1998; Hall 1999). Without the trophic support of surrounding Schwann cells, axons cannot survive. This suggests that there is a brief window of opportunity in which denervated Schwann cells are axon responsive and thus when transected nerves can regrow. Thus, the nerve repair process should not be delayed. This is clinically important in pain management because many possible treatments to reduce pain also have the potential to delay the repair process if administered during this critical regenerative window.

5.2.2. Central Cellular Activation Following Nerve Injury

In neurons of the dorsal horn, ERK activation can be seen within minutes of spinal nerve injury or high intensity nociceptor stimulation; however, its upregulation is transient in nature and lasts less than six hours (Ji, Baba et al. 1999; Zhuang, Gerner et al. 2005). ERK activation is mediated by various types of post-synaptic receptors, including AMPA, NMDA, NK-1 (the substance P receptor) and TrkB (a brain derived neurotrophic factor (BDNF) receptor), all of which can be released from nociceptive primary afferents (Ji, Gereau et al. 2009). The convergence of multiple signaling pathways, including PKC, Ras, PI3K and PKA, on ERK activation indicates that ERK may play a critical role in signal transduction in dorsal horn nociceptive neurons (Ji, Gereau et al. 2009). ERK activation produces post-translational effects that mediate increased sensitivity of dorsal horn neurons, including increases in NMDA receptor activity, inhibition of Kv4.2 potassium channel activity and

induction AMPA receptor trafficking (Hu, Carrasquillo et al. 2006; Ji, Gereau et al. 2009; Latremoliere and Woolf 2009). ERK activation also increases the transcription of pronociceptive genes, including NK-1, TrkB, Zif268, proDyn, c-fos and cyclooxygenase 2 (COX2), which may mediate the long-term hypersensitivity of dorsal horn neurons (Ji, Gereau et al. 2009). Due to its many pronociceptive effects, ERK activity is often thought to serve as a marker for central sensitization.

Microglial activation involves several changes, including changes in morphology (from ramified to amoeboid), gene expression (enhanced MCH I and II, CD11b and Iba1), function (phagocytosis) or number (proliferation). Microglial activation following nerve injury coincides with its expression of phospho-p38 (p-p38). Several neuropathic pain models induce a robust increase in p-p38 in microglia of the dorsal horn beginning at 12 hours, peaking at 3 days and slowly declining over several weeks (Jin, Zhuang et al. 2003; Tsuda, Mizokoshi et al. 2004; Katsura, Obata et al. 2006; Wen, Suter et al. 2007). The intrathecal administration of p38 inhibitors attenuates neuropathic pain (Jin, Zhuang et al. 2003; Tsuda, Mizokoshi et al. 2004); however, these inhibitors are unable to reverse existing pain states (Svensson, Hua et al. 2003). p-p38 can be activated by a host of molecules already known to increase pain sensitivity, including TNFα, IL-1β, MCP-1, CX3CR1, inducible nitric oxide synthase and MMP-9, as well as the ATP receptors P2X and P2Y12 (Abbadie, Lindia et al. 2003; Sung, Wen et al. 2005; Svensson, Schafers et al. 2005; Ikeda, Tsuda et al. 2007; Tang, Svensson et al. 2007; Zhuang, Kawasaki et al. 2007; Kawasaki, Xu et al. 2008; Kobayashi, Yamanaka et al. 2008). The p38 pathway enhances the activity of several inflammatory mediators, including NFκB, COX2, IL-1β and iNOS (Svensson, Marsala et al. 2003; Sung, Wen et al. 2005; Ji and Suter 2007). This pathway also activates phospholipase

A2 to generate arachidonic acid for prostaglandin production, and the intrathecal administration of a p38 inhibitor reduces PGE_2 release in the dorsal horn following an inflammatory insult (Svensson, Hua et al. 2003). Minocycline is a broad-spectrum anti-microbial tetracycline compound that inhibits microglial activation and decreases pain behavior possibly through a reduction in the expression of proinflammatory cytokines from microglia (Raghavendra, Tanga et al. 2003; Ledeboer, Sloane et al. 2005).

Under conditions of chronic neuropathic pain, astrogliosis begins as early as seven days post-neuropathic injury and remains for the duration of behavioral allodynia, for up to four weeks (Tanga, Raghavendra et al. 2004). Transgenic inhibition of inflammatory pathways selectively in astrocytes lessened behavioral sensitization following CCI (Fu, Zhang et al. 2010). Decreases in glutamate transporter expression following the development of astrogliosis (Sung, Lim et al. 2003; Binns, Huang et al. 2005) may account for the increase in glutamate levels in the spinal cord during chronic pain and may produce the AMPA and NMDA receptor activation underlying the hyperexcitability of secondary pain transmission neurons (Cavaliere, Cirillo et al. 2007). Furthermore, the administration of glial metabolic inhibitors decreases behavioral hypersensitivity after the induction of neuropathic pain (Tawfik, Nutile-McMenemy et al. 2007).

MAPKs are differentially activated in dorsal horn neurons, microglia and astrocytes following neuropathic injury. ERK is briefly activated in dorsal horn neurons following noxious stimulation or neuropathic injury, lasting only from ten minutes to six hours post-stimulus (Ji, Baba et al. 1999; Jin and Gereau 2006). By one week post-injury, p38 MAPK is still active in microglia; however, at this time point, its levels begin to fall, approaching basal levels by 21 days post-nerve injury (Zhuang, Wen et al. 2006). In contrast, increased

phosphorylation of c-jun-N-terminal kinase (JNK), primarily observed in astrocytes but not in neurons or microglia, is just beginning (Zhuang, Gerner et al. 2005). SNL induces delayed and persistent activation of JNK; SNI-induced JNK activation begins three days post-injury and remains past 21 days post-injury in GFAP-expressing astrocytes of the dorsal horn. TNFα is only capable of inducing a transient activation of JNK in astrocytes, while fibroblast growth factor-2 (FGF-2) can induce persistent JNK activation in the spinal cord *in vivo* and *in vitro* (Ji, Kawasaki et al. 2006). Intrathecal infusion of JNK inhibitors attenuates behavioral pain in several models of neuropathic pain (Obata, Yamanaka et al. 2004; Zhuang, Wen et al. 2006). Intrathecal infusion of JNK inhibitors can also attenuate established allodynia when given 10 days post-injury (Zhuang, Wen et al. 2006). TNFα-induced JNK signaling produces an increase in MCP-1, which can sensitize dorsal horn neurons (Gao, Zhang et al. 2009).

5.3. TEMPORALLY PHASIC RESPONSES OF SPECIFIC MOLECULES TO PERIPHERAL NERVE INJURY

5.3.1. Matrix metalloproteases 9 and 2

Matrix metalloproteases (MMPs) play two major roles post-nerve injury. First, they are responsible for the degradation of extracellular matrix proteins, a process that helps to clear the path for the infiltration of non-resident macrophages. Second, they are capable of cleaving and activating a variety of cytokines, including TNFα and IL-1β (Myers, Campana et al. 2006; Kawasaki, Xu et al. 2008). Peripheral nerve injury produces a biphasic peak in MMPs that correlates closely with that of TNFα.

In the nerve, MMP-9 (also known as gelatinase B) is elevated 1 day post-crush or axotomy injury and remains elevated above baseline, although at substantially lower levels than at the 1 day time point, until 60 days post-injury (Chattopadhyay, Myers et al. 2007;

Chattopadhyay and Shubayev 2009). MMP-9 is found in adult nerves only after injury and is primarily localized to Schwann cells (Chattopadhyay, Myers et al. 2007). Dominant-negative MMP-9 transgenic mice show remarkable protection from peripheral Wallerian degeneration due to MMP-9-dependent control of myelin protein degradation and macrophage migration into injured sciatic nerves (Shubayev, Angert et al. 2006; Chattopadhyay, Myers et al. 2007; Kobayashi, Chattopadhyay et al. 2008). While interference caused by macrophage invasion usually reduces the degree of nerve degeneration and pain (Myers, Heckman et al. 1996), in neuropathic pain models, dominant-negative MMP-9 mice only show a modest reduction or delay of mechanical allodynia (Chattopadhyay, Myers et al. 2007). In Schwann cells, MMP-9 selectively activates ERK, but not p38 or JNK. MMP-9 can activate the Ras/Raf/MEK-ERK1/2 signal transduction pathway to promote Schwann cell proliferation via the erbB, IGF-1 and PDGF tyrosine kinase receptors (Chattopadhyay and Shubayev 2009).

A second MMP peak occurs 5 days post nerve injury; however, this peak is mediated by MMP-2 (gelatinase A) (Shubayev and Myers 2000). Both the MMP-9 and MMP-2 peaks coincide with the peak levels of TNFα. The cytokines tumor necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β), as well as nerve growth factor (NGF), have all been implicated in the induction of MMP-9 following peripheral nerve injury (Shubayev, Angert et al. 2006; Chattopadhyay, Myers et al. 2007). This induction process can become a feed-forward mechanism, as MMP-9 also promotes the cleavage and activation of TNFα and IL-1β.

In the DRG and spinal cord, there is also a biphasic response of MMPs to nerve injury that first involves a transient increase in MMP-9 followed by a persistent increase in MMP-2. Spinal nerve ligation induces a rapid (less than one day post-injury) but transient

(less than three days) upregulation of MMP-9 in DRG neurons (Ji, Xu et al. 2009). MMP-9 is transported to the central terminals of DRG neurons and released into the spinal cord where it is capable of cleaving IL-1β and activating microglia. MMP-9 inhibition by intrathecal injection of small synthetic inhibitors, peptide inhibitors or siRNA attenuates neuropathic pain only if administered during this early phase (Ji, Xu et al. 2008). A late phase response occurs from seven through 21 days post-injury, in which DRG satellite cells and spinal cord astrocytes show a delayed but persistent upregulation of MMP-2. MMP-2 is thought to maintain neuropathic pain through IL-1β cleavage and the activation of astrocytes. The intrathecal injection of small molecule inhibitors, endogenous inhibitors (TIMP-2) and MMP-2-targeted siRNA can block the upregulation of ERK in spinal cord astrocytes and prevent late-phase neuropathic pain behavior (Ji RR, Xu ZZ, 2009 Trends in Pharmacological Science).

5.3.2. Cytokine TNFalpha

Cytokines, which are a large group of small proteins and are released from a wide variety of cells, regulate various inflammatory and immune functions. These proteins also have pleiotropic effects that can trigger a variety of cellular responses depending on the cell type, timing and molecular environment. Cytokines are predominantly produced by immune and glial cells, rather than neurons, and are grouped into the following classes: interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), chemokines, transforming growth factors (e.g., TGF-beta) and colony-stimulating factors. Most ILs, IFNs and colony-stimulating factors mediate their effects through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway; however, IL-1 and TNF activate the nuclear

factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) signaling pathways. Further, TGF-beta members act through the Smad family (Shubayev, Kato et al. 2010).

TNFα is a proinflammatory cytokine and is one of the most studied cytokines involved in the generation of neuropathic pain. In its transmembrane form, TNFα, similar to MMP-9, is mainly expressed by Schwann cells (Wagner and Myers 1996). In both crush and CCI models of neuropathic pain, TNFα increases almost 3-fold by 12 hours post-nerve injury, returns to near baseline levels and then exhibits a second smaller peak five days following nerve injury (George, Schmidt et al. 1999; Shubayev and Myers 2000; George, Buehl et al. 2004). Release and activation from its 26 kD transmembrane form to its 17 or 14 kD active forms is performed by MMPs (Gearing, Beckett et al. 1994). TNFα is also capable of inducing the expression of MMPs in peripheral glia (Shubayev, Angert et al. 2006; Chattopadhyay, Myers et al. 2007). Following neuropathic lesion, the first peak in TNFα coincides with the peak phase of MMP-9, and the second TNFα peak corresponds with the peak phase of MMP-2 (Shubayev and Myers 2000).

Direct injection of TNFα into the endoneurial cavity produces painful neuropathy and degenerative changes, including endoneurial inflammation, primary demyelination and axonal degeneration (Wagner and Myers 1996). TNFα activity depends on its expression level, its release and activation from its precursor transmembrane form and the availability of its receptors, TNFR1 and TNFR2; however, most of its effects in pain models are thought to be mediated by TNFR1. On neurons, TNFR1 signaling activates the transcription factors NFκB and AP1, which leads to induction of proinflammatory and immunomodulatory genes (Feuerstein, Liu et al. 1994; Darnay and Aggarwal 1997). In DRG neurons, the TNFR1-induced activation of p38 MAPK increased tetrodotoxin-resistant Na^+ currents (Jin and

Gereau 2006). In macrophages, TNFα activation of the TNFR1 receptor can increase the expression of intercellular adhesion molecules, thereby increasing the efficiency of macrophage-neuron interactions (Schafers, Schmidt et al. 2002). In Schwann cell cultures, TNFα can dose-dependently increase monocyte chemoattractant protein-1 (MCP-1) through activation of the NFκB and p38 MAPK pathways (Subang and Richardson 2001). (The response of MCP-1 in neuropathic pain is discussed in more detail below.)

TNFα is retrogradely transported from the injury site to the DRG within one day of nerve damage and returns to basal levels within a week following CCI injury (Shubayev and Myers 2001). This transport pattern matches the temporal pattern for the axonal transport of trophic factors following nerve injury (Curtis 1998; Tonra, 1998). TNFα has many central effects, many of which have yet to be studied in the dorsal horn. In the central nervous system, glial TNFα has been shown to enhance synaptic efficacy by increasing the surface expression of GluR1-possessing AMPA receptors via TNFR1-mediated PI3K activation (Beattie, Stellwagen et al. 2002; Stellwagen, Beattie et al. 2005). By changing the AMPA receptor subtype density, glial TNFα was shown to regulate synaptic scaling (Stellwagen and Malenka 2006). TNFα can affect astrocytes by reducing their expression of GLT-1 glutamate transporters (Sitcheran, Gupta et al. 2005) and reducing PKC-dependent K^+ conductance, which affects their ability to buffer K^+ from active neurons (Koller, Allert et al. 1998). In dorsal horn slices, TNFα increased the frequency of spontaneous EPSCs and enhanced AMPA and NMDA currents (Kawasaki, Zhang et al. 2008). The intrathecal application of TNFα induces CREB activation and heat hyperalgesia (Kawasaki, Zhang et al. 2008). TNFα is also capable of activating ERK, p38 and JNK signaling pathways. As discussed earlier,

these pathways are sequentially activated in neurons, followed by microglia and astrocytes of the dorsal horn after nerve injury.

