ELUCIDATING MECHANISM IN PATHOLOGICAL PAIN CONDITIONS

by

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(Under the Direction of Han-Rong Weng)

ABSTRACT

Pathological pain is a debilitating condition affecting millions of people worldwide and is poorly treated by conventional therapeutics. In this dissertation, a series of mechanistic studies are presented to identify novel therapeutic strategies to develop analgesic for treatment of pain. This dissertation comprises four chapters. Chapter one a literature review of the role of neuronal dysfunction in chronic pain conditions.

In the chronic pain condition, hyperexcitation of neuronal cells and neuroinflammation are hallmark events in the nociceptive pathway. Homeostasis between excitatory and inhibitory receptor activities is crucial to maintain normal neuronal activities in the central nervous system (CNS**).** In chapter two we have successfully shown in the paclitaxel-induced neuropathic pain model that local application of GAT-1 transporter activity inhibitors at the spinal enlargement reverses the attenuated GABAergic disinhibition and ameliorates neuropathic pain. Thus, targeting GAT-1 transporters for reversing GABAergic disinhibition in the spinal dorsal horn could be a useful approach for treating paclitaxel-induced neuropathic pain.

Tissue and nerve injury produce neuroinflammation which is a critical component, contributing to the genesis of pathological pain. Activation of glial cells, accumulation of proinflammatory cytokines and release of various algesic mediators in

the spinal cord produces long-lasting cellular and behavior changes as a consequence of widespread alterations in gene expression profile. Epigenetics mechanism such as post translation modification of histone tails residues plays important role in various gene activation and suppression. In chapter three we have presented compelling evidence demonstrating global change of enhancer zeste homolog-2 (EZH2) activities and methylation levels of H3K27 in the spinal dorsal horn is associated with glia cells activation and neuroinflammation following nerve injury. Inhibition of EZH2 prevented and attenuated the neuropathic pain condition thus provide compelling evidence that targeting signaling pathways regulating gene expression could prove to be an effective approach for the development of new analgesics for the treatment of neuropathic pain. Lastly chapter four provides a discussion of pharmacological management of pain with a focus on the remaining challenges in the translation of pain therapeutics.

INDEX WORDS: Paclitaxel; paclitaxel-induced neuropathic pain; GABA; GAT-1; GAT-3; chronic pain; pSNL; gliosis; neuroinflammation; enhancer of zeste homolog; histone modification

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DEDICATION

I would like to dedicate this dissertation to my father Jaipal Yadav, my mother Sushila Yadav, my father-in-law Om-prakash Yadav, and my mother-in-law Kailash Yadav for their endless support. This Dissertation is also dedicated to my lovely husband Parveen Yadav, my brother-in-law's Nishant Yadav and Prashant Yadav and my sisters Chitvan Yadav and Hema Yadav for giving me the confidence and faith to succeed. I owe my kids Reneka and Akshay Yadav for their unique support, to bring the best out of me and to convert my smiles to endless happiness. Additionally, I would like to dedicate this dissertation to my sister's kids, Devanshi, Arnav, Aryan and Tanvi for the joy they bring into my life.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The somatosensory nociceptive system's multidimensional ability to perceive and differentiate noxious stimuli as pain in an experience-dependent manner (plasticity) has protected us from harm (Bushnell et al 2013, Schaible & Richter 2004). The perception of pain, brought together by fine tuning of excitatory and inhibitory transmission along the nociceptive pathway, constitutes acute physiological pain responses, which go away once the harmful stimuli subside. However, in chronic pain conditions evoked by tissue/nerve injury, inflammation, or other metabolic disorders, patients often complain of persistent, spontaneous and exaggerated painful sensation in response to noxious (hyperalgesia) and non–noxious (allodynia) stimuli (Dubin & Patapoutian 2010).

Over 100 million adults suffer from chronic pain condition in the United States, which puts a huge burden ($300 billion) on America's economy (Medicine 2011). The current standard care for treatment of chronic pain condition includes classes of opioid and non-opioid prescription drugs and longer-term usage of these drugs causes serious side effects (Schug et al 2003). According to the data presented in 2014 by the Centers for Disease Control and Prevention (CDC), almost 2 million people were addicted to or abusing prescription opioids, and nearly 46 Americans die every day due to prescription opioid overdose (Rudd et al 2016). Hence, there is an urgent need to discover novel therapeutics to address the treatment and management of chronic pathological pain conditions.

Pain condition based on the duration of onset can be divided into acute pain (less than 3-6 months) and chronic pain (more than 3-6 months). Furthermore, based on the etiology, it can be classified as nociceptive pain (normal pain response to noxious stimuli and tissue injury); inflammatory pain (activation and sensitization of nociceptive pathways by inflammatory mediators); and neuropathic pain (caused by a primary lesion to the somatosensory nervous system) Figure 1.1). Because of the complex etiology, some chronic pain conditions are difficult to categorize into the above classes, for example, cancer pain, migraine, headache, fibromyalgia pain, phantom pain, etc. Patients suffering from neuropathic pain often complain of multiple symptoms including numbness, hypersensitivity/allodynia, and burning and/or tingling sensation (Schaible & Richter 2004).

In order to discover novel therapeutic targets to treat various chronic pain conditions, it is critical to understand the pathophysiology of pain. In this chapter, I have summarized mechanism known to cause spinal neuronal dysfunction including several mediators, receptors, ion channels, inhibitory neuronal signals, glia activation, cytokines & chemokines, and epigenetic mechanisms involving in neuropathic pain conditions.

Anatomical Overview of the Nociceptive Circuit

In general, nociceptive circuits can be divided into four major components: 1) primary afferent neurons, 2) projection neurons, 3) interneurons, and 4) descending neurons. The primary focus of this chapter is on the primary and secondary order neurons present in the spinal cord. The primary afferent neurons carry the sensory input from the peripheral body to the spinal cord. These neurons can be divided, based on the speed of transmission

of signals and myelination pattern, into the following categories: fast signal conducting myelinated Aβ and intermediate signal conducting myelinated Aδ fibers, and slow signal conducting unmyelinated C fibers. The cell bodies of primary sensory neurons collectively form a nodular structure known as the dorsal root ganglion, which resides in the neural foramen of the vertebrae. One branch of axons of these pseudo-unipolar primary sensory neurons extends from the cell body to the periphery (peripheral axons), and another branch of axons extends from the cell body to the spinal dorsal horn (central axons).

In the somatosensory nervous system, Aβ fibers convey tactile and proprioceptive innocuous and mechanical stimuli and terminate its signals in the Rexed laminae III-VI of the spinal dorsal horn. In the spinal dorsal horn, Aδ and C primary sensory fibers terminate into the (Rexed laminae I and II) superficial dorsal horn of spinal cord and carry the thermal and nociceptive signaling (Liu et al 2007a) (Figure 1.2). After primary afferent neurons transduction by peripheral stimuli, the nociceptive signal transmits to the dorsal root ganglion. From there, signals are transmitted to postsynaptic projection neurons and interneurons of the spinal dorsal horn and sent to the higher center at the nociceptive signaling pathways in the cortex. The projection neurons are primarily concentrated in laminae I and disseminated in laminae III-VI in the spinal dorsal horn. The axon of these projection neurons crosses the midline and ascends through the anterolateral tract to a higher center. At the higher center, these neurons carry signals to the thalamus, periaqueductal gray matter, lateral parabrachial area and several medullary nuclei. Once perceived these signal , these nociceptive output can modulate the function and biochemical reaction of the spinal dorsal horn and peripheral nervous system via the

descending nociceptive (Todd 2010) (Figure 1.3). Maladaptive processing of primary afferent (Aβ and C) sensory fibers of the spinal cord has been implicated in the development and maintenance of neuropathic pain condition (Devor 2009).

Neuropathic Pain

A primary lesion or dysfunction in the peripheral or central nervous system can result in the development of peripheral neuropathic pain or central neuropathic pain. Damage caused by neurotoxic chemicals, nerve injury, metabolic disorder, infection or tumor invasion leads to peripheral neuropathic pain development (Woolf & Mannion 1999). Patients suffering from peripheral neuropathic pain syndromes often show one or more clinical symptoms such as paranesthesia, dysesthesia, allodynia, hyperalgesias, loss of sensation, and spontaneous or persistent pain sensation. Clinical experience and decades of research related to neuropathic pain have shown that empirical symptom management is not sufficient to bring relief to patients. Therefore, to identify novel therapeutic targets for the development of mechanism-based therapeutic approaches to treat patients suffering from neuropathic pain conditions, it is essential to understand the underlying neurobiological mechanisms.

Noxious stimuli can evoke transient to persistent biochemical changes in the nociceptive pathway. Lesions to the primary neuron axons, cell body or central axons result in disruption in the continuity of somatosensory signals or neuronal cell death in peripheral and central nervous system. Dysfunction of injured primary neurons affects other non-injured neuronal cell function along the sensory signaling pathway at the spinal and supraspinal levels in the central nervous system. Although the mechanism of pain

varies and depends upon the site of pathology and etiology of noxious stimuli, sensitization of the nociceptive pathway may occur at the periphery, dorsal root ganglion (DRG), spinal and/or supraspinal levels of the nervous system.

Dorsal Root Ganglion (DRG)

Peripheral nerve injury incites a reaction by the immune system and glia cells along the nociceptive pathways. In response to the peripheral nerve injury, neuregulin, a differentiation and growth factor, is released within a fraction of a second from the membrane of damaged neurons and activates Schwann cells (Carroll et al 1997). In parallel, immune cells such as macrophages, lymphocytes neutrophils, granulocytes and mast cells also infiltrate the site of nerve lesion (Figure 1.4).

Activated immune and Schwann cells release prostaglandins, IL-1β, IL-6, IL-12 IL-18, tumor necrosis factors-α, interferon- γ, and leukemia inhibitory proinflammatory cytokines (Ma & Eisenach 2003, Perrin et al 2005, Tofaris et al 2002). Macrophages' phagocytic process aids in clearing debris from the injured tissue. A unique set of antigens presented on the surface of activated macrophages helps recruit more macrophages and other immune cells to the site of injury. Peripheral immune macrophages and Schwann cells presented on the distal site of the injured axons promote Wallerian degeneration (Stoll et al 2002). On the mesial side of the nerve injury, activated Schwann cells proliferate and release chemical signals to promote axonal growth and re-myelination (Stoll et al 2002). Neurotropic growth factor such as glial cell-derived neurotropic growth factor released by activated Schwann cells can directly sensitize nociceptors and contributes to pain sensation in response to nerve injury (Malin et al 2006).

On the one hand, the release of proinflammatory cytokines and chemokines eliminates impaired cells and promotes healing, but on the other hand, progressive and/or chronic inflammation dysregulates neuronal function and hampers tissue recovery (Cunha et al 2005) (Figure 1.1). Inflammatory mediators released by neurons, as well as the glial cells of the central and peripheral nervous system, contribute to hyperexcitability and sensitization of primary sensory neurons and increases in excitatory synaptic transmission in the spinal dorsal horn and at higher centers (Basbaum et al 2009, Gensel et al 2012). Macrophages, lymphocytes, Schwann cells and injured axons on the mesial side provoke a reaction in the dorsal root ganglions (Figure 1.4).

The dorsal root ganglion (DRG) houses the cellular bodies of primary sensory afferent fibers. The peripheral ganglion cells are pseudo-unipolar. From the main processes, two branches emerge, one of which goes from the cellular body to the periphery and the other towards the central nervous system (spinal dorsal horn). Based on the cellular body size, DRG neurons can be classified into large and small neuronal cells. The DRG cell bodies are generally separated from each other by satellite glial cells (Figure 1.3). Interestingly, the DRG is not protected by a blood-nerve barrier but only by a thin envelope of satellite glial cells, and therefore cells such as macrophages from the periphery can infiltrate easily upon noxious stimulation (Hu & McLachlan 2002, Shimizu et al 2011). Due to its unique physical location and anatomical characteristics, the DRG has been manipulated to treat neuropathic pain (Acar et al 2008, Deer et al 2013, Manchikanti 2000, Nash 1986, Van Zundert et al 2007).

Like other glial cells in the central nervous system, the satellite glia cells of the DRG carry many receptors and thereby influence the DRG neurons signaling in response

to the various mediators (Hanani 2005). Following peripheral nerve injury, substance P, histamine, prostaglandins, bradykinins, serotonin, neutrophins, growth factors, cytokines (interleukins, TNF-α, interferons) chemokines, ATP and reactive oxygen species are released by local tissue and immune cells.

Additionally, primary sensory neurons are activated via G-protein coupled receptors and initiate the secondary messengers' cascade, resulting in change in intracellular calcium level. The rise in intracellular calcium within a primary nociceptive neuron, leads to changes in the variety of gene and protein expression and, consequently, results in excessive releases of neurotransmitters including glutamate, substance P, and brain-derived neurotrophic factor (BDNF) that further stimulate higher-order neurons present in the nociceptive pathways. Enhanced sensitization and ectopic stimulation of primary afferent fibers are influenced by various factors, including expression of ion channels and receptors present on primary afferents fibers.

Following nerve injury, increased release of neurotrophic growth factor (NGF) increases the synthesis of substance P and calcitonin gene-related peptide (CGRP) via interacting with its receptor tyrosine kinase A (trk A) and leads to further enhanced release of inflammatory mediators (Lewin et al 1994). Additionally several endogenous analgesic biochemical mediators are also secreted Endogenous analgesic mediators such as anti-inflammatory factors IL-4 and IL-10 and transforming growth factor-β1 play important roles in the suppression of excitatory neuronal signals and the reduction of pain sensation (Dubovy et al 2014) (Table 1 Figure-1). However, the excessive release of algesic mediators after nerve injury throws off the balance between the algesic and

analgesic mediators and consequently leads to hypersensitization of the nociceptive pathway.

Table 1.1: List of endogenous algesics/proalgesics and analgesics mediators associated with pain

Endogenous Algesic/Proalgesic	Endogenous Analgesic
Immune cells: Mast cells: Hist, 5HT, LTs, TNF,IL-6 Neutrophils: ROS, TNF, IL-1β, 2, 6, 17 Macrophages: TNF, Il-1β, 6, 15,18 Lymphocytes: TNF, IFNγ, Il-17 **Glial Cells:** Schwann cells: MMP9, TNF, IL-1β, NGF-β ATP, PGE2 Satellite cells: TNF, IL-1β, NGF-β Microglia: TNF, IL-1β, 6, NO, PGE2, BDNF, LTs, MCP-1 Astrocytes: TNF, ATP, NO, Glutamate, IL-1β, 6,IFNγ**,** Fractalkine, MCP-1, MIP-1α	**Immune cells:** Mast cells: IL4, 10 Neutrophils: Endorphin/Enkephalin Lymphocytes: IL-4, 10 9th2), B7-Ht (t-cells)' TGF-β (tregs), Endorphin- containing –T cells **Glial cells:** Schwann cells: Epo, GDNF Satellite cells: GDNF Microglia: GDNF, IGF-1 Astrocytes: GDNF

Afferent sensory input evoked by nerve injury and noxious stimuli increases the excitability of nociceptive neurons in the spinal cord. However, the mechanisms of central sensitization in the spinal dorsal horn remain elusive. The primary focus of this chapter is to present a literature review on the role of neuronal dysfunction, activation of glia cells, proinflammatory cytokines, chemokines and the epigenetic transcription mechanism involved in neuropathic pain development and maintenance in the spinal dorsal horn following nerve injury.

Spinal Cord-Dorsal Horn

The spinal cord dorsal horn serves as a gateway for the nociceptive signaling from the periphery to the central nervous system and vice versa. Neuronal dysfunction, overproduction of pro-inflammatory cytokines, and activation of glia (reactive gliosis) cells are hallmark events that take place in the spinal dorsal horn and contribute to the pathology of neuropathic pain (Figure 1. 3). The biochemical changes in the nociceptive circuitry can be viewed at the cellular, signaling pathway, and gene expression levels. At the cellular level, it is essential to understand how altered neuronal and glial cell function in neuropathic pain leads to aberrant neuronal dysfunction (Scholz & Woolf 2007a, Vartak-Sharma & Ghorpade 2012, Xu et al 2007, Zhang et al 2011, Zhang et al 2012a). All these biochemical changes occurring in the nociceptive pathways require either activation or suppression of various mediators. The literature has suggested that more than one mechanism is involved in the non-epigenetic and epigenetic mechanisms of persistent pain development and maintenance. In chronic pain conditions, noxious stimuli activate primary afferent sensory neurons and incite the release of proinflammatory cytokines and many neuropeptides such as substance P, neurokinin, calcitonin gene-related peptide, brain-derived neurotrophic factor ATP, glutamate, etc., which have been found to be implicated in the peripheral and central sensitization of the nervous system (Duggan et al 1990, Morton & Hutchison 1989).

Spinal Neuronal Dysfunction in Neuropathic Pain

A fine balance of excitatory and inhibitory synaptic transmission is critical for proper processing of nociceptive information in the central nervous system (CNS). After

peripheral nerve injury, primary afferent excitatory neurons release excessive glutamate neurotransmitter and other mediators including neuropeptides (substance-P, calcitonin gene-related peptide), and adenosine triphosphate and proinflammatory cytokines into the synaptic cleft. These neurochemical events contribute to neuronal hyperexcitability and pain transmission at the spinal and supraspinal level. However, under normal physiological conditions, the inhibitory synaptic transmission limits the excitability of neurons and thereby facilitates spatial and temporal synchronization of the neuronal network, information processing (Dykes 1997, Krnjevic 1997) and neuronal plasticity (Dykes 1997). In contrast, in chronic pain condition, the loss of GABAergic inhibition, along with enhanced excitatory neurochemical release and neuroanatomical changes, results in neuronal dysfunction in the spinal dorsal horn. Studies have shown that hyperexcitation of neurons are associated with increased glutamate release (Nie et al 2010a), increased upregulation and/or activation of glutamate receptors (Bhangoo & Swanson 2013, Mills et al 2002, Yan et al 2013) and sodium channels (Hains & Waxman 2007), enhanced activation of glia cells and inflammation (Ji et al 2013) and decreased endogenous inhibitory activity (Gwak & Hulsebosch 2011). Altogether, it has been shown that hyperexcitability is an important event occurring in the spinal dorsal horn after nerve injury and plays pivotal role in chronic pain development and maintenance.

Role of Spinal Excitatory Neuronal Cells in Neuropathic Pain

In neuropathic pain, the abnormal ectopic excitability of myelinated Aβ fibers of the spinal dorsal horn leads to the sensations of paranesthesia and dysesthesia, whereas

the altered excitability property of Aδ and C fibers results in experiencing burning and lancinating pain (Djouhri & Lawson 2004, Tal et al 1999). Aberrant neuronal firing pattern of peripheral sensory fibers and dorsal root ganglion neurons leads to hypersensitization of the spinal dorsal horn neurons. Several spinal mechanism have been recognized which contributes to dysfunction of neurons in the spinal dorsal horn in the neuropathic pain state. Nerve injury induces increased response to the stimuli, and enhanced spontaneous activity of the spinal dorsal horn has been recorded (Bavencoffe et al 2016, Bedi et al 2010, Carlton et al 2009). Nerve injury causes an increased level of extracellular glutamate, increased production of proinflammatory cytokines and reactive oxygen species, which in turn activates the release of numerous protein kinases of signaling pathways resulting in the initiation and maintenance of a hyperexcitable state of spinal dorsal horn neurons. The activation of multiple signaling pathways impact the functional activity and expression of various receptors, ion-channels, and transporters present on neuronal and glial cells. This alteration in activity of spinal cells essentially begins in response to noxious stimuli which neutralizes and limits their damaging effects. However in chronic pain stage these dysfunctional changes persist for a long period of time.

In the pathological pain state, the distinct distribution and expression level of sodium channels have been linked to the development and maintenance of pathological pain conditions (Liu & Wood 2011, Rogers et al 2006). Voltage -gated sodium channels found in diverse a population of neurons can be generally categorized into the fast-acting, tetrodotoxin-sensitive (TTX-S) sodium channels, and the slow-acting, tetrodotoxin-resistant (TTX-R) sodium channels. Several isoforms of sodium channels have been

identified ($Na_V1.1$ to Na_V 1.9). Studies have demonstrated that high expression and function levels of $Na_V1.7$, Na_V 1.8 and Na_V 1.9 on sensory fibers are associated with pain nociception, and have further suggested that they may be manipulated as therapeutic targets to treat pain (Cummins & Rush 2007, Priest & Kaczorowski 2007). Genetic mutation in the alpha subunit of $Na_V.1.7$ (SCN9A gene) leads to loss of function and results in congenital insensitivity to pain (Cox et al 2006, Nilsen et al 2009). On the other hand, mutation in the $Na_V1.7$ sodium channels, an autosomal–dominant inherited erythromelalgia disease, is associated with a gain in function in patients, resulting in severe burning pain sensation and redness of skin (Han et al 2006, Yang et al 2004b).

The excitatory amino acids, glutamate and aspartate, exerts their effects by binding to both metabotropic (mGlu) and inotropic receptors (NMDA, AMPA, Kainate). Several lines of evidence have shown that nerve injury induces a high concentration of intracellular calcium ions, which leads to the activation of protein kinase A (PKA), protein kinase C (PKC) and the calcium-calmodulin-dependent kinase II (CAmK II) pathway, followed by activation of the MAPK/ERK signaling pathway (Xu et al 2006). These protein kinases phosphorylate the NMDARs and potentiate the excitatory activity of the spinal dorsal horn's neurons. An experiment performed in our lab has further shown that the functional coupling of IL-1β receptors and presynaptic NMDA receptors leads to enhanced glutamate release, activation of non-NMDA receptors and the hyperexcitation of primary afferent neurons in the spinal dorsal horn (Yan & Weng 2013b). Suppression of presynaptic NMDAR activity in primary sensory neurons prevents enhanced glutamatergic responses (Yan et al 2013). Mediators such as substance P and calcitonin-gene related peptide (CGRP) released from the terminals of the injured

C fibers terminal lead to the activation of NMDA receptors and induce activity-dependent sensitization known as long-term potentiation (LTP). These adaptive mechanisms result in increased thermal hyperalgesia and mechanical allodynia in neuropathic pain conditions (D'Mello & Dickenson 2008).

Like NMDARs, AMPARs are also ionotropic receptors and become activated via interacting with the glutamate excitatory neurotransmitter. Blocking the AMPA receptor at the spinal level attenuates neuropathic pain condition (Chen et al 2013c). Protein kinase A (PKA) and A-kinase anchoring protein 79/150 (AKAP 79/150) are released at the synapse and phosphorylate Glu1 subunit of AMPA receptors at ser843. Phosphorylation of AMPA at ser843 activates calcium-permeable AMPARs and leads to the sensitization of neurons (Qiu et al 2014). In the chronic constriction nerve injury model, it has been demonstrated that loss of the GluA2 subunit of the *substantial gelatinosa* AMPA receptors alters channel conductance and calcium permeability. This alteration leads to decreases in the single-channel conductance of AMPA receptors on inhibitory neurons and an increase in single-channel conductance of AMPA receptors on excitatory neurons, which contributes to the overall hyperexcitability properties of the sensory neuron after injury (Chen et al 2016).

Glial glutamate transporters (GLT-1, GLAST) in the spinal dorsal horn play a crucial role in clearing of glutamate neurotransmitter from the synaptic cleft. Impaired glial glutamate uptake leads to excessive extra-synaptic glutamate spillover from the synaptic cleft of the spinal dorsal horn neurons and causes excessive activation of NMDA receptors (Nie & Weng 2009a, Nie & Weng 2010a). Multiple mechanisms have been investigated in relation to the expression and function of glial glutamate transporters. In

the nerve-injured pain model, we found that IL-1β activated the PKC pathway and thereby increased glial glutamate transporter endocytosis (Yan et al 2014). We have also demonstrated that glycogen synthase kinase 3 beta (GSK3β) is partially responsible for increased neuroinflammation and decreased protein expression of glia glutamate transporters in the nerve-injured spinal dorsal horn (Weng et al 2014b). We and others have shown that restoring the function of glial glutamate transporters significantly reduces hyperalgesia in the nerve-injured model (Liaw et al 2005, Weng et al 2006, Yan et al 2013). Pharmacological inhibition of glia activation by minocycline, an antibiotic, restored deficient glial glutamate uptake and normalized activation of NMDA receptors at the synapse in the spinal dorsal horn, along with attenuation of mechanical allodynia in rats in the pSNL nerve injury model (Nie et al 2010b). Selective increased expression of GLT-1 via treatments of ceftriaxone (an antibiotics) (Hu et al 2010) or gene transfer (Maeda et al 2008) significantly reduces hyperalgesia induced by nerve injury (Weng et al 2006). Furthermore, a study from our lab has unraveled another signaling pathway involved in hypersensitization. We have demonstrated that an agonist of adenosine monophosphate-activated protein kinase (AMPK) reduces inflammation and restores basal expression level of glutamate transporters (Maixner et al 2015b).

Reactive oxygen species (ROS) are another endogenous mediators that plays an important role in various diseases, including chronic pain. Reactive oxygen species are free radicals produced during cellular metabolic processes. We and others have shown their important role in the genesis of pain. ROS increase inflammatory cellular signaling cascades and affect many downstream targeting proteins' (Maixner et al 2016, Salvemini et al 2011). Electrophysiological studies in the spinal dorsal horn have demonstrated that,

through activation of TRPA1 and TRPV1 channels, ROS increase the spontaneous release of glutamate neurotransmitter from presynaptic terminals and enhance excitatory synaptic transmission (Nishio et al 2013). Research work done in our lab on AMPKα1 gene knockout mice has demonstrated that ROS inhibition reduces glutamatergic synaptic activity and attenuates allodynia (Maixner et al 2016). Increases in neuronal nitric oxide synthase (nNOS) lead to increases in oxidase 2 (Nox2), which result in ROS-induced increases in the expression of PKC-dependent NMDARs and pain hypersensitization (Choi et al 2016).

Spinal projection neurons carry the spinal output signals to the brain's higher centers through ascending pathways (Figure 1.5). A majority of these projection neurons can be found in the superficial dorsal horn lamina I which expresses the neurokinin 1 receptor (NK1). A neuropeptide, substance P (Todd 2002), is released from the presynaptic terminals of sensory neurons and interacts with NK1 receptors, sensitizing the projection neurons and sending signals to higher centers such as in the thalamus, periaqueductal grey (PAG) and the parabrachial region of the brain (Doyle & Hunt 1999). These NKI positive cells also send the signals to the rostral ventromedial medulla (RVM) region of brain stem (Doyle & Hunt 1999, Suzuki et al 2002). From the RVM region, descending tracts to the spinal cord can be modulate signals in the dorsal horn. In addition to the above-mentioned projection neurons, more of the projection neurons can be found in lamina III-VI of the spinal dorsal horn, which also send signals to the thalamus through the spinothalamic ascending tract. These ascending and descending pathways modulate the primary sensory input signals and influence how we perceive pain sensations, playing a critical role in neuropathic pain development (Seybold 2009).

Norepinephrine (NE) released from the descending inhibitory pathway primarily interacts with α2-adrenoceptor receptors (Millan 2002) and suppresses neurotransmitter release from the primary afferent nerve terminal, thereby inhibiting the firing of projection neurons present in the spinal dorsal horn (Jones 1991).

Role of Spinal Disinhibition in Neuropathic Pain

Nerve injury causes a loss of intrinsic inhibition . Loss of balance between the excitatory and inhibitory systems leads to hypersensitization in chronic pain condition. Modulation of the excitatory pattern of dorsal horn projection neurons by descending inhibitory serotonergic, noradrenergic and dopaminergic pathways originates from periaqueductal gray (PAG), locus coeruleus, the raphe nuclei, and the rostral ventral medulla (RVM) playing an essential role in pain perception (Lau & Vaughan 2014) (Figure 1.2). Below is the summary of various altered inhibitory peptides, neurotransmitters, receptors, channels, transporters and intermediates composing the spinal inhibitory system in the development of chronic pain conditions.

Endogenous opioids and receptors

There are more than 20 known opioidergic peptides released throughout the brain, with unique but overlapping distribution patterns (Charnay et al 1984, Miller & Pickel 1980, Stengaard-Pedersen & Larsson 1981). Opioid-peptides such as β-endorphin, met-enkephalin, nociceptin and dynorphin influence the dynamic function of nociception, reward behavior, emotional center reaction, and learning and memory processing in the central nervous system. These opioids exert their effects via interacting with μ-opoids receptors (MOPrs), δ-opioid receptors (DOPrs) and κ-opioids receptors (KOPrs). Opioid

peptides binding to their recptors intiate the coupling of Gi/Go proteins and subsquently lead to the inhibition of adenylyl cyclase and voltage gated Ca^{2+} channels, resulting in activation of inward rectifying K^{+} channels and the MAPK pathway (Polakiewicz et al 1998, Simonds 1988, Ueda 1989). Many subtypes of the μ,δ and κ opioid receptors have been identified and are the key targets in the treatment of acute and chronic pain. MOPr1 mainly mediates analgesis effects and is primarily involved with opioid addication and tolerance, whereas MOPr2 has been identified to be involved in causing respiratory and cardiac depression.

GABA/Glycine

In the spinal dorsal horn, from laminae I-III, 30% of the interneurons are inhibitory in nature (Yasaka et al 2010). These inhibitory neurons are either GABA- and/or glycine-positive cells and secrete GABA and/or glycine inhibitory neurotransmitters. It has been observed that from the ventromedial medulla some GABAergic/glycinergic projection neurons can also be found throughout the dorsal horn of the spinal cord (Antal et al 1996a, Cho & Basbaum 1991) (Figure 1.5). In the somatosensory system, the modulation of peripheral stimuli first occurs at the level of the spinal dorsal horn (SDH) through the GABAergic inhibitory neurons and the inhibitory descending fibers (Antal et al 1996b, Carlton & Hayes 1990, Todd 1990). The Inhibitory interneurons are stimulated by the activation of Aβ and low-threshold C sensory afferent fibers, thus preventing the initiation of noxious pain signaling (Daniele & MacDermott 2009, Lu & Perl 2003, Lu & Perl 2005, Todd 1996, Zheng et al 2010).

In the spinal cord injury animal model, it has been shown that there is a loss of GABAergic interneurons in the spinal cord of laminae I-III, which leads to depression of

the GABAergic tone and contributes to facilitating pain signaling in the SDH (Meisner et al 2010). In addition, the application of bicuculline, an antagonist of the $GABA_A$ receptor, reduces the excitation of dorsal horn neurons. Furthermore, manipulation of a positive allosteric compound such as midazolam weakens the noxious activity evoked by wide dynamic range neurons of the spinal cord (Clavier et al 1992, Sumida et al 1995).

Several neurological disorders, such as epilepsy, neonatal seizures, anxiety, depression, neuropathic pain, etc., are characterized by the excessive excitatory activity of neurons (Kahle et al 2008). Aberrant activation of neurons in these diseases can be attributed to the loss of action of an inhibitory neurotransmitter. Amino acid neurotransmitters γ-Aminobutyric acid (GABA) and glycine mediate the fast inhibitory neurotransmission in the CNS (Zeilhofer et al 2012). Of these two amino acids, GABA is the major inhibitory neurotransmitter in brain areas and serves a pivotal role in sensory network synchronization. GABA, released from GABAergic neurons, contributes to somatosensory information processing and has been characterized by the mechanism of feed-forward and feed-backward inhibition. In chronic neuropathic pain, loss of inhibitory synaptic transmission by GABA and glycine have been associated with pain sensitivity (Zeilhofer 2008). The inhibitory control exerted by the GABA can be initiated through binding to the anion permeable receptor $GABA_A$ receptor and the G protein–coupled $GABA_B$ receptor. In neuropathic pain, activation of $GABA_A$ receptor alleviates pain in a rodent model (Knabl et al 2008). The transmembrane sodium gradient influences extra synaptic $GABA_A$ receptors altering the tonic current inhibition by affecting the GABA transporter equilibrium (Wu et al 2006b). Aberrant processing of Aβ and C sensory fibers has been linked to the neuropathic pain (Baron 2000). The

concentration and duration of GABA inhibition is partially dependent on the high-affinity Na+- dependent GABA transporters. Dysfunction of GABA transporters occurs in several central nervous system disorders, such as strokes, epilepsy, neuropathic pain, etc. (Kersante et al 2013). Several studies have demonstrated that, not only do these transporters play an important role in the uptake of GABA at the fast inhibitory synapses and modulate the inhibitory postsynaptic responses (Kersante et al 2013), they are also involved in the complex process of the maturation of the cerebral cortex during development.