5.3.3. Chemokine MCP-1

Monocyte chemoattractant protein (MCP)-1 is upregulated within three hours of nerve transection in Schwann cells and remains increased for up to eight days (Subang and Richardson 2001). In Schwann cell cultures, MCP-1 production can be triggered by TNFα through the NFκB and p38 pathways (Subang and Richardson 2001). The receptor for MCP-1, also known as chemokine C-C motif ligand 2 (CCL2), is chemokine C-C motif receptor 2 (CCR2), which is selectively expressed on macrophages in the periphery (Banisadr, Queraud-Lesaux et al. 2002). MCP-1 release recruits CCR2-positive non-resident macrophages (Toews, Barrett et al. 1998; Siebert, Sachse et al. 2000). The infiltration of peripheral macrophages marks a major shift in the axonal environment, as they are the major producers of cytokines, primarily due to their large numbers.

In under four hours, MCP-1 increases rapidly in DRG neurons (Tanaka, Minami et al. 2004; Jeon, Lee et al. 2009) and in the satellite cells of the DRG (Jung, Toth et al. 2008). Its receptor, CCR2, is also upregulated in both neuronal and glial cells of the DRG (White, Sun et al. 2005). MCP-1 sensitizes nociceptors through the activation of TRP channels and the inhibition of K^+ conductance (White, Sun et al. 2005; Sun, Yang et al. 2006; Jung, Toth et al. 2008). MCP-1 can be transported to the central terminals of primary afferents in the superficial dorsal horn and colocalizes with markers for substance P and CGRP (Dansereau, Gosselin et al. 2008; Gao, Zhang et al. 2009). MCP-1 can be released from primary afferent terminals in an activity-dependent manner; however, this only occurs after neuropathic lesion

and does not occur in sham-operated animals (Van Steenwinckel, Reaux-Le Goazigo et al. 2011).

The release of MCP-1 from primary afferents is involved in the activation of dorsal horn microglia (Thacker, Clark et al. 2009). CCR2 is upregulated three days following nerve injury in dorsal horn neurons and by one week post-lesion in microglia (Abbadie, Lindia et al. 2003; Gao, Zhang et al. 2009). After peaking at day seven, MCP-1 levels rapidly decline but do not return to baseline until several months later (Zhang and De Koninck 2006). Nerve injury-induced microgliosis and pain behavior can both be prevented with an intrathecal injection of an MCP-1 neutralizing antibody; additionally, CCR2 knockout mice lack nerve injury-induced microgliosis and pain behavior (Zhang, Shi et al. 2007; Thacker, Clark et al. 2009). Presumably, this inhibition is due to the neuronal release of MCP-1, as astrocytes are not activated at this stage. At later stages of neuropathic pain, astrocytes produce large amounts of MCP-1 in a JNK-dependent manner (Gao, Zhang et al. 2009). At this stage, microglia are beginning to show declining activation; however, MCP-1 may have direct effects on neurons as well. Patch-clamp recordings in lamina II neurons show that MCP-1 enhances spontaneous EPSCs and potentiates both NMDA- and AMPA-induced currents (Gao, Zhang et al. 2009).

5.3.4. Transcription factor NFκB

Nuclear factor κB (NFκB) is a ubiquitous transcription factor that is usually held inactive in the cytosol. Activation by cytokines, such as TNFα, leads to its translocation to the nucleus where it promotes the transcription several proinflammatory molecules, including nitric oxide, dynorphin and COX2 (Adcock, Newton et al. 1997; O'Neill and Kaltschmidt 1997). Both NFκB and COX2 have been shown to play a major role in inflammatory pain;

however, for the purposes of this review, I have only discussed the literature on neuropathic pain. Following CCI, the transgenic inhibition of NFκB, specifically in astrocytes, reduces hyperalgesia and macrophage recruitment (Fu, Zhang et al. 2010; Zhang, Fu et al. 2011). At one day post-injury, Fu et al. found peripheral decreases in IL-1β, TNFα, MCP-1, CCR2 and CXCL10; however, these differences were gone by three days post-injury, and no later time points were examined. At two weeks post nerve injury, NFκB activation increased in DRG neurons and Schwann cells surrounding the proximal nerve ending (Ma and Bisby 1998). NFκB is also activated centrally at three and five days post-nerve injury in ipsilateral dorsal horn neurons, but not in glial cells (Pollock, Pennypacker et al. 2005). However, it has not been examined centrally at later time points when astrocyte activation occurs.

A major downstream product of NFκB activation is COX2, the rate-limiting enzyme converting arachidonic acid to prostaglandins and a key player in neuroinflammation. Following spinal nerve injury, COX2 is up-regulated in a biphasic pattern. There is a brief transient increase in COX2 in Schwann cells from days one to three post-injury. This transient increase is followed by a persistent increase in COX2 in macrophages beginning at seven days and lasting through four weeks post-injury (Takahashi, Kawaguchi et al. 2004). Prostaglandin E2 (PGE2) is the major downstream product of COX2 activation and is known to play a role in peripheral sensitization during neuropathic pain. Peripheral injection of PGE2 causes pronounced hyperalgesia (Ferreira 1972; Ferreira, Nakamura et al. 1978). By 21 days post-injury, immunohistochemical staining for the PGE2 receptors EP1 and EP4 doubles. EP1 predominantly colocalizes with macrophage markers, while EP4 is localized in both macrophages and Schwann cells. Lower levels of all four EP receptors are found on neurons; however, there is no change in expression post-injury (Woodhams, MacDonald et

al. 2007).The mechanisms of EP receptor-mediated sensitization following nerve injury are unknown. However, an endoneurial injection of a COX2 inhibitor following nerve injury has been shown to produce a dose-dependent relief of mechanical hyperalgesia (Syriatowicz, Hu et al. 1999).

There is a small transient increase in COX2 mRNA in the deep layers of the dorsal horn starting at ten hours and peaking at 24 hours post-SNI (Broom, Samad et al. 2004). Enhanced COX2 activation may lead to the release of prostaglandins, which can increase neuronal excitability in the spinal cord (Samad, Moore et al. 2001). However, the intrathecal injection of COX2 inhibitors was inconsistent in reducing pain behavior and was only effective when given prior to or within one day of the neuropathic lesion (Zhao, Chen et al. 2000; Broom, Samad et al. 2004; Takeda, Sawamura et al. 2005). Consistently, the administration of systemic COX2 inhibitors performed better than intrathecal COX2 inhibitors at decreasing neuropathic pain behavior (Takeda, Sawamura et al. 2005). Collectively, it seems that COX2 expression in the spinal cord may play a role in the initiation of neuropathic pain but not in its maintenance.

5.4. SUMMARY

Many of the molecules involved in the development of persistent pain are common between the peripheral and central responses. However, it is evident that most of these molecules initially increase peripherally and subsequently increase centrally over a period of hours or days. There is a temporary biphasic pattern to their increase both peripherally and centrally. Peripherally, this biphasic response is mediated first by Schwann cells and second by macrophages and usually occurs over a period of days. Centrally, this is mediated first by microglia and second by astrocytes and often occurs over a period of weeks. Both peripherally and centrally, glial cells are important in establishing the neuronal environment

that can lead to both peripheral and central sensitization. Most importantly, these phasic responses demonstrate that persistent pain is not only the extension of acute pain but also the result of ongoing neuroplastic changes in pain processing. Studying both neuropathic pain development and long-term maintenance will be important in producing clinically successful drugs.

CHAPTER 6.

THE INVOLVEMENT OF PERIPHERAL GLIA IN THE ALLEVIATION OF MECHANICAL ALLODYNIA BY INFLAMMATORY PATHWAY SUPPRESSION

6.1. OVERVIEW

Chronic neuropathic pain leads to long-term changes in the sensitivity of both peripheral and central nociceptive neurons. Glial fibrillary acidic protein (GFAP)-positive glial cells are closely associated with these nociceptive neurons as astrocytes in the central nervous system and as non-myelinating Schwann cells in the peripheral nerves. Central and peripheral GFAP-positive cells are reported to be involved in the maintenance of chronic pain through a host of inflammatory cytokines, many of which are under control of the transcription factor nuclear factor κB (NFκB) and the enzyme cyclooxygenase 2 (COX2).

We used a conditional knockout (cKO) mouse expressing Cre recombinase under the hGFAP promoter and a floxed COX2 gene in order to inactivate the COX2 gene specifically in GFAP-positive cells. In COX2 cKO mice, we found alleviation of pain behavior for several weeks following spared nerve injury, a model of neuropathic pain. Next, we used the tet-off tetracycline transactivator system to suppress NFκB activation in GFAP-positive cells and found a similar phasic pattern of relief from pain behavior following nerve-injury. Experiments were performed to determine whether NFκB was exerting its effects on chronic pain behavior peripherally or centrally. We used oxytetracycline, a blood-brain barrier impermeable analog of doxycycline, to allow transgene expression centrally but not

peripherally to address this question. Peripheral inhibition of the transgene abolished the pain relief in transgenic mice. This implicates non-myelinating Schwann cells, not astrocytes, in the maintenance of neuropathic pain behavior.

6.2. INTRODUCTION

Neuropathic pain resulting from peripheral nerve insult is the result of both peripheral and central sensitization of nociceptive neurons. Furthermore, neuropathic pain is associated with the activation of both peripheral and central glia. Peripherally, Schwann cells immediately detect nerve injury and coordinate the recruitment of blood-born macrophages to the injury site through their release of cytokines and chemokines. Centrally, nerve injury produces the sequential activation of microglia and astrocytes. Activated glia produce proinflammatory cytokines, including interleukin-1β and tumor necrosis factor α, which exert at least part of their effect through the activation of the transcription factor nuclear factor kappa B (NFκB) (Tegeder, Niederberger et al. 2004; Niederberger, Schmidtko et al. 2007).

In its inactive form, NFκB is sequestered in the cytoplasm, bound to inhibitory factors of the IκB family. Upon stimulation by inflammatory mediators, IκB is phosphorylated by the IκB kinase (IKKβ) complex leading to its ubiquitination and degradation, allowing NFκB to translocate to the nucleus. In the nucleus, NFκB promotes the transcription of other cytokines, chemokines and proinflammatory molecules. Studies have shown that inhibition of NFκB in GFAP-positive glia reduces hyperalgesia in the formalin model of inflammatory pain and in the chronic constriction injury model of neuropathic pain (Fu, Zhang et al. 2007; Meunier, Latremoliere et al. 2007; Fu, Zhang et al. 2010).

NFκB positively regulates the expression of several proinflammatory cytokines, including nitric oxide and dynorphin as well as increasing the expression of cycloxygenase-2 (COX2) (Adcock, Newton et al. 1997; O'Neill and Kaltschmidt 1997). The major

downstream product of COX2 activation is prostaglandin E2 (PGE2). Peripheral or intrathecal injection of PGE2 causes pronounced hyperalgesia (Ferreira 1972; Ferreira, Nakamura et al. 1978). However, the administration of systemic COX2 inhibitors performs better than intrathecal COX2 inhibitors at decreasing pain behavior (Takeda, Sawamura et al. 2005). While COX2 seems to be involved in the generation of persistent pain, the cellular source of the COX2 increases remains unidentified.

Here we use transgenic mouse models to dissect the cellular origins of inflammatory mediators that sensitize neurons following injury. We used three mouse lines to manipulate the NFκB-COX2 inflammatory pathways. First, we used conditional knockout transgenic mice expressing Cre recombinase under the hGFAP promoter and a floxed COX2 gene in order to inactivate the COX2 gene specifically in GFAP-positive cells. Next, to constitutively activate NFκB in astrocytes, we used a mouse line expressing consitutively active IKKβ driven by the tetO promoter (Huber, Denk et al. 2002). Thus, IKKβ will continuously phosphorylate IκB, allowing NFκB to translocate to the nucleus (IKKca mice). To inactivate NFκB in astrocytes, a dominant negative version of IKKβ driven by the tetO promoter was used (IKKdn mice) (Huber, Denk et al. 2002). These NFκB modulator lines are crossed to a line carrying the tetracycline transactivator (tTA) driven by the hGFAP promoter, which is localized to astrocytes centrally and non-myelinating Schwann cells peripherally.

We found that transgenic suppression of the NFκB and COX2 inflammatory pathways in GFAP-positive cells produced a temporary alleviation of mechanical sensitivity in the spared nerve injury (SNI) model of neuropathic pain. This phenotype was abolished when transgenic suppression of the NFκB inflammatory pathway was limited to astrocytes.

This finding suggests an important role for peripheral glia in the maintenance of mechanical sensitivity following nerve injury.

6.3. METHODS

6.3.1. Mice

All procedures and behavioral experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Mice were raised under a 12:12 light:dark cycle and fed Prolab RMH (LabDiet) *ad libitum*. All mice were backcrossed to C57/bl6 for greater than ten generations.

6.3.2. Generation of GFAP-specific COX2-deficient mice.

Floxed COX2 mice were generated as previously described (Yu, Fan et al. 2006). Briefly, floxed Cox2 mice were generated by inserting *loxP* sites, using homologous recombination, into introns 5 and 8 to enable deletion of exons 6–8. The insertion of the two *loxP* sites did not affect normal COX2 expression and induction (Yu, Fan et al. 2006; Vardeh, Wang et al. 2009). GFAP-CreERT floxed COX2 mice were induced with a course of 16 tamoxifen injections (3 mg/40 g), starting at post-natal day (p) 21. Littermate floxed, Cre-negative mice were used as wild-type (WT) controls. A subset of littermate floxed, Cre-negative mice were given tamoxifen to control for tamoxifen injection in behavioral experiments.

6.3.3. Generation of GFAP-specific NFκB constitutive activator or suppressor mice

tetO-IKKca and IKKdn mice were generated as previously described (Herrmann, Baumann et al. 2005). Both lines were bred to mice in which the tTA is driven by a 2.2 kb fragment of the human GFAP promoter. GFAP-tTA mice were maintained on doxycycline (25 μg/mL in the drinking water) to suppress transgene expression throughout the central and peripheral nervous system while in utero and until p21. At weaning, the mice were taken off

doxycycline and given drinking water either without drug to allow the expression of the transgene in all GFAP-positive cells or with oxytetracycline (Oxy) (200 mg/mL) to suppress peripheral transgene expression. The mice were maintained on this treatment for 30 days or greater prior to experimental use.

6.3.4. Spared nerve injury model of neuropathic pain

Using isoflurane, mice at least 60 days old were deeply anesthetized, as monitored by toe pinch and eye blink reflexes. The left hind leg was secured with tape, the hair removed with Nair, and the leg was cleaned with iodine and alcohol. The three divisions of the sciatic nerve were exposed. The common peroneal and sural nerves were ligated, and a 1-3 mm portion of the nerve was removed distal to the ligation to prevent regrowth. The musculature was returned to its original position, and the skin closed with wound clips. The area was again cleaned with iodine and alcohol, and anti-bacterial and anti-fungal agents were applied. The animals were housed in cages with soft bedding.

6.3.5. Behavior

Animals were singly placed in a Plexiglas enclosure on top a metal grate. Paw withdrawal frequency was used a measure of mechanical allodynia. A von Frey hair (IITC Life Sciences, Woodland Hills, CA) calibrated to 3.61 log force was applied to the center of the foot pad ten times at an interval of at least one minute between applications. The number of times the paw was withdrawn due to the stimulus was recorded. The paw withdrawal threshold was measured using the up-down method of von Frey hair application (Chaplan, Bach et al. 1994). Mechanical sensitivity was measured for two days prior to surgery. Testing then occurred at four days, seven days and every subsequent week until six weeks post-injury.

6.3.6. Immunohistochemistry

Animals were deeply anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Once deeply anesthetized, as monitored by the toe-pinch and eye-blink reflexes, the animals were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Lumbar segments 4-6 of the spinal cord, dorsal root ganglia (DRG) and sciatic nerves were removed and post-fixed for two hours. The tissue was cryoprotected with 30% sucrose in PBS and subsequently frozen in OCT. Fourteen micrometer-thick sections were made on a cryostat (Leica, Wetzlar, Germany). Immunohistochemistry was performed with primary antibodies against mouse GFAP (1:500; Dako), chicken GFAP (1:500; Abcam), rabbit Iba-1 (1:500; Wako), mouse NeuN (1:500; Millipore), rabbit peripherin (1:1000; Millipore), rabbit neurofilament 200 (1:500; Sigma), mouse S100β (1:1000; Sigma), rabbit pNFκB (1:400; Cell Signaling) and biotinylated IB4 lectin (1:100; Vector). Secondary antibodies were either anti-mouse, anti-rabbit or anti-chicken Alexa 568, Alexa 488, Alexa 647 or avidin-conjugated Alexa 568. The slides were coverslipped with Vectashield with DAPI. Images were collect on a Zeiss Axioskope fluorescent microscope with a Dage XL16 camera or on a Zeiss LSM 710 confocal microscope. Quantification of immunopositive cells was performed manually.

6.3.7. Electrophysiology

To electrophysiologically characterize the primary afferent nociceptive fibers, we used a skin-nerve preparation. Animals were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). The tibial nerve was dissected free from the sciatic notch to the distal termination in the shaved skin of the hind paw. The skin was placed dermal side up in the organ bath and superfused with oxygenated, modified synthetic interstitial fluid (SIF: 123 mM NaCl, 3.5 mM KCl, 0.70 mM $MgSO_4$, 2.0 mM $CaCl_2$, 9.50 mM sodium gluconate, 1.70 mM NaH_2PO_4, 5.50 mM glucose, 7.50 mM sucrose, and 10.0 mM HEPES, pH adjusted

to 7.4 with NaOH) at a 10 ml/min flow rate. The bath temperature was maintained at 31–32 °C by a Peltier cooling/heating module (Cell MicroControls, Norfolk, VA), and the temperature was measured by a thermocouple (T-type, Bailey Instruments, Saddle Brook, NJ) placed in the bath. The nerve trunk was passed through a channel into a second compartment filled with a bilayer of SIF and mineral oil. The portion of the nerve most proximal to the sciatic notch was dissected free of connective tissue and elevated onto a mirrored stage.

Nerve fiber bundles were progressively split until single units or small groups of fibers could be suspended on a gold wire electrode. Single units were isolated as described by Reeh et al. (1986) and Zimmermann et al. 2009. Extracellular recordings were filtered and digitized by a differential amplifier (World Precision Instruments, Sarasota, FL) and a Digidata 1440A data system (Molecular Devices, Sunnyvale, CA). The receptive field was located and mapped by electrical stimulation with a search electrode (tapered, 0.25 mm, 5 MΩ, epoxy-insulated tungsten, A-M Systems, Inc., Sequim, WA) and by mechanical stimulation with a hand-held monofilament or blunt glass probe.

The length of nerve between the receptive field and the recording electrode, as well as the time interval between the stimulus artifact and the upsweep of action potential, were measured to calculate conduction velocity (m/s). C-fibers were classified by their conduction velocity of less than 1 m/s. C-fibers were characterized by mechanical threshold, heat threshold, cold and acid responsiveness. Calibrated von Frey monofilaments (Semmes Weinstein Von Frey Aesthesiometer, Stoelting, Wood Dale, IL) were used to estimate mechanical thresholds. Heat threshold was determined using a 980 nm, 7.5 W, continuous wave, class IV diode laser (Lass/DLD-7-NM3, LASMED, Mountain View, CA) (Pribisko

and Perl 2011). Cold and acid responsiveness were tested using chilled (1 °C) and acidified (pH adjusted to 3.6 with HCl) SIF. A milled plastic cylindrical reservoir (5 mm internal diameter, 0.3 ml capacity) with a molded Sylgard (Dow Corning, Midland, MI) lip was applied over the receptive field. Iced and acidified SIF were sequentially transferred by a handheld micropipette. A fiber was described as responsive when at least 2 action potentials were consistently evoked on repetitive stimulation.

6.3.8. Statistical analysis

All data are presented as the mean ±SEM. Significant differences were assessed by Student's *t*-test or an analysis of variance followed by a Bonferroni post hoc analysis. The criteria for significance were as follows: $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

6.4. RESULTS

6.4.1. Glial-specific transgenic COX2 knockout temporarily reduces mechanical sensitivity following peripheral nerve injury

GFAP-positive glia are thought to play a role in the maintenance of neuropathic pain through the release of inflammatory molecules (Ma and Bisby 1998; Milligan and Watkins 2009; Fu, Zhang et al. 2010; Shubayev, Kato et al. 2010). The major product of COX2 activation is PGE2, which can sensitize peripheral or central neurons (Ferreira 1972; Ferreira, Nakamura et al. 1978). We used tamoxifen inducible GFAP-specific Cre mice to drive the removal of the floxed COX2 gene specifically in GFAP-positive glia. Tamoxifen injections were given post-weaning to avoid any potential developmental confounds of transgene expression. To control for possible effects of tamoxifen, a group of WT animals were also injected with the same time course of tamoxifen.

To assess the role of COX2 in GFAP-positive glial cells on pain behavior, we measured mechanical allodynia and paw withdrawal threshold for two days prior to SNI and for six weeks following peripheral nerve injury in COX2 cKO, WT littermate controls and tamoxifen (Tam)-injected WT littermate controls (male COX2 cKO n=10, female COX2 cKO n=10, male WT n=10, female WT n=10, male WT+Tam n=10, female WT+Tam n=10).

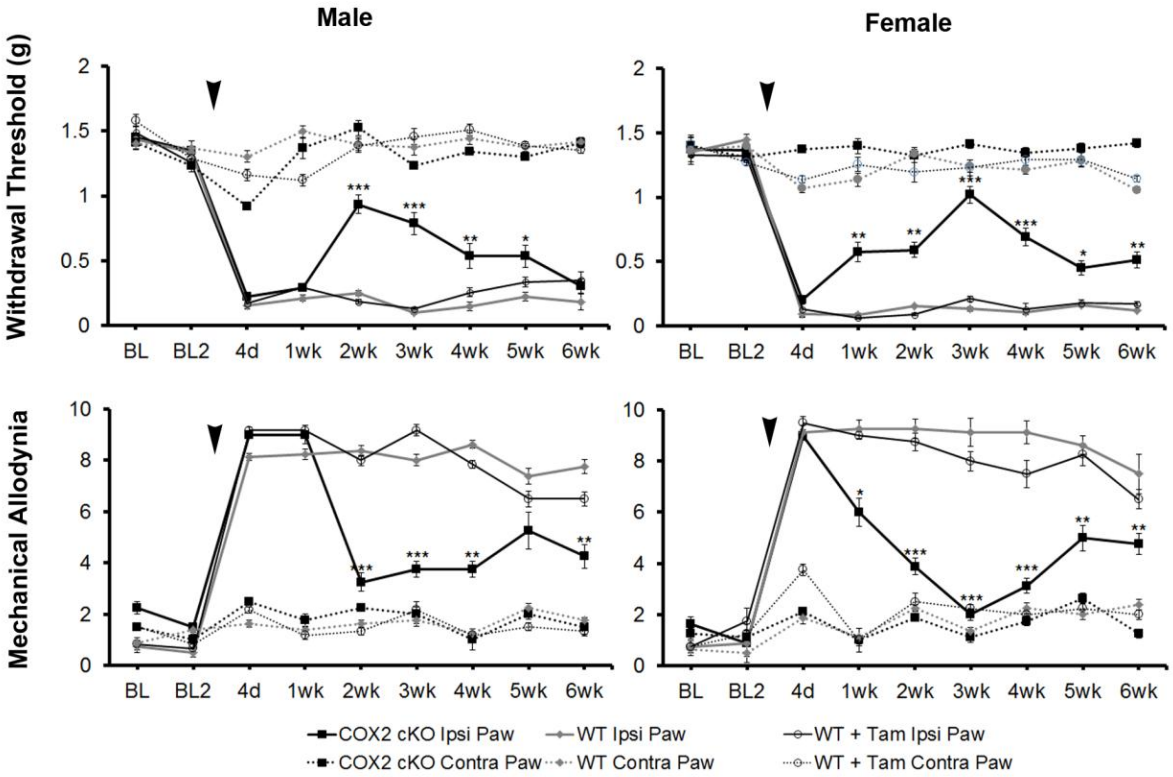

Figure 7. Withdrawal threshold and mechanical allodynia following SNI in male and female COX2 cKO mice (male n=10; female n=10), **WT littermate controls** (male n=10; female n=10) **and WT littermate animals given** tamoxifen (male n=10; female n=10). There was no difference in basal mechanical sensitivity between any of the groups. All groups developed mechanical allodynia in the paw ipsilateral to nerve lesion at four days post-injury. WT and WT + Tam groups maintained mechanical sensitivity though the six-week time point. COX2 cKO animals displayed temporary alleviation of mechanical sensitivity for four or more weeks. In males, mechanical sensitivity decreased at two weeks post injury, while in females, sensitivity decreased at one week post-injury. Bonferroni post hoc analysis: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for COX2 cKO vs. WT ipsilateral hindpaws.

As shown in figure 7, there was no difference in mechanical sensitivity in male or female naïve mice. By four days post-SNI, both males and females developed mechanical allodynia in the paw ipsilateral to the lesion, but not in the contralateral paw, as assessed by paw withdrawal frequency and threshold measurements. In WT and tamoxifen injected WT control animals, mechanical sensitivity of the ipsilateral paw was maintained through the six-week testing period. There was no significant impact of tamoxifen injection on WT animals at any time point. At two weeks post-injury, male COX2 cKO mice showed an abrupt and dramatic reduction in mechanical sensitivity ($P < 0.001$ compared to the WT ipsilateral paw). The reduction in mechanical sensitivity peaked at two weeks and slowly returned to WT levels by six weeks post-injury ($P < 0.05$ for weeks two through five). Female COX2 cKO mice showed a similar reduction in pain sensitivity that began slowly at one week post-injury and peaked at three weeks post injury. The reduction in pain sensitivity was maintained throughout our six week testing period, however following the peak of pain alleviation at week three, values slowly began to return to WT levels.

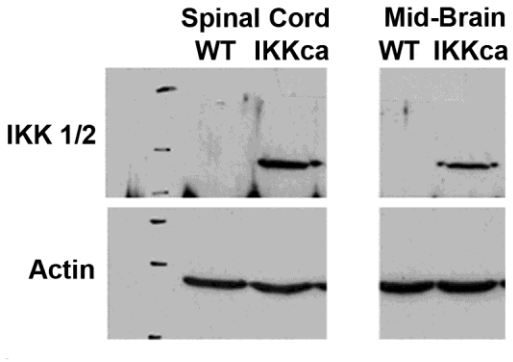

Figure 8. A western blot showing higher protein levels of IKK in the spinal cord and mid-brain lysates of IKKca mice compared to WT controls. Each lane was normalized to actin as a loading control.