It has been demonstrated that, following an episode of spontaneous seizures in the case of temporal lobe epilepsy, GAT-1 transporter downregulation occurs (Ueda & Willmore 2000). Further, GAT-1 transporter knock out mice showed the development of abnormal motor function (ataxia and tremor), anxiety, abnormality in the regulation of body temperature and sedation along with overall body weight loss (Chiu et al 2005). On the other hand, if functional GABA transporters in the synaptic cleft increase the rate of GABA reuptake increases thus reducing the concentration of GABA in the vicinity and consequently decreases the probability of its interaction with GABA receptors.

By utilizing molecular cloning techniques, four distinct genes encoding GABA transporters (GATs) have been identified (Guastella et al 1990). The four GABA transporters are known as GAT-1, GAT-2, GAT-3 and BGT-1; GAT-1 and GAT-3 are widely expressed in the cerebral cortex. GAT-1 is the most abundantly expressed in the cortex neurons and, in some places, is also expressed by astrocytes. GAT-1 expressing mRNA neurons are predominantly found in the layer IV and II of the neocortex region. All the GABAergic neurons (GAD67 positive cells) express the GAT-1 mRNA.

Surprisingly, some of the pyramidal glutamatergic neurons also express the GAT-1 protein. GAT-1 positive fibers are present in the white matter beneath the neocortex and the corpus callosum regions. Immunocytochemical studies have demonstrated that GAT-1 is widely associated with axon terminals and fibers known as punctuate structures near the soma and proximal dendrites of cortical neurons in the rodent as well as in mammalian neocortex (Minelli et al 1995). However, GAT-3 transporters are found exclusively in astrocytes of the central nervous system (Minelli et al 1996).

Selective GAT-1 inhibition has been shown to exert antinociceptive effects in rodent models of acute, facilitated and chronic neuropathic pain. Local administration of selective GAT-1 inhibitor (NO-711) in anesthetized rats induces an increase in GABA levels and modulates the evoked spinal EAA release, which contributes to the analgesic action of this class of drugs (de Almeida et al 2002). While impairment in GABAergic inhibitory synaptic activities in the spinal dorsal horn is an important mechanism contributing to the genesis of neuropathic pain after nerve injury (Bonin & De Koninck 2013, Coull et al 2005, Coull et al 2003b, Moore et al 2002), currently, whether and how the spinal inhibitory system is altered in neuropathic pain induced by paclitaxel remains not fully understood.

Paclitaxel is a taxanes family derivate extracted from the bark from the Pacific yew and is a first-line chemotherapy drug used to treat many solid tumors such as breast (Henderson et al 2003), lung (Socinski & Shea 1997) and ovarian cancer (Fader et al 2008). Cancer cells are highly proliferative. Paclitaxel's ability to destabilize microtubules' lattice structure via directly binding to microtubules consequently leads to cellular cell cycle arrest in dividing cells (Derry et al 1995). However, paclitaxel's anti-

neoplastic properties are nonspecific and can also affect normal cells' microtubules structure also.

Treatment with paclitaxel can damage vulnerable sensory and motor neurons of the peripheral nervous system. The loss of functional microtubules' in neurons can lead to axonal degeneration, secondary demyelination, and loss of sensory fibers (Dougherty et al 2004). Patients suffering from paclitaxel-induced neuropathic pain exhibit symptoms bilaterally in both hands and feet and often complain of loss of sensory sensation, paresthesia and occasionally pain. Paclitaxel-induced neuropathic pain and sensory dysfunction is dose-limiting and affects the patient's quality of life, thus hampering the use of this life-saving drug in cancer treatments (Argyriou et al 2008, Cata et al 2006b, Dougherty et al 2004). Over the past decade, it has been suggested that paclitaxel impairs axoplasmic transportation in neuronal cells, thereby leading to axonal degeneration (Scripture et al 2006). Other studies have suggested a mechanism involved in vacuolated and swollen mitochondria in peripheral neurons, such as depletion of ATP (Zheng et al 2011b), impaired Ca^{2+} release (Kidd et al 2002), and oxidative stress (Varbiro et al 2001).

In chapter two of this dissertation, we present the evidence that animals treated with paclitaxel develop neuropathic pain and have enhancements of GABA transporter-1 protein expression and global GABA uptake, as well as suppression of GABAergic tonic inhibition in the spinal dorsal horn. Pharmacological inhibition of GABA transporter-1 ameliorates the paclitaxel-induced suppression of GABAergic tonic inhibition and neuropathic pain. Thus, targeting GAT-1 transporters for reversing GABAergic disinhibition in the spinal dorsal horn could be a useful approach for treating paclitaxel-induced neuropathic pain.

Recognition of the important role played by immune and glial cells in the modulation of neuronal cells' function and their involvement in the development of persistent pain have opened new avenues for the identification of novel therapeutic targets for optimal pain therapy (Scholz & Woolf 2007b). Research in this field has grown significantly during the past two decades. Here, I have summarized the important function of non-neuronal cells in relation to chronic pain conditions.

Role of Spinal Glial Cells in Neuropathic Pain

Glial cells are non-neuronal cells and comprise more than 50% of the cellular population of the nervous system (Azevedo et al 2009, Watkins et al 2007). Glia cells such as microglia, astrocytes, and oligodendrocytes play a wide variety of roles, including synthesis, release, and uptake of neurotransmitter, and protection of neuronal cells and helps in maintaining physiological homeostasis in the central nervous system (Figure 1.4). It has been demonstrated that glial cells expresses many similar receptors and ion transporters as a neuron, such as the ionotropic and metabotropic glutamate receptors, gamma-aminobutyric acid receptor, purinergic receptor, acetylcholine receptors, adrenergic receptors, etc. (Eulenburg & Gomeza 2010, Jarvis 2010, Porter & McCarthy 1997). Close interaction between a neuron and glial cells and their role in neuroinflammation and neurodegenerative diseases has made them attractive therapeutic targets to treat nervous system-related pathologies. Neuronal cells can generate and propagate action potentials, and glial cells actively participate in modulating local synaptic transmission (Takahashi & Tsuruhara 1987, Yang et al 2009). Hence, glial cells are important players in contributing to normal physiological and pathophysiological

conditions in the nervous system. After nerve injury, the spinal dorsal horn glia cells become reactive (Colburn et al 1999a, Ji et al 2013). In neuropathic pain conditions, reactive gliosis has been observed in the spinal dorsal horn. Reactive gliosis is the process that includes the activation of glial cells, alterations of glial cell morphology and changes in the expression of pro- and antinociceptive genes in the SDH of the spinal cord.

Microglia cells in neuropathic pain

Microglia cells are an immune monocyte/macrophage type of cells in the central nervous system and are responsible for innate immune responses. Microglia precursor cells originate from the mesodermal lining and enter the central nervous system during early stages of embryogenesis (Ginhoux et al 2010). In normal physiological conditions, these microglia cells continuously surveys the central nervous system microenvironment for any potentially damaging substance or pathogen via its long and fine processes (Nimmerjahn et al 2005). In normal conditions, these cells divide at a minimal rate to maintain the number of resident microglia cell population (Lawson et al 1992). Upon presentation by noxious stimuli, resident microglia cells become activated. Activated microglia cells undergo changes in cellular morphology (thickened processes, somatic hypertrophy), proliferation, gene expression profile and functional changes that are known as the "microgliosis" response (Colburn et al 1999b, Coyle 1998). After nerve injury, microglia cells are the first to respond (Tanga et al 2004) and stay activated for long periods of time (2-10 days) (Coyle 1998).

Nerve injury activates the microglia cells, which leads to the production and proliferation of pro-inflammatory cytokines and chemokines that facilitate neuropathic

pain development and maintenance (Colburn et al 1997, Coyle 1998). Inhibition of microglia activation by pharmacological agents such as pentoxifylline (Raghavendra et al 2003b), minocycline (Ledeboer et al 2005) and ibudilast (Ledeboer et al 2006) suppresses inflammation and activation of glial cells (microglia and astrocytes), thereby attenuating neuropathic pain development. After peripheral nerve injury, proliferation of microglia cells and enhanced migration of microglia cells from distant sites have been observed, increasing the local microglia cell population of the spinal dorsal horn. The microglia cells' proliferation in the spinal dorsal horn transiently increases the number of microglia cells at the early stages of nerve injury and usually peaks after two days of peripheral nerve injury (Echeverry et al 2008, Liu et al 2000). Several mediators have been identified that stimulate microglia cell proliferation. One such example is Neuroglin-1 (NRG-1), which is released from primary afferent neurons and binds to the tyrosine kinase heterotrimeric receptor (erB2, erB3, and erB4) in the spinal dorsal horn, stimulating microglia proliferation via activating the MEK/ERK1/2 pathway (Calvo et al 2011, Calvo et al 2010). Up-regulation of the macrophage-colony stimulating factor (M-CSF) following nerve injury coincides with the proliferation of microglia cells. Blocking of M-CSF expression by a mutation in or neutralization of the axotomized rat facial nucleus model prevented glial cells' proliferation (Yamamoto et al 2010). Additionally, activated microglia also expressed interferon-γ (IFN-γ) receptors, which increases microglial proliferation, and produce morphological changes following peripheral nerve injury. Blocking of the IFN-γ receptor prevented microglial activation and reversed tactile allodynia (Tsuda et al 2009).

We can recognize activated microglia cells by using various enhanced specific markers such as clusters of differentiation 11b (CD11b) ionized calcium binding adaptor (Iba1), toll-like receptor 4 (TLR4), activation of p38-mitogen activated protein kinase pathway, etc. (Lehnardt et al 2003, Tanga et al 2004). Once the glia cells become activated, they release cytokines, chemokines, growth factors and various proteases into the extracellular space. In the spinal dorsal horn of a nerve-injured model, activation of microglial cells may manifest in two polarized forms: classical activation M1, a pro-inflammatory secretary type, and an alternative M2 form, an anti-inflammatory type (Popiolek-Barczyk et al 2015, Xu et al 2016). Studies have shown a significant increase in the M1 type of activated microglia over 14 days of nerve injury in SDH (Popiolek-Barczyk et al 2015, Xu et al 2016). Following nerve injury, the activation of microglia contributes to the development of neuropathic pain. Inhibition of microglia activation by administering minocycline prevents nociceptive hypersensitization and chronic pain development (Lin et al 2007). Robust activation of microglial cells within a few hours of nerve injury has been observed and can persist for days. Interestingly, prevention of microglia activation by minocycline during the development of pain has proven to have therapeutic value but failed to attenuate preexisting mechanical allodynia and hyperalgesias in neuropathic pain condition (Raghavendra et al 2003a). This suggests that mediators released by activated microglia cells at the early stages of nerve injury are predominantly responsible for the development of pain.

Spinal microglia become reactive to the mediators released by injured sensory neurons and microglia themselves (Beggs & Salter 2007, Hathway et al 2009, Suter et al 2009). Mediators released from glia and neurons affect the functions of both cell types.

Secreted proteins, such as neuregulin-1 (NRG1) (Calvo et al 2011), metalloproteinase-2 & 9 (MMP-2 & 9) (Kawasaki et al 2008a), chemokine monocytes chemoattractant protein 1(MCP1) (Abbadie et al 2003), chemokine CCL-21 (Biber et al 2011), chemokine fractalkine (Milligan et al 2005) and growth factors (NGF, BDNF, NT-3, NT-4), are released from neurons and enhance neuron-glial cell interaction in response to the nerve injury. Upregulation of neurotransmitter receptors, such as the NMDA (glutamate), AMPA (glutamate), NK1 (SP), CGRP , TrkB (BDNF), P2X7 and P2X4 (purinergic), expression on the microglia cells' surface have been observed following peripheral nerve injury (McMahon & Malcangio 2009, Pezet et al 2002, Ransohoff & Perry 2009, Rasley et al 2002). The neurotransmitter released from neurons can modulate microglia cells via acting through these receptors. Similarly, mediators have been found in the microenvironment near the vicinity of the microglia after nerve injury. ATP, ROS, NO, interferons, chemokines (CCRs, CXCRs) and proinflammatory cytokines (IL, TNF family members) are among the several mediators that have been identified in the chemical soup of the spinal dorsal horn following nerve injury.

ATP

Excessive release of ATP in the nerve-injured spinal dorsal horn activates microglia cells by interacting with ionotropic (P2X) and metabotropic (P2Y) receptors present on the microglia cells' surface (Inoue & Tsuda 2012). A dramatic increase in the expression of cell-surface receptors for neurotransmitters such as purinergic receptor -2 plays an important role in neuropathic pain. Pharmacological inhibitions of the P2X4 receptor attenuated pain in existing neuropathic pain condition and delivery of P2X4 receptors activated microglia cells in naïve rats' spinal cord, showing the development of

allodynia, which suggests the important role played by the P2X4 receptor in chronic pain development and maintenance (Tsuda et al 2003).

Nitric oxide

Nitric oxide (NO) is a byproduct of the catabolic reaction of L-arginine by NO synthase enzyme to L-citrulline and is produced by a wide variety of cells, including neurons and microglia (Zhang & Snyder 1995). Increased NO production is associated with sensitization of dorsal horn neurons and chronic pain condition (Meller et al 1992, Stanfa et al 1996). In response to the peripheral nerve injury in the spinal dorsal horn, an increase of Ca^{+2} ions (Clementi & Meldolesi 1997), cytokines, neurotrophins, prostaglandins and stimulation of NMDA receptors (Kitto et al 1992) triggers the production of NO (Brenman & Bredt 1997, Millan 1999). NO mediates several intracellular transduction pathways via the mechanism of cyclic guanidine monophosphate activation (Wang & Robinson 1997). NO-triggered signaling pathways can alter the activity of neuronal and non-neuronal cells by modifying gene expression and enhance the release of glutamate (Sorkin 1993), substance P (Inoue et al 1997), calcitonine-gene-related peptides (Garry et al 1994), cytokines and prostaglandins (Bezzi et al 1998).

Neurotrophic Factors

Neurotrophic factors, including nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), are important players in neuronal survival, function and synaptic plasticity(Lu et al 2005, Poo 2001). These factors exert their function by binding to two distinct classes of receptors, which include the p75 neurotrophin receptor ($p75^{NTR}$) and the receptor tyrosine kinases Trk family

(TrKA, TrKB, and TrKC) (Reichardt 2006). NGF binds to TrKA receptors, BDNF and NT-4 bind to TrKB receptors, and NT-3 binds to TrKC receptors selectively, while all the neurotrophins can bind non-selectively to $p75^{NTR}$ (Lu et al 2005). The precursor pro-neurotrophins cleaved by matrix metalloproteinases and plasmin mature into neurotrophins secretary protein (Mowla et al 2001). Interestingly, precursor pro-neurotrophins protein via $p75^{NTR}$ receptor interaction has deleterious effects on neurons, whereas mature neurotrophins via Trk receptors promote survival of neurons (Lee et al 2001). Neurotrophic factors primarily activate phosphatidylinositol 3-kinases (P13K) – Akt, which inhibits apoptotic-associated proteins (Datta et al 1997). Additionally, the MAPK-MEK/ERK pathway is activated by neurotrophins via Trk receptors and stimulates anti-apoptotic proteins (Riccio et al 1999). On the other hand, neurotrophins receptors present on neuronal and microglial cells become sensitized and activated by the release of neurotrophin factors and contribute to the development of hypersensitivity and pain maintenance (Calvo et al 2010, Obata & Noguchi 2006, Petersen et al 1998). The protective and damaging effects of neurotrophins in chronic pain are determined by the expression level of the p75 neurotrophin-4 receptor (Barrett et al 1998).

BDNF

In peripheral nerve injury-induced neuropathic pain condition, brain-derived neurotrophic factor (BDNF) has been recognized as essential mediators of the development of neuronal plasticity. BDNF is released from microglia cells upon activation of purinergic signaling by ATP. More specifically, activation of P2X4 purinoreceptors by ATP-evoked enhanced Ca2+ and phosphorylation of p38-MAPK result in more production of BDNF (Trang et al 2009, Zhou et al 2011a). BDNF are

essential components of synaptic plasticity and memory (Bramham & Messaoudi 2005). Studies have showed that BDNF play an important role in development of synaptic plasticity in peripheral nerve injury via modulating spinal dorsal horn C-fiber, which evoked late-long-term potentiation (L-LTP) (Zhou et al 2011a). This modulation by BDNF occurs through activation of ERK, p38MAPK and NF-κ B signaling pathway (Zhou et al 2008). Additionally, after peripheral nerve injury in the spinal dorsal horn, BDNF from microglia cause a depolarizating shift in the anion gradient of spinal lamina I neurons, leading to disinhibition and hyperexcitation of neurons and consequently contributing to tactile allodynia (Coull et al 2005, Coull et al 2003a). Blocking of BDNF or TrkB receptors reversed the allodynia and the anion shift in the nerve-injured model (Coull et al 2005).

Astrocyte cells in neuropathic pain

Astrocyte cells are one of the three major cellular (microglia, astrocytes, and oligodendrocytes) types and constitute 40% to 50% of the entire glial cell population in the central nervous system (Montgomery 1994) (Figure 1.4). Like microglia cells, astrocytes are also an important player in maintaining neuronal survival and homeostasis (Montgomery 1994). In general, astrocytes and neurons express similar receptors and ion channels. Due to unique electrophysiological membrane properties, neurons can generate and propagate action potentials, whereas astrocytes cannot do so. Nonetheless, astrocyte cells are crucial in the development of the CNS. They contribute to neurotransmission, cerebral angiogenesis (Beck et al 1986), regulation of pH and ion concentrations, detoxification, and also produce neurotrophins, cytokines, and chemokines.

Astrocytes' ability to connect with other astrocytes through physically coupled networks mediated by gap junctions such as connexin-43 (Cx43) helps in facilitating intercellular transmission of mediators such as Ca^{2+} ions and other cytosolic contents (Chen et al 2012, Giaume & McCarthy 1996). Astrocyte cells also communicate with neighboring neurons via tight junctions (Nedergaard 1994) and synapses (Theriault et al 1997). Through computational analysis, it is estimated that in rodents, single astrocytes can connect to 4 to 6 neuron cell bodies via approximately 140,000 synaptic connections and 300 to 600 neuronal dendrites (Oberheim et al 2009). All these characteristics of astrocytes make them principal modulators of neuronal cell functions in health and diseases. Interestingly, astrocytes present in gray matter (protoplasmic) in the human neocortex are more than 2.5 times larger in diameter and extend their primary process 10 times more than rodents' protoplasmic astrocytes, which have been suggested as key attributes in phylogenetically advanced species (Halassa et al 2007).

Astrocytes' interaction with microglia, presynaptic neurons, and postsynaptic neurons constitutes tetrapartite synapses in the spinal cord and signifies their important role in health and diseases (Scholz & Woolf 2007b). Following peripheral nerve injury generally, microglia activation preceds the astrocytes activation (Echeverry et al 2008). Taken together, morphological and biochemical changes in astrocyte cells can alter sensory transmission signals in the spinal dorsal horn (Crown et al 2008, Gwak et al 2008). Literature evidence has demonstrated that nerve injury induces astrocyte activation (Gwak & Hulsebosch 2009). For example, after peripheral nerve injury, microglial cells become activated within 24 hours, followed by astrocyte cell activation. Astrocyte activation can last more than 3 months in the spinal dorsal horn upon

peripheral afferent nerve injury (Deumens et al 2009, Gwak et al 2012). Activation of astrocytes constitutes morphological changes such as swelling and hypertrophy-hyperplasia (astrogliosis) and proliferation of astrocytes in response to noxious stimuli (Watson et al 2014). Up-regulation of glial fibrillary acidic protein (GFAP), S100β and vimentin are indicative of astrocyte activation and have been associated with neuropathic pain condition (Coyle 1998, Garrison et al 1991, Ridet et al 1997). Intrathecal administration of GFAP antisense oligonucleotides reduces hyperalgesia in nerve-injured neuropathic pain (Kim et al 2009).

Mediators such as ATP, substance P, excitatory amino acids neurotransmitters, NO and fractalkine from primary afferent neurons and mobilization of intracellular Ca^{2+} in the astrocytes activate several signaling molecules in the dorsal horn, resulting in the activation of astrocyte cells (Hide et al 2000, Marriott & Wilkin 1993, Takuma et al 1996). Astrocytes possess various activation stages. Transient activation of astrocytes usually occurs within minutes of noxious stimuli and is mediated through Ca2+ ions and/or the activation of signaling pathway via phosphorylation. The intermediate state of activation encompasses changes happening after several minutes to hours and is mediated by post-translation mechanisms. In contrast, long-term activation of astrocytes is generally mediated via changes in transcriptional mechanisms and persists from several hours to days. Inhibition of astrocyte activation by fluorocitrate, fluoroacetate, and L-alpha-aminoadipate alleviates neuropathic pain condition in rodent models (Lefevre et al 2015, Wang et al 2009, Zhang et al 2012b, Zhuang et al 2006).

Marked changes in extracellular ionic concentration, growth factors, chemokines and proinflammatory cytokines levels activates astrocytes in the spinal dorsal horn

following neuropathic pain (Mollace et al 1998, Watkins & Maier 2000, Zhang et al 2013b). The release of neurotrophic factors such as glial cell-derived neurotrophic factors (GDNF) protects neurons (Boucher & McMahon 2001). An elevated level of GDNF achieved by spinal injection of GDNF or overexpression by lentiviral vector prevented mechanical and thermal hyperalgesia in chronic pain condition (Adler et al 2009). GDNF mediated the increased level of somatostatin and contributed to its neuroprotective effects (Adler et al 2009).

Cytokines such as IL-1β, TNF-α and chemokines (Monocytes chemoattractant protein-1 / MCP-1) released from activated astrocytes in the spinal cord have been implicated in the development and maintenance of neuropathic pain. Activation of spinal cord astrocytes is sufficient to elicit hyperalgesia and allodynia nociceptive behavior. Nerve injury induces signaling pathways in spinal cord astrocytes, such as the phosphorylation of the C-jun N-terminal kinase (JNK) and the MAP kinase pathway, implicated in the development and maintenance of persistent chronic pain (Gao et al 2009b, Moon et al 2014, Zhuang et al 2006). Upregulation of spinal sigma non-opioid intracellular receptor 1 (σ1 receptor) following nerve injury was mediated by p38 MAPK phosphorylation and activation of astrocytes, which are associated with the development of mechanical allodynia in a chronic constriction nerve injury model (Moon et al 2014).

D-serine, an endogenous ligand for N-methyl-D-aspartate (NMDA) receptor, contributes to central sensitization and synaptic plasticity in neuropathic pain (Latremoliere & Woolf 2009). In normal conditions, D-serine is secreted from neuronal cells in the spinal cord. However upon noxious stimulation, activated astrocytes from the superficial laminae of the dorsal horn are mainly responsible for the elevated D-serine

levels, which is a key player in neuropathic pain development (Kartvelishvily et al 2006, Lefevre et al 2015, Schell et al 1995, Wolosker et al 1999). Depletion of spinal D-serine by intrathecal administration of D-aminoacid oxidases or NMDA receptor antagonist D-AP5 significantly attenuated mechanical allodynia following peripheral nerve injury (Lefevre et al 2015).

The glia glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST) present on astrocytes play essential roles in clearing the synaptic and extra-synaptic excitatory neurotransmitters, thereby aiding the termination of excitatory neuronal signals (Huang et al 2004, Nie & Weng 2009a). Downregulation of GLT-1 and GLAST transcription and translation contributes to hyperexcitability of dorsal spinal neurons and, consequently, the development and maintenance of chronic neuropathic pain condition. In the spinal dorsal horn, substantial gelatinosa neurons have a prolonged excitatory postsynaptic current upon pharmacological inhibition of GLT-1 (Weng et al 2007). Mechanistic studies show that GLT-1 and GLAST transporters' inhibition by TBOA or dihydrokainic acid (a selective inhibitor of GLT-1) increases metabotropic glutamate receptors-1 alpha and AMPA receptor-mediated excitatory postsynaptic currents (EPSCs), respectively (Huang et al 2004, Weng et al 2007). Blocking of IL-1β and ROS upregulates glutamate transporters' expression levels and prevents hypersensitization of spinal dorsal horn neurons in chronic pain (Kim et al 2010). Increased IL-1 β level activates the calcium/PKC pathway, which further enhances the dynamin-dependent endocytosis of GLT-1 and GLAST glial glutamate transporters and depletes their spinal expression after peripheral nerve injury (Yan et al 2014). Similarly, increased levels of IL-1 β were associated with upregulated phosphorylation of GSK3β

and downregulated GLT-1 protein expression after nerve injury. Pharmacological suppression of GSK3β reversed proinflammatory cytokines levels and restored GLT-1 expression, along with attenuation of neuropathic pain conditions (Weng et al 2014b).

In response to noxious stimuli, spinal astrocytes undergo a proliferation process. Mediators such as ATP, cytokines, chemokines, etc., released from activated astrocytes and/or other cellular types present in the spinal dorsal horn stimulate the proliferation of astrocytes, which contributes to neuropathic pain development and maintenance (Franke & Illes 2014, Ke et al 2016). The study showed that activation of Janus kinase-signal transducers and activators of the transcription 3 signaling pathway (JNK/STAT3) resulted in spinal astrocyte proliferation following nerve injury (Tsuda et al 2011). Intrathecal administration of JNK/STAT3, or the cell cycle inhibitor flavopiridol, prevented astrocyte activation and proliferation and attenuated preexisting chronic pain (Byrnes et al 2007, Tsuda et al 2011). Intraperitoneal administration of the immunosuppressive agent methotrexate in a spinal root-injured model prevented glial cell activation and proliferation, and it also prevents the development of mechanical allodynia (Hashizume et al 2000). This result suggests that inflammatory mediators evoke glia activation in neuropathic pain. Furthermore, in a spinal cord injury model, suppression of the cell cycle by olomucine prevented neuronal cell death and astroglia proliferation and enhanced axonal regeneration (Tian et al 2006).

Altogether, recognition of the important role played by immune and glial cells in the modulation of neuronal cells' function and their involvement in the development of persistent pain has opened new avenues for the identification of novel therapeutic targets for optimal pain therapy (Scholz & Woolf 2007b).

Role of Spinal Cytokines and Chemokines mediators in neuropathic Pain

It has been increasingly recognized that the release of neuromodulators such as cytokines and chemokines plays an essential role in the development and maintenance of pain. Therefore, it is important to identify molecular and cellular key modulators and their underlying mechanism in pain control. Cytokines and chemokines are secreted proteins that affect the survival, differentiation, proliferation and functional activity of most of the body's cells. These soluble mediators bind to specific transmembrane receptors presented on various cells and activate many secondary messengers and downstream signaling pathways. The term "neuroinflammation" encompasses inflammation of the affected part of the central nervous system. Innate and adaptive immune reactions in response to stress, toxin, infection, and trauma have both beneficial and deleterious effects on central nervous system health. In neuropathic pain, proinflammatory cytokines such as IL-1β, TNF- α, IL-6, TGF-β, IL-10, etc., are some endogenous modulators that have been heavily investigated to understand how they can alter neuronal function in chronic pain condition.

IL-1β

Upon nerve injury, the release of ATP, CXCL1, and excessive excitatory neurotransmitter release activates microglia cells to release IL-1β. 1L-1β is a glia-derived cytokine mediator that can affect microglia, astrocytes, and neurons. IL-1β exerts its effects by binding to type I IL-1 receptors (IL-1RI). The release of IL-1 receptor antagonist (IL-1Ra) and protein expression of IL-1R type II receptor in microglia cells negatively affects the release IL-1β from microglia cells (Mantovani et al 2003). IL-1Ra

can bind to IL-1RII without transmitting any signal via acting as a decoy ligand (Mantovani et al 2003). The relative level of IL-1and IL-1Ra determines the extent of nerve tissue injury by controlling the level of IL-1β (Smith et al 1999). Studies have demonstrated that administration of IL-4, IL-10 and the antagonist of the IL-1 receptor suppresses microglial activation by lowering the level of IL-1β and TNF-α pro-inflammatory cytokines (Pousset et al 1999, Relton & Rothwell 1992, Sawada et al 1999). Microglia, astrocytes, and oligodendrocytes express IL-1R-I (D'Souza et al 1994, Molina-Holgado et al 2000, Pinteaux et al 2002). The release of IL-1 activates the NF-κB signaling pathway and induces immune and inflammatory gene expression, which further affects the expression of several cytokines, cytokine receptors, growth factors, tissue remodeling enzymes, extracellular matrix components, and adhesion molecules (O'Neill & Greene 1998). Several key proteins have been coimmunoprecipitated along the IL-receptor, including IL-1-dependent kinase (IRAK-1, IRAK-2), which has been implicated as an upstream mediator of NF-κB. Furthermore, it has been demonstrated in mouse that bone marrow-derived macrophage cells that p16Ink4a positive cells, a cell cycle and aging associated protein, suppresses IL-6 production in macrophages by promoting degradation of IL-1R-associated kinase 1 (IRAK1). This is the apparent an anti-mechanism of action of p16Ink4a in suppressing rheumatoid arthritis. (Kellermayer 2012).

The release of IL-1β triggers astrogliosis in the spinal dorsal horn following nerve injury and further enhances the activated astrocyte expression level of TNF-α, MCP-1, IL-6, Il-8, 1P10 and adhesion molecules (ICAM-1, VCAM-1) (John et al 2003). The release of IL-1β, TNF α and IL-6 can sensitize spinal dorsal horn neurons and plays an

important role in synaptic plasticity and neuronal excitability in neuropathic pain (Gustafson-Vickers et al 2008). IL- 1β activates inducible nitric oxide (iNOS) expression level, and a higher level of NOS and NO in the spinal cord contributes to hypersensitization of nociceptors and pain (Sung et al 2004). The activation of PKC and a higher level of IL-1β promote endocytosis of glial glutamate transporters in the spinal dorsal horn following nerve injury and contribute to neuropathic pain development (Yan et al 2014). Excessive endogenous release of IL-1β in neuropathic rats increased the release of the glutamate neurotransmitter from the primary afferent neurons (Yan & Weng 2013b). Whole cell patch clamp recording from rat spinal cord slices has shown that IL-1β enhances AMPA- and NMDA (Glutamate receptors)-induced current and hypersensitization of substantia gelatinosa neurons of the spinal dorsal horn (Liu et al 2013). Altogether, the essential role played by IL-1β in the initiation and maintenance of neuropathic pain suggests the importance of evaluating it further for therapeutic manipulation of this condition.

TNF-α

TNF-α is a cytokine released from neuronal and glial cells, including microglia and astrocytes (Chung & Benveniste 1990, Wei et al 2007). Microglia cells are the major source of TNF-α secretion. It has been implicated in the initiation and maintenance of neuropathic pain (Sommer & Kress 2004, Sommer et al 1998). TNF-α exerts two different effects by binding to p55 (TNFR1) and p75 (TNFR2) receptors expressed on most cells, including microglia, astrocytes and neuronal cells (Vallejo et al 2010). Literature evidence has shown that TNF-α upregulation during neuroregeneration and neurodegeneration demonstrated a unique ability to induce both neuroprotection as well

as pathology of the CNS (Golan et al 2004, Leung & Cahill 2010, Marchetti et al 2004, Mogi et al 1994, Pozniak et al 2016). Immunosuppression's, along with neuroregeneration effects, have been observed in TNF-α receptor knockout mice (Arnett et al 2001, Wang et al 2002). The diverse outcome of TNF-α in various cellular processes is attributed to different receptor profiles present on the targeted cells (Bruce et al 1996). TNF-α interaction mediated through TNFR receptors induces production of several chemokines and adhesion molecules and regulates inflammation, proliferation, differentiation and the apoptotic process of cells (Badiola et al 2009, Chen & Goeddel 2002, Rousselet et al 2012). In general, TNFR1 receptor association with diseases and TNFR2 receptor association with neuroprotection have been observed (Arnett et al 2001).

In relation to neuropathic pain, upregulation of TNF-α and its receptors (TNFR1, TNFR2) have been detected at the peripheral nerve injury site as well as in the spinal dorsal horn (George et al 1999, Ohtori et al 2004, Schafers et al 2003a). In a spinal cord contused injury model, the level of TNFR1 increase can be detected within 15 min of injury and can persist up from one to three days after injury. In the same study, upregulation of TNFR2 was detected as early as TNFR1, but peaked at 4 hrs after injury (Holmes et al 2004). Inhibition of TNF-α synthesis or blocking its receptors prevents the development of hyperalgesia and allodynia behaviors (Ribeiro et al 2000, Sommer et al 2001). Upon TNF-α release, activation of NF-κB, JNK and p38MAPK signaling pathways are associated with neuronal plasticity and neuropathic pain (De Smaele et al 2001, Schafers et al 2003b, Tang et al 2001). Exogenous TNF-α application on C-fibers suggested that long-term potentiation (LTP) associated with hyperexcitation of C-fibers neurons can be inhibited by pharmacological application of NF-κB, JNK, and p38MAPK

pathway inhibitors (Liu et al 2007b). A randomized clinical trial administration of infliximab for disc herniation-induced sciatica chronic pain condition failed to support the beneficial effects of systemic anti-TNF-α treatment (Korhonen et al 2004, Korhonen et al 2006). The detriment and beneficial effects of TNF-α in the CNS make it a less than ideal target for therapeutic manipulation to treat neuropathic pain

IL-6

IL-6 is a soluble cytokine composed of many family members, including leukemia inhibitory factor (LIF), IL-6, IL-11, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin-1 and various growth-promoting factors (Taga & Kishimoto 1997). IL-6 exerts both neurotrophic and neuroprotective functions. In relation to neuroinflammation and neurodegeneration, IL-6 has been extensively studied. In classic signaling pathways, IL-6 cytokines form a complex by binding to membrane-bound IL-6 receptors (mIL-6R)(Scheller et al 2014), promote binding of signal transducing membrane protein gp130, and induce dimerization of IL-6 complex. By recruiting other associated proteins, it can further activate the signaling cascade of Janus-activated kinase/signal transducer activation of transcription (JAK/STAT), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) (Boulanger et al 2003, Heinrich et al 2003, Kishimoto 2005, Zhou et al 2016). In contrast, in the IL-6 trans-signaling pathway, IL-6 can bind to a soluble form of the IL-6 receptor and subsequently interact with h gp130 protein, which activates the pro-inflammatory signaling cascade (Rose-John 2012).

Administration of IL-6 or overexpression of both IL-6 and the soluble IL-6 receptor reduces brain damage (Loddick et al 1998) and accelerates the regeneration of damaged nerves, respectively (Hirota et al 1996). Genetic knockdown of IL-6 causes sensory nerve regeneration and impairment of function (Zhong et al 1999). Increased IL-6 production, along with other cytokines, activates microglia cells and stimulates astrocyte proliferation (Fattori et al 1995, Selmaj et al 1990). Astrocyte cells are the main contributors to elevated IL-6 levels in the CNS (Van Wagoner & Benveniste 1999). Upregulation of IL-1β, TNF-α, and IFN-γ enhanced the production of IL-6 level in the CNS (LeMay et al 1990, List et al 1992, Tada et al 1993). An elevated level of IL-6 and IL6 receptors in the dorsal root ganglion and spinal dorsal horn are found in various pathological pain models (Melemedjian et al 2010, Melemedjian et al 2014). Injection of IL-6 in rat hind paw hyper-sensitizes the nociceptive fibers and induces mechanical allodynia.Blocking of IL-6 synthesis by using a protein inhibitor, reverses the mechanical allodynia behavior (Melemedjian et al 2010). Further mechanistic studies has revealed that activation of cyclic AMP response element binding protein (CREB) mediates the upregulation of IL-6 gene transcription after sciatic nerveinjury (Melemedjian et al 2010). Collectively, all these studies suggest that IL-6 cytokines can prove to be a therapeutic target to manage neuropathic pain.

TGF-β

Transforming growth factor-β (TGF-β) is a cytokine superfamily composed of more than 30 families of proteins. Some of the subfamily members are from TGF-βs, morphogenetic proteins, and activins, and these molecules regulate many biological functions such as proliferation, migration, differentiation and apoptosis (Lawrence 1996).

Although the TGF-β superfamily is widely distributed throughout the body, in the central nervous system TGF-β isoforms are primarily present (Gomes et al 2005). The TGF-β1, TGF-β2 and TGF-β3 isoforms in the central nervous system activate Smad2/3 and Smad4 transcription factors via interacting with the TGF receptor I (TGFRI) and TGF receptor II (TGFRII) family (Vivien et al 1998). Upon binding to its ligands, TGF-β receptors can also activate non-Smad signaling pathways (non-canonical TGF-β signaling) such as JNK, MAPK P13/AKT (Mu et al 2012).

The TGF-βs are secreted by both neuronal and glial cells in the central nervous system and have been implicated in CNS development and diseases. In vitro studies have shown that astrocytes, Schwann, and oligodendrocyte cells express all TGF-β isoforms, but microglia expresses only TGF-β1 [255]. In a model of chronic pathological pain, the TGF-β family prevents inflammation and provides neuroprotection (Krieglstein et al 2002). Upregulation of TGF-β isoform in response to noxious stimuli has been observed. Examples of this response include: increased level of TGF-β in infiltrating leukocytes and microglia; as well as activated astrocytes in multiple sclerosis, peripheral nerve injury, Alzheimer's, Parkinson's, prion diseases and in AIDS (Pratt & McPherson 1997). Nevertheless, it is still unclear that an increase of TGF-βs in neurodegenerative disease is a compensatory mechanism or aids in disease progression. Two opposite effects of TGF-β1 have been documented: it can induce beta-amyloid deposition and can decrease the beta-amyloid deposition by enhancing the microglia-mediated clearance (Lesne et al 2003, Wyss-Coray et al 2001).

The bone morphogenic protein (BMP) and activins membrane-bound inhibitor (BAMBI) act as decoy TGFR1 receptors and negatively regulate the TGF-

β/BMP/activins signaling pathways (Onichtchouk et al 1999). A neuropathic pain model using BAMBI genetic knocked out mice exhibited attenuated nociceptive behaviors in response to noxious stimuli. In the spinal cord, BAMBI-KO mice induction of gain in TGF-β signaling contributes to higher levels of endogenous opioids peptides, including β-endorphins and enkephalins, resulting in attenuated nocifensive behavior responses (Tramullas et al 2010). TGF-βs isoforms provide neuroprotection against glutamate cytotoxicity, control astrocyte cells functions (morphology, differentiation, motility, cytoskeleton, and proliferation), promote wound healing and immunosuppression, and induce microglia proliferation (Bruno et al 1998, Buisson et al 1998, de Sampaio e Spohr et al 2002, Dobbertin et al 1997, Gagelin et al 1995, Lindholm et al 1992, Pratt & McPherson 1997). TGF-β 1 increased production of IL-6, leukocyte inhibitory factor, nerve growth factor, platelet-derived growth factor and monocyte chemoattractant protein (Benveniste 1998). In a partial sciatic nerve ligation model, intrathecal delivery of TGF-β1 attenuated neuropathic pain-associated nociceptive behaviors (Echeverry et al 2009). Similarly, systemic administration of TGF-β1 reverses mechanical allodynia and thermal hyperalgesia following nerve injury (McLennan et al 2005). Overall, studies have demonstrated that TGF-β as a therapeutic analgesic can prove to be beneficial for managing neuropathic pain condition.

IL-10

Interleukin-10 is composed of two approximately 18 kDa cytokine proteins that suppress inflammation and immune cells. Monocytes, macrophages, subsets of T-lymphocytes and B cells primarily produce IL-10 (Glocker et al 2011). IL-10 promotes cell survival by activating JAK1/STAT3, PI3 kinase, MAPK, SOCs and NFκB pathways.

Upon nerve injury, more inflammatory cytokines are released than anti-inflammatory cytokines. This has been recognized as a key culprit in the genesis and maintenance of neuropathic pain. IL-10 binds to its receptor IL-10R to exert its immunosuppressive effects. By preventing the secretion of TNFα, IL-1β, IL-I6, IL-2 and interferon –γ, IL-10 prevents differentiation and proliferation of macrophages, thereby limiting cellular damage and promoting healing (Moore et al 2001). IL-10R is present in the peripheral and central nervous system. Following nerve injury, higher production of IL-10 occurs for a short duration of time, but decreases as the chronic pain develops (Mika et al 2008). Interestingly, application of low-concentrations of IL-10 at the injured sites promotes regeneration of damaged axons (Atkins et al 2007, Sakalidou et al 2011). Despite its promise, however, the therapeutic potential of utilizing IL-10 to treat neuropathic pain condition requires more investigation.

Chemokines

Chemokines are chemotactic cytokines that are potent neuromodulators and have been implicated in the enhancement and maintenance of neuropathic pain. Chemokines are small secretary proteins (8-14 KD), exhibiting leukocyte chemoattractant and cytokine-like properties. More than 50 chemokines have been identified in humans, with sequence similarity ranges from 25-95%. Based on the conserved cysteine-rich sequences, the chemokine ligands (L) have been categorized into CCL, CXCL, XCL, and CX3CL subfamilies (Bonecchi et al 2009, Zlotnik & Yoshie 2000). These chemokine ligands attract a wide variety of cells, including monocytes, eosinophils, basophils, T lymphocytes, natural killer (NK) cells, and dendritic cells. Additionally, the role of chemokines in development, homeostasis, and synaptic transmission puts forth a complex

picture of their diverse roles in cellular function (Asensio & Campbell 1999). Neurons and glial cells can produce chemokines. Although several chemokines, such as CXCL12 (SDF-1, stromal cell derived factor-1) (Bhangoo et al 2009, Oh et al 2001), CCL5 (Malon & Cao 2016) and CCL21 (Biber & Boddeke 2014, Biber et al 2011), have been implicated in the hypersensitization of nociceptive neurons, CX3CL1/fractalkine and CCL2/MCP1(Zhu et al 2014a) are two chemokines that are well-investigated in neuropathic pain. Chemokines exert their effect by interacting with their receptors. These receptors are seven transmembrane-domain G-protein-coupled receptors (GPCR) (Charo & Ransohoff 2006). Activation of several signaling pathways, including phospholipase C (PLC), MAP kinase, phosphatidyl inositol-3 kinase (PI-3K) and JAK/STAT, occurs and leads to the functional outcome initiated by chemokines (Cartier et al 2005, Mellado et al 2001, Soriano et al 2003).

Fractalkine/CX3CL1

An increased level of fractalkine chemokines in the dorsal root ganglion and spinal dorsal horn have been implicated in neuropathic pain (Verge et al 2004). Fractalkine released from primary afferent neurons binds to CXCR1 receptors, which are predominantly present on the microglia cells membrane (Milligan et al 2004). The protease cathepsin S, which is excreted by activated microglia, cleaves the fractalkine chemokines domain present on neurons (Clark et al 2009). A high level of fractalkine is detected in cerebrospinal fluids in neuropathic pain. Spinal application of cathepsin S in fractalkine genetic knockout mice failed to elicit hyperalgesia, whereas in normal rats it induces nociceptive hypersensitization (Clark et al 2007, Staniland et al 2010). Blockade of the CXCR1 receptor or neutralizing the fractalkine by means of an antibody prevents

mechanical and thermal hypersensitivity development in chronic pain (Milligan et al 2004). Blockage of the IL-1receptor, neutralization of IL-6 and application of nitric oxide synthase inhibitor prevented fractalkine-induced mechanical allodynia. Further fractalkine-induced pain was prevented by minocycline administration and glial cell activation (Milligan et al 2005).

CCL2/MCP-1

CCL2, also known as monocytes chemoattractant protein-1 (MCP-1), is a chemokine that attracts monocytes, macrophages, and microglia to the site of inflammation and injury. CCL2 is released from small and large injured and uninjured neurons of the DRG following nerve injury (White et al 2005). However, in the spinal cord, CCL2 is not only expressed in the neurons of the spinal dorsal horn but also can be found in astrocyte cells (Gao et al 2009b). CCL2 elicits the signaling cascades by binding to CCR1, CCR2 and CCR3 receptors. Among these three receptors, the binding affinity of CCL2 to the CCR2 receptor is higher than to the CCR1 and CCR3 receptors (Kurihara & Bravo 1996). Upon nerve injury, the correlation of CCL2 release with microgliosis in the spinal cord demonstrated its important role in microglial activation (Thacker et al 2009). Expression of the CCR2 receptor in the spinal dorsal horn is controversial. It is well established that CCR2 receptors are present on the surface of microglia cells (Abbadie et al 2003). However, several studies reported that CCR2 receptors are present on astrocytes (Knerlich-Lukoschus et al 2008) as well as on neuronal cell surfaces (Gao et al 2009b). In situ hybridization studies showed a weak level of CCR2 in the DRG and spinal cord in normal rats; however, three days after injury, the CCR2 mRNA level was upregulated in spinal dorsal horn neurons (Gao et al 2009b).

Peripheral nerve injury increases the expression of CCL2 and CCR2 in the dorsal root ganglion and spinal dorsal horn (Jung et al 2008, Zhang & De Koninck 2006). In genetic knockdown CCR2 mouse, the mechanical threshold for nociceptive behavior was decreased following nerve injury (Abbadie et al 2003). Additionally, spinal application of a CCR2 antagonist attenuated pre-existing tactile allodynia developed in a rodent nerve-injured model (Bhangoo et al 2007). Similarly another study has also shown that neutralization of CCL2 prevented microglial activation (Zhang et al 2007), and application of CCL2 in normal rats induced microgliosis (Thacker et al 2009). In vitro studies have shown that TNF-α exposure induces astrocytes' CCL2 expression mediated by JNK pathway activation (Gao et al 2009b). CCL2 can sensitize the neurons via enhancing NMDA- and AMPA-induced inward current. Central sensitization of neurons by CCL2 is mediated by activation of the ERK pathway (Gao & Ji 2009).

Studies have demonstrated that chemokines mediate neuron-glia interaction and have also shown their role in the development and maintenance of neuropathic pain. Thus, clinical trials targeting chemokine receptors were an obvious choice. However most of the chemokine receptors antagonists have failed in clinical trials due to their lack of potency and efficacy.

The lack of potent analgesics with minimum side effects to combat pathological pain condition still poses challenges to relieving patients suffering from chronic pain condition. Further investigations are needed in this field to better understand the roles of chemokines and cytokines in chronic pain in order to find novel therapeutic targets. In the pain research field, scientists have begun to explore new avenues and mechanisms (genetic/epigenetic) behind the development and maintenance of chronic pain state. In

recent decades, the field of epigenetics and its role in CNS pathology, including pain, has attracted scientists' attention. Emerging evidence suggest that epigenetic mechanisms play important roles in the development and maintenance of chronic pain condition.

Role of Epigenetic Mechanism in Neuropathic Pain

Peripheral nerve injury can alter gene expression profiles at the spinal and supraspinal levels in the nociceptive pathways and can cause long-lasting cellular and behavior changes (Austin et al 2015, Dominguez et al 2010, Kalous et al 2007, Suzuki et al 2005, Xiao et al 2010, Xu & Yaksh 2011). Widespread perturbations in gene expression profiles that are caused by or are a response to nerve injury stimuli mediate neuronal plasticity and lead to chronic pain (Li et al 2016, Morioka et al 2016, Morioka et al 2015, Obradovic et al 2015). One way of controlling gene expression is by limiting access to transcription factors and other enzymes necessary for gene transcription. The genome is packed in the highly organized structure of chromosome. A chromosome consists of chromatin, which is basically made of repeating nucleosome units. A nucleosome unit is composed of about 140 base pairs of DNA wrapped around histone octamer proteins. Chromatin is a highly dynamic structure and is generally found in two higher-order forms: euchromatin (open chromatin structure accessible to chromatin remodeling factors) and heterochromatin (closed, compact chromatin structure associated with gene silencing). The gene transcription activation or suppression in euchromatin's chromatin structure is further determined by a combination of various enzymes (Orphanides & Reinberg 2002). Another way of controlling gene expression is after gene transcription, by means of suppressing or degrading the mRNA before it gets translated to

protein. Non-genetic mechanisms such as epigenetics can regulate gene expression at the transcription and post-transcription levels.

The term “epigenetic” encompasses those mechanisms that involve stable and/or heritable changes in gene expression without any changes in DNA nucleotide sequences. In response to developmental and environmental cues, epigenetic mechanisms regulate a broader range of genes and protein expression involved in various cellular signaling pathways of cell-specific function to the development of diseases. Environmental factors such as diet, maternal care, stress, injury, chemical agent, neuroinflammation, drug addiction, drug tolerance, alcohol abuse, etc., are among those shown to remodel epigenetics (Basavarajappa & Subbanna 2016, Doura & Unterwald 2016, Landgrave-Gomez et al 2015, Papavasiliou et al 2015, Shimazu et al 2013, Weiss et al 2001). Literature evidence suggests that in the majority of neurological and neurodegenerative diseases, long-term gene transcription regulation is mediated by re-modulation of chromatin structure or controlling the mRNA expression level (Landgrave-Gomez et al 2015). For example, at the gene transcription level of epigenetic gene regulation, DNA methylation and histone modification are two well-recognized epigenetic mechanisms that play important roles in remodeling the chromatin structure. At the level of post-transcription epigenetic gene regulation, small non-coding RNA-like microRNAs play significant roles in gene suppression. MicroRNAs binding to specific mRNA templates either led to its degradation or prevent its translation to protein. Epigenetic mechanisms, including DNA methylation, histone modification, and non-coding RNAs, are involved in the activation and suppression of gene expression and central sensitization of nociceptive pathways (Imai et al 2011, Kami et al 2016, Laumet et al 2015, Li et al 2014, Lin et al

2016, Maiaru et al 2016, Sun et al 2015, Willis et al 2015, Zhang et al 2016). This role has obvious relevance to the development and maintenance of chronic pain.

DNA methylation in neuropathic pain

Studies have shown that chronic pain induces a long-lasting alteration in gene expression in the nociceptive pathways mediated by DNA methylation, which is partially responsible for functional and structural changes (plasticity) that result in the increased production of pro-inflammatory cytokines, dysfunction of neurons and activation of glial cells (Geranton et al 2007, Griffin et al 2007, Lacroix-Fralish et al 2007, Lacroix-Fralish et al 2006). Gene expression is regulated by either adding or removing methylation chemical marks on the 5-position of the cytosine pyrimidine ring of dinucleotides (Bird 1980, Wyatt 1951). On the gene promoter region, CpG-rich islands are major targets of methylation covalent modification (Bird et al 1985). In general, hypermethylation (more 5-methylcytosine) marks at the promoter region results in gene transcription repression and vice versa (Bird 1986). The enzymes that transfer the methyl group are known as DNA methyltransferases (DNA methyltransferases 1, 2, 3a, 3b) (Rishi et al 2010) and the enzymes that remove these methylation marks from the cytosine are identified as DNA demethylation enzymes (ten-eleven translocation 1, 2, 3) (van der Wijst et al 2015). Following spared nerve injury in rat dorsal root ganglion in a time-dependent manner, there was the induction of DNA methyltransferase (DNMT) expression in a time - dependent manner -, suggesting its role in neuropathic pain (Pollema-Mays et al 2014). In a mouse model suffering from neuropathic pain, maternal feeding behavior contributed to the increase in expression of DNA methyltransferase-1 in the amygdala region of the brain (Zhong et al 2015). However, another group showed that a combination of nerve

injury and environmental factors incites a global decrease in DNA methylation levels at the prefrontal cortex and amygdala region of the brain in a neuropathic pain model (Tajerian et al 2013). The different timeline for DNMT protein expression and additional effect from environmental factors could contribute to the opposing findings in the above studies.

Methylation of mammalian DNA requires interaction between methylated sites and methyl-CpG-binding proteins (MeCP 1, 2 and 3) (Lewis et al 1992, Meehan et al 1989). Administration of DNA methyltransferase inhibitors such as 5-azacytidene attenuated pain behavior in a chronic constriction nerve injured rodent model. These changes were associated with a decreased global DNA methylation levels and MeCP2 expression in the lumbar spinal cord (Wang et al 2011). In nerve-injured mice, increased activity of DNA methyltransferase 3a and 3b contributes to increased DNA methylation marks at the GAD-1 (GAD 67 is coded by GAD-1 gene) gene promoter region in the spinal cord leading to decreased glutamate decarboxylase expression (Wang et al 2016). The release of ATP from injured sensory neurons sensitizes the nociceptive pathways through interacting with purinergic receptors (P2Xrs), which are present on neuron and glia cell surfaces. The upregulation of these purinergic ligand-gated ion channel 3 receptors contributes to neuropathic pain development (Burnstock 2016). In diabetic neuropathic pain, hypomethylation at the promoter region of purinergic ligand-gated ion channel-3 receptors resulted in enhanced gene expression and contributed to diabetic peripheral neuropathic pain development (Zhang et al 2015a). Together, these studies suggest DNA methylation's important role at the peripheral, spinal and supraspinal levels in chronic neuropathic pain development.

Micro-RNA in neuropathic pain

MicroRNAs are small non-coding RNAs endogenously expressed by cells to regulate gene transcription at the translation level. In general, these miRNAs are 20-26 nucleotides in length. Based on the sequence complementarity to the 3′ untranslated region of a specifically targeted strand of mRNA, they can either represses mRNA translation or completely degrade it. In humans, more than 30% of protein-encoding genes are regulated by microRNAs (Chaudhuri & Chatterjee 2007). MicroRNA gene regulation has been identified in many physiological conditions, including the development and normal cell function of the central nervous system (Cao et al 2016, Davis et al 2015) as well as the development and maintenance of pathological diseases such as neurodegeneration and pathological pain condition (Basak et al 2016, Jagot & Davoust 2016, Sakai & Suzuki 2015). Massive changes in miRNAs expression profiles at the peripheral and spinal cord levels in various nerve-injured pain models, revealed by microarray and deep sequencing analyses, suggest their essential roles in neuropathic pain condition (Bali et al 2014, Brandenburger et al 2012). In inflammatory pain induced by CFA, downregulation of miR-16 and mir-1 were observed in the dorsal root ganglion. In contrast, after sciatic nerve ligation there was no change in miR-16 level at any time point, but there was downregulation of miR-1 levels in the dorsal root ganglion at 3, 7 and 14 days post-peripheral nerve injury. However, no change in the expression profile of miRNAs in the spinal dorsal horn has been reported in either of the above models (Kusuda et al 2011). Similarly, in a bilateral sciatic nerve chronic constriction injury model, upregulation of miR-341 has been found in the dorsal root ganglion but not in the spinal dorsal horn (Li et al 2013a). Furthermore, miR-203, miR-181a-1 and miR-541

were downregulated in the spinal dorsal horn of all these models. Taken together, these studies suggest that, depending on the etiology of pain, differential temporal and spatial miRNAs patterns result in various phenotypic characteristics changes

miRNAs in neuropathic pain: Dorsal Root Ganglion

Different miRNAs profile signatures have been identified following in early and late stages of nerve injury in the dorsal root ganglion, suggesting an important role in the development and maintenance of neuropathic pain. For example, following the early stages (1h, 3h, 6h and 9 h) of sciatic nerve transaction injury, miR-188 level remained upregulated while miR-500 level remained downregulated in the dorsal root ganglion (Zhou et al 2011b). In contrast, during the late stages of nerve injury, distinct miRNAs profile patterns have been observed, such as the persistence of miR-21 upregulation even 6 months after nerve injury (Strickland et al 2011).

Depending on the type of nerve injury, the miR-1 level in the dorsal root ganglion fluctuates accordingly. For example, in a partial sciatic nerve ligated model, mir-1 level decreased, whereas after sciatic nerve axotomy, miR-1 levels increased (Kusuda et al 2011). Similarly, following sural nerve injury, there was also upregulation of miR-1 level, and after tibial nerve injury, its expression level was enhanced (Norcini et al 2014). However, the validation of miR-1 as a therapeutic target so far has been shown primarily in a cancer pain model, in which upregulated miR-1 was knocked down by administrating small interfering RNAs targeting miR-1 to DRG in order to suppress bone cancer pain (Bali et al 2013). All these studies suggest that the role of miR-1 in pain development is varied and depends on the etiology of the pain.

Hyperexcitability of the dorsal role ganglion has been linked to the late phase development and maintenance of neuropathic pain. A lower expression of miR-7a in small DRGs neurons after spinal nerve ligation injury was identified to be associated with pain maintenance and neuronal dysfunction (Sakai et al 2013). Mechanistic studies have shown that suppression of β2 subunits of voltage-gated sodium channels by miR-7a decreases the excitability of these neurons and suppresses pain (Isom et al 1995, Lopez-Santiago et al 2006, Sakai et al 2013). An analgesic effect obtained by overexpressing miR-7a in the dorsal root ganglion was observed during maintenance of pain but not during the development of pain, suggesting its unique role in neuropathic pain. On the other hand, miR-21 experssion was upregulated in all DRG neurons after various types of nerve injury, including spinal nerve injury, axotomy (Strickland et al 2011), chronic constriction sciatic nerve injury and nerve crush. Increased levels of IL-1β and Il-6 pro-inflammatory cytokines in the dorsal root ganglion have been identified as an underlying molecular mechanism for miR-21 upregulation in neuropathic pain (Sakai & Suzuki 2013, Zhou et al 2015). It has been further identified as involved in promoting axon growth of the DRG after axotomy, suggesting its involvement in neuronal repair (Strickland et al 2011).

Specific clusters of miR-182-96-183 miRNAs found in sensory organs/tissue are also present in the dorsal root ganglion. The three distinct miRNAs arising from these clusters include miR-182, miR-96 and miR-183, and their expression was downregulated in the dorsal root ganglion neurons following spinal nerve ligation injury (Aldrich et al 2009).

In chronic constriction, sciatic nerve-injury suppressed miR-96 was linked to the high expression of Nav1.3 sodium channels and contributed to neuropathic pain. Expression of miR-96 after three days of chronic constriction sciatic nerve injury suppressed the level of Nav1.3 in dorsal root ganglion and alleviated neuropathic pain (Chen et al 2014a). Accordingly, miR-183 level decreases after peripheral nerve injury in DRG neurons. Intrathecal administration of lentiviral vector carrying miR-183 encoding sequence contributed to suppressed Nav1.3 and BDNF mRNA expression and consequently suppressed mechanical allodynia (Lin et al 2014).

miRNAs in neuropathic pain: The Spinal cord

Identification of tissue- and stage-specific miRNAs expression profiles in the spinal cord after nerve injury has highlighted their important role in neuropathic pain development and maintenance. Robust synaptic inputs and release of several mediators from injured primary afferent neurons and glia cells induce an exaggerated neuronal response and glial activation in the spinal dorsal horn. Sensory neuron excitations is regulated by voltage-gated Ca2+ channels (VGCCs), which help in maintaining the intracellular Ca2+ concentration and play an important role in synaptic plasticity (Catterall & Few 2008). L-type voltage-gated calcium channels, specifically Cav1.2, have been implicated in sensory processing and in the maintenance of chronic pain condition (Fossat et al 2010). MiR-103 has been identified as a small non-coding RNA that can directly interact with Cav1.2 mRNA and regulate its translation. MiR-103's lower expression leads to higher Cav1.2, in the spinal dorsal horn and has been implicated in chronic pain condition; intrathecal administration of miR-103 has been shown to alleviate neuropathic pain (Favereaux et al 2011).

The release of proinflammatory cytokines and activation of glial cells in the spinal dorsal horn are two hallmark events that take place in the spinal dorsal horn after peripheral nerve injury. MiR-155 can directly bind at the 3′ untranslated region of the suppressor of cytokine signaling 1 in microglia cells and suppresses its expression, resulting in increased production of cytokine levels in the spinal cord and the development of nociceptive behavior following nerve injury. Furthermore, it has been demonstrated that intrathecal administration of miR-155 inhibitor suppresses the expression of IL-1β, IL-6, IκBα and p38MAPK phosphorylation levels (Dragone et al 2014). Suppression of miR-155 reverses the level of suppressor of cytokines signaling 1 and also reverses neuropathic pain behavior (Tan et al 2015).

The autophagy process, which is the ability of cells to clean up cellular debris and recycle biomolecules such as proteins, lipids and organelles of their cytoplasm, is critical for maintaining homeostasis. The autophagy process can incite innate and adaptive immune responses in response to appropriate stimuli (Arroyo et al 2014). Dysregulation in the autophagy process has been implicated in the modulation of the neuroinflammation process and has been identified as one of the contributors in the development and maintenance of chronic pain condition (Berliocchi et al 2015). In an L5 spinal nerve ligation (SNL) nerve-injured model, the suppression of the autophagy process in the spinal dorsal horn was associated with enhanced miR-195 expression. When miR-195-overexpressing primary microglial cells were stimulated with lipopolysaccharide, production of IL-1β, TNF-α, and iNOS increased. Application of an miR-195 inhibitor reversed painful nociceptive behavior in an SNL model (Shi et al 2013). Further mechanistic study demonstrated that suppression of autophagy process related protein

(eg. ATG14) by miR-195 contributes to enhanced neuroinflammation and central sensitization of the nociceptive pathway in neuropathic pain condition (Shi et al 2013).

Histone modification in neuropathic pain

A nucleosome is a fundamental subunit of chromatin, composed of histone proteins around which DNA is circumscribed. Four globular histone subunits, H2A, H2B, H3, and H4, form an octamer structure serving as a spool center around which approximately 146 nucleotides pairs are wrapped. The N-terminal tail region of histone proteins, consisting of approximately 25-40 amino acid residues, protrudes outside and beyond the nucleosome surface. The amino acid residues of N-terminal tails are subjected to posttranslational modification and play a dynamic role in chromatin structural changes (Figure 1.7). Two proposed mechanisms have been put forward through which histone modification can affect chromosome function. The first mechanism is based on the electrostatic charges brought together by these histone post-translation modifications, resulting in altered binding of histone proteins and DNA. The second mechanism proposes that a different pattern of post-translation modification presented at the N-terminal tail of histones can be identified by specific protein recognition modules and, accordingly, can turn "on" or "off" gene transcription (Narlikar et al 2002). The post-translation modification at the N-terminal tail most frequently occurs at lysine, arginine, serine, tyrosine and threonine amino acid residues. Post-translation modification in the form of methylation, acetylation, phosphorylation, ubiquitination and ADP-ribosylation brings diverse changes in chromatin structure and can have activating or silencing effects on gene expression (Jenuwein & Allis 2001).