6.4.2. Glial-specific constitutive activation of NFκB has no effect on mechanical sensitivity following peripheral nerve injury

NFκB is upstream of COX2 and is a key signaling molecule in many inflammatory pathways. To investigate the role of NFκB in GFAP-positive glial cells, we generated mice using the tet-Off system. In these mice, the GFAP promoter was used to drive glial-specific constitutive activation of the NFκB pathway (IKKca mice). Figure 8 shows that IKKca mice have more IKK protein in the spinal cord than WT littermate controls. To determine whether

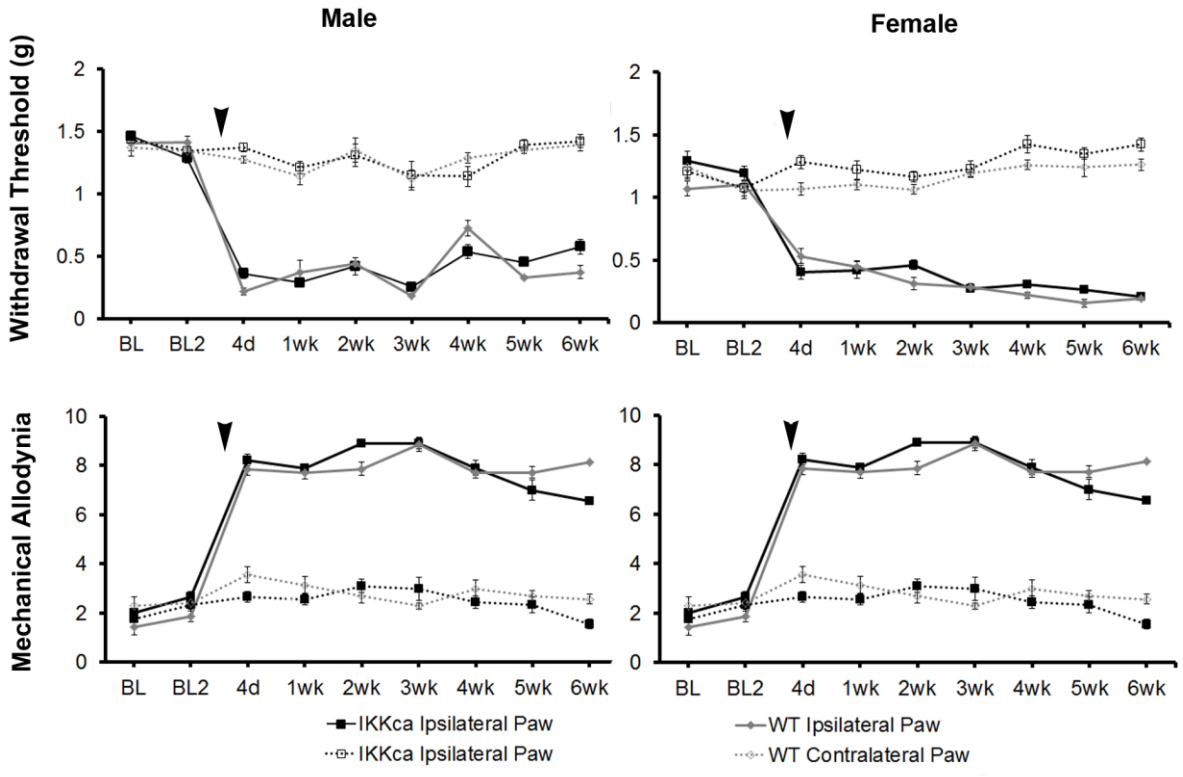

Figure 9. Withdrawal threshold and mechanical allodynia following SNI in male and female IKKca mice (male n=10; female n=10) **and WT littermate controls** (male n=10; female n=10). There was no difference in basal mechanical sensitivity between the groups. All mice developed mechanical allodynia in the paw ipsilateral to nerve lesion beginning four days post-injury, which lasted throughout the duration of our testing period. There was no difference in the development of mechanical sensitivity following SNI between IKKca and WT mice.

constitutive activation of NFκB in GFAP-positive glial cells plays a role in pain behavior, we measured mechanical sensitivity for two days prior and for six weeks following SNI in IKKca mice and littermate controls (male IKKca n=10, female IKKca n=10, male WT n=10, female WT n=10) (Fig. 9). We found no difference in basal sensitivity between these groups of mice. Both transgenic and WT mice developed robust mechanical allodynia in the paw ipsilateral to the nerve lesion; there was no difference in mechanical allodynia between the groups.

6.4.3. Glial-specific transgenic suppression of NFκB temporarily reduces mechanical sensitivity following peripheral nerve injury

To suppress NFκB signaling, we used the tet-Off system driven by the GFAP promoter to induce glial-specific expression of a dominant negative IKK. To characterize expression, GFAP-tTA mice were bred to a tetO-eGFP reporter line of mice. We observed strong native eGFP expression in the dorsal horn, which colocalized with the astrocyte marker GFAP, but not with markers for microglia (Iba-1) or neurons (NeuN) (Fig. 10). In the sciatic nerve, we observed eGFP expression in non-myelinating Schwann cells (GFAP-positive) of the sciatic nerve. eGFP did not colocalize with any markers for macrophages (Iba-1), large diameter axons (NF200) or small diameter axons (peripherin). Surprisingly, there was no eGFP expression in GFAP-positive satellite cells of the dorsal root ganglion (DRG) nor in any other cells of the DRG. Immunohistochemistry was also performed two weeks post-SNI to detect changes in eGFP localization after nerve lesion and gliosis; however, no changes were evident.

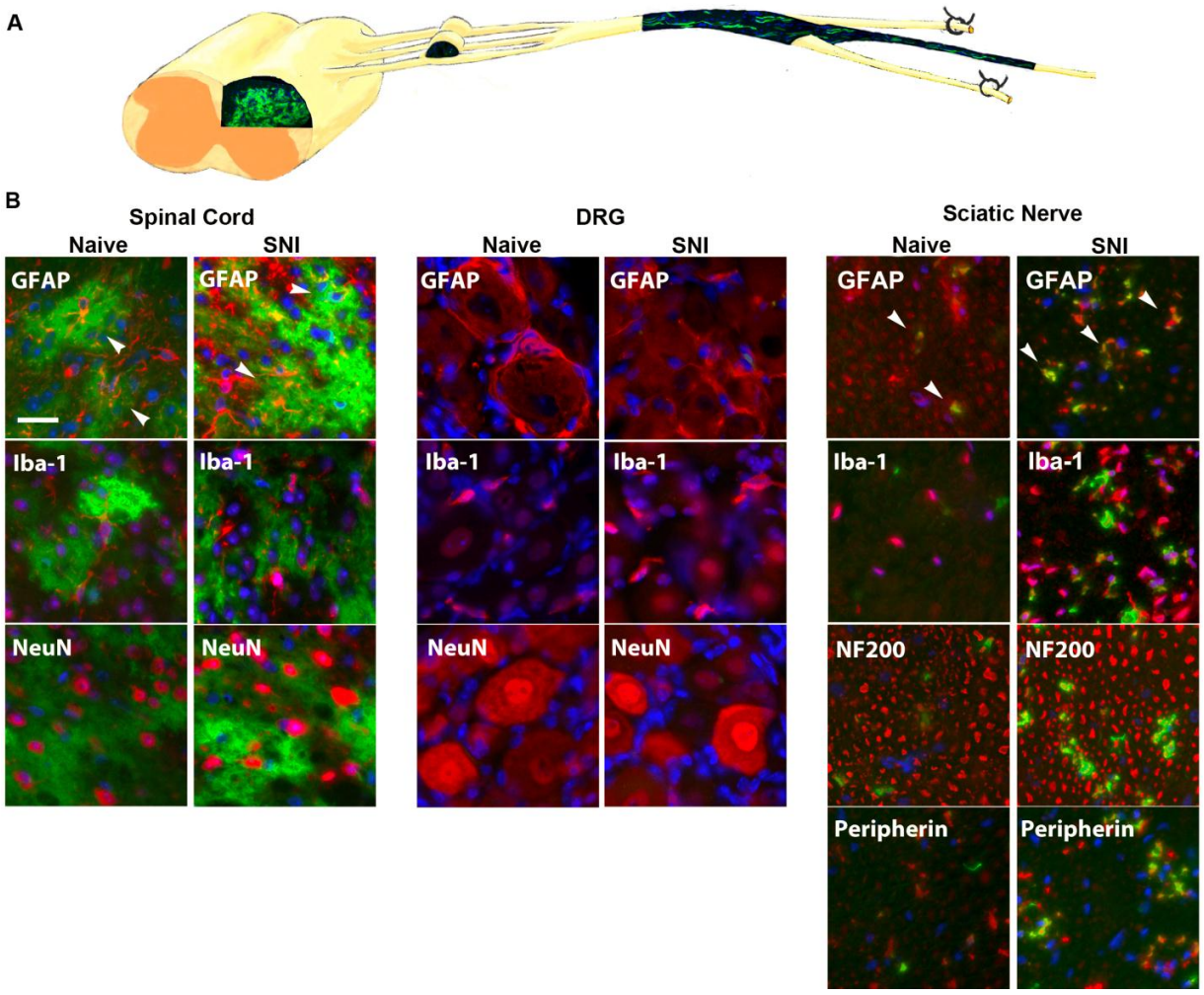

Figure 10. GFAP-tTA tetO-eGFP reporter expression in naïve and SNI animals. A. A diagram of the spinal cord, DRG and sciatic nerve from GFAP-tTA eGFP reporter mice. The diagram shows the expression of eGFP in the spinal cord and long stripes of eGFP expression in the sciatic nerve (longitudinal section). **B.** The native eGFP reporter (*green*) colocalized with immunohistochemical staining for astrocytes (GFAP, *red*) in the dorsal horn and with non-myelinating Schwann cells (GFAP, *red*) in the sciatic nerve (transverse sections), but not with satellite cells (GFAP, *red*) in the DRG. eGFP did not colocalize with any markers for microglia or macrophages (Iba-1), neurons (NeuN) or large or small axonal markers (NF200 or peripherin). The second column in each region, spinal cord, DRG and sciatic nerve, shows staining from that region two weeks post-nerve SNI. There was no change in eGFP localization two weeks post-injury. All cellular and axonal markers are red and are listed on the individual images. Native eGFP is shown in green. DAPI is shown in blue. White arrows indicate colocalized cells. The scale bar is 20 µm.

To investigate the role of NFκB in GFAP-positive glial cells on pain behavior, we measured mechanical allodynia and paw withdrawal threshold for two days prior to SNI and for six weeks following peripheral nerve injury in IKKdn and littermate control animals male IKKdn n=6, female IKKdn n=5, male WT n=9, female WT n=9). There was no difference in basal mechanical sensitivity in the male or female groups (Fig. 11). Following SNI, all animals developed mechanical allodynia ipsilateral to the nerve-lesion, as assessed by paw

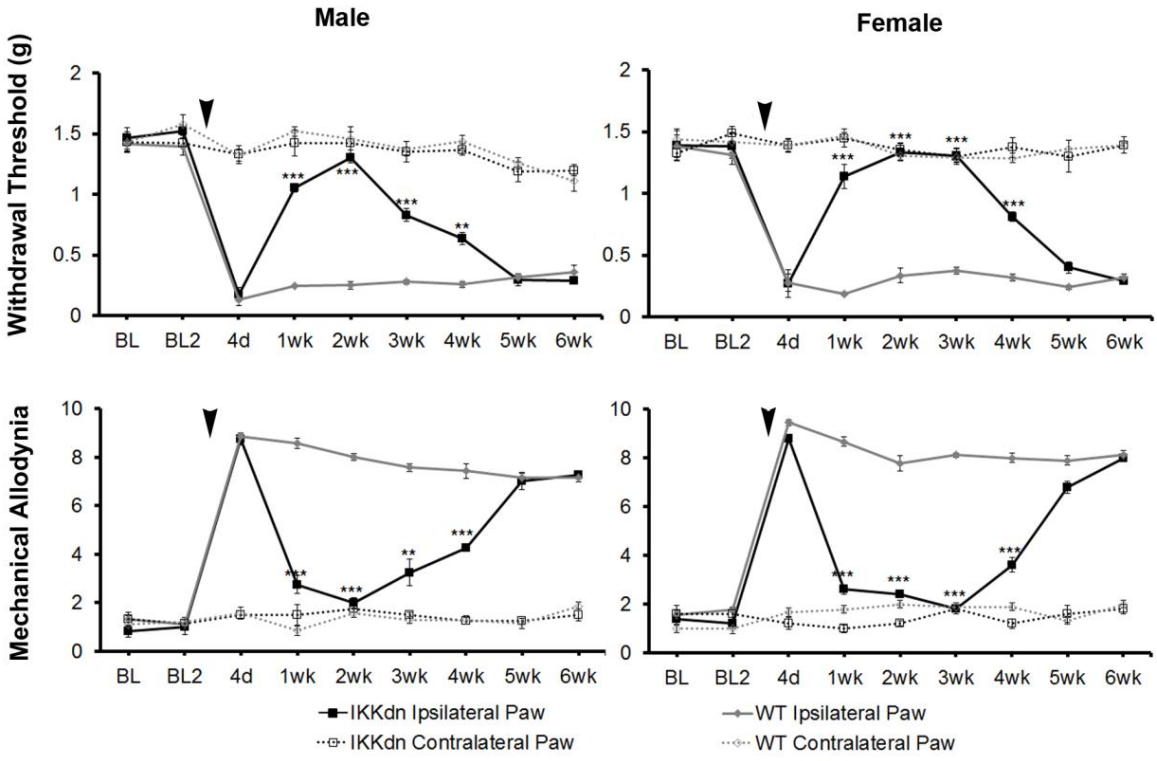

Figure 11. Withdrawal threshold and mechanical allodynia following SNI in male and female IKKdn mice (male n=6; female n=5) **and WT littermate controls** (male n=9; female n=9). There was no difference in basal mechanical sensitivity between the groups. Both groups developed mechanical allodynia in the paw ipsilateral to nerve lesion at four days post-injury. WT animals maintained mechanical sensitivity though the six-week time point. IKKdn animals displayed temporary alleviation of mechanical sensitivity for four or more weeks. In both males and females, mechanical sensitivity decreased at one week post injury and returned to WT levels by week five. Bonferroni post hoc analysis: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, IKKdn vs. WT ipsilateral hind paws.

withdrawal frequency and threshold. In control animals, mechanical sensitivity of the ipsilateral paw was maintained through the six-week testing period. In IKKdn transgenic animals, pain behavior began to subside one week post-injury ($P < 0.001$) and was alleviated by two weeks post-injury in both the male and female IKKdn mice ($P < 0.001$).