Histone acetylation in neuropathic pain

In general, histone lysine/arginine acetylation covalent modification promotes gene transcription (Ruthenburg et al 2007). Histone acetylation marks can be put on the set of enzymes known as histone acetyltransferases (HATs) and can be erased by another group of the set of enzymes known as histone deacetylases (HDACs) (de Ruijter et al 2003, Morrison et al 2007). Primary afferent neurons' voltage-gated potassium channel K_v4 subunits control neuron excitability. After nerve injury, reduction in messenger RNA expression of the Kv4.3 gene was associated with neuropathic pain (Vydyanathan et al 2005). Enhanced neuron-restrictive silencer factor (NRSF) binding to the $K_v4.3$ gene neuron-restrictive silencer element (NRSE) region was promoted by hypoacetylation of histone H4, resulting in K_v-4.3 downregulation in the dorsal root ganglion (Uchida et al 2010b). Similarly, NRSF gene hypoacetylation resulted in upregulation of NRSF gene expression. Therefore, more NRSF was available to bind to the NRSE conserve sequence at Nav 1.8, and μ-opioids receptor genes consequently downregulated their expression and caused pathological and pharmacological dysfunction in the C-fibers of dorsal root ganglion following nerve injury (Uchida et al 2010a).

Chronic constriction injury prompted increased global acetyl-histone H3 and decreased silent information regulator 1 (Sirt1) class II HDAC enzyme in the spinal dorsal horn and was implicated in neuropathic pain development. Intrathecal administration of a pharmacological activator of Sirt1 activator reverses painful nociceptive behavior, Sirt1 protein expression and histone H3 acetyl level in the spinal dorsal horn (Shao et al 2014, Yin et al 2013). Excessive release of brain-derived neurotrophic factor (BDNF) from injured primary afferent neurons in DRG and spinal

dorsal horn plays an important role in the central sensitization process via modulating neurotransmission in the spinal dorsal horn (Biggs et al 2010). Nerve injury caused acetylation at histone H3 and H4 near the BDNF promoter region, which induces BDNF transcription and neuropathic pain development (Uchida et al 2013). Several studies have demonstrated that nerve injury increased HDACs enzymes in the DRG and spinal dorsal horn and contributes to neuropathic pain condition. Administration of histone deacetylase inhibitors such as trichostatin A, valproic acid, suberoylanilide hydroxamic acid, biacalin, etc., have shown analgesic effects in different neuropathic pain models (Cherng et al 2014, Denk et al 2013, Matsushita et al 2013, Zammataro et al 2014). The study has shown that an HDAC inhibitor drives activation of synaptic δ opioid receptors in the brain stem nucleus raphe Magnus and enhances δ-opioids analgesia in chronic neuropathic persistent pain condition (Tao et al 2016).

Increased release of pro-inflammatory cytokines and chemokines is a hallmark of neuropathic pain condition. In a partial sciatic nerve ligation model, higher expression of macrophage inflammatory protein2 (MIP-2) and C-X-C chemokines receptor type 2 (CXCR2) proteins on infiltrating neutrophils and macrophages in the surrounding epineurium area further aid in the accumulation of neutrophils and macrophages and up-regulation of inflammatory cytokines (Sonoda et al 1998, Yan et al 1998). The presence of higher levels of acetylation on histone 3 at lysine 9 at the promoter region of MIP-2 and CXCR2 genes enhance transcription. The administration of acetyltransferase inhibitor anacardic acid suppressed the MIP-2 and CXCR2 expression level, reversed proinflammatory cytokines level and attenuated nerve injury-induced neuropathic pain (Kiguchi et al 2012). Similarly, hyperacetylated histone H3 at lysine 9 on the CXCR2

promoter region in the spinal cord contributed to enhanced gene transcription upon rodent hind paw incision. Surgical incision incited upregulation of CXCR2 expression in the spinal cord and was suppressed by administration of anardic acid (Sun et al 2013).

Following sciatic nerve injury, increased expressions of CCL2 and CCL3 receptors in infiltrated macrophage from bone marrow in the injured peripheral nerve are due to enhanced histone H3 acetylation at lysine 9 and histone H3 tri-methylation at lysine 4 (Kiguchi et al 2013). Additionally, in a chronic constriction injury model, administration of curcumin exerted its therapeutic effects by reducing the acetyl H3 acetyl histone H4 in the promoter of BDNF and cox-2 genes and consequently down-regulated their expression in neuropathic pain (Zhu et al 2014b).

The advantage of utilizing HADC inhibitors in combination with opioids has been suggested. Loss of mu opioids' receptor function due to enhanced binding of neuron-restrictive silencer factors in the dorsal root ganglion reduces the effectiveness of morphine drugs in treating neuropathic pain. HDAC inhibitors such as trichostatin A and valproic acid enhanced morphine analgesia effects by suppressing the neuron-restrictive silencer factor via restoring histone hypoacetylation levels in its promoter region (Uchida et al 2015). The study demonstrated that the HDAC inhibitor drives activation of synaptic δ opioid receptors in the brain stem nucleus raphe Magnus and enhances δ-opioids analgesia in chronic neuropathic persistent pain condition (Tao et al 2016).

Histone Methylation in neuropathic pain

In general, depending upon the specific lysine/arginine amino acid residues of the histone tail involved in covalent methylation chemical marks, change can either activate or suppress gene expression. Recently, several studies have reported the role of histone

methylation in the development and maintenance of neuropathic pain. A study showed that pannexin1, a large pore membrane pore channel present in neuron and glia cells, is highly expressed in the dorsal root ganglion after nerve injury and has been recognized as a key player in central sensitization during persistent pain (Bravo et al 2014). The presence of two gene-activating methylation marks, H3k4me2 and H3K9ac histone marks, around the promoter region of the pannexin-1 gene contribute to its higher expression in the DRG and neuropathic pain (Zhang et al 2015c). Activation of the Wnt signaling pathway has been implicated in the development and maintenance of pain (Zhang et al 2013a). The study demonstrated that hyperacetylation of the promoter region of Wnt gene hyperacetylation leads to activation of its gene transcription and the Wnt-mediated signaling pathway in the spinal dorsal horn after nerve injury (Feng et al 2015).

Gene suppression by means of the histone modification tail requires the coordination of multiple types of machinery, such as histone methyltransferases and demethylases (Alam et al 2015, Burgold et al 2008, Burgold et al 2012, Del Rizzo & Trievel 2014, Martinez & Simeonov 2015, Xu et al 2010). The Polycomb repressive complexes 2 (PRC2) is one part of an essential machinery that can suppress gene expression by transferring the mono, di or trimethyl groups on lysine 27 of histone3 (H3K27). The methyltransferase function of PRC2 is performed by multi-complex units, including an enhancer of zeste homolog-2 (EZH2), a suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED) and retinoblastoma (Rb)-associated proteins 46/48 (RbAp46/48). Although the methyltransferase activity is performed by the catalytic subunit of EZH2, it is inactive on its own and requires assembly of all these subunits, including SUZ12, RbAp46/48, and EED to transfer the methyl group to H3 on lysine 27

(Justin et al 2016, Kim & Roberts 2016b, Margueron et al 2009, Yuan et al 2012). Traditionally, EZH2 and histone methylation at lysine 27 of H3 have been implicated in increased production of pro-inflammatory cytokines and the proliferation of cells in various diseases such as cancer, Crohn's disease, ulcerative colitis, in other organs (Agherbi et al 2009, Berger 2007, Gupta et al 2010, Kim & Roberts 2016b). Emerging evidence suggests the role of EZH2 in the development of the central nervous system (CNS) and various other pathologies associated with it (Berke & Snel 2014, Hossein-Nezhad et al 2016, Kakkar et al 2015, Li et al 2013b, Shen et al 2016).

During neocortical development, the PRC2 complex checks the multipotent neuronal progenitor cells and controls their early neurogenic-to-astrogenic cellular fate (Hirabayashi et al 2009b). The EZH2 protein prevents the premature onset of neurogenesis in a developing brain (Sparmann et al 2013). The balance between the cells' self-renewal and the proliferation of neuronal stem cells is controlled by the EZH2 protein. In another series of experiments, H3K27me3 and EZH2 have been shown to be involved in the differentiation and proliferation of neurons, astrocyte and oligodendrocyte cells (Feng et al 2016b, Heinen et al 2012, Hirabayashi et al 2009b, Hwang et al 2014, Iida et al 2015, Lee et al 2008, Palomer et al 2016, Pereira et al 2010, Sher et al 2012, Sher et al 2008). In a partial sciatic nerve ligation model (pSNL), the epigenetic transcriptional activation of MCP-3 (proinflammatory cytokines) was due to a reduced level of the H3K27me3 suppressive mark at the MCP-3 promoter, which contributes to long-lasting neuropathic pain development (Imai et al 2013). Upon nerve injury, increased activity of euchromatic histone-lysine N-methyltransferase-2 known as G9a, HDACs and enhancer of zeste homolog 2 (EZH2), along with increased demethylation of

lysine 9 on histone H3 at the promoter region of K (+) channel genes (Kcnd2, Kcnd4, Kcnma1 and Kcnq2 genes), occurred in the dorsal root ganglion. Selective knockout of G9a by siRNA in the dorsal root ganglion activates K (+) channel expression and attenuates chronic pain (Laumet et al 2015). Both studies presented compelling evidence and signaled the significance of EZH2 and H3k27TM levels in neuropathic pain. However, while utilization of a pharmacological epigenetic reversal agent to restore individual gene expression proved to be beneficial in understanding the mechanism of their expression, the global effects of these epigenetic reversing agents in managing pain conditions at the spinal cord level are still unknown. In the present study, we are the first to report an altered global level of H3K27TM and EZH2 protein expression in the spinal dorsal horn of a nerve injury model.

A common histone methylation associated with gene silencing in a biological system is histone H3 lysine 27 trimethylation (H3K27TM) (Bannister & Kouzarides 2011, Kirmizis et al 2004). The enhancer of zeste homolog 2 (EZH2) is a key enzyme with the ability to transfer the methyl group to specific histone H3 lysine 27 at the promoter region of the specific genes, thereby suppressing their expression (Bannister & Kouzarides 2011, Kirmizis et al 2004). Studies observing the development of the nervous system have shown that EZH2 expression is necessary for maintaining cell identity (Feng et al 2016a, Zhang et al 2015b). Studies have also demonstrated the role of EZH2 in the differentiation of immune cells and neuronal cells. Forced expression of EZH2 in postnatal mouse astrocytes demonstrated their ability to proliferate. Additionally, these cells began showing downregulation of astrocyte markers, such as GFAP and s100, and upregulation of neural stem cell-like genes such as nestin, Sox2, musashi and CD133.

These cells began to demonstrate stem cell-like characteristics, but lacked complete dedifferentiation (Sher et al 2011) into stem cells. Interestingly, during the neocortical development phase, the neuronal progenitor cells (NPC) while switching from neurogenic to astrogenic fate, EZH2 knockdown promoted the neurogenic phase and delayed the astrogenic phase entry of cells (Hirabayashi et al 2009a). EZH2 expression plays a key role in maintaining the balance between the progenitor cells self-renewal process and differentiation (Pereira et al 2010). All these studies suggest that EZH2 plays an important role in determining cellular fate and rate of proliferation.

Our current study (chapter-3) reveals that EZH2 plays a critical role in the development and maintenance of neuropathic pain through regulating the neuroinflammation process in the spinal dorsal horn. Hence, suppressing the spinal EZH2 and H3K27TM levels may be a novel strategy for the development of new analgesics for the treatment of neuropathic pain.

Conclusion and Future Direction

Altogether, epigenetic and genetic mechanisms plays important role in regulating mediators' expression levels in physiological and pathological diseased state including neuropathic pain. Upon nerve injury there is enhanced neuronal excitability has been observed along with increased synaptic strength and reduced inhibitory signaling, which resulted into enhanced spinal dorsal horn neuronal function in nociceptive pathways. Identifying a drug-able target unique to specific types of pain that can be further manipulated to effectively treat chronic pain condition with minimum side effects is both desirable and worthy of exploration.

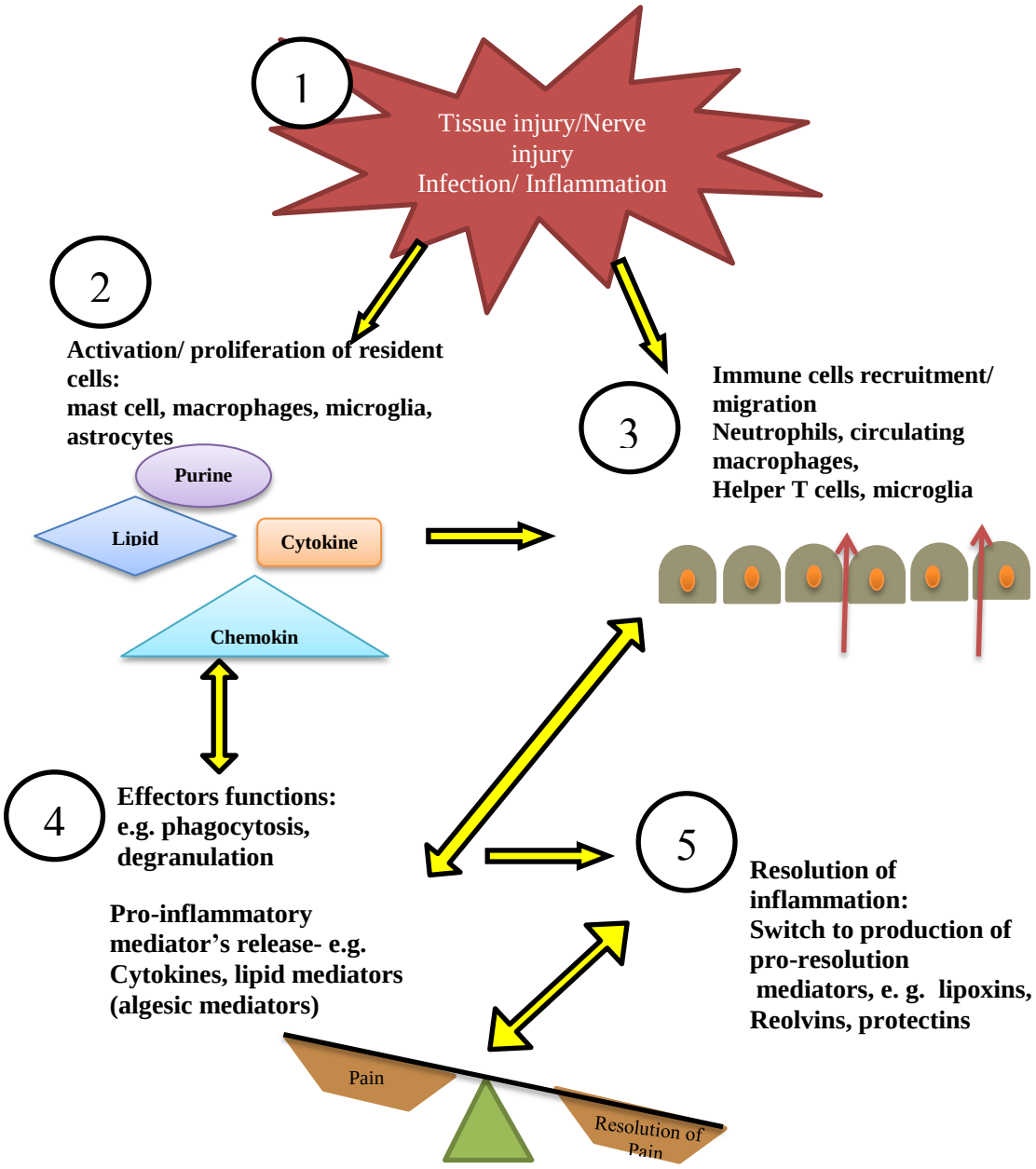

Figure 1.1: **Schematic illustration providing general overview of various stages of the inflammatory response following noxious stimuli.** Upon peripheral tissues / nerve damage, activation/ proliferation of mast cells, macrophages, Schwann cells, satellite glia cells, microglia and astrocytes to peripheral and central sensitization. Several pro-resolution mediators also get released to limit the damage and to resolves the noxious stimuli state. However higher concentration of algesic mediators results in the development of pain. In chronic pain states multiple mediators such as inflammatory cytokines, chemokines, lipids, drives the development and maintenance of pain.

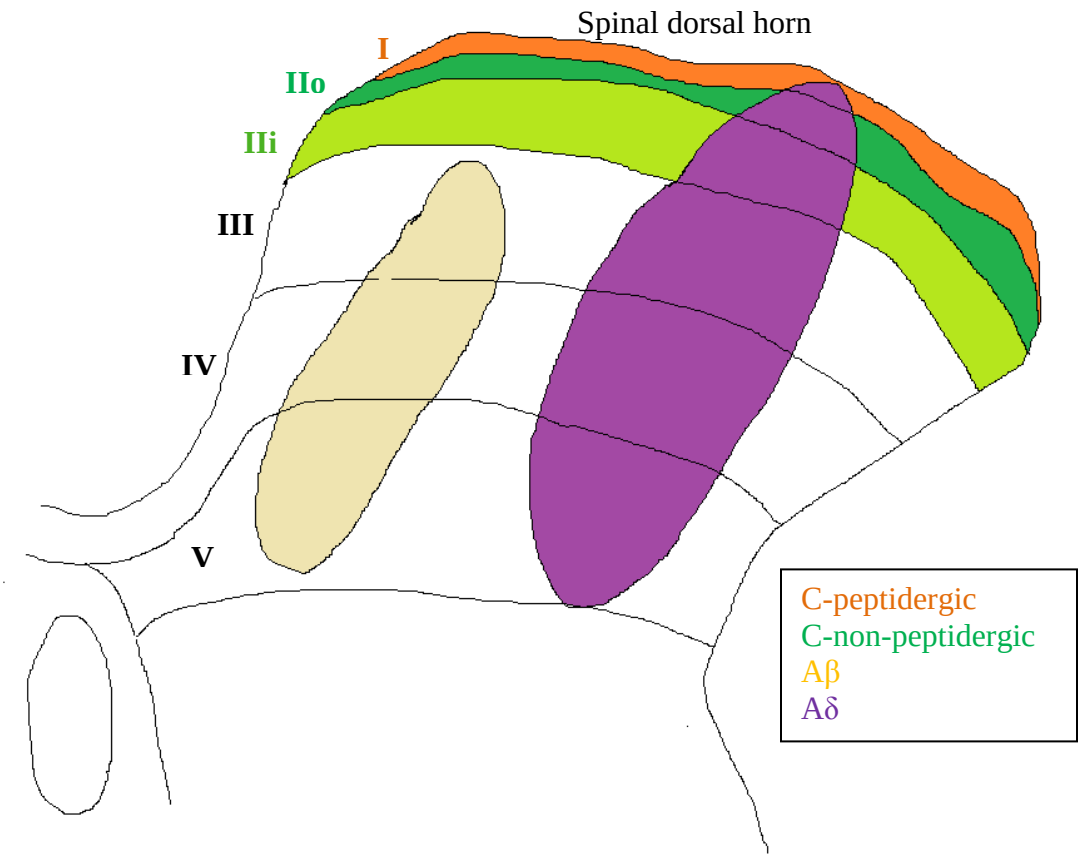

Figure 1.2: **Primary nociceptive afferent fibers and laminae of spinal dorsal horn**. Based on the different cellular types, the spinal dorsal horn can be divided into ten separate regions know as laminae (I- X). Various types of primary afferent fiber terminate in distinct regions of the spinal dorsal horn laminae.

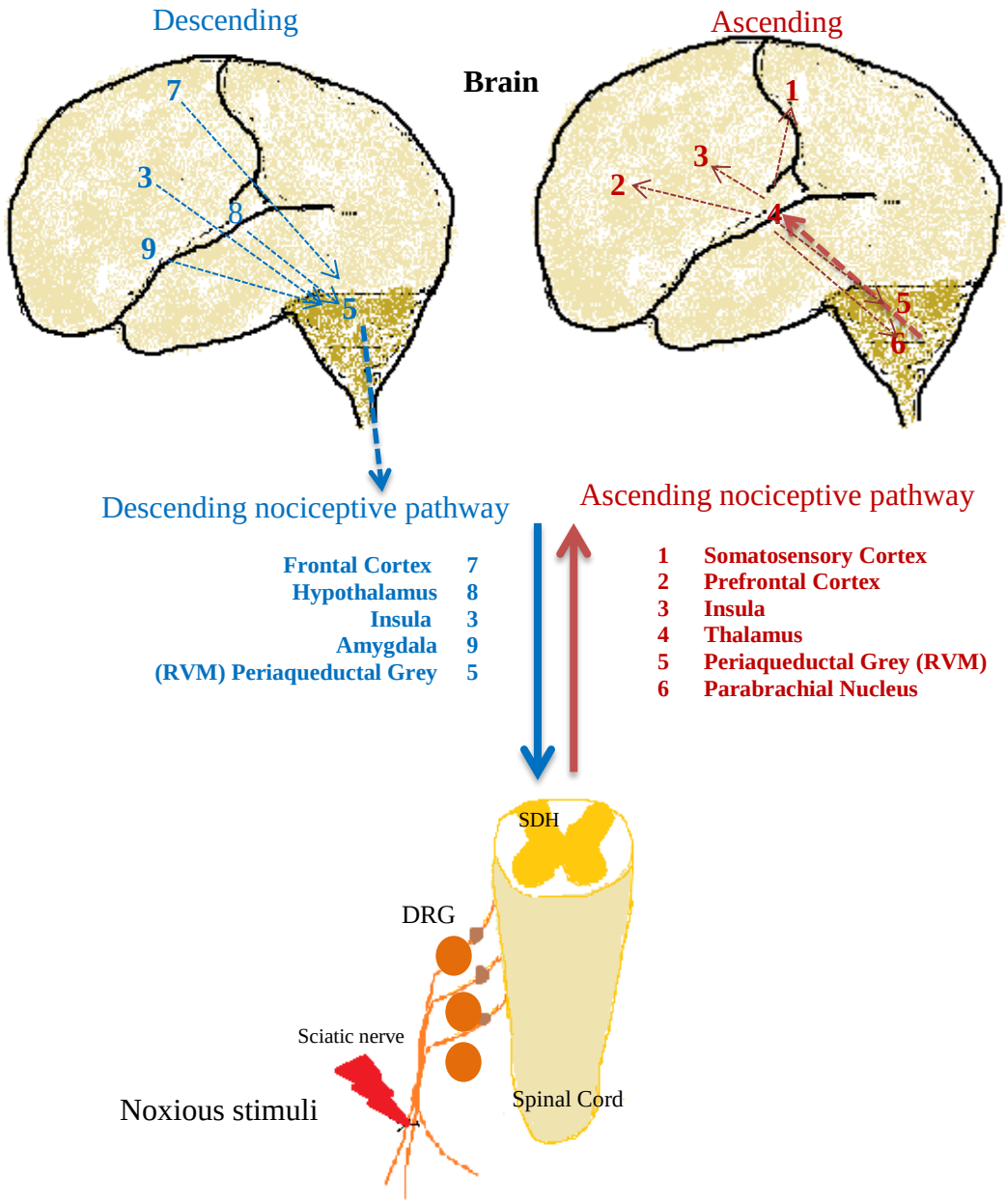

Figure 1.3: **Schematic illustration of some of the brain regions involved in ascending and descending pathways and nociceptive signal processing.** Ascending pathways (marked in red) carry signals from the spinal dorsal horn (SDH) to the central nervous system. The nociceptive signals from SDH primarily go to thalamus, with additional connections to parabrachial nucleus and periaqueductal gray. From there the pain sensations signals go to the somatosensory cortex, insula and prefrontal cortex for central signal integradation. The descending nociceptive pathways (marked in blue) carry the signals from the frontal cortex, insula, amygdala and hypothalamus region of the central nervous system and converge on the periaqueductal gray prior to modulation of activity in the spinal dorsal horn.

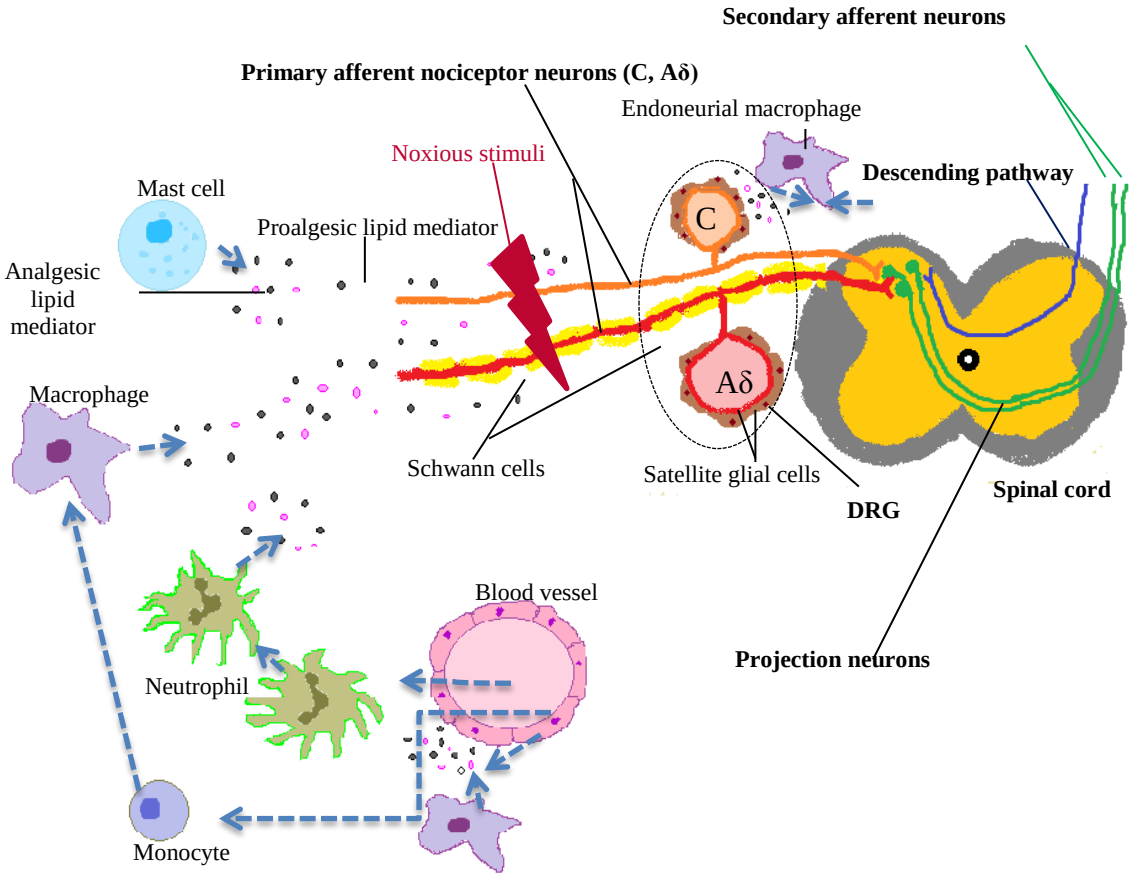

Figure 1.4: **Endogenous algesic/proalgesic and analgesic mediators released by resident nociptive fibers and immune cells in peripheral nervous system in response to the noxious stimuli.** In dorsal root ganglion (DRG), the cell bodies of primary nociceptor neurons (pseudo unipolar neurons) are located. DRG receives signals from peripherally (skin, bone etc.) and transmits them to the spinal cord. Various types of receptors present on the primary afferent nociceptor fibers are capable to detect harmful physical and chemical stimuli. Through these receptors peripheral nociceptor terminal sense the proalgesic (pain enhancing) mediators released from activated immune cells and damaged cells (nerve/ surrounding cells). Analgesic endogenous mediators released either from damaged neurons and resident cells (microglial cells, satellite glial cells of DRG, mast cells, macrophages, epidermal keratinocytes, blood–bone leukocytes recruited to the injury site) have potential to neutralize the noxious stimuli and pain sensation. The primary afferent neurons integrate the input signal in response to the analgesic (inhibitory) and proalgesic (excitatory) mediators and carry the signals to the secondary projection neurons of the spinal dorsal horn, which further transmits the signal via the ascending pathway to the brain. The descending pathway from the brain and brain stem modulates nociceptive processing.