The pattern of alleviation in the IKKdn mice was similar to that observed in COX2 cKO mice. Mice initially displayed mechanical allodynia, followed by approximately three weeks of pain alleviation. The mechanical allodynia in the IKKdn mice then slowly returned to WT levels. No change was observed in the contralateral paws of the IKKdn or WT littermate controls.

6.4.4. Oxytetracycline suppresses peripheral transgene expression using the tet-Off system while leaving central expression intact

Persistent pain manifests itself as an expression of neuronal plasticity in both primary sensory neurons and spinal dorsal horn neurons. There is both a peripheral and central contribution to sensitization in persistent pain. Likewise, using the GFAP promoter, our system drives the expression of transgenes both centrally, in astrocytes, and peripherally, in non-myelinating Schwann cells. The tet-Off system is typically regulated by the tetracycline derivative doxycycline, which, similar to tetracycline, binds the tTA, preventing it from binding to the tetO promoter and blocking transcription of the downstream transgene. Doxycycline is highly bioavailable and easily crosses the blood-brain barrier. To tease apart the central and peripheral roles of GFAP-positive glia we used oxytetracycline (Oxy), a tetracycline derivative that does not cross the blood-brain barrier.

GFAP-tTA tetO-eGFP reporter mice were administered Oxy in their drinking water from weaning until p60. At p60, they were sacrificed to examine transgene expression using

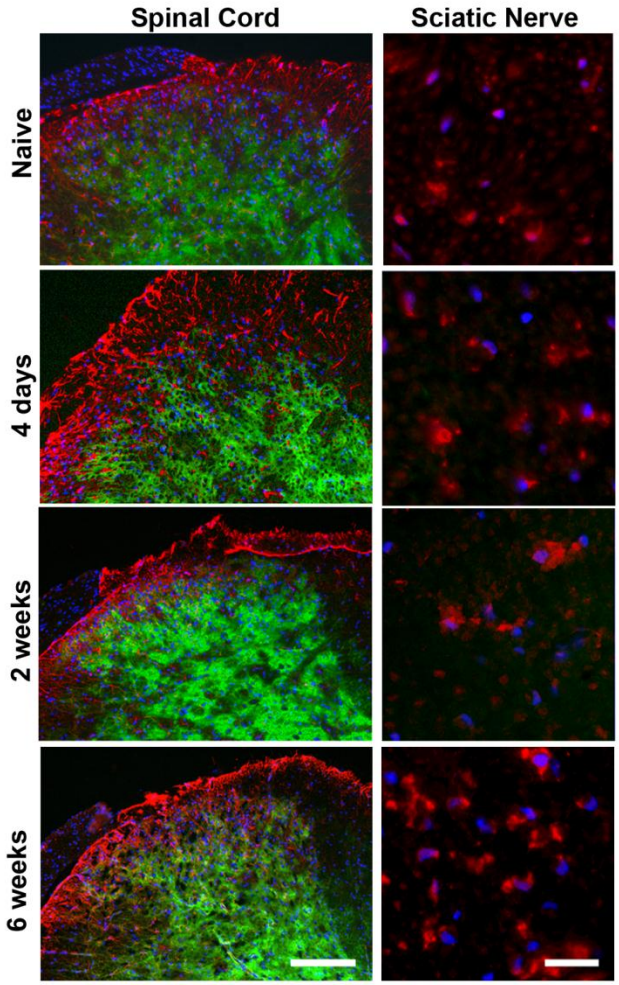

Figure 12. A pre- and post-nerve injury timeline showing transgenic, eGFP expression (*green*) and GFAP immunohistochemical staining (*red*) from mice on Oxy, which demonstrates that Oxy is not capable of crossing the blood-brain barrier to inhibit reporter gene expression at any point post-surgery (four days, two weeks or six weeks) (n=3 animals per time point). Oxy does inhibit transgene expression in the sciatic nerve in naïve mice through six weeks post-nerve injury. The scale bar on left is100 μm. The scale bar on right is 20 μm.

immunohistochemistry. We found that Oxy effectively inhibited transgene expression peripherally while leaving central expression intact (see Fig 6) (n=3 per group). eGFP was not detected in the sciatic nerve either in naïve animals or in animals two weeks post-injury. In the spinal cord, eGFP remained colocalized with the astrocyte marker GFAP in both naïve and nerve-injured animals. eGFP was not colocalized with any markers for microglia or macrophages (Iba-1), neurons (NeuN) or large or small peripheral nerves (NF200 or peripherin).

The permeability of the blood-brain barrier increases for one week post-nerve injury (Beggs, Liu et al. 2010). To determine whether Oxy entered the spinal cord in a great enough quantity to regulate transgene expression, we performed time-course of immunohistochemical staining following SNI. Adult animals on Oxy for at least one month prior to surgery underwent SNI and were sacrificed at 4 days, 2 weeks or 6 weeks post-injury (n=3 per time point). eGFP expression was suppressed in all sciatic nerve sections, while its central expression in the spinal cord was preserved at every time point (Fig. 7). These findings indicate that Oxy was not able to cross the blood-brain barrier in sufficient concentration to block gene expression post-nerve injury.

Next, we evaluated the ability of Oxy to regulate the expression of the IKKdn transgene by examining the downstream marker pNFκB. If effective, Oxy should peripherally inhibit IKKdn expression thus allowing pNFκB activation in non-myelinating Schwann cells of the sciatic nerve. Activated or phosphorylated NFκB is localized to the cell nucleus, whereas the non-myelinating Schwann cell marker GFAP is often localized to cellular processes, making colocalization of these two markers difficult. S100β is a marker for both myelinating and non-myelinating Schwann cells and fills the cytosol of the cell,

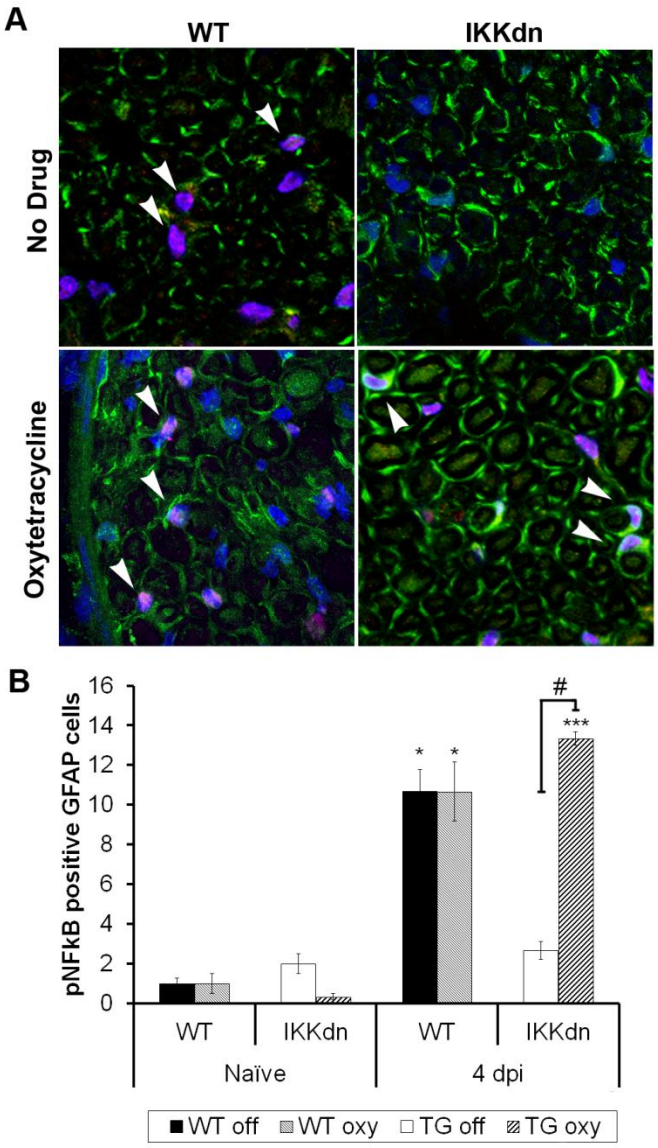

Figure 13. SNI activates NFκB in non-myelinating Schwann cells which is inhibited by the expression of IKKdn and reversed with Oxy administration. **A**. Immunostaining for S100β (green), pNFκB (red) and DAPI (blue) in the sciatic nerve four days post-SNI. WT animals showed increases in nuclear pNFκB staining both in the drug-free and Oxy groups. IKKdn animals without Oxy showed decreased pNFκB staining, whereas the pNFκB expression of IKKdn animals administered Oxy returned to WT levels. **B**. Cells positive for GFAP, S100β and nuclear pNFκB were quantified. In naïve animals, both WT and IKKdn animals showed low levels of pNFκB-positive cells regardless of drug treatment. In animals four days post-SNI, WT animals showed a significant increase in the number of pNFκB-positive cells. This increase was not affected by treatment with Oxy. In IKKdn animals, SNI did not produce an increase in pNFκB-positive cells compared to naïve animals. However, in IKKdn animals, SNI produced an increase in pNFκB-positive cells similar to that observed in WT animals. (In all groups, nine images were analyzed from three different animals.) The number of positive cells displayed is the mean per image ± SEM. * $P < 0.05$, ** $P < 0.01$,

*** $P < 0.001$ for the comparison of the SNI group to its naïve counterpart. # $P < 0.001$ IKKdn four days post-injury off drug vs. on Oxy.allowing for colocalization with nuclear markers. We therefore performed immunohistochemical staining with the markers for GFAP and S100β in conjunction with the nuclear marker DAPI and the pNFκB antibody, to quantify the number of pNFκB-positive non-myelinating Schwann cells.

Quantification was performed in naïve WT and transgenic animals, both on and off Oxy. Naïve animals from all treatment groups displayed little pNFκB staining and few cells containing all four colocalized markers (Fig. 8B) (n=3 animals per group, 3 sections per animal). To drive the expression of pNFκB, we performed SNI and sacrificed the animals four days post-injury. Figure 8A shows representative sections from all four treatments at the four days post-SNI time point (GFAP labeling was omitted for clarity). Following injury, pNFκB was significantly elevated in WT animals compared to the naïve WT animals ($P < 0.05$). The administration of Oxy to WT animals had no effect on pNFκB expression. In transgenic animals, the IKKdn transgene completely prevented the SNI-induced increase in pNFκB-positive cells observed in WT animals. The administration of Oxy to IKKdn animals reversed this effect. IKKdn animals on Oxy had significantly higher levels of pNFκB positive non-myelinating Schwann cells compared to IKKdn animals without drug ($P < 0.001$).

Oxy effectively inhibited peripheral transgene expression while leaving central expression intact. The peripheral expression of IKKdn suppressed NFκB signaling in non-myelinating Schwann cells. The administration of Oxy inhibited peripheral expression of IKKdn, allowing NFκB signaling to occur. Therefore, in IKKdn animals on Oxy, the IKKdn protein should only inhibit NFκB signaling centrally in astrocytes.

6.4.5. Astrocyte-specific transgenic inhibition of NFκB has no effect on mechanical sensitivity following peripheral nerve injury

To tease apart the role of NFκB in astrocytes vs non-myelinating Schwann cells in nerve injury, we measured mechanical sensitivity following SNI in IKKdn and littermate controls both on and off Oxy (IKKdn Oxy n=10, IKKdn Off n=10, WT Oxy n=9, WT Off n=8). Male and female mice were tested separately, but the data were pooled because there were no significant sex differences in the previous cohort. We measured mechanical allodynia and paw withdrawal threshold for two days prior to SNI and for six weeks following peripheral nerve injury. There was no difference in basal mechanical sensitivity in the off drug or Oxy groups (Fig. 9). Following SNI, all animals developed mechanical allodynia as assessed by paw withdrawal frequency and threshold. In WT control animals, mechanical sensitivity of the ipsilateral paw was maintained through the six-week testing period. Transgenic IKKdn animals showed the same pattern of temporary alleviation of mechanical sensitivity starting at one week following nerve injury ($P < 0.001$) and lasting until four weeks post-injury. Surprisingly, this effect was completely reversed in transgenic animals administered Oxy which blocks transgene expression peripherally but not centrally. Transgenic animals on Oxy exhibited the same development of mechanical sensitivity as WT animals following SNI.

In the transgenic mice off drug, the IKKdn transgene suppressed NFκB signaling in both astrocytes and non-myelinating Schwann cells. This resulted in the temporary alleviation of pain behavior following SNI. Oxy inhibited peripheral but not central transgene expression (Fig 7). As a result, in transgenic mice on Oxy, NFκB signaling is only suppressed centrally in astrocytes. The suppression of NFκB in astrocytes alone was not sufficient to relieve pain behavior.