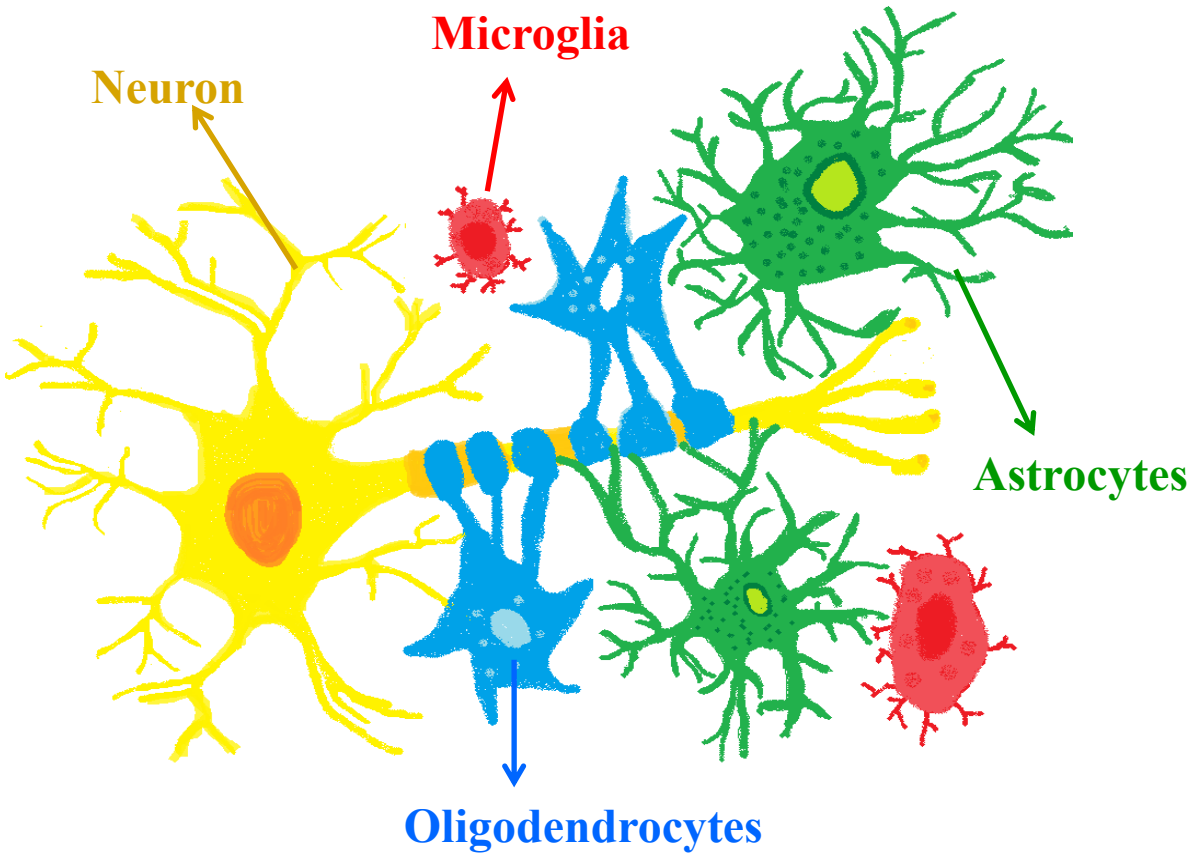

Figure 1.5: **Illustration of neurons and different types of glia (microglia, astrocytes, and oligodendrocytes) cells found in central nervous system.**

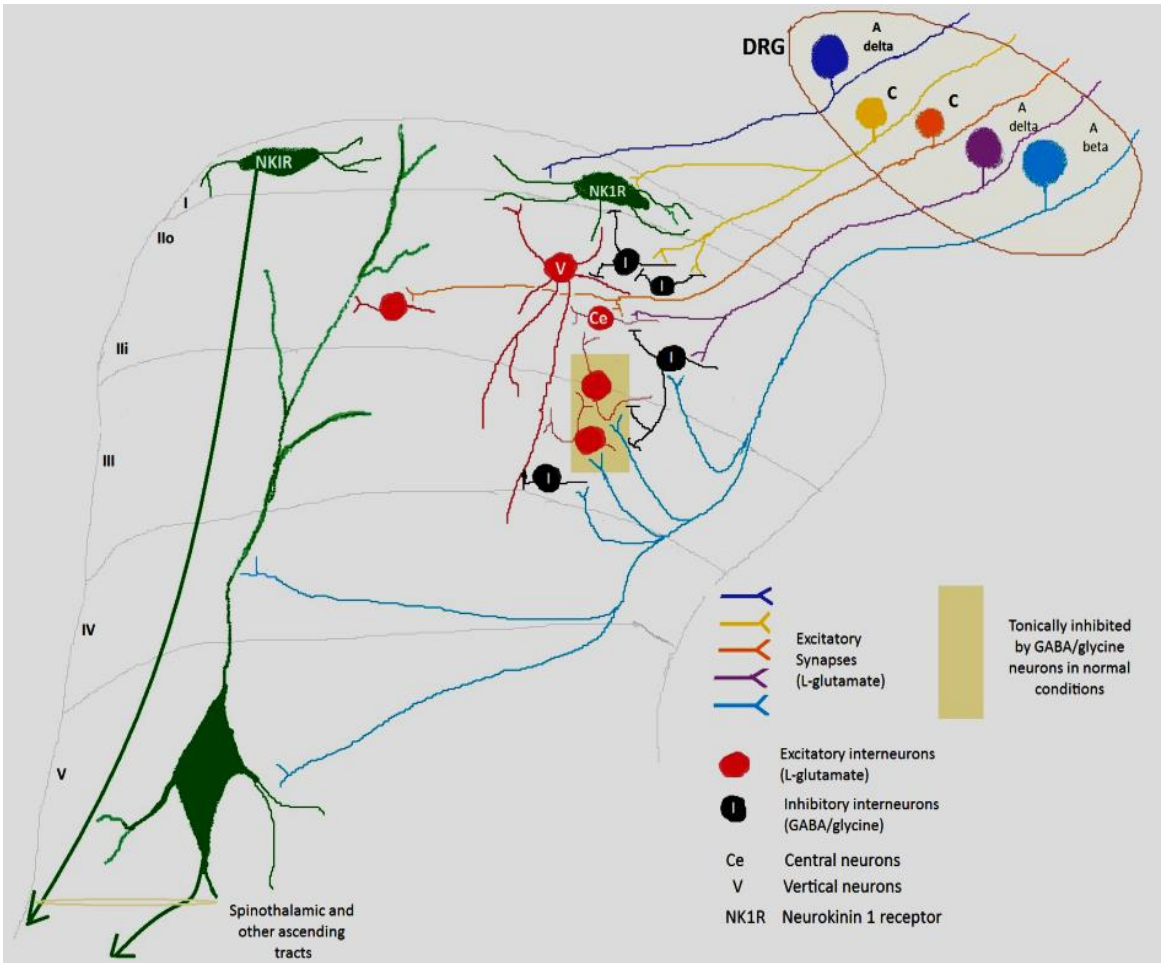

Figure 1.6: **Nociceptive fibers signaling pathway in spinal cord.** Peripheral, heat, mechanical & touch signals are carried by peptidergic/nonpeptidergic Aδ and C nociceptor primary afferent fibers to projection neurons and interneurons of spinal dorsal horn. Projection neurons can be found primarily in spinal lamina I, III, IV and V. The nociceptive projection neurons can be classified into neurokinin 1 receptor positive (laminae I) and wide dynamic range neurons (laminae III, IV and V). Excitatory and inhibitory interneurons of laminae II get activated after receiving input from nonpetidergic (mechanical stimuli)/ peptidergic (heat stimuli) fibers and spread the signals to projection neurons of central neurons (Ce) and vertical neurons (V) via polysynaptic excitatory pathway. Excitatory interneurons excite the neurons while inhibitory interneurons serve as gate transmission of nociceptive input into dorsal horn by controlling the projection neurons excitatory signals. Inhibitory neurons (GABAergic/glycinergic) also receive the signal from the low threshold mechanoreceptors Aβ fibers. Additionally, inhibitory interneurons control the input to the excitatory interneurons of spinal dorsal horn (Adapted and modified from (Benarroch 2016))

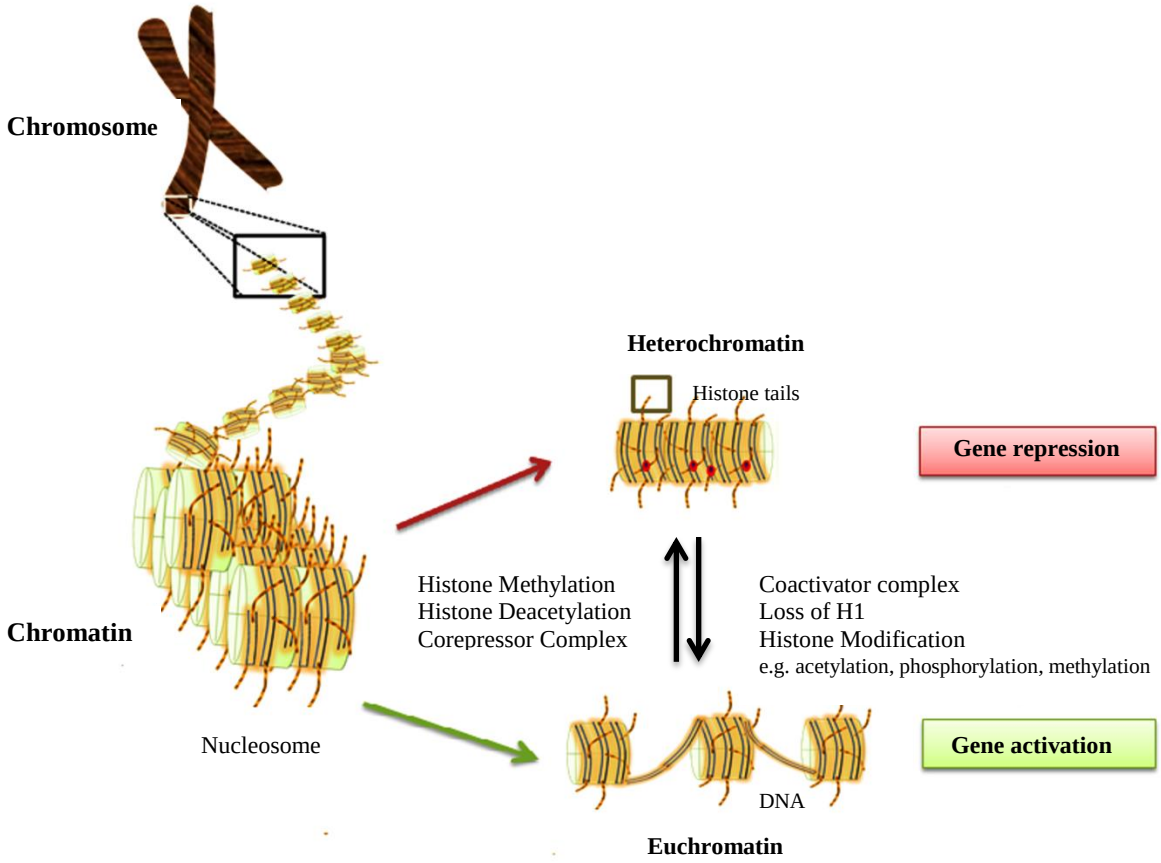

Figure 1.7: **Role of histone modification in the chromatin structure organization**. Chromosome is consisting of chromatin which is comprised of nucleosome (a fundamental packing unit of Chromatin). A single nucleosome composed of double stranded helical DNA (`146 bp) wrapped around the spool of histone proteins (an octamer). From the histone proteins, histone tails protrude. The amino acids residues presented on histone tails are subjected to various post translation modification (PMT) such as methylation, acetylation phosphorylation etc. and control the chromatin structures. Depending upon the combination of PMT and various coactivator/corepressor complexes present near the nucleosome it could cause closed (heterochromatin)/ open (euchromatin) chromatin structures and subsequently effects gene repression or gene activation, respectively.

CHAPTER 2

BLOCKING THE GABA TRANSPORTER GAT-1 AMELIORATES SPINAL GABAERGIC DISINHIBITION AND NEUROPATHIC PAIN INDUCED BY PACLITAXEL

Ruchi Yadav, Xisheng Yan, Dylan W. Maixner , Mei Gao , and Han-Rong Weng: Blocking the GABA transporter GAT-1 ameliorates spinal GABAergic disinhibition and neuropathic pain induced by paclitaxel. J Neurochem, 2015. 133(6): p. 857-69.
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Abstract

Paclitaxel is a chemotherapeutic agent widely used for treating carcinomas. Patients receiving paclitaxel often develop neuropathic pain and have a reduced quality of life which hinders the use of this life-saving drug. In this study, we determined that the role of GABA transporters in the genesis of paclitaxel-induced neuropathic pain using behavioral tests, electrophysiology, and biochemical techniques. We found that tonic GABA receptor activities in the spinal dorsal horn were reduced in rats with neuropathic pain induced by paclitaxel. In normal controls, tonic GABA receptor activities were mainly controlled by the GABA transporter GAT-1 but not GAT-3. In the spinal dorsal horn, GAT-1 was expressed at presynaptic terminals and astrocytes while GAT-3 was only expressed in astrocytes. In rats with paclitaxel-induced neuropathic pain, the protein expression of GAT-1 was increased while GAT-3 was decreased. This was concurrently associated with an increase of global GABA uptake. The paclitaxel-induced attenuation of GABAergic tonic inhibition was ameliorated by blocking GAT-1 but not GAT-3 transporters. Paclitaxel-induced neuropathic pain was significantly attenuated by the intrathecal injection of a GAT-1 inhibitor. These findings suggest targeting GAT-1 transporters for reversing disinhibition in the spinal dorsal horn could be a useful approach for treating paclitaxel-induced neuropathic pain.

Introduction

Paclitaxel (taxol) is a potent anti-tumor drug used for the treatment of carcinomas in a wide range of organs including lung, breast, ovaries, prostate and others. The clinical application of this life-saving agent is hampered by paclitaxel induced neuropathic pain (P-INP). Currently, effective treatments for P-INP are not available. It is known that pathological pain originates from aberrant neuronal activities along the pain signalling pathway, including peripheral nociceptors, neurons in the spinal dorsal horn and supraspinal pain centres. Homeostasis between excitatory and inhibitory receptor activities is crucial to maintain normal neuronal activities in the central nervous system (CNS**).** In the spinal dorsal horn, attenuation in glycinergic and/or γ-aminobutyric acid (GABA) inhibitory system contributes to the genesis of pathological pain. For example, reduction of glycinergic receptor activities in the spinal superficial dorsal horn is associated with inflammatory pain (Bonin & De Koninck 2013, Harvey et al 2004) while impairment in GABAergic inhibitory synaptic activities in the spinal dorsal horn is an important mechanism contributing to the genesis of neuropathic pain induced by nerve injury (Bonin & De Koninck 2013, Coull et al 2005, Coull et al 2003b, Moore et al 2002). Currently, whether and how the spinal inhibitory system is altered in neuropathic pain induced by paclitaxel remains not fully understood.

GABA is the major inhibitory neurotransmitter released from GABAergic interneurons in the spinal dorsal horn (Bardoni et al 2013, Bonin & De Koninck 2013). GABA exerts its inhibitory effects through acting on ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors at presynaptic terminals to reduce presynaptic glutamate release (Bardoni et al 2013). Activation of presynaptic $GABA_B$ receptors with baclofen

can ameliorate pathological pain (Fukuhara et al 2013, Gaillard et al 2014). GABA also acts on $GABA_A$ receptors at postsynaptic neurons to cause influx of Cl^- and membrane hyperpolarization in postsynaptic neurons (Bardoni et al 2013). Activation of synaptic $GABA_A$ receptors by GABA released presynaptically produces phasic inhibition while activation of extrasynaptic $GABA_A$ receptors by ambient GABA is related to tonic inhibition of neurons (Belelli et al 2009, Lee & Maguire 2014). Studies of GABAergic receptor activities in the spinal dorsal horn have mainly concentrated on understanding the fast synaptic (phasic) inhibition. Little is known about the regulation of GABAergic tonic inhibition in the spinal dorsal horn in normal and pathological pain conditions.

One important factor that regulates the clearance and maintenance of the homeostasis of extracellular inhibitory transmitters is the GABA transporter system (Zhou & Danbolt 2013). GABA transporters are located on the plasma membrane in neurons and astrocytes, which transport extracellular GABA into the cell as it is not metabolized extracellularly (Zhou & Danbolt 2013). In the CNS, there are mainly two types of GABA transporters, GABA transporter-1 (GAT-1) and GABA transporter-3 (GAT-3). Studies in the forebrain show that GABAergic tonic inhibition is controlled by GABA transporters. The regulation of GABA receptor activities by GAT-1 and GAT-3 and cellular types expressing GAT-1 and GAT-3 are region-specific (Belelli et al 2009, Kersante et al 2013, Lee & Maguire 2014, Park et al 2009). Previous studies have suggested that the protein expression of GABA transporters is altered in animals with pathological pain induced by inflammation or nerve injury (Daemen et al 2008, Ng & Ong 2001, Ng & Ong 2002). Currently, it is unclear whether changes of tonic

GABAergic inhibition and GABA transporters contribute to the genesis of paclitaxel induced neuropathic pain.

In this study, we for the first time revealed that GABAergic tonic inhibition in the spinal dorsal horn of rats with P-INP is reduced. We defined the cellular location of GAT-1 and GAT-3 and the role of these transporters in GABAergic tonic inhibition in normal and P-INP. We demonstrated that blocking GAT-1 in the spinal dorsal horn is a powerful approach to ameliorate P-INP.

Methods and materials

Animals

Adult male Sprague-Dawley rats (body weight: 170-220 g, Harlan Laboratories) were used. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia and were fully compliant with the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals.

Paclitaxel-induced neuropathic pain model in rats

P-INP was induced in rats by intraperitoneal (i.p.) injection of paclitaxel (Taxol, Bristol-Myers Squibb) at a dose of 2 mg/kg on four alternate days (days 1, 3, 5, and 7) with a cumulative dose of 8 mg/kg. Paclitaxel and vehicle were prepared and injected as previously described (Gao et al 2013).

Behavioral tests

All the behavioral testing were conducted in a quiet room with room temperature at 22 ^{o}C (Weng et al 2003). To test mechanical sensitivity, rats were placed on a wire mesh, loosely restrained under a plexiglass cage (12 x 20 x 15 cm^3) and allowed to

acclimate for at least 30 min. Hind paw withdrawal response thresholds to mechanical stimuli were defined with a set of von Frey monofilaments (bending force from 0.58 to 14.68 g), which were applied in an ascending order to evoked a 50% or greater withdrawal responses (Yan et al 2014). This value was later averaged across all animals in each group to yield the group response threshold. To determine thermal sensitivity, rats were placed on a smooth glass plate pre-heated at 30 ^{o}C. A radiant heat beam (diameter 5 mm^2) was directed onto the mid-plantar surface of the hind paw from beneath (Hargreaves et al 1988). The withdrawal latency was recorded as previously described (Maixner et al 2015a). When the behavioral tests and i.p. injection took place on the same day, the i.p. injection was made after the behavioral tests. The experimenters conducting the behavior tests were blinded to the type of treatments given to the animals.

To determine whether the tested agents cause impairment in motor functions or sedation, the rotarod test was conducted as described previously (Hara et al 2014, Stone et al 2014). Animals were placed on a rotating drum and the drum was set to rotate from 4 to 40 rpm over a period of 5 min. The period of time in seconds at which the animal fell from the drum was recorded. The mean time for each treatment group of animals was taken for statistical analysis.

Intrathecal catheter implantation

A polyethylene (PE-10) catheter that ended at the spinal L4 segment was intrathecally placed as previously described (Yaksh & Rudy 1976). Briefly, rats were anesthetized with 2-3% isoflurane. A PE-10 catheter was carefully inserted into an opening at the atlanto-occipital membrane and advanced to the lumber enlargement. Behavior tests and intrathecal drug administration were conducted 7 days after the

intrathecal implantation of the catheter. At the end of the behavior experiments, 50 μL of 2% lidocaine was injected into the catheter. If hind paw paralysis did not occur after the lidocaine injection, rats were omitted from the data analysis.

Western blotting

Animals were deeply anesthetized with urethane (1.3–1.5 g/kg, i.p.). The L4-L5 spinal segment was exposed and removed from the rats. The dorsal half of the spinal segment was isolated and quickly frozen in liquid nitrogen and stored at -80 °C for later use. Frozen tissues were homogenized and protein was isolated and quantitated as previously described (Gao et al 2013, Maixner et al 2015a). Protein samples (40 μg) were electrophoresed in 10 % SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% milk or 5% BSA, and then incubated respectively overnight at 4 °C with polyclonal rabbit anti- GAT-1 (1:1000, Abcam) polyclonal rabbit anti-GAT-3 (1:1000, Abcam) primary antibodies, or a monoclonal mouse anti-β-actin (1:2000, Sigma-Aldrich, St. Louis, USA) primary antibody as a loading control. Then the blots were incubated for 1 hr at room temperature with corresponding HRP-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, CA, USA), visualized in ECL solution (Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) for 1 min, and exposed onto FluorChem HD2 System. The intensity of immunoreactive bands was quantified as previously described (Gao et al 2013). Levels of each biomarker were expressed as the ratio to the loading control protein (β-actin).

GABA uptake assay

GABA uptake activity in the lumbar spinal dorsal horn of rats was measured using

synaptosome preparations according to previously established protocols with modifications (Hu et al 2003, Ozkan et al 1997, Wonnemann et al 2000, Yan et al 2014). The L4-L5 spinal segment was removed from the rat anesthetized with urethane (1.3–1.5 g/kg, i.p) and the dorsal half of the spinal cord was isolated. Synaptosome preparations were prepared immediately in Syn Per TM synaptic protein extraction reagent (Thermo scientific) and quantified by the BCA assay (Thermo scientific). Briefly the homogenates were centrifuged at 10,500 x g for 10 min at 4 °C, and the supernatant was collected. The remaining pellets were re-suspended in the same solution and re-centrifuged at 9,200 x g for 10 min at 4°C. The two supernatant were combined and centrifuged again at 9,200 x g for 10 min at 4°C to obtain the synaptosomal pellets, which contained both neuronal and glial GABA transporters. The synaptosome protein (50 μg) was incubated in Locke's buffer solution containing: 0.5 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.32 M sucrose, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 4 μg/ml leupeptin, and 0.01 M phosphate-buffered saline. GABA uptake activity was determined by incubating the synaptosome preparation with a solution containing 0.4 μCi of - Aminobutyric Acid (GABA), γ-[2,3-3H(N)] (Perkin Elmer Life Sciences, Boston, MA) at room temperature for 10 min. The reaction was terminated by filtering the synaptosomes through a Whatman GF/B filter presoaked with the same buffer solution. The filter was washed two times with ice-cold Locke's buffer (2ml) and was then transferred to a vial containing a scintillation cocktail. The radioactivity of the final samples was measured by a liquid scintillation counter (Beckman, LS6500).

Immunohistochemical Analysis

Male Sprague-Dawley rats were deeply anesthetized with urethane (1.3-1.5 g/kg,i.p.) and the L4 and L5 spinal cord was removed, fixed, and cyrosectioned as previously described (Weng et al 2014a) . Sections were incubated overnight at 4 °C in 2% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS containing primary antibodies against the following targets: rabbit anti- GAT-1 (1:200, Abcam), rabbit anti-GAT-3 (1:200, Abcam), mouse anti-GFAP (a marker for astrocytes, 1:500, Cell Signaling), rat anti-OX-42 (a marker for microglia, 1:500, AbD Serotec), and mouse anti MAP2 (a marker for neuronal cytoskeleton 1:500, Cell Signaling) antibodies. After washing three times with 0.1 M PBS, the sections were incubated for 2 hr at room temperature with the corresponding Texas Red antibody (1:500 Vector Laboratories), Alexa Fluor 488 antibody (1:500 Life Technologies), or the Mouse Anti-NeuN Alexa Fluor 488 conjugated antibody (the neuronal cell body marker, 1:200, Millipore). After rinsing three times with 0.1M PBS, the sections were mounted onto gelatin-coated slides, air-dried, and cover-slipped with Vectashield mounting medium (Vector Laboratories). Non-adjacent sections were selected randomly, and the immunostaining for each antibody were viewed under an Olympus BX43 microscope with an Olympus U-CMAD3 camera. Images were processed using Olympus-cellSens Dimensions.

Recording of GABAergic currents

Spinal slices were obtained from rats as previously described (Weng et al 2007). Briefly, rats were deeply anesthetized under isoflurane, and a laminectomy was then performed to remove the lumbar spinal cord. The L4 to L5 spinal segment was placed in ice-cold sucrose-based artificial cerebrospinal fluid (aCSF) pre-saturated with 95% O_2

and 5% CO_2. The sucrose-based aCSF contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 12.0 mM glucose, and 25.0 mM $NaHCO_3$. Transverse spinal cord slices (400 μm thick) were cut in the ice-cold sucrose aCSF and then pre-incubated for at least 2 hours in Krebs solution oxygenated with 95% O_2 and 5% CO_2 at 35 °C before they were transferred to the recording chamber. The Krebs solution contained 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 11.0 mM glucose, and 25.0 mM $NaHCO_3$. Following pre-incubation, a rat spinal slice was placed in the recording chamber (1.5 ml in volume), perfused with Krebs solution at 35 °C, and saturated with 95% O_2 and 5% CO_2. Borosilicate glass recording electrodes (resistance, 3–5 MΩ) were made and filled with an internal solution containing 110 mM Cs_2SO_4, 5 mM KCl, 2.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5mM Na-GTP, and 10 mM lidocaine *N*-ethyl bromide (QX314), adjusted to pH 7.2–7.4 with 1 M CsOH (290–300 mOsm) (Jiang et al 2012). The recording electrodes were directed to the out layer of spinal dorsal horn lamina II. Whole-cell configurations were established by applying moderate negative pressure after electrode contact (Weng et al 2007). A seal resistance of ≥ 2 GΩ and an access resistance of about 20 MΩ were considered acceptable. GABAergic currents were recorded in the presence of 0.5 μM strychnine (a glycine receptor inhibitor), 10 μM DNQX (an AMPA/kainate receptor inhibitor) and 25 μM D-AP5 (NMDA receptor inhibitor) at a holding potential of 0 mV (Moore et al 2002). The depth of the recorded cell in the slice (about 50 μm below the surface) was kept constant across all experiments.

Materials

NO-711 and SNAP5114 were obtained from Sigma-Aldrich (St. Louis, MO).

Data analysis

All data are presented as the mean ± SEM. One way or two way ANOVA with repeated measures over time or different treatments was used to detect differences on paw-withdrawal response thresholds and latencies, followed by the Bonferroni post hoc test to determine sources of differences. Student's t-tests were used to make comparison between groups (non-paired t) and within the same group (paired t). A *P* value less than 0.05 was considered statistically significant.

Results

In this study, rats used for the mass spectrometry, electrophysiology, and Western blot experiments were assigned into two groups, paclitaxel treated group and vehicle treated group. Paclitaxel (2 mg/kg. i.p.) or vehicle was respectively injected into animals in the paclitaxel group or vehicle group on days 1, 3, 5, and 7. Hind paw withdrawal response thresholds to mechanical and thermal stimuli were measured on days 1, 5 and 10. Consistent with previous findings by us (Gao et al 2013) and others (Okubo et al 2011, Polomano et al 2001), rats receiving paclitaxel in this scheme developed mechanical allodynia and thermal hyperalgesia. These were evident by a significant reduction (51 vehicle-treated rats versus 48 paclitaxel-treated rats) of mechanical thresholds ($F_{(2, 46)} = 196$, $P < 0.001$) and latencies ($F_{(2, 46)} = 98.65$, $P < 0.001$) of hind paw withdrawal responses on day 5 and day 10 after the first injection (Fig.1.1). Electrophysiology and Western blot experiments were performed on rats 10 days after the first i.p. injection, when all rats treated with paclitaxel developed mechanical allodynia and thermal hyperalgesia.

GABAergic tonic inhibition in the spinal dorsal horn is weakened in paclitaxel-treated rats

To determine whether altered GABAergic receptor activities in the spinal cord contribute to the genesis of P-INP, we compared GABAergic tonic inhibition in spinal dorsal horn neurons in rats treated with paclitaxel and rats treated with vehicle. Following previously published protocols (Bai et al 2001, Clarkson et al 2010, Jia et al 2005), we analyzed GABAergic tonic inhibition by bath-perfusing the $GABA_A$ receptor inhibitor bicuculline (25 μM) and measuring changes of holding currents when GABAergic currents in the spinal dorsal horn neurons were recorded. We found that perfusion of bicuculline into the recording chamber blocked spontaneous GABAergic IPSCs and revealed a shift in the holding currents in slices taken from vehicle control rats (tested in 11 neurons) (Fig. 1.2A) and paclitaxel-induced neuropathic rats (tested in 11 neurons) (Fig. 1.2B). The changes of holding currents induced by bicuculline in rats with P-INP (23.93 ± 2.76 pA) were significantly less (Fig. 2C, $P < 0.001$) than those in vehicle control rats (63.93 ± 3.53 pA) (Fig. 1.2C). These data indicated that GABAergic tonic inhibition is reduced in the spinal dorsal horn of rats with P-INP. Thus, enhancing GABAergic tonic inhibition may be an effective approach to alleviate P-INP.

Tonic GABA receptor activities in the spinal dorsal horn are mainly regulated by GAT-1 under normal conditions

Studies in the forebrain have shown that tonic GABAergic inhibition is regulated both by GABA synthesis (Fatemi et al 2005, Nishikawa et al 2011) and by GABA transporters (Kirmse et al 2008, Nishikawa et al 2011, Semyanov et al 2004, Smith et al 2007, Wu et al 2006a). To determine the role of GABA transporters in the regulation of

GABAergic receptor activities in the spinal dorsal horn, we first determined the cellular location of the GABA transporters GAT-1 and GAT-3 in the spinal dorsal horn of normal control animals using immunohistochemistry technique. As shown in Figure 3A and B, GAT-1 and GAT-3 were widely expressed in the entire spinal dorsal horn with more dense expression in the superficial dorsal horn (laminae I and II). Furthermore, expression of GAT-1 was co-localized with a marker for neuronal cytoskeleton (MAP2) and the astrocyte marker (GFAP), but not with the neuronal cell body marker (NeuN) or microglia marker (OX42) (Fig. 2.3C). In contrast, GAT-3 was predominantly co-localized with GFAP, but not OX42, MAP2, or NeuN (Fig. 2.3D). These data indicate that: a) GAT-1 is expressed in neuronal presynaptic terminals and astrocytes; b) GAT-3 is only present in astrocytes; c) neither GAT-1 nor GAT-3 is expressed in microglia.

We next determined whether GABAergic tonic inhibition in the spinal dorsal horn is regulated by GAT-1 and GAT-3 in normal controls. NO-711 and SNAP5114 are widely used to selectively block GAT-1 and GAT-3 respectively (Keros & Hablitz 2005, Nusser & Mody 2002, Rossi et al 2003). We recorded GABAergic tonic currents before and during bath-perfusion of the GAT-1 inhibitor (NO-711, 10 μM). As shown in Figure 2.4A and C, the GAT-1 inhibitor significantly ($P < 0.001$) increased the holding currents (which reflects tonically active GABA conductance) by -37.05 ± 2.36 pA (n = 8). Under the condition when GAT-1 was inhibited by NO-711 (10 μM), the addition of the GAT-3 inhibitor SNAP5114 (100 μM) resulted in a further increase of holding currents (n = 8, $P < 0.001$) (Fig. 2.4 A and C). Interestingly, when GAT-3 was blocked alone by bath-perfusion containing SNAP5114 (100 μM), we did not observe a significant change in

the holding currents (n = 9, P = 0.58) (Fig. 2.4B). These data indicated that GAT-1 is critical in maintaining tonic GABA inhibition in the spinal dorsal horn.

Expression of GAT-1 is increased while expression of GAT-3 is reduced in the spinal dorsal horn in rats with P-INP, which is concurrently associated with increased GABA uptake activities

We then determined whether altered functions of GAT-1 and GAT-3 are implicated in the genesis of P-INP. We examined protein expressions of GAT-1 and GAT-3 in the dorsal half of the L4-5 spinal segment in the paclitaxel group or vehicle group. As shown in Figure 2.5A, in comparison with rats treated with vehicles (n = 10), the expression of GAT-1 in the spinal dorsal horn was significantly increased (n = 11, P < 0.001) while the expression of GAT-3 in the spinal dorsal horn was significantly reduced in rats with P-INP. We next determined the global GABA uptake activities carried by both GAT-1 and GAT-3 in the spinal dorsal horn of rats with P-INP and rats receiving vehicle treatment using synaptosome preparations (Mitrovic et al 1999, Sung et al 2003). We found that GABA uptake activities were significantly increased in rats with P-INP (n = 4, P < 0.001) in comparison with rats (n = 3) in the vehicle group (Fig. 2.5B). These data indicate that in the spinal dorsal horn of rats with P-INP GABA uptake activities are increased, which is ascribed to the increased GAT-1 function.

The paclitaxel-induced GABAergic disinhibition is ameliorated by blocking GAT-1 but not GAT-3

We then investigated whether the paclitaxel-induced suppression of GABAergic tonic inhibition in the spinal dorsal horn can be ameliorated by blocking GAT-1 and GAT-3. As shown in Figure 6A and C, the GAT-1 inhibitor (NO-711, 10 μM)

significantly increased the holding currents. The degree of increases induced by the GAT-1 inhibitor on the holding currents in the paclitaxel-treated rats (-66.27 ± 3.80 pA, n = 7) (Fig. 2.6C) was significantly ($P < 0.001$) higher than those in vehicle treated rats (-37.05 ± 2.36 pA, n = 8) (Fig. 4C). Furthermore, we did not observe significant ($P < 0.05$) alterations in the holding current in paclitaxel treated rats when the GAT-3 inhibitor (SNAP5114, 100 μM) was perfused into the bath in the presence of the GAT-1 inhibitor (NO-711, 10 μM) (Fig. 2.6 A and C). The holding current was not significantly altered by the GAT-3 inhibitor alone (Fig. 1.6B) (n = 9, $P = 0.39$). These data indicate that inhibition of GAT-1 is a potent approach to restore the paclitaxel-induced suppression of GABA receptor tonic activities in the spinal dorsal horn.