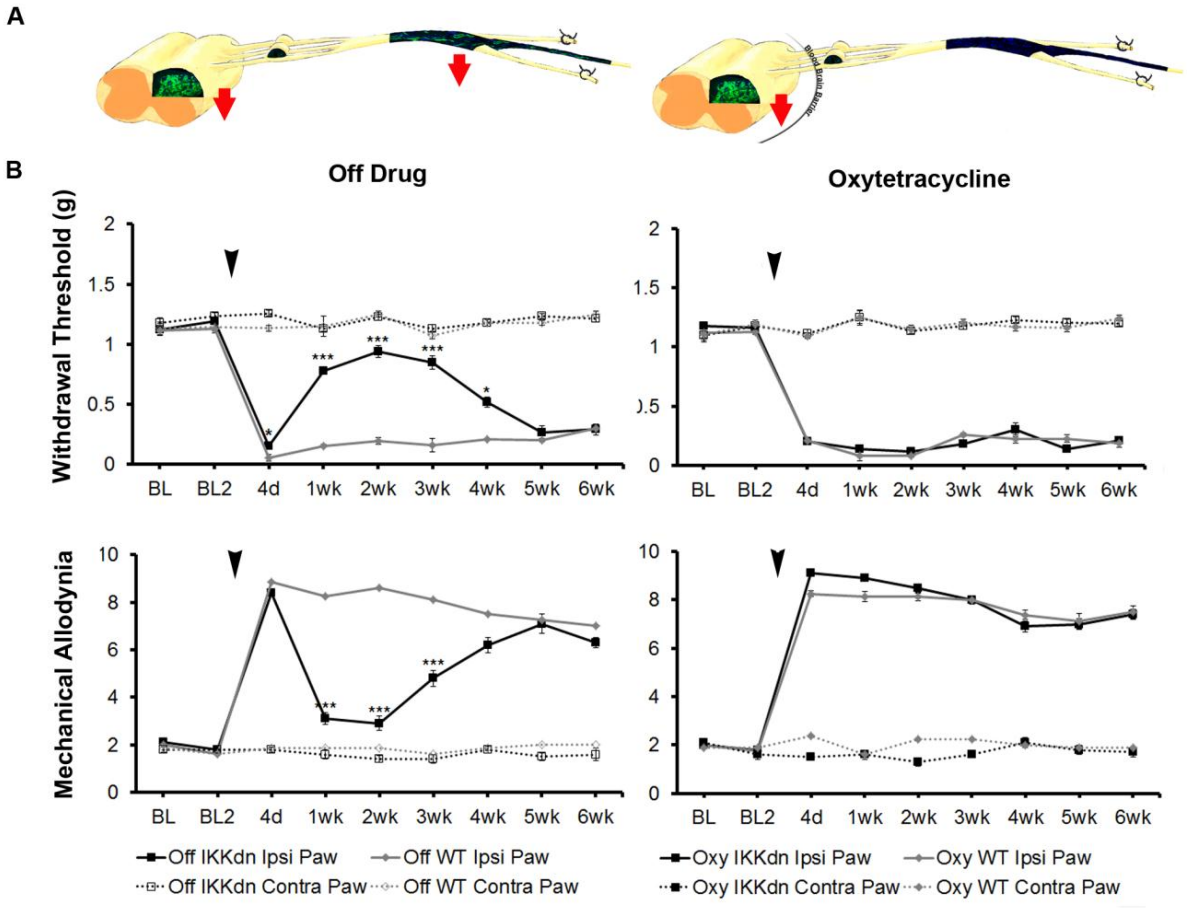

Figure 14. Withdrawal threshold and mechanical allodynia following SNI in male and female IKKdn mice and WT littermate controls with and without Oxy. **A.** A diagram of the spinal cord, DRG and sciatic nerve from GFAP-tTA eGFP reporter mice following treatment with or without oxytetracycline. In animals without oxytetracycline administration, we observed expression of eGFP in the spinal cord and sciatic nerve, but not in the DRG. The red arrows indicate the regions of NFκB suppression in transgenic animals without oxytetracycline. In animals administered oxytetracycline, we observed eGFP in the spinal cord, but not the DRG or sciatic nerve. The red arrows indicate the regions of NFκB suppression in transgenic animals administered oxytetracycline. **B.** Withdrawal threshold and mechanical allodynia following SNI in IKKdn and WT littermate control animals with and without administration of the transgene inhibitor oxytetracycline (IKKdn Off n=10; IKKdn Oxy n=10; WT Off n=8; WT Oxy n=9). There was no difference in basal mechanical sensitivity between any of the groups. All groups developed mechanical allodynia in the paw ipsilateral to the nerve lesion at four days post-injury. WT animals maintained mechanical sensitivity of the ipsilateral paw though the six-week time point. IKKdn animals without oxytetracycline displayed temporary alleviation of mechanical sensitivity for four or more weeks. IKKdn animals on oxytetracycline displayed no difference in the development or maintenance of mechanical sensitivity compared to littermate controls. Bonferroni post hoc analysis: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, for IKKdn vs WT ipsilateral hind paws.

6.4.6. Glial NF-κB inhibition does not change the threshold or spontaneous firing of unmyelinated C-fibers

GFAP-positive non-myelinating Schwann cells are closely associated with nociceptive C-fibers. To obtain a physiological measure of changes in the primary afferents, we electrophysiologically characterized them using a skin-nerve preparation. Figure 10A shows a schematic in which the skin of the foot pad is pinned to the base of the organ bath on the left with nerves attached. The distal portion of the nerve penetrates the portal between the compartments, and the proximal nerve is elevated onto a mirrored stage on the right (see Fig 10A). Individual or small groups of fibers are teased apart and placed on a recording electrode. In this manner, we were able to provide stimulation to the receptive field of single nerve units to characterize their conduction velocity, mechanical threshold, heat threshold, cold and acid responsiveness and spontaneous activity.

Recordings were made from single C-fibers of the tibial nerve, at the level of the sciatic notch, which is proximal to the nerve injury site. We characterized C-fibers in IKKdn mice and their WT littermates at two weeks post-SNI, the time point at which we saw the greatest behavioral differences (11 IKKdn fibers, 2 animals; 10 WT fibers, 2 animals). Following SNI, there were no differences in the mechanical or heat threshold, cold or acid responsiveness or spontaneous firing of unmyelinated C-fibers between IKKdn and littermate controls.

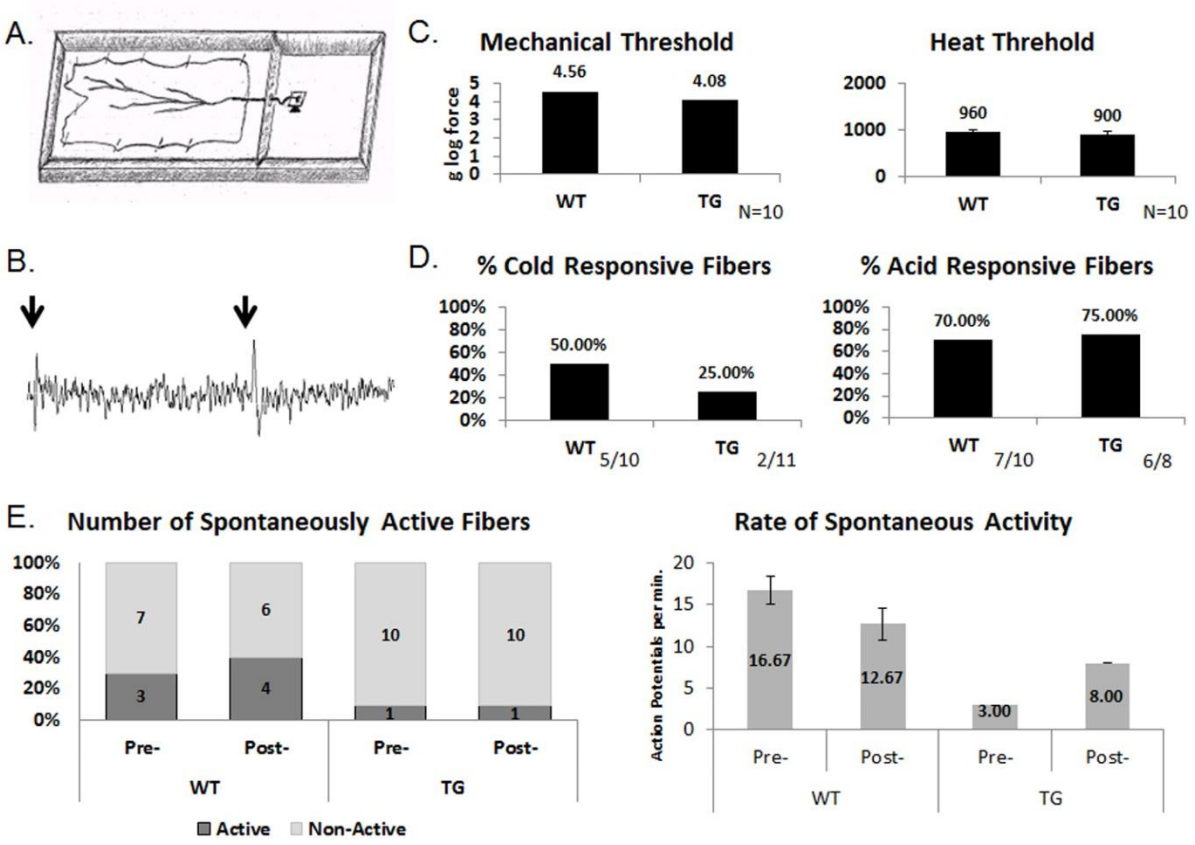

Figure 15. Skin-nerve preparation in IKKdn and WT controls. A. Schematic representations of a dual compartment recording chamber for the mouse skin-nerve preparation. The skin is pinned to base of the organ bath on the left. The distal nerve penetrates the portal between the compartments. The proximal nerve is elevated onto a mirrored stage on the right. **B.** The length of nerve between the receptive field and the recording electrode, as well as the time interval between stimulus artifact (first tag on trace) and the upsweep of the action potential (second tag), are measured to calculate the conduction velocity (m/s). The C-fibers were classified by their conduction velocity of less than 1 m/s. **C.** There was no significant difference in mechanical or heat threshold between IKKdn and WT littermate controls. **D.** There was no significant difference between the percent of cold responsive or acid responsive C-fibers between IKKdn and WT littermate controls. **E.** There was no significant difference between the number of spontaneously active fibers pre- or post-stimulation or their rates of spontaneous activity.

6.5. DISCUSSION

The development of neuropathic pain following peripheral nerve damage involves a coordinated peripheral and central response from both neurons and glia. We used a transgenic mouse model approach to dissect the cellular origins of the inflammatory mediators that sensitize neurons following nerve injury.

The activation of NFκB results in the transcription of a variety of cytokines and chemokines involved in the inflammatory response. Here, we used mice capable of activating or suppressing the classical NFκB pathway specifically in GFAP-positive cells. First, we found that constitutive activation of NFκB in GFAP-positive cells had no effect on pain behavior following neuropathic injury. This may be due to a ceiling effect of NFκB activation following nerve injury. Second, we found that the inhibition of NFκB in GFAP-positive cells temporarily alleviates pain beginning at one week and lasts until four weeks post-injury in both male and female mice.

In the chronic constriction injury (CCI) model of nerve injury, Fu et al. (2010) also found that transgenic in inhibition of NFκB in GFAP-positive cells produced a decrease in pain behavior following nerve injury. However, the decrease in pain behavior was consistent and non-phasic throughout the testing period, as monitored out to three weeks post-injury. Although many chronic neuropathic pain studies conclude around three weeks post-injury, by continued behavioral monitoring of mechanical sensitivity, we observed a return of pain behavior at five weeks post-injury.

In addition to the transcription of a host of cytokines and chemokines, the activation of NFκB results in the transcription of COX2, the rate-limiting enzyme converting arachidonic acid to prostaglandins. Following spinal nerve injury, peripheral COX2 is

upregulated in a biphasic pattern. There is a brief transient increase in COX2 in Schwann cells from one to three days post-injury, followed by a persistent increase in macrophages beginning at seven days and lasting through four weeks (Takahashi, Kawaguchi et al. 2004). Transgenic knockout of COX2 in the central and peripheral nervous system using the nestin promoter is known to be effective in reducing pain behavior in inflammatory pain models (Vardeh, Wang et al. 2009); however, its cellular source was previously unknown. In this study, however, we demonstrated that Schwann cells are a cellular source of COX2 activation which contributes to mechanical hypersensitivity following nerve-injury. Prostaglandin E2 (PGE2) is the major downstream product of COX2 activation and is known to play a role in neuropathic pain. Peripheral injection of PGE2 causes pronounced hyperalgesia (Ferreira, Nakamura et al. 1978). By three weeks post-nerve injury, the PGE2 receptors EP1 and EP4 are upregulated in macrophages and Schwann cells (Woodhams, MacDonald et al. 2007). Furthermore, endoneurial injection of a COX2 inhibitor following nerve injury has been shown to produce a dose-dependent relief of mechanical hyperalgesia (Syriatowicz, Hu et al. 1999). Centrally, there is also a brief transient increase in COX2 mRNA in the deep layers of the dorsal horn starting at ten hours and peaking at 24 hours post-SNI (Broom, Samad et al. 2004). Enhanced COX2 activation may lead to release of prostaglandins, which can increase neuronal excitability in the spinal cord (Samad, Moore et al. 2001). However, the administration of systemic COX2 inhibitors performed better than intrathecal COX2 inhibitors at decreasing pain behavior in neuropathic pain models (Takeda, Sawamura et al. 2005).

We show that transgenic knockout of COX2 specifically in GFAP-positive cells results in the same behavioral phenotype following spared nerve injury as in animals with

NFκB suppression in the same cellular population. COX2 conditional knock-out animals showed no significant difference in pain sensitization at four days following spared nerve injury. However, these animals showed temporary inhibition of pain behavior two to five weeks post injury. The onset of persistent pain was delayed until almost six weeks post-injury. Mechanical sensitivity developed in the same temporal pattern as in IKKdn transgenic animals, in which NFκB was suppressed.

GFAP-positive cells are in key positions both peripherally and centrally to affect nociceptive neurons. In the peripheral nervous system, small diameter nociceptive fibers are specifically wrapped by non-myelinating Schwann cells. Both myelinating and non-myelinating Schwann cells are S100β positive, but only non-myelinating Schwann cells are GFAP positive (Wewetzer, Grothe et al. 1997). Schwann cells become activated almost immediately upon nerve injury. Initially, their major role is to coordinate the recruitment of non-resident macrophages to the injury site (Toews, Barrett et al. 1998; Siebert, Sachse et al. 2000). Through their release of cytokines, chemokines, growth factors and matrix metalloproteases, they establish the endoneurial environment. In the central nervous system astrocytes ensheath neuronal synapses and play a role in the regulation of neuronal excitability both in physiology and pathophysiology. Following peripheral nerve injury, astrocytes become reactive and produce a host of cytokines and chemokines, which can promote the sensitization of neurons. Furthermore, blockade of astrogliosis by the intrathecal application of fluorocitrate or JNK inhibitors reduces pain behavior (Meller, Dykstra et al. 1994; Milligan, Twining et al. 2003; Zhuang, Wen et al. 2006).

To tease apart the relative contribution of non-myelinating Schwann cells and astrocytes, we used Oxy, a novel regulator of the tet-Off system, which is similar to

doxycycline, except that it does not cross the blood-brain barrier. We have shown that Oxy is capable of turning off the tet-Off system of transgene expression peripherally while leaving central transgene expression intact. This finding allowed us to further dissect the cellular origins of our phenotype.

We used Oxy to inhibit transgene expression peripherally while allowing central expression of the IKKdn transgene in astrocytes. Transgenic suppression of NFκB in CNS astrocytes was not sufficient to alleviate pain behavior, whereas peripheral and central suppression was sufficient. This result implicates the peripheral, GFAP-positive, non-myelinating Schwann cells in this phase of chronic pain.

To obtain a physiological measure of the changes occurring in the peripheral nerves, we performed electrophysiological recordings using a skin-nerve preparation. We did not detect any differences between IKKdn and WT animals in mechanical or heat threshold, the number of cold or acid responsive fibers or in spontaneous activity. However, the skin-nerve preparation has had variable results in detecting differences in threshold and spontaneous firing between nerve-injured and naïve animals without drug application (Flatters, Fox et al. 2003; Flatters, Fox et al. 2004; Walczak, Pichette et al. 2006).

In either case, transgenic suppression of the NFκB or knockout of COX2 resulted in the significantly delayed development of persistent mechanical sensitivity until five weeks post-SNI. This finding could indicate that by suppressing inflammatory pathways in GFAP-positive cells, we are slowing but not stopping a process key to the development of neuropathic pain. In addition to the possible PGE2-related mechanisms discussed above, the suppression of inflammatory signaling in Schwann cells can also lead to a decrease in MCP-1 and a decrease in macrophage recruitment following CCI in the sciatic nerve (Fu, Zhang et

al. 2010; Zhang, Fu et al. 2011). Activated NFκB may also regulate the gene expression of extracellular matrix proteins in Schwann cells, thereby influencing their ability to migrate into nerve-injured areas (Anton, Weskamp et al. 1994). Interference with macrophage invasion of the injured area usually reduces the degree of nerve degeneration and pain (Myers, Heckman et al. 1996). Overall, our findings suggest that non-myelinating Schwann cells play a key role in neuropathic pain.

CHAPTER 7.

SUMMARY AND DISCUSSION

7.1. OVERVIEW

The present work is the first known work to establish the structural relationship between astrocytes and the primary afferents in the dorsal horn. The present work was performed concurrently with the first published attempt to examine the role of gliotransmission in the development of pain (Foley, McIver et al. 2011). The present work is also the first known work to utilize the tet-Off system to tease apart the peripheral and central components of a transgenic behavioral phenotype. Lastly, it is the first known work to demonstrate the impact of the phasic nature of the non-myelinating Schwann cell inflammatory contribution to persistent neuropathic pain behavior.

Persistent neuropathic pain is the coordinated activation and sensitization of glial and neuronal elements both peripherally and centrally. This work, in addition to a growing body of literature, demonstrates that considering solely neuronal activity, solely peripheral sensitization or solely central sensitization following nerve injury would provide an incomplete understanding of the development and maintenance of persistent neuropathic pain. Here, we have investigated one piece of the puzzle by examining the GFAP-positive glial cell contribution to persistent neuropathic pain.

7.2. TRIPARTITE SYNAPSE STRUCTURE SURROUNDING GLOMERULAR SYNAPSES OF INCOMING PRIMARY AFFERENTS IN THE DORSAL HORN

7.2.1. Summary

Although there has been a great deal of work performed on astrocyte involvement in neuropathic pain over the last two decades, particularly in the dorsal horn, the structural relationship between astrocytes and neurons in the dorsal horn has not been addressed. Many astrocyte functions are dependent upon their proximity to the synapse. Astrocyte proximity to, or ensheathment of, different synapses varies widely between brain regions and even between types of synapses within a given brain region. Among single synapses, with one presynaptic and one post-synaptic unit, synapses range from those that are not contacted at all by astrocytes processes to those that are up to 87% ensheathed by these processes, such as the climbing fiber population in the cerebellum. Complex synapses, or synaptic glomeruli, differ from single synapses in that multiple synaptic contacts are made and they are often enclosed in a common glial sheath (Chao, Rickmann et al. 2002). Due to their large size serial section reconstruction of whole glomeruli and quantitative measurement of the degree of ensheathment is difficult and it our knowledge, has not previously been published from any region of the central nervous system.

As the architecture of the dorsal horn is quite complex, we chose to examine two populations of incoming nociceptive sensory fibers: C1-type primary afferents, which correspond to the non-peptidergic C fiber population, and C2-type primary afferents, which correspond to the A-delta fiber population. Both form glomerular synapses, with a single primary afferent terminal synapsing with many second order neurons. We found that every C1- and C2-type glomerular synapse was contacted by an astrocyte process. Both C1- and C2-type glomeruli were highly ensheathed by astrocyte processes, with the C1-type showing

slightly higher degrees of ensheathment (87.59% ±3.16% ensheathment of C1 type glomeruli vs 77.20% ±6.98% ensheathement of C2-type glomeruli) (Table 3.2). Astrocyte processes rarely penetrated the interior of the glomeruli and seemed to serve as a barrier between the glomerulus and the neuropil. The areas that were devoid of astrocyte contact often contained large axons passing through and only rarely contained small fibers or unrelated synapses. This type of glomerular insulation likely protects primary afferent synaptic signaling from extrasynaptic spillover and changes in the extracellular milieu.

The astrocytic content of the neuropil varies by brain region. In the stratum radiatum of the CA1 region of the hippocampus, measurements vary between 4 and 10% (Ventura and Harris 1999; Lehre and Rusakov 2002). In the cerebellar cortex, the Bergmann glial content is much greater, with a value of approximately 33% astrocyte content (Lehre and Rusakov 2002). We found that, in lamina II of the dorsal horn, astrocyte processes occupy 17.6 ±0.8% of the local neuropil (Table 3.1).

The astrocyte content of the dorsal horn (17.6%) is much lower than the degree of ensheathment of glomerular synapses (C1 glomeruli 87%; C2 glomeruli 77%) demonstrating that contact with, and the ensheathment of, glomerular synapses is not a random event. If all tissues were equally attractive to astrocytes, then the association between astrocyte processes and synaptic elements should follow statistical rules. With random contact formation, the fraction of synaptic contact should equal the proportion of astrocyte membranes among neuropil membranes. However, the contact of synaptic glomeruli by astrocytic processes is much greater than the proportion astrocytic content in the neuropil, demonstrating that contact is not a random event and more than likely serves a specific purpose.

7.2.2. Future Directions

Astrocyte processes are highly mobile. For instance, ultrastructural studies in the barrel cortex have shown that 24 hours of whisker stimulation can induce increases in astrocyte coverage of synapses (Genoud, Quairiaux et al. 2006). This finding implies that stimulation encourages an increase astrocytic coverage of active synapses. Under conditions of persistent pain, nociceptive primary afferents become hypersensitized, with increased firing to normally non-noxious stimuli and sometimes spontaneous firing.

In response to pathological pain from a wide number of pain syndromes, including diabetic neuropathy, chemotherapy-induced neuropathy, peripheral nerve injury or inflammation and spinal cord inflammation, astrocytes become reactive and display hypertrophy of the large processes, as detected by immunohistochemical staining for GFAP (Milligan and Watkins 2009). However, what occurs at the scale of a single synapse and its surrounding fine astrocytic processes is still largely unknown. Hypertrophy of the fine processes could result in process extension or retraction from the synapse, either of which could have profound effects on neuronal signaling. Ultrastructural studies of the fine processes of reactive astrocytes have yet to be accomplished in persistent pain conditions. To our knowledge, changes in the astrocyte ensheathment of synapses following the development of gliosis have never been studied under any conditions in the CNS.

7.3. TRANSGENIC MANIPULATION OF GLIOTRANSMISSION DURING PERSISTENT NEUROPATHIC PAIN

7.3.1. Summary

The activation of astrocytes, or astrogliosis, has been shown to coincide with the long-term maintenance phase of chronic neuropathic pain. Blockade of this activation through glial metabolic inhibitors inhibits astrogliosis and reduces pain behaviors. However,

the astrocyte-specific contribution to neuropathic pain has yet to be identified. Gliotransmission may represent a mechanism by which astrocytes contribute to neuropathic pain. Studies have shown that astrocytes participate in the modulation of neuronal networks through the release of gliotransmitters (Araque, Parpura et al. 1999; Haydon and Carmignoto 2006; Montana, Malarkey et al. 2006), such as glutamate (Parpura, Basarsky et al. 1994), D-serine (Schell, Molliver et al. 1995) and ATP (Guthrie, Knappenberger et al. 1999).

However, because gliotransmission uses the same host of cellular machinery as neurotransmission, the use pharmacological agents to tease apart the effects of gliotransmission vs neurotransmission is often limited due to a lack cellular specificity. We have taken advantage of the glial fibrillary acidic protein (GFAP) promoter to drive transgenes or remove floxed genes, which within the central nervous system is specific to astrocytes. This technique allows us to clarify the distinct roles that neurons and astrocytes play in physiology and pathophysiology.

Vesicular gliotransmission is thought to occur upon the stimulation of Gq GPCRs, triggering the production of IP_3, which then activates the IP_3R2 receptors on the endoplasmic reticulum, resulting in calcium release. Increases in intracellular calcium interact with astrocytic SNARE machinery to promote vesicle fusion and gliotransmitter release. We developed knockout and transgenic mice to inhibit gliotransmission at two critical points in the Gq GPCR pathway. In the IP_3R2 knockout mouse, removal of the IP_3R2 gene prevents spontaneous and Gq GPCR-mediated calcium release from internal stores in astrocytes in the hippocampus (Petravicz, Fiacco et al. 2008). In the dnSNARE transgenic mouse, the introduction of a dominant negative SNARE (dnSNARE) protein driven by the GFAP promoter has been shown to inhibit the vesicular release of ATP from astrocytes in the

hippocampus (Pascual, Casper et al. 2005). Prior to this work, neither line had been examined in the spinal cord.

In the present work, we found that IP_3R2 full knockout (KO) mice displayed spontaneous and Gq GPCR-mediated calcium increases from internal stores from astrocytes in the dorsal horn. To circumvent any potential confounds of knocking out IP_3R2 in neurons, we bred these mice to GFAP-Cre mice to produce conditional knockout mice (cKO) in which IP_3R2 is selectively knocked out in GFAP-positive cells. In the dorsal horn, the IP_3R2 cKO transgenic mice had greatly attenuated spontaneous and Gq GPCR-mediated increases in astrocyte calcium and would therefore be expected to have decreased calcium-dependent gliotransmitter release. However, there was no difference in basal mechanical sensitivity between IP_3R2 cKO mice and littermate controls. To determine whether IP_3R2-mediated calcium release is involved in the development or maintenance of persistent pain, IP_3R2 cKO mice underwent spared nerve injury, a robust model of neuropathic pain. Astrogliosis developed normally, and there was no difference in the development or maintenance of mechanical allodynia following nerve injury between these transgenic mice and the littermate controls.

We also used dnSNARE mice, in which SNARE-dependent vesicle release is selectively inhibited in GFAP-positive cells, to determine whether vesicular release from astrocytes plays a role in the development of maintenance of neuropathic pain. In these mice, the GFAP promoter drives both the dnSNARE transgene and eGFP reporter. These transgenic mice and their littermate controls underwent spared nerve injury surgery to induce neuropathic pain. Although we found high levels of the eGFP reporter in the dorsal horn both

before and after nerve-lesion we found no difference in basal mechanical sensitivity nor the development or maintenance of mechanical allodynia following nerve-injury.

Together the findings from the IP_3R2 cKO and dnSNARE transgenic mouse models suggest that vesicular mediated gliotransmitter release does not play a role in mechanical nociception. Furthermore, vesicular mediated gliotransmitter release from astrocytes does not play a role in the development or maintenance of mechanical sensitivity following SNI.

7.3.2. Future Directions

Although we found no evidence of changes in basal mechanical sensitivity or the development of mechanical allodynia following nerve injury in either the IP_3R2 knockout or dnSNARE models, it is possible that other modalities of pain, such as thermal sensitivity, could be altered in these mice. In future studies, thermal sensitivity could be monitored using the Hargreaves test. However, there is no evidence for the thermal-specific involvement of glia in nociception using any sort of glial modulating agent, including fluorocitrate, a non-selective glial metabolic inhibitor (Meller, Dykstra et al. 1994), methionine sulfoximine, an astrocytic glutamine synthetase inhibitor (Chiang, Wang et al. 2007), or propentofyllin, a methylaxthine (Sweitzer, Schubert et al. 2001). In future studies, these transgenic lines of mice could also be tested using different models of pain, such as the complete Freund's adjuvant or formalin models of inflammatory pain.