Mechanical allodynia and thermal hyperalgesia in rats with P-INP are attenuated by intrathecal injection of GAT-1 inhibitors

Finally, we determined whether inhibition of GAT-1 can ameliorate mechanical allodynia and thermal hyperalgesia induced by paclitaxel. Rats pre-implanted with intrathecal catheters were assigned into 4 groups: paclitaxel+saline group, paclitaxel+GAT-1 inhibitor group, vehicle+saline group, and vehicle+GAT-1 inhibitor group. After taking baseline withdrawal response thresholds to radiant heat and mechanical stimuli, rats received either 4 injection of paclitaxel (2 mg/kg, i.p) or vehicle on alternative days. Ten days following the first injection, mechanical allodynia and thermal hyeralgesia were confirmed by behavioral tests using von Frey monofilament (Figure 2.7A) and radiant heat stimuli (Figure 2.7B). Meanwhile mechanical and thermal thresholds in the vehicle+saline group and naïve+GAT-1 inhibitor group remained stable. We then topically applied the GAT-1 inhibitor NO-711 (10 μg in a volume of 10 μl) onto

the spinal lumbar enlargement in the paclitaxel+GAT-1 inhibitor group and naïve+GAT-1 inhibitor group through the pre-implanted intrathecal (i.t.) catheter. Rats in the paclitaxel+saline group and vehicle+saline group received 10 μl of saline via intrathecal injection. Changes in thermal and mechanical sensitivities were determined at 0.5, 1, 3, and 24 hours after the i.t. injection. The paclitaxel-induced mechanical allodynia and thermal hyperalgesia were significantly attenuated by a single intrathecal injection of the GAT-1 inhibitor NO-711(10 μg). As shown in Figure 2.7A, NO-711 significantly increased the mechanical thresholds in the paclitaxel+GAT-1 group at 30 min (10.00 ± 0.01 g) and 60 min (8.00 ± 0.89 g) in comparison with their own values (4.00 ± 0.89 g) before the NO-711 injection ($F_{(4,20)} = 30.18$, $P < 0.001$), or in comparison with their counterparts in the paclitaxel+saline group at 30 min (5.2 ± 0.87 g) and 60 min (3.6 ± 0.97 g) (main effects of drug: $F_{(1,45)} = 12.39$, $P < 0.01$; main effect of time: $F_{(4,45)} = 8.90$, $P < 0.001$; interaction: $F_{(4,45)} = 4.30$, $P < 0.005$; 5 to 6 rats/group). Similarly, latencies of withdrawal responses to radiant heat stimuli in the paclitaxel+GAT-1 group were also significantly increased at 30 min (13.50 ± 1.07 s) and 60 min (11.51 ± 0.87 s) after the injection of NO-711(10 μg) in comparison with the values (7.45 ± 0.50 s) prior to the NO-711 injection ($F_{(4,20)} = 14.33$, $P < 0.001$), and their counterparts in the paclitaxel+saline group at 30 min (8.13 ± 0.38 s) and 60 min (7.97 ± 0.77 s, Figure 2.8B) (main effects of drug: $F_{(1,45)} = 19.97$, $P < 0.001$; main effect of time: $F_{(4,45)} = 5.52$, $P < 0.01$; interaction: $F_{(4,45)} = 6.46$, $P < 0.003$; 5 to 6 rats/group).

The effects of GAT-1 inhibitors on mechanical and thermal thresholds disappeared by 3 hours after drug administration. These results indicated that the blockade of GAT-1 activities is a powerful approach to reverse P-INP. In contrast,

administration of saline in the vehicle+saline group or the GAT-1 inhibitor (10 μg in a volume of 10 μl) in the naive+GAT-1 inhibitor group did not significantly alter mechanical or thermal thresholds in these two groups (Figure 7A and B). To further determine whether the GAT-1 inhibitor at the tested dose (10 μg) cause impairment on motor functions and/or sedation, the rotarod test was performed. Retention time on an accelerating rotarod is a widely used index to monitor motor functions and/or sedation in animals, since sedation also causes the animals to fall (Hara et al 2014, Stone et al 2014). We found that in comparison with naïve rats (n = 8) receiving intrathecal saline injection (10 μl), intrathecal administration of the GAT-1 inhibitor NO-711 (10 μg in a volume of 10 μl) to naïve rats (n = 8) did not significantly (P = 0.22 to 0.77) alter the retention time on the rotarod over the observation period (0.5 to 24 hours after the intrathecal injection) (data not shown). These results indicate that NO-711 at a dose of 10 μg does not cause motor impairment or sedation.

Discussion

Mechanisms underlying paclitaxel-induced neuropathic pain

Pathological changes at both peripheral nerves and the spinal dorsal horn have been reported in animals with P-INP. For example, P-INP in rats is associated with the degeneration of intraepidermal terminal arbors of sensory neurons and activation of Langerhans cells in the skin (Boyette-Davis et al 2011, Siau et al 2006). Furthermore, the incidence of swollen and vacuolated axonal mitochondria is increased in myelinated and unmyelinated sensory axons (Xiao & Bennett 2012, Zheng et al 2011a). Agents that enhance mitochondrial function have been shown to be effective in the prevention or

attenuation of P-INP (Jin et al 2008). The protein and mRNA expressions of TRPV1 in dorsal root ganglion sensory neurons are increased in rats receiving repeated paclitaxel treatments (Hara et al 2013). More recently, it was reported that chronic paclitaxel significantly increases the protein expression of the chemokine CX3CL1 in A-fiber primary sensory neurons and infiltration of macrophages into the dorsal root ganglion (DRG) in rats, which contributes to the paclitaxel-induced DRG neuronal apoptosis of A fibers and pathological pain (Huang et al 2014). At the spinal cord level, we have previously shown that wide-dynamic range neurons in the spinal dorsal horn in rats with paclitaxel-induced neuropathic pain display a significant increase in both spontaneous activities (action potentials) and responses to noxious mechanical, heating, and cooling stimuli, as well as an abnormal wind-up to transcutaneous electrical stimuli (Cata et al 2006a). Loss of homeostasis between excitatory and inhibitory inputs to neurons is a major factor leading to exaggerated neuronal activation. In this regard, the down-regulation of glial glutamate transporters in the spinal dorsal horn has been demonstrated to be causal in the genesis of P-INP (Doyle et al 2012, Gao et al 2013, Weng et al 2005). Glial glutamate transporters control neuronal activation by promptly clearing the excitatory neuronal transmitter glutamate released from presynaptic terminals in the spinal dorsal horn (Nie & Weng 2009b, Nie & Weng 2010b, Weng et al 2007). Improving glial glutamate transporter functions by reducing levels of peroxynitrite (Doyle et al 2012) or suppression of GSK3β activities (Gao et al 2013) in the spinal dorsal horn effectively prevents and attenuates P-INP. It was recently reported that up-regulation of NKCC1 in the spinal dorsal horn causes a depolarizing shift and reduces GABA induced membrane hyperpolarization in GABAergic neurons following P-INP

(Chen et al 2014b). Whether the tonic GABAergic inhibition is altered in P-INP has not been explored. Our current study provided direct evidence that GABAergic tonic disinhibition in the spinal dorsal horn is implicated in the genesis of P-INP.

Regulation of GABAergic receptor activities in the spinal dorsal horn in pathological pain conditions

Disinhibition in the spinal dorsal horn is a critical factor leading to excessive neuronal activation in animals with neuropathic pain induced by mechanical injury. Extracellular GABA level in the lumbar dorsal horn is reduced by nerve injury (Castro-Lopes et al 1993, Stiller et al 1996). In neuropathic rats, amplitudes and frequencies of $GABA_A$ receptor–mediated IPSCs in neurons in the superficial dorsal horn are attenuated (Moore et al 2002). Additionally, the spinal application of GABA agonists attenuate mechanical allodynia and thermal hyperalgesia induced by nerve injury (Malan et al 2002). It is known that GABAergic receptor activities are governed by at least the following factors: the amount of GABA released from presynaptic terminals, the function of GABA transporters, and the function of GABA receptors at the postsynaptic neuron (Zhou & Danbolt 2013). GABA produces phasic inhibition by acting on $GABA_A$ receptors inside the synapse, and tonic inhibition by acting on extrasynaptic $GABA_A$ receptors (Belelli et al 2009). Studies of GABA receptor activities in the spinal dorsal horn have been mainly focused on the phasic GABA inhibition. For example, synaptic GABAergic activities (phasic inhibition) are reduced when the release probability of GABA neurotransmitters is decreased by activation of presynaptic A1 adenosine-receptors or $GABA_B$ receptors (Hugel & Schlichter 2003, Yang et al 2004a). Activation of neuronal acetylcholine receptors (Takeda et al 2003) or M3 muscarinic acetylcholine

receptors (Zhang et al 2006) at presynaptic terminals increases the GABA release from the presynaptic terminals and GABAergic phasic inhibition. Reduction of GABA synthesis through the glutamate-glutamine cycle at the presynaptic terminals also decreases GABAergic phasic inhibition (Jiang et al 2012). At the post-synaptic site, phasic inhibition induced by the activation of GABA receptors are reduced by the down-regulation of the K^+-Cl^-cotransporter KCC2, which disrupts Cl^- homeostasis in neurons (Coull et al 2005, Coull et al 2003b).

Previous studies show that GABAergic tonic inhibition is present in the spinal dorsal horn (Ataka & Gu 2006, Maeda et al 2010, Takahashi et al 2006). It is generally believed that extrasynaptic $GABA_A$ receptors (which contain the δ subunit) are responsible for the generation of GABAergic tonic inhibition. It was demonstrated that selective activation of the extrasynaptic $GABA_A$ receptors with the δ$GABA_A$ receptor-preferring agonist 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridine-3-ol (THIP) increases the tonic GABA inhibition, suppresses neuronal excitability in the spinal dorsal horn, and acute nociception in mice (Bonin et al 2011). Currently, whether GABAergic tonic inhibition in the spinal dorsal horn is regulated by GABA transporters remains unexplored. Our study filled this gap by demonstrating that tonic GABA receptor activities in the spinal dorsal horn are regulated by GABA transporters both in normal and neuropathic pain induced by paclitaxel treatment.

Role of GABA transporters in the regulation of GABA receptor activities in the CNS

GABA transporters play an important role in the clearance and homeostasis of extracellular GABA in the CNS because no enzymes are available in the extracellular space to convert GABA into a biologically inert molecule (Zhou & Danbolt 2013).

Among the four GABA transporters, GAT-1 and GAT-3 are widely expressed in the CNS (Guastella et al 1990, Melone et al 2013). Expression of GAT-1 and GAT-3 transporters is region-specific. In the forebrain area, GAT-1 is abundantly expressed in terminals of cortical neurons but not in thalamic, Purkinje and striatonigral synapses where GAT-3 is expressed mainly in astrocytes (Zhou & Danbolt 2013). Studies on the cellular location of GAT-1 and GAT-3 in the spinal dorsal horn are limited. In one study on the rat spinal trigeminal nucleus, GAT-1 and GAT-3 are expressed in astrocytes (Ng & Ong 2001). Expressions of GAT-1 in presynaptic terminals and GAT-3 in astrocytes in the mouse spinal dorsal horn were recently reported (Kim et al 2014). Our current study on rats further confirmed that: a) GAT-1 is positioned at the presynaptic terminals but not at the neuronal cell body; b) GAT-1 is also expressed in astrocytes; c) GAT-3 is only expressed in astrocytes; d) neither GAT-1 nor GAT-3 is expressed in microglia.

Regulation of GABAergic tonic inhibition by GAT-1 and GAT-3 varies depending on different regions. For example, pharmacological inhibition of GAT-1 increases GABAergic tonic currents in the hippocampus (Nusser & Mody 2002) and cerebellum (Rossi et al 2003) but not in the sensorimotor cortex (Keros & Hablitz 2005). When GAT-3 is inhibited, GABAergic tonic currents are increased in the supraoptic nucleus (Park et al 2006) but not in the sensorimotor cortex (Keros & Hablitz 2005). A combined inhibition of GAT-1 and GAT-3 is required to significantly enhance tonic inhibition in the sensorimotor cortex (Keros & Hablitz 2005). The regulation of tonic inhibition in the spinal dorsal horn is unknown. Our study showed that under normal conditions, GABAergic tonic inhibition in the spinal dorsal horn was increased when GAT-1 but not GAT-3 was inhibited. Only under the condition when GAT-1 had been

inhibited, further inhibition of GAT-3 increased GABAergic tonic inhibition. These data suggest that in comparison with GAT-3, GAT-1 is positioned in proximity to the presynaptic terminals, and the GABA uptake by GAT-1 is more powerful, which may result from the higher efficacy or number of GAT-1 transporters. Consistent with this notion, GAT-1 was found to be expressed in presynaptic terminals by us (Figure 2.3) and others (Kim et al 2014), and the global GABA uptake is increased in rats with the up-regulation of GAT-1 protein expression and down-regulation of GAT-3 expression (Figure 2.5).

Protein expressions of GAT-1 and GAT-3 in the spinal dorsal horn are altered under different pathological pain conditions but controversy remains. Animals with neuropathic pain induced by chronic constriction of the sciatic nerve or spared nerve injury have an increased protein expression of GAT-1 in the spinal dorsal horn (Daemen et al 2008) or the gracile nucleus (Gosselin et al 2010). Using the same animal model, others reported that the protein expression of GAT-1 in the same area is reduced (Miletic et al 2003, Shih et al 2008). Expressions of GAT-1 and GAT-3 transporters are increased in the spinal trigeminal nucleus in rats with inflammatory pain induced by carrageenan injection (Ng & Ong 2001). In another inflammatory pain model induced by formalin injection, global GABA uptake in the mouse spinal cord is increased at 20 min and 120 min after formalin injection (Hu et al 2003). Our present study demonstrated that GAT-1 protein expression is increased while GAT-3 protein expression is reduced in the spinal dorsal horn in rats with P-INP, and these changes are concurrently associated with an increase of global GABA uptake at the same region. These data suggest that enhanced GABA uptake may in part contribute to the reduced GABAergic tonic inhibition in rats

treated with paclitaxel. Targeting GABA transporters to produce analgesic effects has been reported. It was reported that latencies in the tail flick reflex (Hu et al 2003) or the hot plate test (Kubo et al 2009) were prolonged in mice receiving intraperitoneal injection of NO-711 (3 to 10 mg/kg). However, others showed that intrathecal application of GAT-1 inhibitor NO-711 (up to 100 μg) did not produce analgesic effects in the sham-operated control rats (Li et al 2011a). In agreement with this study, our findings show that intrathecal application of NO-711 (10 μg) did not alter mechanical and thermal thresholds of paw withdrawal responses, or motor functions in rats. Systemic or intrathecal administration of GAT-1 inhibitors ameliorates neuropathic pain induced by chronic constriction of the sciatic nerve (Daemen et al 2008, Li et al 2011a), and the second phase nociceptive behaviors in the formalin pain model (Hu et al 2003). Similarly, spinal inhibition of GAT-3 suppresses the second-phase response in the formalin pain model and attenuates mechanical allodynia induced by chronic constriction injury in rats (Kataoka et al 2013). Currently, the regulation of GABAergic tonic inhibition by GABA transporters in pathological pain conditions has not been investigated. In this study, we found that the attenuation of GABAergic tonic inhibition in the spinal dorsal horn of rats with P-INP is significantly reversed by the GAT-1 inhibitor but not GAT-3 inhibitor. In intact animals, we demonstrated that inhibition of spinal GAT-1 ameliorates mechanical allodynia and thermal hyperalgesia induced by paclitaxel treatment.

Conclusions and Future Directions

In this study, we found that disinhibition in the spinal dorsal horn contributes to the genesis of P-INP. This is at least in part due to enhanced GABA uptake by GAT-1 in

the spinal dorsal horn. Suppression of GAT-1 activities reverses disinhibition in the spinal dorsal horn and attenuates mechanical allodynia and thermal hyperalgesia induced by paclitaxel treatment. Together, these results suggest targeting GAT-1 activity may be useful for the development of therapeutics in P-INP.

Through P-INP animal models we have shown that single local injection of GAT-1 inhibitor attenuates mechanical allodynia and thermal hyperalgesia for few hours. However it would be worth to investigate multiple dosage effects of GAT-1 inhibitor on P-INP model. It would be interesting to learn about any preventive effects of GAT-1 inhibitor on development and maintenance of paclitaxel induced neuropathic pain in animal models.

Acknowledgements

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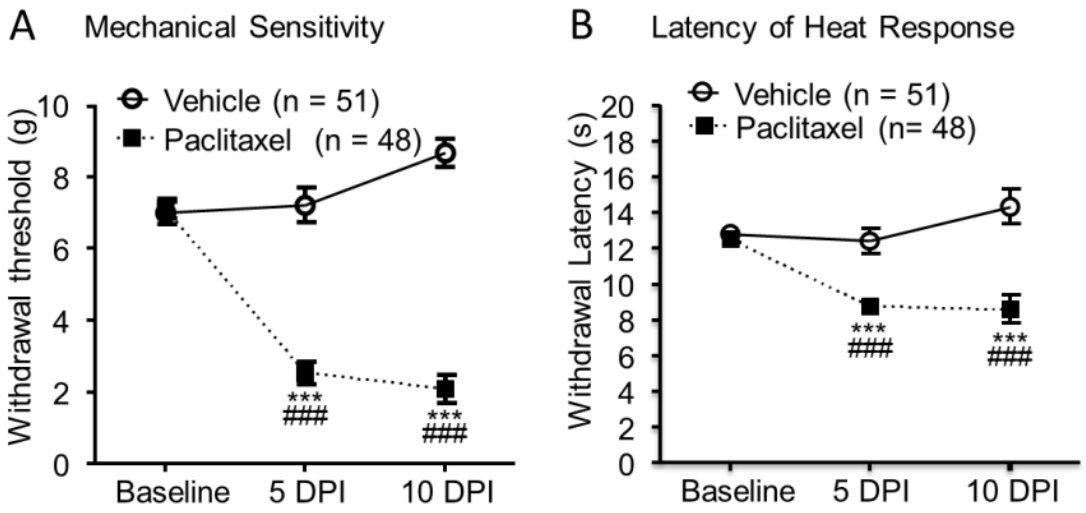

Figure 2.1: **Rats treated with paclitaxel develop mechanical allodynia and thermal hyperalgesia.** Rats were injected (i.p.) with either vehicle or paclitaxel (2.0 mg/kg) on four alternate days (days 1, 3, 5 and 7). The mean (± SEM) mechanical thresholds of hind paw withdrawal responses *(A)* and latencies of the withdrawal response to heat stimuli *(B)* prior to the i.p. injection (Baseline), and on 5 days (5 DPI) and 10 days (10 DPI) post the first injection were plotted. Comparisons of data between baseline and each time point in the paclitaxel group are labeled with *. Comparisons of data between the vehicle group and the paclitaxel group at each time point are indicated with #. Three symbols, $P < 0.001$.

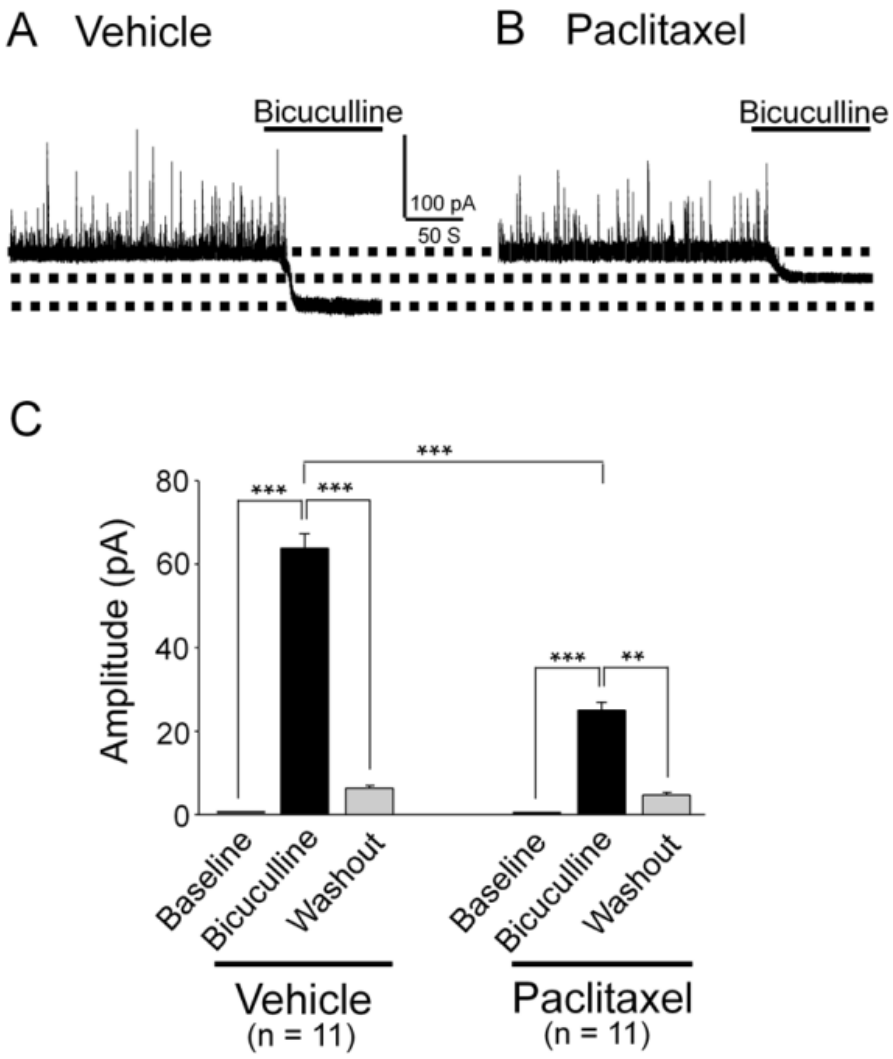

Figure 2.2: **GABAergic tonic inhibition in the spinal dorsal horn is weakened in the paclitaxel-treated rats**. Raw data show recordings of GABAergic currents before and after blocking of $GABA_A$ receptors with bicuculline (25 μM) obtained from vehicle *(A)* and paclitaxel *(B)* treated rats. *(C)* Shows the mean (± SEM) changes of holding currents induced by bicuculline in vehicle and paclitaxel treated rats. ** $P < 0.01$; *** $P < 0.001$.

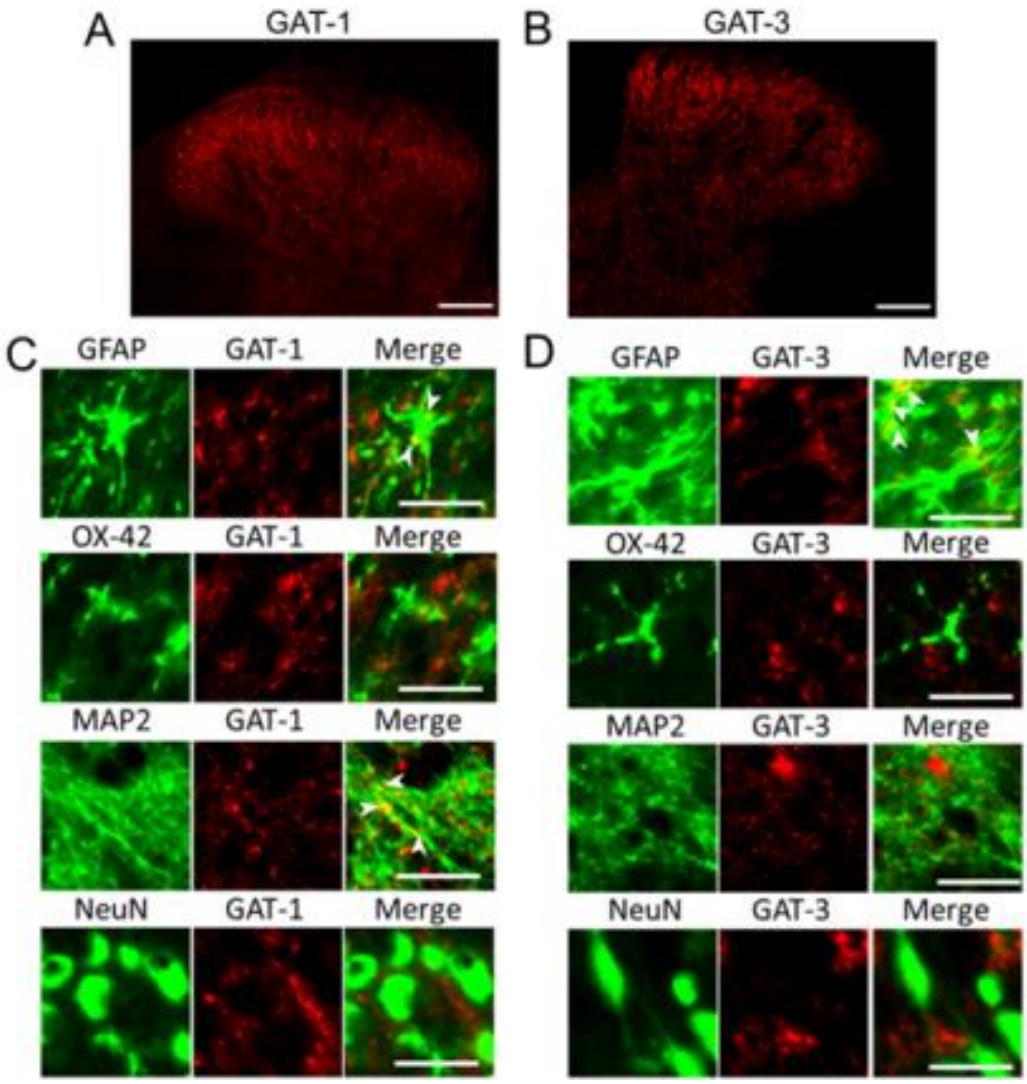

Figure 2.3: **GAT-1 is expressed in neurons and astrocytes and GAT-3 only is present in astrocytes.** Samples were obtained from the spinal dorsal horn of normal control rats. *(A)* and *(B)* respectively show staining of GAT-1 and GAT-3 in the spinal dorsal horn, note higher expressions of GAT-1 and GAT-3 at the superficial dorsal horn (Scale bar = 100 μM). *(C)* Shows that expression of GAT-1 is colocalized with MAP2 (a marker for neuronal cytoskeleton) and GFAP (an astrocyte marker), but not with NeuN (a neuronal cell body marker) or OX42 (a microglia marker) (Scale bar = 20 μM). *(D)* Shows that GAT-3 is predominantly colocalized with GFAP, but not OX42, MAP2, or NeuN (Scale bar = 20 μM).

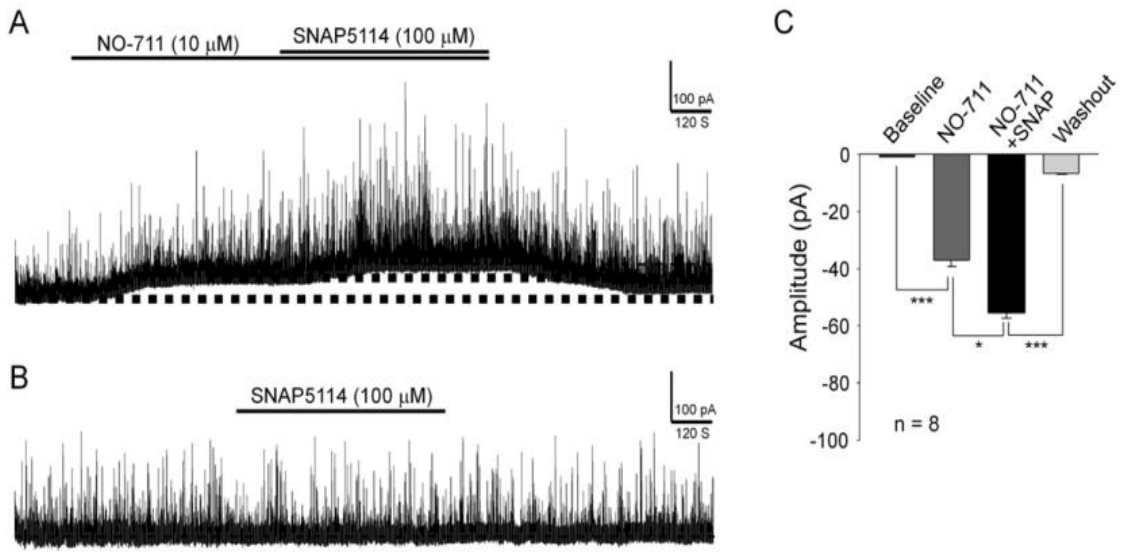

Figure 2.4: **Tonic GABA receptor activities in the spinal dorsal horn are mainly regulated by GAT-1 under normal conditions**. Data were obtained from normal control rats. *(A)* Shows recordings of GABAergic currents before and after blocking of GAT-1 with NO-711 (10 μM) and then further inhibition of GAT-3 with SNAP5114 (100 μM). *(B)* Shows recordings of GABAergic currents before and after blocking of GAT-3 alone with SNAP5114 (100 μM). *(C)* Shows the mean (± SEM) changes of holding currents induced by NO-711 and then NO-711 plus SNAP5114. ** $P < 0.01$; *** $P < 0.001$.

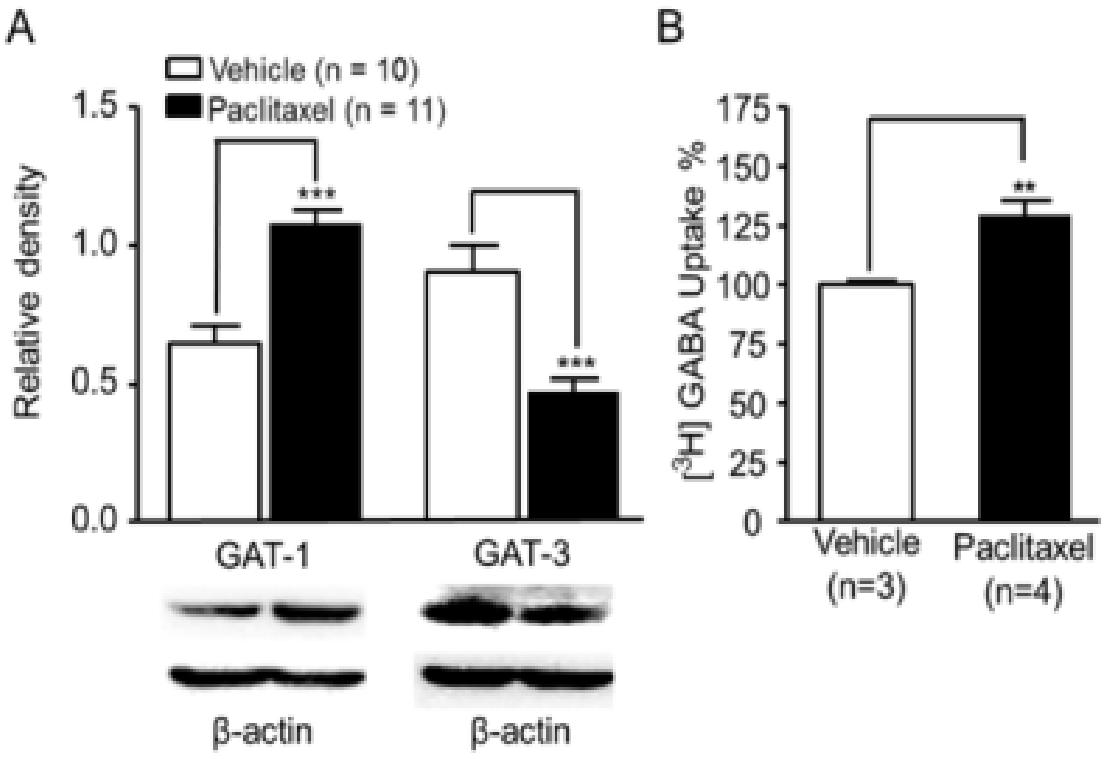

Figure 2.5: **Rats with P-INP have an increased protein expression of GAT-1 and reduced protein expression of GAT-3, which was accompanied with increased GABA uptake activities.** *(A)* Shows the mean (± SEM) relative density of GAT-1 and GAT-3 to β-Actin in the spinal dorsal horn of rats treated with vehicle and paclitaxel. Samples of GAT-1 and GAT-3 expressions in the spinal dorsal horn at the L4 to L5 segment in paclitaxel and vehicle treated rats are shown. *(B)* Shows the mean (± SEM) GABA uptake activities obtained from the spinal dorsal horn of rats receiving vehicle and paclitaxel treatment. *The GABA* uptake activities in the synaptosome preparation from rats treated with paclitaxel treatment were normalized with those treated with vehicle measured at the same time. ** $P < 0.01$; *** $P < 0.001$.

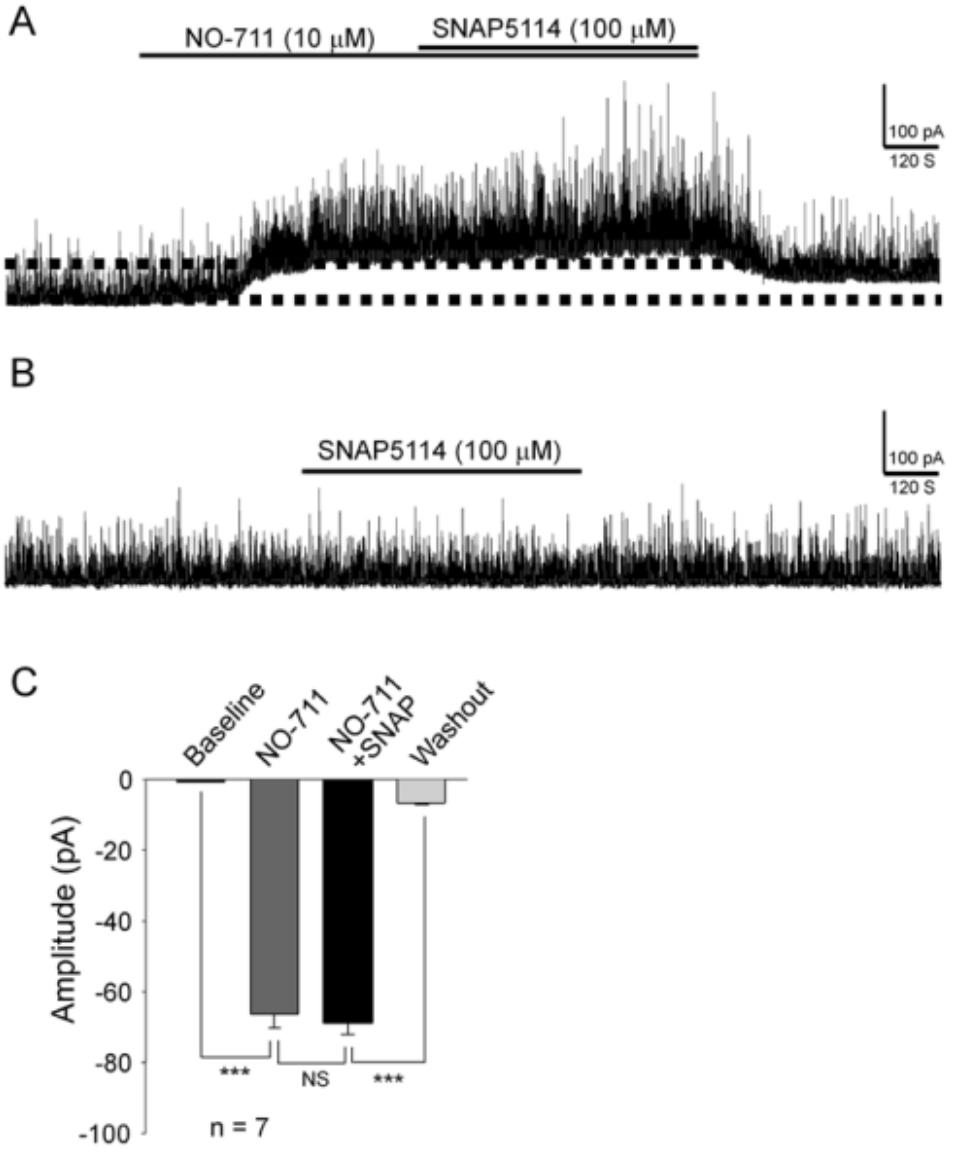

Figure 2.6: **The paclitaxel-induced GABAergic disinhibition is ameliorated by blocking GAT-1 but not GAT-3**. The original recordings in *(A)* and *(B)* were obtained from rats with P-INP. *(A)* Shows recordings of GABAergic currents before and after blocking of GAT-1 with NO711 (10 μM) and then further inhibition of GAT-3 with SNAP5114 (100 μM). *(B)* Shows recordings of GABAergic currents before and after blocking of GAT-3 alone with SNAP5114 (100 μM). *(C)* Shows mean (± SEM) changes of holding currents induced by NO-711 and then NO-711 plus SNAP5114 in rats treated with paclitaxel. ** $P < 0.01$; *** $P < 0.001$.

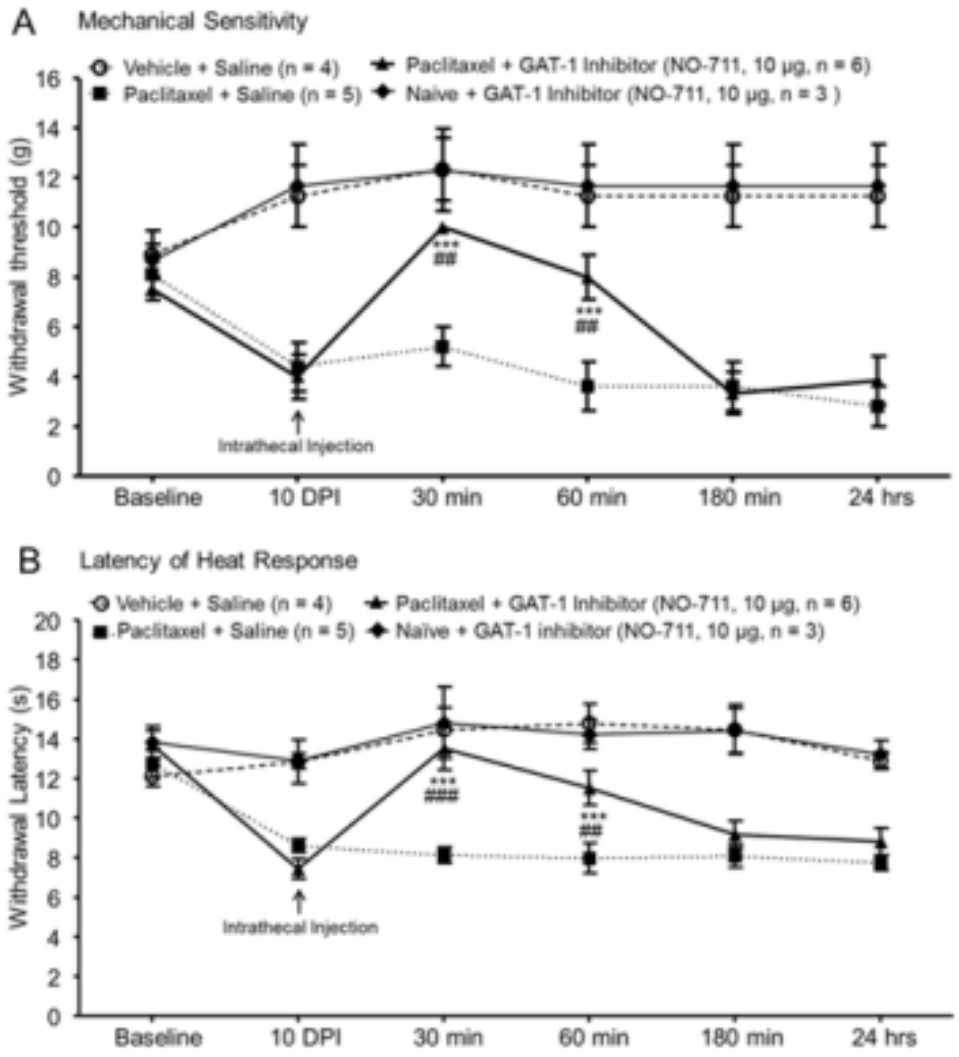

Figure 2.7: **Mechanical allodynia and thermal hyperalgesia in rats with P-INP are attenuated by an intrathecal injection of a GAT-1 inhibitor**. Line plots show measurements of mechanical thresholds of hind paw withdrawal responses *(A)* and latencies of the withdrawal response to heat stimuli *(B)* collected at baseline, 10 days post-i.p. vehicle or paclitaxel injection (10 DPI), and then 30, 60, 180 min and 24 hours after the intrathecal administration of the tested agent. Baseline indicates the measurement before animals received i.p. vehicle or paclitaxel injection. Comparisons between data collected on 10 DPI and at following each time point are indicated with * for the paclitaxel+GAT-1 inhibitor group. Comparisons between the paclitaxel+saline group and paclitaxel+GAT-1 inhibitor group are labeled with #. Two symbols: $P < 0.01$, three symbols: $P < 0.001$, data shows mean (± SEM).

CHAPTER 3

EZH2 REGULATES SPINAL NEUROINFLAMMATION IN RATS WITH NEUROPATHIC PAIN

Ruchi Yadav and Han-Rong Weng: EZH2 regulates spinal neuroinflammation in rats with neuropathic pain. Neuroscience, 2017 May 4; 349:106-117. doi: 10.1016
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Abstract

Alteration in gene expression along the pain signaling pathway is a key mechanism contributing to the genesis of neuropathic pain. Accumulating studies have shown that epigenetic regulation plays a crucial role in nociceptive process in the spinal dorsal horn. In this present study, we investigated the role of enhancer of zeste homolog-2 (EZH2), a subunit of the polycomb repressive complex 2, in the spinal dorsal horn in the genesis of neuropathic pain in rats induced by partial sciatic nerve ligation. EZH2 is a histone methyltransferase, which catalyzes the methylation of histone H3 on K27 (H3K27), resulting in gene silencing. We found that levels of EZH2 and tri-methylated H3K27 (H3K27TM) in the spinal dorsal horn were increased in rats with neuropathic pain on day 3 and day 10 post nerve injuries. EZH2 was predominantly expressed in neurons in the spinal dorsal horn under normal conditions. The number of neurons with EZH2 expression was increased after nerve injury. More strikingly, nerve injury drastically increased the number of microglia with EZH2 expression by more than 7 fold. Intrathecal injection of the EZH2 inhibitor attenuated the development and maintenance of mechanical and thermal hyperalgesia in rats with nerve injury. Such analgesic effects were concurrently associated with the reduced levels of EZH2, H3K27TM, Iba1, GFAP, TNF-α, IL-1β, and MCP-1 in the spinal dorsal horn in rats with nerve injury. Our results highly suggest that targeting the EZH2 signaling pathway could be an effective approach for the management of neuropathic pain.

Introduction

Neuropathic pain is a debilitating condition affecting millions of people and poorly managed by the current therapeutics such as opioids or nonsteroidal anti-inflammatory drugs. Developing more potent and safe analgesics is an urgent need to combat such devastating condition. It is known that alterations of protein expression via transcriptional and post transcriptional regulation in spinal neurons and glial cells are critical mechanisms contributing to the genesis of neuropathic pain (Befort et al 2001, Suzuki & Dickenson 2000, Taylor 2001, Woolf & Salter 2000). Identifying signaling molecules regulating gene expression would provide potential targets for the development of analgesics.

One key component leading to the development and maintenance of neuropathic pain is excessive activation of spinal dorsal horn neurons following nerve injury (Suzuki & Dickenson 2000, Taylor 2001). Activation and proliferation of microglia and astrocytes, and subsequent over-production of inflammatory mediators such as TNF-α, IL-1β and MCP-1 are causative factors causing excessive activation of spinal dorsal horn neurons (Grace et al 2014, Ren & Dubner 2016). In this context, TNF-α, IL-1β (Kawasaki et al 2008b, Yan & Weng 2013a) and MCP-1 (Gao et al 2009a) enhance excitatory glutamatergic synaptic activities in the spinal dorsal horn while inhibitory GABAergic synaptic activities in the same region are suppressed by TNF-α, IL-1β (Kawasaki et al 2008b, Yan et al 2015). Suppression of microglial activation with minocycline (Nie et al 2010b), or astrocytic activation with propentofylline (Tawfik et al 2008) can effectively attenuate neuropathic pain. Recent studies have demonstrated that regulation of gene expression profile via epigenetic mechanisms play an important role in

the genesis of chronic pain conditions (Descalzi et al 2015, Ligon et al 2016a). Currently, it remains unclear whether and how epigenetic mechanisms are engaged in the regulation of glial activation and production of TNF-α, IL-1β and MCP-1 in the spinal dorsal horn in animals with neuropathic pain.

Epigenetics mechanisms including DNA methylation, histone modification, and non-coding RNAs are implicated in activation and suppression of various gene expressions in the development and maintenance of chronic pain in animals (Bai et al 2015, Liang et al 2015, Ligon et al 2016a). Among the many histone modification mechanisms, N-terminal tail of histone is subjected to various post translation modification such as acetylation, methylation, phosphorylation, ubiquitination. In general, histone acetylation at the lysine residue promotes gene transcription. Whereas, histone methylation can either activate or represses gene transcription, depending upon which histone lysine residue is methylated (Margueron et al 2009, Martinez & Simeonov 2015). It is generally believed that methylation of histone H3 on K27 results in gene silencing (Margueron et al 2009). Polycomb complex 2 (PRC2) is one class of gene repressive epigenetic transcriptional regulators. PRC2 induces gene silencing by transferring the mono, di or tri-methyl groups onto lysine 27 of histone3 (H3K27) via its subunit the enhancer of zeste homolog-2 (EZH2), a histone methyltransferase (Alam et al 2015, Burgold et al 2008). Previous studies have shown that EZH2 plays an important role in controlling cell differentiation and proliferation. Increased levels of tri-methylated H3K27 (H3K27TM) and EZH2 promote the differentiation and proliferation of neurons, astrocytes and oligodendrocytes cells (Pereira et al 2010). During neocortical development PRC2 complex checks the multipotent neuronal progenitor cells and

controls their early neurogenic- and astrogenic cellular fate (Hirabayashi et al 2009b). EZH2 protein prevents the premature onset of neurogenesis in a developing brain (Sparmann et al 2013). More recently, it was shown that EZH2 is engaged in the production of inflammatory cytokines and proliferation of cells in various diseases such as rheumatoid arthritis (Miao et al 2013) and breast cancer (Hartman et al 2013, Kim & Roberts 2016a). Given that glial cell proliferation and over-production of inflammatory mediators are salient mechanisms implicated in the generation of chronic pain, the role of EZH2 in the spinal pain system was investigated in this study.

We demonstrated that increased global levels of EZH2 and H3K27TM in the L4-L5 spinal dorsal horn are critical epigenetic mechanisms contributing to the regulation of activation of microglia and astrocytes, and over-production of TNF-α, IL1-β, and MCP-1 in the spinal dorsal horn, which plays a key role in the genesis of neuropathic pain induced by partial sciatic nerve ligation.

Methods and materials

Animals

Adult male Sprague-Dawley rats (body weight range: 210 to 280 g, Harlan Laboratories) were used. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Georgia and were fully compliant with the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals.

Partial sciatic nerve ligation (pSNL) neuropathic pain model

Partial sciatic nerve ligation was made as previously described (Seltzer et al 1990, Yan et al 2014). Briefly, animals were anesthetized with 2-3% isoflurane. Under sterile

conditions, the left sciatic nerve was exposed at the upper thigh and ligated approximately to one-thirds to one-half of its diameter with a 5-0 silk suture. In sham-operated rats, the left sciatic nerve was exposed but not ligated. Following the surgery, the wounds were closed with skin staples.

Behavioral tests

Behavioral tests were conducted in a quiet room with the room temperature at 22°C as we previously described (Weng et al., 2006; Yan et al., 2013). To verify the changes in mechanical sensitivity before and after surgery, rats were placed on a wire mesh under a plexiglass cage (12 x 20 x 15 cm^3), loosely restrained and allowed to acclimate for at least 30 min. A series of von Frey monofilaments (bending force from 0.6 to 26.00 g) were tested in ascending order to generate response-frequency function for each animal. Each von Frey filament was applied 10 times to the mid planter area of the left hind paw for about 1 s to measure hind paw withdrawal response. The response-frequency function for each von Frey filament was defined as [(number of withdrawal responses of the hind paw/10) x 100%]. Withdrawal responses mechanical threshold was determined by the lowest force filament that evoked a 50% or greater withdrawal responses frequency (Weng et al., 2006; Yan et al., 2013). This value was later averaged across all animals in each group to yield the group response threshold (g).

To determine thermal sensitivity, rats were placed on a smooth glass plate pre-heated at 30 ^oC. A radiant heat beam (diameter 5 mm) was directed onto the mid-plantar area of the left hind paw to evoke a paw withdrawal response from beneath (Hargreaves et al 1988). The withdrawal latency (defined as the time between the onset of radiant heat and the time when the rat withdrew his paw) was recorded. Each hind paw was

stimulated three times with an interval of at least 2 min. A cutoff time of 20 s was used to avoid damage to the skin. Three paw withdrawal latencies recorded from each individual rat were averaged.

Intrathecal catheter implantation and drug administration

Intrathecal (i.t.) injection of tested drugs to the spinal enlargement was made through the pre-implanted intrathecal catheter. Briefly, a polyethylene (PE-10) catheter that ended at the spinal L4 segment was intrathecally placed as previously described (Yadav et al 2015, Yaksh & Rudy 1976). Rats were anesthetized with 2-3% isoflurane and a PE-10 catheter was carefully inserted through the atlanto-occipital membrane opening and advanced to the lumbar enlargement (L4-L5). The muscles were then sutured in layers and the skin edges were closed with skin staples. The animals were allowed to recover for 7 days before the baseline behavior test were conducted. Animals with the i.t. catheter were then randomly divided into pSNL or sham groups to receive pSNL and sham operation respectively. Drugs or vehicle in a volume of 10 μL was injected into the spinal lumbar enlargement region through the intrathecal catheter, followed by 20 μL of saline to flush. The EZH2 inhibitors (DZNep and GSK126) were prepared in DMSO and saline. The final concentration of DMSO in the injected solution was less than 1%. Saline with the same concentration of DMSO was used as a vehicle control. When the drug administration fell on same day as the behavior analysis, the behavior tests were completed prior to the drug administration. At the end of the behavior experiments, rats were intrathecally injected with 50 μL of 2% lidocaine. If hind paw paralysis did not occur, rats were removed from the experiment. The experimenter who conducted the behavioral tests was blinded to the treatments given to the animals.

Western blotting

Animals were deeply anesthetized with urethane (1.3–1.5 g/kg, i.p.). The L4-L5 spinal segment was exposed and removed from the rats. The dorsal quadrant of the spinal segment ipsilateral to the operation side was isolated and quickly frozen in liquid nitrogen and stored at -80 °C for later use. Frozen tissues were homogenized with a hand-held pellet in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% deoxycholic acid, 2 mM orthovanadate, 100 mM NaF, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 100 IU mL−1 aprotinin) for about 30 min on ice. The samples were then centrifuged for 20 min at 12,000 g at 4 °C and the supernatants containing proteins were collected. The quantification of protein contents was made by the BCA method. Protein samples (50 μg) were electrophoresed in 12.5 % SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% milk or 5% BSA in TBST, and then incubated respectively overnight at 4 °C with polyclonal rabbit anti-GFAP (1:1,000, cell signaling), polyclonal rabbit anti-Iba1 (1:1,000; Wako), rabbit anti-TNF-α (1:500; Millipore), rabbit anti-IL-1β (1:500; Millipore), rabbit anti-MCP-1 (1:500; Abcam), rabbit anti-EZH2 (1:500; Abcam), rabbit anti-H3K27TM (1:500; Epigentek), rabbit anti-total histone H3 (1;1,000; cell signaling) primary antibodies, or a monoclonal rabbit anti-β-actin (1:2,000; Millipore) primary antibody as a loading control. The blots were then incubated for 2 hr at room temperature with corresponding HRP-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology, CA, USA), visualized in ECL solution (Super Signal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) for 2.5 min, and exposed onto Odyssey imaging system-LI-COR biosciences. The

bands intensity of immunoreactivity was quantified using ImageJ 1.46 software (NIH). Levels of each biomarker were expressed as the relative ratio to the loading control protein (β-actin) unless otherwise indicated.

Immunohistochemical Analysis

Animals were deeply anesthetized with urethane (1.3-1.5 g/kg, i.p.) and perfused through the ascending aorta with 200 ml heparinized phosphate-buffered solution (0.1 M PBS, pH = 7.35) followed by a solution of 4% formaldehyde in (0.1 M PBS, pH = 7.35). The L4 and L5 spinal cord was removed and fixed for next 24 hrs at 4 °C in fresh 4% formaldehyde. The L4- L5 spinal lumbar region was dehydrated with gradient ethanol, and embedded in paraffin. The transverse sections of the spinal cord were sliced at a 10 µM thickness and mounted on superfrost plus slides. After dewaxing and hydration, slices were subjected for an antigen-retrieval method by sodium citrate (pH = 6) at 90^{o}C for 30 min followed by bench-cooling for 20 min. After washing with 0.1 M PBS, slices were then blocked in 10% normal goat serum with 0.05% BSA, 0.3% Triton X-100 in 0.1 M PBS for 3 hrs at room temperature. The sections were then incubated overnight at 4 °C with primary antibodies. Either simultaneous or sequential incubation of primary antibodies mixture in 2% normal goat serum with 0.0 5% BSA, 0.3% Triton X-100 in 0.1 M PBS against the following targets: mouse anti-NeuN (a marker for neuronal cells,1:200, Cell Signaling); mouse anti-GFAP (a marker for astrocytes, 1:500, Cell Signaling); mouse anti- Iba1 (1:200, Millipore) antibodies. After washing three times with 0.1 M PBS, the sections were incubated for 4 hrs at room temperature with the corresponding Texas Red antibody (1:500, Vector Laboratories) or Alexa Fluor 488 antibody (1:500, Life Technologies). After rinsing three times with 0.1 M PBS, the

sections were air-dried and cover-slipped with Vectashield mounting medium (Vector Laboratories). Non-adjacent sections were randomly selected, and the immunostaining for each antibody were viewed under an Olympus BX43 microscope with an Olympus U-CMAD3 camera. Images were processed using Olympus-cell Sens Dimensions. Four to five sections from each rodent's L4-L5 spinal cord segments (4-5 animals per group) were randomly selected and used for analysis. The number of $GFAP^+/EZH2^+$, $Iba1^+/EZH2^+$ microglia and $NeuN^+/EZH2^+$ neurons with clear, visible cell body and nuclei in the spinal dorsal horn were counted.

Materials

DZnep and GSK126 were purchased from Cayman chemicals (USA).

Data analysis

All data are presented as the mean ± S.E. One-way and two-way ANOVA with repeated measures were used to respectively detect difference in behavioral data over different time points in the same group, and detect difference in behavioral data between groups over different time points. Once difference was found, the Bonferroni post hoc test was used to determine sources of differences. Non-paired Student's t-tests were used to make comparison between groups for data collected from western blots. A *p* value less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism (v5.03, GraphPad Software, Inc.).

Results

Nerve Injury induced allodynia and thermal hyperalgesia

In order to define the role of EZH2 in the genesis of neuropathic pain, expressions of

multiple molecules in the spinal dorsal horn ipsilateral to the operation side were analyzed in rats on days 3 and 10 after pSNL or sham operation. Adult male animals were randomly assigned into 4 groups, two groups receiving pSNL, and two groups receiving sham operation. Behavioral tests were performed to confirm that animals receiving pSNL developed mechanical allodynia and thermal hyperalgesia before the animals were used for any biochemical assays. The mechanical thresholds of hind paw withdrawal responses (5.75 ± 2.7 g, n = 10) in rats with pSNL on day 3 after surgery were significantly lower than their own baseline (prior to the surgery) (14.18 ± 2.3 g, n = 10, $p < 0.001$) or the sham operated rats (14.96 ± 2.4 g, n = 8, $p < 0.001$) on the same day. At the same time, latencies of hind paw withdrawal responses to radiant heat stimuli in rats with pSNL (7.49 ± 0.71 s, n = 10) were significantly shorter than their own baseline values (14.29 ± .64 s, n = 10, $p < 0.001$) or the sham-operated rats (15.82 ± 1.17 s, n = 8, $p < 0.001$). We also found that mechanical thresholds (6.05 ± 1.18 g, n = 15) in rats with pSNL on day 10 after surgery were significantly reduced in comparison with their baseline values (15.45 ± 0.90 g, n = 15, $p < 0.001$) or rats with sham-operation (17.33 ± 0.64 g, n = 12, $p < 0.001$). Meanwhile, latencies of hind paw withdrawal responses to radiant heat stimuli in rats with pSNL were significantly ($p < 0.01$) reduced in comparison with their baseline 15.23 ± 0.50 s to 6.91 ± 0.32 s (n = 15) and with the sham-operated rats (16.15 ± 0.77 s, n = 12). Mechanical thresholds (n = 8) or thermal thresholds (n = 12) in rats with sham-operation measured on day 3 or day 10 after surgery were similar to their own baseline prior to the surgery. These data indicate that rats receiving pSNL had clear signs of neuropathic pain (mechanical allodynia and thermal hyperalgesia) both on day 3 and day 10 after the surgery. Unless otherwise stated, all data

in the rest of this paper were obtained from animals that completed the behavioral assessment stated above.

Global levels of EZH2 and H3K27TM were increased in the spinal dorsal horn in rats with neuropathic pain

To determine the role of EZH2 in the genesis of neuropathic pain, we examined global EZH2 protein expression in the spinal dorsal horn ipsilateral to the operation side in rats receiving pSNL and rats receiving sham operation on days 3 and 10 after the surgery. Using western blot techniques, we found that in comparison with sham-operated rats (n = 4) EZH2 protein expression in the spinal dorsal horn was significantly ($p < 0.05$) increased in animals (n = 5) with pSNL on days 3 (Figure 3.1A) and 10 (Figure 3.1B) after surgery. EZH2 is a methyltransferase that causes methylation of H3K27 (Kuzmichev et al 2002). To determine whether the increased protein expression of EZH2 is accompanied with an increase of its functional activity, we measured levels of H3K27TM using western blots. We found that in comparison with the sham-operated groups, animals in the pSNL groups had a higher level of global H3K27TM protein in the spinal dorsal horn on days 3 (Figure 3.1A) and 10 (Figure 3.1B) after surgery. Together these data indicate that the EZH2 activity is increased in the spinal dorsal horn of rats with neuropathic pain.

EZH2 was predominantly expressed in neurons in the spinal dorsal horn under normal conditions, and nerve injury increased the number of neurons and microglia with EZH2 expression

To identify the cellular types responsible for the globally increased expression of EZH2, we investigated the cellular location of EZH2 since the cellular location of EZH2

in the spinal cord has not been reported. To define the cellular location of EZH2 protein, the L4- L5 spinal segment obtained from rats receiving sham operation and pSNL on day 3 and day 10 (four to five rats per group) after surgery were used. We observed a stronger EZH2 staining in the dorsal horn of rats with pSNL on days 3 and 10 than that in rats with sham-operation (Figure 3.2A). Furthermore, microglia, astrocytes, and neurons in spinal slices were respectively labeled with Iba1, GFAP, and NeuN antibodies. The slices were doubled-labeled with the EZH2 antibody. We found that EZH2 was predominantly co-localized with NeuN in sham operated rats (Figure 3.2B and C). The number of neurons with EZH2 expression was significantly ($p < 0.001$) increased after nerve injury (Figure 3.2B and C). More strikingly, nerve injury drastically increased ($p < 0.001$) the number of microglia with EZH2 expression by more than 7 fold on day 3 and day 10 after nerve injury in comparison with the sham operated rats on the same days (Figure 3.2B and C). Consequently, the ratios between cells with $NeuN^+$ and $EZH2^+$ and cells with $Iba1^+$ and $EZH2^+$ were significantly reduced from 8.77 ± 2.04 (n = 5) in the sham operated rats to 1.23 ± 0.06 (n = 5, $p < 0.01$) in rats on day 3 after nerve injury, and from 7.86 ± 1.23 (n = 5) in the sham operated rats to 2.21 ± 0.18 (n = 5, $p < 0.01$) in rats on day 10 after nerve injury.

No significant change in the number of $GFAP^+/EZH2^+$ cells was observed in rats with pSNL on days 3 and 10 after surgery compared to rats with sham operation (Figure 3.2B and C). These data indicate that: a. EZH2 is mainly expressed in neurons under normal conditions; b. nerve injury increases the number of both microglia and neurons with EZH2 expression, with a much more drastic increase in microglia with EZH2 expression. Furthermore, the expression of Iba1 on day 3 and day 10 after nerve injury

were about 2 fold of those in sham operated animals on the same day, suggesting that the number of microglia in animals with nerve injury was about 2 fold of that in sham animals. The more than 7 fold increase in number of microglia with EZH2 expression in animals on day 3 and day 10 after nerve injury suggests that the increase in number of microglia with EZH2 expression is not purely due to the increase in the number of microglia, rather, it suggests that at least some of the original microglia prior to nerve injury have EZH2 expression increased.

The increased levels of EZH2 and H3K27TM was temporally associated with neuroinflammation in the spinal cord in neuropathic rats

The drastic increased number of microglia with EZH2 expression prompted us to explore the potential link between EZH2 and neuroinflammation in the spinal dorsal horn. We examined whether the increased levels of EZH2 and H3K27TM in the spinal cord are temporally associated with activation of microglia and astrocytes, and over-production of TNF-α, IL-1β and MCP-1. Protein expressions of the microglial marker (Iba1), astrocytic marker (GFAP), TNF-α, IL-1β and MCP-1 in the spinal dorsal horn of rats with pSNL on day 3 (Figure 3.3A; n = 5) and day 10 (Figure 3.3B; n = 5) were significantly higher than the sham-operated animals on day 3 (Figure 3.3A; n = 4) and day 10 (Figure 3.3B; n = 4). The temporal association between the increased levels of EZH2 and H3K27TM and spinal neuroinflammation highly suggest that EZH2 and H3K27TM may be implicated in the spinal neuroinflammation process under neuropathic conditions.

Pharmacological inhibition of EZH2 prevented the development of neuropathic pain

To determine whether the increased EZH2 activity contributes to the development of chronic pain induced by pSNL, we examined whether pharmacological inhibition of EZH2 can prevent the development of neuropathic pain. In the first set of experiments, a widely used EZH2 inhibitor, 3-Deazaneplanocin (DZNep) (Girard et al 2014, Glazer et al 1986, Miranda et al 2009, Tan et al 2007) was used. DZNep is an S-adenosylhomocysteine hydrolase inhibitor, known to suppress the protein expression of EZH2 and levels of H3K27TM without effects on DNA methyltransferases (Fiskus et al 2009, Fujiwara et al 2014, Tan et al 2007). Rats were randomly assigned into four groups: Sham + Vehicle, Sham + DZNep, pSNL + Vehicle, and pSNL + DZNep groups. Behavior analysis was performed on day 1 before the surgery and then on days 2, 4, 6, 8, and 10 after the surgery. Rats in the pSNL + DZNep group and Sham + DZNep group received DZNep (20 nM in 10 μL) through the pre-implanted intrathecal catheter on day 1 immediately prior to the surgery and then daily till day 9 after the surgery. Vehicles (10 μL) were administered to rats in the pSNL + Vehicle and Sham + Vehicle groups in the same fashion. As shown in Figure 4A, mechanical thresholds of withdrawal responses were significantly ($p < 0.001$) reduced in the pSNL + vehicle group (n = 12) starting from day 2 through day 10 following the nerve injury compared to their baseline prior to the surgery and those from the sham + vehicle group (n = 9). Mechanical thresholds of withdrawal responses in the pSNL + DZNep group (n = 11) at baseline, and on days 2 and 4 were similar to those in the pSNL + Vehicle group (n = 12), but became significantly higher than those in the pSNL + vehicle group from day 6 through day 10 ($p < 0.001$). Meanwhile, mechanical thresholds in the Sham + Vehicle group and Sham +

DZNep group remained stable during the 10 day observation period. Similarly, we also observed that latencies of withdrawal responses to radiant heat stimuli were significantly prolonged at the same time point in the pSNL + DZNep group than those in the pSNL + Vehicle group (Figure 3.4B).