7.4. NEUROINFLAMMATORY INTERACTIONS BETWEEN NEURONS AND GLIA DURING PERSISTENT NEUROPATHIC PAIN

7.4.1. Summary

Neuropathic pain resulting from peripheral nerve insult is the result of both the peripheral and central sensitization of nociceptive neurons and is associated with the activation of both peripheral and central glia. Peripherally, Schwann cells immediately detect

nerve injury and coordinate the recruitment of non-resident macrophages to the injury site through their release of cytokines and chemokines. Centrally, nerve injury produces the sequential activation of microglia and astrocytes, as detected by increases in cellular hypertrophy, as well as the activation of the mitogen associated protein kinases (MAPKs) p38 and JNK. Glia, both central and peripheral, are the main producers of proinflammatory cytokines and chemokines, many of which are under the control of the NFκB transcription factor.

To determine the cellular origins of the inflammatory response following nerve injury, we used IKKdn mice that express a dominant negative IKK protein in order to suppress the NFκB signaling pathway under the control of the GFAP promoter. We found that the GFAP promoter targeted peripheral non-myelinating Schwann cells and central astrocytes, both of which are in key positions to modulate nociceptive neurons. We found no difference in basal mechanical sensation and little difference in the initial development of mechanical allodynia following nerve lesion. However, by one week post-injury, we found that suppression of the NFκB pathway in non-myelinating Schwann cells and astrocytes resulted in a dramatic alleviation of pain behavior that lasted until five weeks post-nerve injury. By six weeks post-nerve injury, the transgenic animals had returned to WT control levels of pain behavior. The results from male and female mice were similar.

To further elucidate the pathways the astrocytic pathways involved in neuropathic pain, we used a second transgenic mouse line in which the GFAP promoter drives Cre-recombinase to remove a floxed COX2 gene. COX2 is directly downstream of NFκB and is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. The phasic pain behavior response found in the IKKdn mice was mimicked by the COX2 cKO

transgenic mice. The COX2 cKO mice showed a robust temporary relief from mechanical sensitivity from two to five weeks post nerve injury (Fig 5.1). The results from male and female mice were similar. This strong phasic response pattern, although similar to the phasic activation of certain cell types and the phasic release of specific molecules following nerve injury, has not been previously demonstrated in animal models of pain.

We have demonstrated that suppression of the NFκB-COX2 pathway specifically in GFAP-positive cells results in a temporary yet robust alleviation of pain behavior following nerve injury. The use of the GFAP promoter, while limiting our transgenic manipulation to specific cell types, is active both peripherally, in non-myelinating Schwann cells, and centrally, in astrocytes (Fig 5.4B). To determine the peripheral versus central contribution of our transgenic phenotype, we used oxytetracycline (Oxy), a novel modulator of the tet-Off system.

The tet-Off transgenic system consists of a tetracycline transactivator (tTA) driven by the promoter of interest, in this case GFAP, which then binds to the tet operon (tetO) to promote transcription of the downstream gene; for example, in the IKKdn mice, binding of the tTA to the tetO minimal promoter drives the expression of dominant negative IKK to suppress NFκB signaling. Originally, the tet-Off system was designed to be used with tetracycline, which binds to the tTA and prevents it from binding to the tetO promoter, thus inhibiting transcription of the downstream transgene. Thus, in the absence of tetracycline, the transgene is expressed, while the addition of tetracycline turns "off" transgene expression. In vivo, the administration of tetracycline to transgenic animals enables the circumvention of any potential confounds of expressing a transgene during development. Upon adulthood, withdrawal of the drug permits transgene expression. The tet-Off system quickly moved to

the use of doxycycline rather than tetracycline, following the discovery that doxycycline is equally as effective as tetracycline at regulating transgene expression in the tet-Off system and has a longer half-life (24 vs 12 hours) (Gossen and Bujard 1995; Gossen, Freundlieb et al. 1995). Additionally, doxycycline has a higher bioavailability in vivo (Agwuh and MacGowan 2006). Oxy is an analog of doxycycline; however, it is much less lipophilic and does not cross the blood-brain barrier in significant quantities (Barza, Brown et al. 1975). Oxytetracycline also has lower bioavailability than doxycycline via oral administration (58% versus 80%) (Agwuh and MacGowan 2006).

To test the use of oxytetracycline in vivo, we used a GFAP-tTA::tetO-eGFP reporter line of mice. We found that oxytetracycline was able to effectively suppress transgene expression in peripheral non-myelinating Schwann cells while permitting transgene expression in central astrocytes. Therefore, the use of oxytetracycline should allow the dissection of peripheral versus central components in any transgenic model that utilizes the tet-Off system.

We used oxytetracycline to tease apart the roles of non-myelinating Schwann cells and astrocytes in IKKdn mice, which display suppressed NFκB expression in GFAP-positive cells, using the tet-Off system. In transgenic animals with NFκB suppression in both peripheral non-myelinating Schwann cells and central astrocytes, we observed a robust temporary alleviation of mechanical allodynia following nerve injury. Peripheral inhibition of the transgene through Oxy administration completely reversed this effect: transgenic animals on oxytetracycline exhibited the same development of mechanical sensitivity as WT animals following nerve-injury. With the transgene inhibited peripherally, in transgenic mice

on oxytetracycline NFκB signaling is only suppressed centrally in astrocytes. Therefore, the central suppression of NFκB in astrocytes alone was not sufficient to relieve pain behavior.

7.4.2. Future Directions

The phasic pattern of pain relief following the inhibition of the NFκB-COX2 pathway is one of the most striking components of this research. The inhibition of inflammatory pathways in GFAP-positive cells had no impact on the initial development of mechanical allodynia at four days post-injury. One possible explanation is that early mechanical allodynia is primarily mediated by neurons and not by the surrounding glial cells. A second explanation is that the mechanical sensitivity at four days post-injury is so robust that even without the glial contribution, the paw withdrawal thresholds are as low as can be scored, representing a basement effect. Supporting this, Fig 5.9B shows that although both groups displayed mechanical allodynia at four days post-injury, there was a significant difference between transgenic IKKdn and WT animals even at the four day time point.

Two major peripheral glial events occur between four days and one week post-injury: Schwann cells undergo massive proliferation, and non-resident macrophages infiltrate the injury area. Schwann cell proliferation is thought to be an erbB2- and B3-dependent process and is not thought to rely on NFκB or COX2 signaling. However, Schwann cells play an important role in the recruitment of macrophages through the expression of the chemokine MCP-1 (Toews, Barrett et al. 1998; Siebert, Sachse et al. 2000), which is mediated by the NFκB and p38 pathways (Subang and Richardson 2001). Although MCP-1 increases within three hours of nerve injury, its effects are not pronounced until non-resident macrophages actually invade the area; this invasion typically occurs around four days post-injury. The infiltration of peripheral macrophages marks a major shift in the axonal environment, as they

are the major producers of the neuronal sensitizing cytokines, primarily due to their large numbers.

The process of macrophage recruitment, however, is not thought to be COX2 dependent. The major signaling molecule downstream of the NFκB-COX2 pathway is PGE2, which is known to play a role in neuropathic pain. Peripheral injection of PGE2 causes pronounced hyperalgesia (Ferreira, Nakamura et al. 1978), while endoneurial injection of a COX2 inhibitor following nerve injury has been shown to produce a dose-dependent relief of mechanical hyperalgesia (Syriatowicz, Hu et al. 1999). There is a brief increase in COX2 in Schwann cells from one to three days post-injury; however, the direct effects of this increase in COX2 expression are unknown (Takahashi, Kawaguchi et al. 2004).

Another feature of the phasic pattern of pain relief found with the suppression of inflammatory signaling in GFAP-positive cells is the return of mechanical sensitivity to WT levels by six weeks post-injury. NFκB is a highly regulated transcription factor that can be activated by both canonical and non-canonical signaling pathways. It is possible that our suppression of the canonical NFκB signaling pathway was insufficient to prevent the development of neuropathic pain and, instead, was only able to delay its development. However, while it is possible to overcome the effect of a dominant negative protein by an increase in the expression of upstream mediators, this is not possible with a knockout animal. The return of mechanical sensitivity by six weeks post-nerve injury also occurred in the COX2 cKO animals. This argues against the return of allodynia being due to simply overwhelming the transgenic mouse model system. Thus, the late development of neuropathic pain may be mediated by factors other than inflammatory signaling in GFAP-positive cells.

In sum, a variety of cells and molecules are involved in the temporal response to neuropathic pain. Non-myelinating Schwann cells may play a critical role in the development and maintenance of neuropathic pain through their activation of the NFκB-COX2 signaling pathway during a specific phase of the neuropathic pain response from one to five weeks post lesion. However, the inhibition of inflammatory pathways in non-myelinating Schwann cells and astrocytes is not sufficient to prevent the eventual development of mechanical sensitivity following nerve-injury. Further studies are needed during these late phases of persistent pain to develop an understanding of the molecular mechanisms involved in the ongoing maintenance of persistent neuropathic pain.

7.5. CONCLUDING REMARKS

There is no doubt that temporal patterns exist in response to nerve injury. The response to nerve injury is not the same at twelve hours as it is at twelve days and much less so at twelve months post-injury. However, most studies gather the majority of their neuropathic pain data within the first week following nerve injury, particularly within the first several days. While there is great clinical validity in studying methods of preventing the development of persistent neuropathic pain, for example, when patients are at high risk of developing persistent pain resulting from dental work or amputation, studies should also be conducted to address the issue of resolving ongoing neuropathic pain. Clinically, neuropathic pain is most commonly associated with diabetic neuropathy or lumbar nerve root compression, neither of which is amenable to a short-term preventative drug regimen. Additionally, it is estimated that 3-4.5% of the global population already suffers from ongoing neuropathic pain (Global Industry Analysts 2011).

Recently, work has been performed in the inflammatory pain field on the final phase of inflammatory pain, resolution. Following the clearance of the inflammatory stimulus, the ongoing inflammatory response must be resolved to avoid excessive tissue damage. Resolution of the inflammatory response was once thought to be a passive process or recession of pro-inflammatory molecules. Recently, however, resolution has been found to involve the active recruitment anti-inflammatory and pro-resolving mediators, which induce biochemical programs to enable inflamed tissues to return to their pre-inflammatory states (Serhan, Chiang et al. 2008). The process of resolution requires signals that turn off macrophage infiltration while promoting the uptake and clearance of apoptotic cells (Soehnlein and Lindbom 2010). Resolvin D1 (RvD1) and resolvin E1 (RvE1) represent a new family of pro-resolution molecules and are synthesized from the omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, respectively (Serhan, Hong et al. 2002). These molecules were originally isolated from the exudates formed during the resolution phase of acute inflammation in both rodents and humans (Serhan, Clish et al. 2000). Resolvins have anti-inflammatory, pro-resolving and anti-allodynic effects and work at both a peripheral and central level.

The peripheral administration of resolvins prior to carrageenan injection, a model of inflammatory pain, results in reduced neutrophil infiltration and paw edema as well as reduced thermal and mechanical sensitivity. Resolvins are also known to inhibit the NFκB pathway to dampen the biosynthesis of proinflammatory mediators (Serhan, Chiang et al. 2008). Resolvin administration has been shown to reduce the expression of proinflammatory cytokines and chemokines, such as TNFα, IL-1β, IL-6 and CCL2, all of which are downstream of NFκB activation, in the inflamed paw (Bang, Yoo et al. 2010; Xu, Zhang et

al. 2010). RvE1 exerts its effects through the activation of the ChemR23 receptor. ChemR23 is expressed on a variety of cell types, including nociceptors, neutrophils, macrophages and microglia, which may account for its pleiotropic effects. The activation of ChemR23 reduces the migration of neutrophils and promotes their clearance. Additionally, it inhibits the production of the pro-inflammatory mediators TNFα, IL-1β, IL-6 and IL-12 and induces the expression of anti-inflammatory cytokines, such as transforming growth factor (TGF)-β and IL-10, in macrophages.

Formalin injection, a model of inflammatory pain, results in two phases of pain behavior, the first of which is attributed to peripheral sensitization and the second of which is attributed to central sensitization. The administration of resolvins decreases the second phase of pain behavior following formalin injection, indicating that in addition to the peripheral mechanisms outlined above, resolvins may also impact the central mechanisms of inflammatory pain. Intrathecal administration of resolvins at sub-nanomolar levels following the induction of pain has been shown to reduce the heat and mechanical hypersensitivity induced by complete Freund's adjuvant (Bang, Yoo et al. 2010; Xu, Zhang et al. 2010). RvE1 also blocks the ERK signaling pathway, which regulates peripheral sensitization, central sensitization and glial activation in the DRG and spinal cord (Ji, Gereau et al. 2009).

The antinociceptive effects of resolvins may be partially attributable to their anti-inflammatory role; however, they also have direct analgesic effects. RvE1 is capable of abolishing TRPV1- and TNFα-induced increases in EPSC frequency in dorsal horn neurons. RvD1 is capable of regulating several TRP channels, including TRPA1, TRPV3 and TRPV4, and RvE1 has been shown to effectively inhibit TRPV1-mediated heat hyperalgesia (Xu, Zhang et al. 2010).

The resolution of neuroinflammation and persistent pain may involve more than the passive diffusion of pro-inflammatory mediators but the active induction of anti-inflammatory cytokines and the active inhibition of nociceptors; resolvins play a role in both these processes. While resolvins are found during the naturally occurring process of the resolution of inflammation, it should be mentioned that they are most effective when used as a pretreatment prior to the inflammatory insult. Furthermore, they have not yet been tested in neuropathic pain models.

If resolution is an active process, it is critical to study this process in the setting of neuropathic pain. Understanding the molecular mechanisms of resolution may assist in shifting ongoing neuropathic pain toward the resolution process. However, the current neuropathic pain models have long duration and few studies have attempted to clarify their resolution. The process of resolution from neuropathic pain is not likely to be studied without the development of animal models of neuropathic pain with a shorter duration than our current models.

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