In the second set of experiments, we used another agent GSK126, which is a selective, S-adenosyl-methionine-competitive small molecule inhibitor of EZH2 methyltransferase activity (McCabe et al 2012). Rats were randomly assigned into five groups: Sham + Vehicle, Sham + GSK-126 (5 nM), pSNL + Vehicle, pSNL + GSK-126 (5 nM), and pSNL + GSK-126 (0.5 nM). Drugs and vehicle were administered in the same fashion and time schedule as those described for the DZNep treatment. We found that mechanical thresholds of withdrawal responses in the pSNL + GSK-126 (5 nM) group (n = 10) but not in the GSK-126 (0.5 nM) group (n = 4) were significantly elevated in comparison with those in the pSNL + vehicle group (n = 11) from day 2 through day 10 ($p < 0.001$) (Figure 3.4C). Mechanical thresholds in the Sham + vehicle group and sham + GSK-126 (5 nM) group were not significantly altered during the same period. We also found that GSK-126 prolonged latencies of withdrawal responses to radiant heat stimuli in rats with pSNL in a dose-dependent manner (Figure 3.4D) from day 4 to day 10 after the surgery. These data demonstrate that inhibition of EZH2 can prevent the development of neuropathic pain.

Pharmacological inhibition of EZH2 attenuated the pre-existing neuropathic pain

Next, we determined whether inhibition of EZH2 can attenuate the pre-existing neuropathic pain. Four groups of rats were used: Sham + Vehicle, Sham + DZNep, pSNL + Vehicle, and pSNL + DZNep groups. DZNep (20 nM in 10 μL) were administered

intrathecally daily from day 3 to day 9 after the nerve injury. Vehicles (10 μL) were administered to rats in the pSNL + Vehicle and Sham + Vehicle in the same fashion. We found that mechanical thresholds and thermal latencies of withdrawal responses in the pSNL + DZNep (n = 6) group were significantly increased ($p < 0.05$ to $p < 0.01$) from day 7 to day 10 after the surgery in comparison with their own values on day 3 and those in the pSNL + Vehicle group (n = 6) (Figure 3.5). These data indicate that blocking spinal EZH2 activity can attenuate the pre-existing neuropathic pain.

Global levels of EZH2 and H3K27TM in the spinal dorsal horn of neuropathic rats were restored by inhibition of EZH2

DZNep treatment is known to suppress protein expression of EZH2 and reduce levels of H3K27TM (Fiskus et al 2009, Fujiwara et al 2014, Tan et al 2007). To determine levels of EZH2 and H3K27TM in the spinal dorsal horn, the spinal dorsal horn of the same animals that had finished the 9 day DZNep and vehicle treatments as well as behavioral tests on day 10 as described in Figure 3.4A and B were used. Using western blot techniques, we found that 9 day treatment of DZNep restored basal protein expressions of EZH2 in rats with pSNL (n = 7) (Fig. 6A). At the same time, the H3K27TM level in the neuropathic rats was also restored as demonstrated by western blots (n = 7) (Figure 3.6A). Similarly, we determined spinal levels of EZH2 and H3K27TM in the rats that had completed the 9 day GSK126 and vehicle treatments as well as behavioral tests on day 10 as described in Fig. 4C and D. We found that GKS126 treatment normalized the levels of EZH2 and H3K27TM in rats with pSNL (Figure 3.6B).

DZNep treatment suppressed neuroinflammation in the spinal dorsal horn of rats with neuropathic pain

Finally, we examined the molecular mechanisms by which EZH2 inhibitors exerts its analgesic effects. The drastic increased number of microglia with expression of EZH2 highly suggests that DZNep treatment may suppress neuroinflammation in the spinal dorsal horn in rats with pSNL. Thus, we determined protein expressions of Iba1, GFAP, TNF-α, IL-1β, and MCP-1 in the same animals that had completed with the behavioral tests as described in Figure 4A and B. We found that the increased expressions of Iba1, GFPA, and TNF-α, IL-1β, and MCP-1 in the spinal dorsal horn of rats with pSNL (n = 8) were significantly attenuated when 20 nM DEZNep was intrathecally injected into the animals immediately prior to the pSNL surgery and then daily for 9 days (n = 7) (Figure 3.7). These results demonstrate that suppression of glial activation and production of TNF-α, IL-1β, and MCP-1 are important mechanisms by which DZNep produces the analgesic effects in the animals with neuropathic pain.

Discussion

In this study, we for the first time demonstrated that EZH2 is a key regulator in the development of neuropathic pain. We found that nerve injury caused increases in levels of EZH2 and H3K27TM, and the number of neurons and microglia with EZH2 expression in the spinal dorsal horn. Importantly, spinal pharmacological inhibition of EZH2 attenuated the development and maintenance of mechanical and thermal hyperalgesia in rats with nerve injury. Such analgesic effects are concurrently

accompanied with the reduced protein expressions of Iba1, GFPA, TNF-α, IL-1β, and MCP-1 in the spinal dorsal horn induced by nerve injury. Our findings highly indicate that EZH2 is implicated in the genesis of neuropathic pain through regulating neuroinflammation in the spinal dorsal horn.

Gene activation and suppression play a critical role in the development of plasticity along the peripheral and central pain signaling pathways in chronic pain conditions (Kuner 2010, Lacroix-Fralish et al 2006, Woolf 2011). Emerging studies have implicated epigenetic mechanisms such as DNA methylation, histone modification, and non-coding micro RNAs in aberrant gene expression in animals with neuropathic pain (Buchheit et al 2012, Doehring et al 2011, Gräff et al 2011, Tajerian et al 2011). For example, rats with neuropathic pain have increases in global DNA methylation and methyl-CpG-binding protein 2 (MeCP2), and suppression of global DNA methylation and MeCP2 expression with 5-azacytidine attenuate mechanical allodynia and thermal hyperalgesia induced by nerve injury (Wang et al 2011). Suppression of miR-7a expression in the dorsal root ganglion induced by nerve injury causes an increased protein expression of β2 subunit of the voltage-gated sodium channel, which contributes importantly to the late phase of neuropathic pain (Sakai et al 2013). Inhibition of histone acetylation with HDAC inhibitors attenuates the development of mechanical and thermal hypersensitivity induced by nerve trauma or antiretroviral drug–induced neuropathy in rats, but do not have effects on the pre-existing neuropathic pain (Denk et al 2013)) Increased histone H3 acetylation in the promoter of MIP-2 and CXCR2 leads to the up-regulation of MIP-2 and CXCR2 following nerve injury, and neuropathic pain (Kiguchi et al 2012). In addition to histone acetylation, histone methylation is another important

mechanism regulating gene expression. Currently, our understanding about the role of histone methylation in the nervous system is limited.

The PRC2 is an essential epigenetic machinery that suppresses the gene expression by transferring the mono, di or tri-methyl groups onto lysine 27 of histone3 (H3K27) via its catalytic subunit EZH2, a methyltransferase (Bannister & Kouzarides 2011, Ferrari et al 2014, Kirmizis et al 2004). It was recently reported that a global increase of H3K27TM and EZH2 protein expression in the dorsal root ganglion of rats with neuropathic pain results in suppression of potassium channel activities in the sensory neurons (Laumet et al 2015). Little is known about the global levels of H3K27TM and its methyltransferase (EZH2) in the spinal cord in pathological conditions. Similarly, the effects induced by pharmacological intervention targeting this signaling pathway at the spinal cord have not been explored. In this study, we found that global protein expression of EZH2 and levels of H3K27TM in the spinal dorsal horn were increased on day 3 and day 10 after never injury and spinal pharmacological EZH2 inhibitors attenuate the development and maintenance of neuropathic pain. Importantly, we validated the targets of the EZH2 inhibitors by demonstrating that the levels of EZH2 and H3K27TM were normalized in the spinal cord of neuropathic rats treated with the EZH2 inhibitors. These findings suggest that normalization of the EZH2 activity in the spinal dorsal horn is effective to attenuate neuropathic pain.

Given that glial activation and over-production of inflammatory mediators are critically implicated in the genesis of neuropathic pain, molecular mechanisms regulating spinal glial activation and neuroinflammation have been highly sought-after as the basis of a new generation of therapeutics for neuropathic pain. Recent studies suggest that

histone modification is engaged in epigenetic regulation of genes related to inflammation along the pain signaling pathway under pathological pain conditions. For example, systemic administration of a HDAC inhibitor, sodium butyrate, significantly attenuated the increased TNF-α level in the injured sciatic nerve (Kukkar et al 2014). Nerve injury promotes histone H3 and H4 acetylation and increases expression of BDNF via activating the BDNF promoter (Uchida et al 2013). Pharmacological inhibition or knockdown of the histone acetyltransferase E1A binding protein p300 in the spinal cord attenuates the COX-2 expression in the spinal cord induced by nerve injury (Zhu et al 2012). While it was reported that an upregulation of MCP-3 gene expression in the spinal cord of neuropathic rats was ascribed to the decreased H3K27TM at the MCP-3 promoter (Imai et al 2013), it remains unknown whether global spinal neuroinflammation under pathological pain conditions can be modulated by altering the global levels of EZH2 and H3K27TM in the spinal dorsal horn. Our results show that nerve injury significantly increases the global levels of EZH2 and H3K27TM in the spinal dorsal horn. Neuropathic rats treated with the EZH2 inhibitors had significant less mechanical allodynia and thermal hyperalgesia and less protein expressions of Iba1, GFAP, and TNF-α, IL-1β, and MCP-1 in the spinal dorsal horn in comparison with those in neuropathic rats receiving vehicle treatment. These data strongly indicate that pharmacological suppression of the increased activities of EZH2 attenuate the development of neuropathic pain via suppressing activation of microglia and astrocytes, and production of TNF-α, IL-1β, and MCP-1. Our findings also reiterate the importance to investigate the global levels and function of a given molecule when considering the therapeutic use of the target molecule.

Mechanisms by which EZH2 regulates neuroinflammation remain not well understood. Neuroinflammation is characterized by glial activation and proliferation and their subsequent over-production of proinflammatory mediators (including TNF-α, IL-1β, and MCP-1). Given that trimethylation of H3K27 by EZH2 results in gene repression (Cao and Zhang, 2004; Hansen et al., 2008; Marqueron et al., 2009), the types of genes repressed by H3K27TM/EZH2 must be negative regulators of neuroinflammation. One gene known to be suppressed by EZH2/H3K27TM is the cyclin-dependent kinase inhibitor p16 (Maertens et al 2009). P16 is a well-known cell-cycle-regulatory protein, which inhibits cyclin dependent kinase (like CDK4) activities and suppresses cell proliferation (Traves et al 2012). Previous studies have shown that cell proliferation and inflammation are regulated positively by EZH2/H3K27TM but negatively by P16 gene expression. For example, embryos with EZH2 deletion display severe defects during gastrulation (O'Carroll et al 2001, Zhang et al 2015b) Over expression of EZH2 in differentiating neural stem cells promotes neural stem cells to be differentiated into oligodendrocytes (Sher et al 2008). EZH2 suppression in dorsal root ganglion cocultures interferes with in vitro myelination by Schwann cells (Heinen et al 2012, Zhang et al 2015b). Down-regulation of P16 leads to cell proliferation (Traves et al 2012). More recent studies show that EZH2 and P16 are involved in the inflammation processes. For example, EZH2 is overexpressed in fibroblast-like synoviocytes in the rheumatoid joint and EZH2 causes changes in fibroblast-like synoviocytes through the Wnt signaling pathway, which plays an important role in regulating rheumatoid arthritis (Miao et al 2013, Trenkmann et al 2011). Activation of NF-kB by lysophosphatidic acid and production of IL-6, IL-8 in breast cancer cells is dependent on high EZH2 expression

(Hartman et al 2013). Enhancement of gene expression of P16 results in reduction of rheumatoid arthritis in animal models, and attenuation in production of proinflammatory mediators from synovial fibroblasts of rheumatoid arthritis, and macrophages in response to lipopolysaccharide (LPS) treatment (Murakami et al 2012). Currently, it is unknown about the role of EZH2 in the regulation of inflammation in the CNS. Our present study fills this gap by investigating the role of EZH2 in the spinal cord. The cellular location of EZH2 in the spinal cord was defined for the first time. We revealed that EZH2 is predominantly expressed in neurons under normal conditions. This is in line with a previous report that EZH2 is only expressed in neurons in the spinal dorsal root ganglion (Laumet et al 2015) . We found that nerve injury causes an increase in the global EZH2 expression and the number of neurons with EZH2 expression in the spinal dorsal horn. Intriguingly, nerve injury results in a tremendous increase in the number of microglia with EZH2 expression on 3 day and 10 day after nerve injury. Given that microglial activation, as evident by an increased expression of Iba1 protein, was found at the same time, it is conceivable that the increased expression of EZH2 in microglia contributes importantly to the microglial activation and its production of inflammatory mediators, which ultimately brings about neuroinflammation in the spinal dorsal horn. This notion is indirectly supported by several previous findings. First, cell proliferation is associated with production of inflammatory mediators (Gong et al 2011, Turgeon et al 2013). Second, microglial proliferation is a form of microglial activation, which has been recently demonstrated to be a critical mechanism contributing to the genesis of neuroinflammation and neuropathic pain in rodents (Guan et al 2016, Okubo et al 2016).

Conclusions and Future Directions

In conclusion, our current study reveals that EZH2 plays a critical role in the development and maintenance of neuropathic pain through regulating the neuroinflammation process in the spinal dorsal horn. Hence, suppressing the spinal EZH2 and H3K27TM levels may be a novel strategy for the development of new analgesics for the treatment of neuropathic pain.

In this study we have predominately focused on glial activation and proiflammatory cytokines with in the spinal dorsal horn in the development and maintenance of neuropathic pain, we have not addressed any upstream regulators of EZH2 proteins and downstream effects of EZH2 and H3K27TM mediated specific gene suppressed by this histone modification. By chip-seq analysis we can get genetic profile of various genes which are suppressed by H3K27TM histone modification and verify their usability as potential drug able targets.

Acknowledgments

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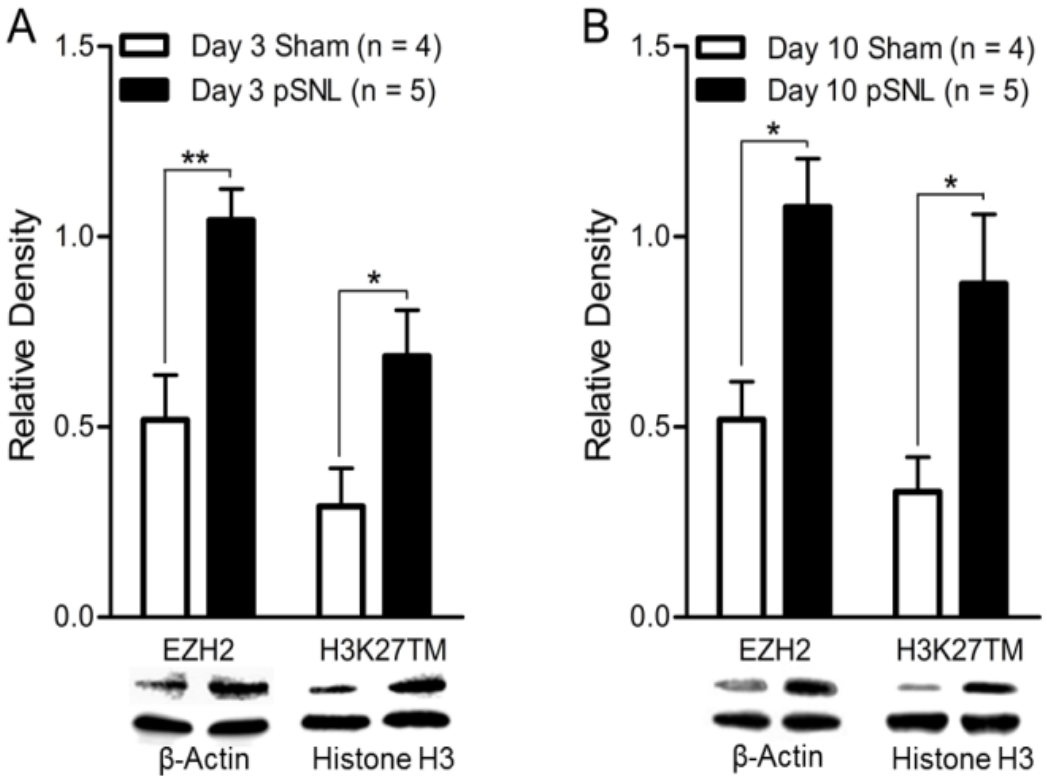

Figure 3.1: **Nerve injury increases EZH2 protein expression and H3K27TM levels in spinal dorsal horn.** Bar graphs show the mean (+S.E.) of relative density ratio of EZH2 over β-actin and H3K27TM over total-histone H3 in the spinal dorsal horn of rats receiving either sham operation or pSNL on day 3 (A) and day 10 (B) after surgery. Western blot samples of each molecular protein in each group are displayed. *$p < 0.05$; **$p < 0.01$.

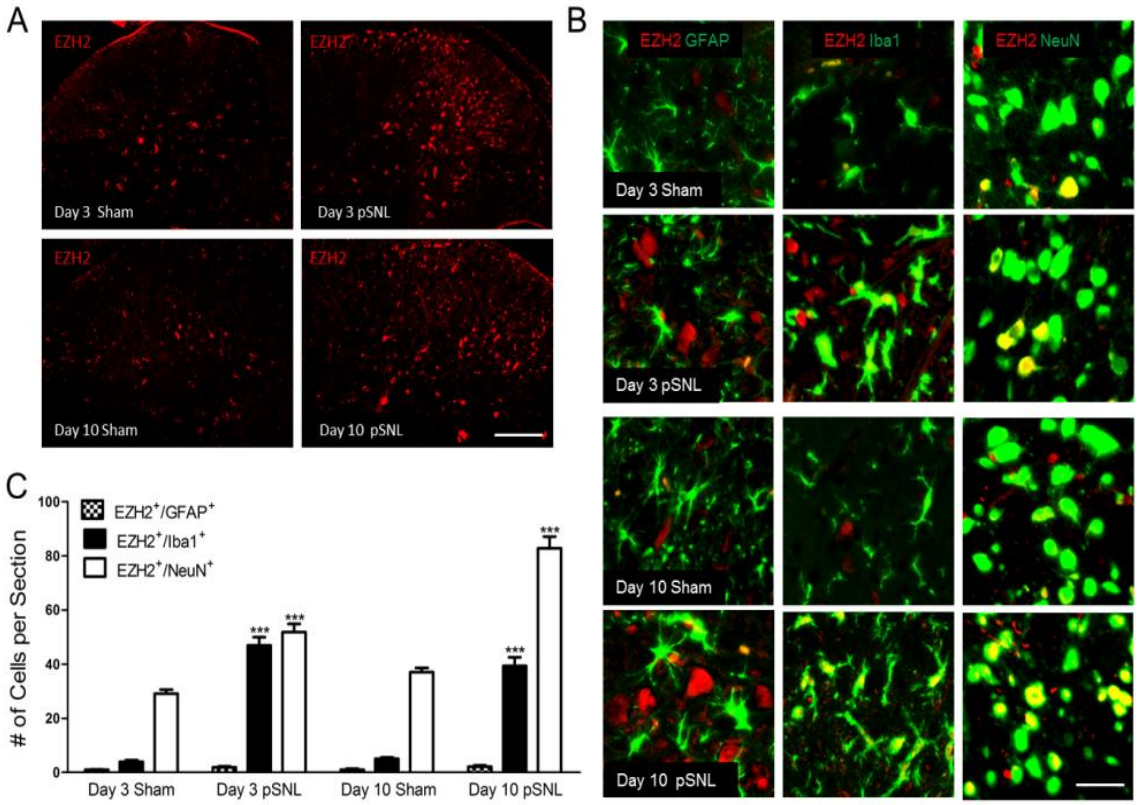

Figure 3.2: **EZH2 is predominantly expressed in neurons in the spinal dorsal horn under normal conditions, and nerve injury increases the number of neurons with EZH2 expression and microglia with EZH2 expression.** (A) shows samples of EZH2 staining in L4-L5 spinal dorsal horn ipsilateral to the operation site in sham and pSNL rats on days 3 and 10 after surgery. Scale bar: 200 µm. (B) shows co-localization of EZH2 staining (red) with different cellular markers (in green): Iba1 for microglia, GFAP for astrocytes, and NeuN for neurons. Scale bar: 50 µm. (C) Bar graphs show the mean (+ S.E.) numbers of EZH2 $^{+}$/Iba1^{+}, EZH2^{+}/GFAP^{+} , and EZH2^{+}/NeuN^{+} cells in the spinal dorsal horn per section in sham operated and pSNL rats on days 3 and 10 after surgery. Four to five rats per group were used for analysis. Comparisons of the same cellular types on the same days after surgery between sham-operated and pSNL rats are shown. *** $p <$ 0.001.

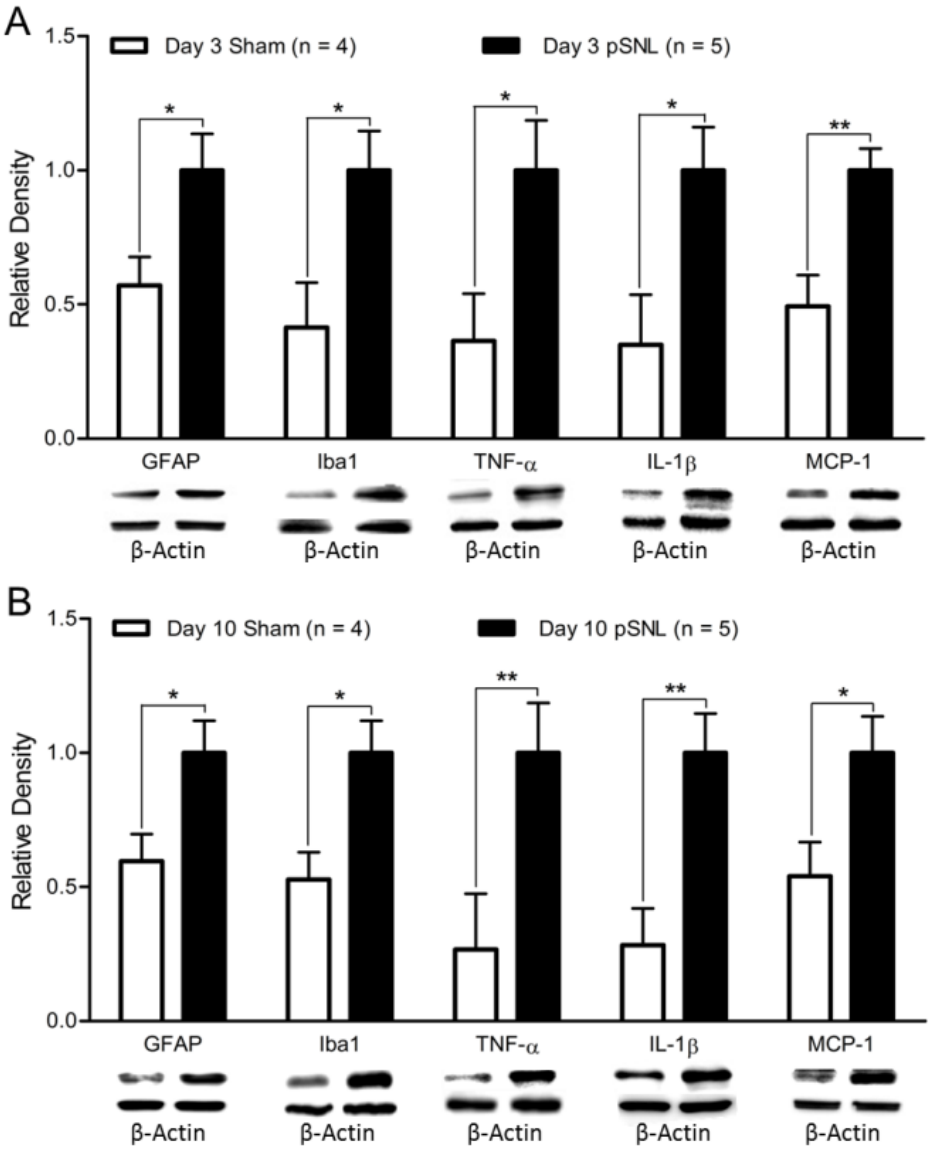

Figure 3.3: **Nerve injury increases protein expressions of Iba1, GFAP, TNF-α, IL-1β, and MCP-1 in the spinal dorsal horn**. Bar graphs show the mean (+ S.E.) density of Iba1, GFAP, TNF-α, IL-1β, and MCP-1 relative to β-actin in the spinal dorsal horn in rats receiving either sham operation or pSNL on day 3 (A) and day 10 (B) after surgery. Samples of each molecular protein expression in each group are displayed. *$p < 0.05$; **$p < 0.01$.

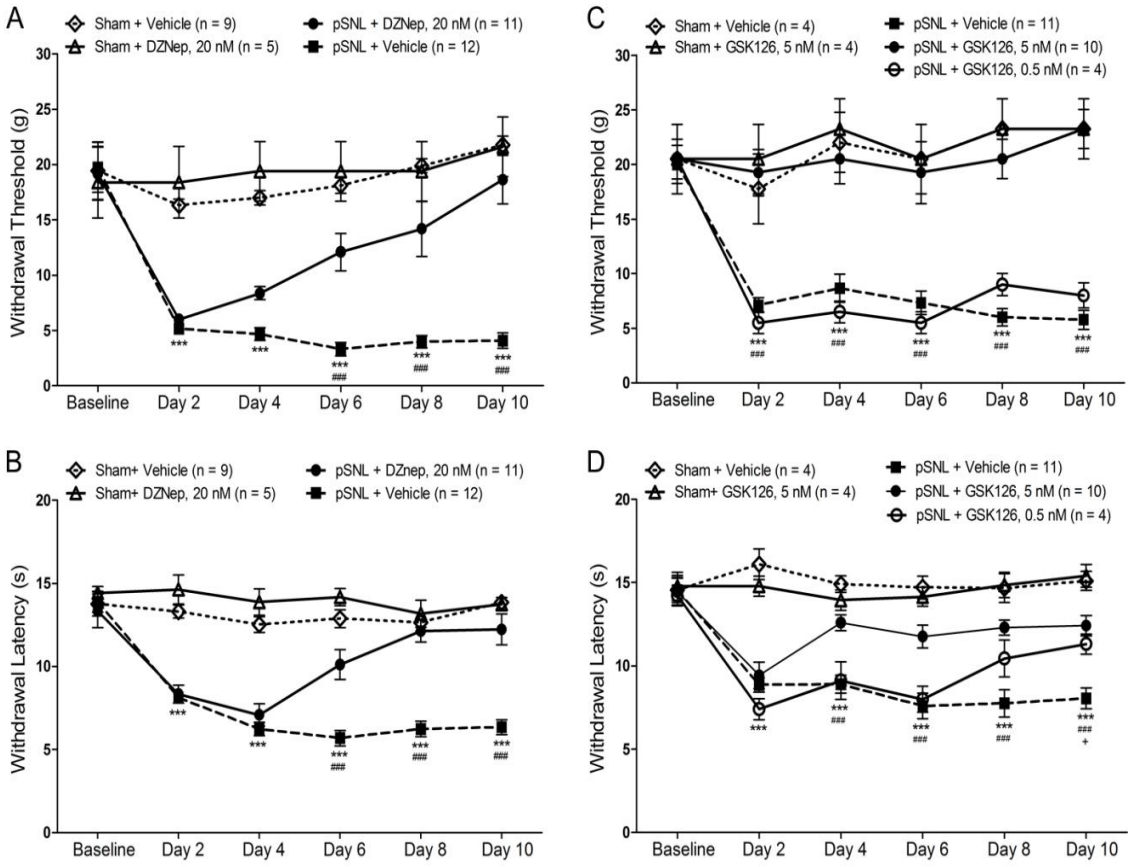

Figure 3.4: **EZH2 inhibitors prevent the pain hypersensitivity induced by nerve injury.** (A) and (B) show the mean (± S.E.) mechanical withdrawal threshold and thermal withdrawal latency during the 10-day observation period in four groups of rats treated with daily intrathecal injection of either 20 nM DZNep (in 10 μL) or vehicle (10 μL) for 9 days. Comparisons between baseline and each time point in the pSNL+ vehicle group are indicated with *; comparisons between the pSNL + 20 nM DEZNep and pSNL + Vehicle groups are indicated with #. (C) and (D) show the mean (± S.E.) mechanical withdrawal threshold and thermal withdrawal latency during the 10-day observation period in five groups of rats treated with daily intrathecal injection of either 5 nM GSK-126 (in 10 μL), 0.5 nM GSK-126 (in 10 μL), or vehicle (10 μL) for 9 days. Comparisons between baseline and each time point in the pSNL+ vehicle group are indicated with *; comparisons between the pSNL + 5 nM GSK-126 and pSNL + Vehicle group are indicated with #; comparisons between the pSNL + 0.5 nM GSK-126 and pSNL + vehicle group are indicated with +. One symbol: $p < 0.05$; three symbols: $p < 0.001$.

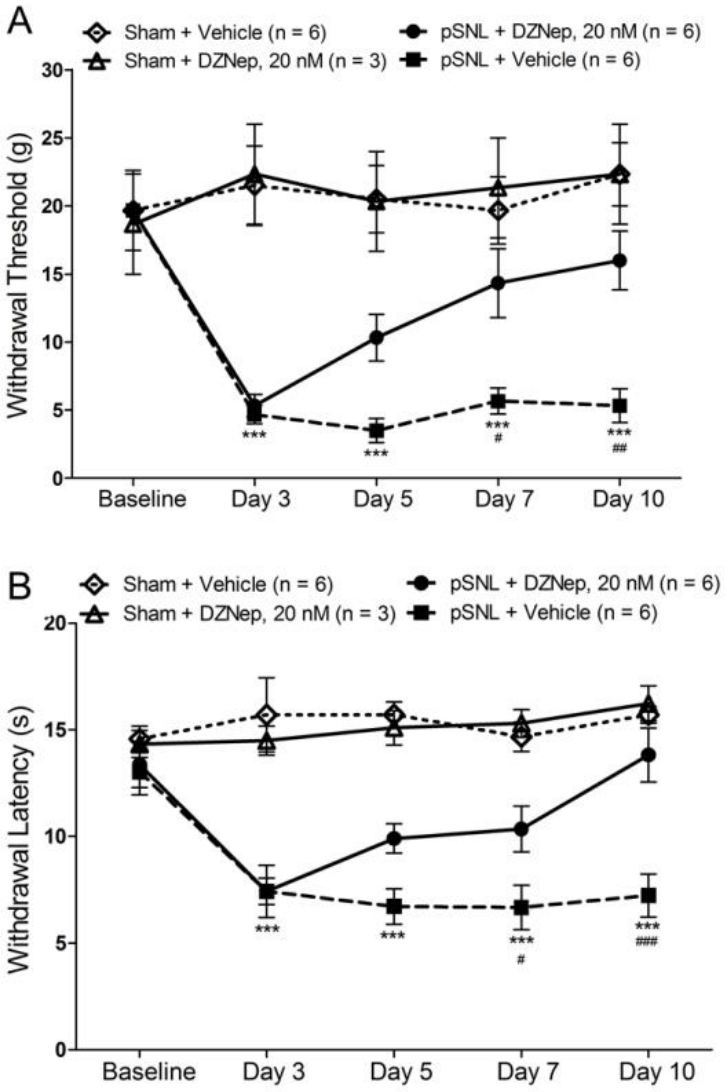

Figure 3.5: **EZH2 inhibitors attenuate the pre-existing pain hypersensitivity induced by nerve injury.** Line plots show the mean (± S.E.) mechanical withdrawal threshold (A) and thermal withdrawal latency (B) during the 10-day observation period in four groups of rats. Daily intrathecal injection of either 20 nM DZNep (in 10 μL) or vehicle (10 μL) was made from days 3 to 9. Comparisons between baseline and each time point in the pSNL+ Vehicle group are indicated with *; comparisons between the pSNL + 20 nM DEZNep and pSNL + vehicle group are indicated with #. One symbol: $p < 0.05$; two symbols: $p < 0.01$; three symbols: $p < 0.001$.

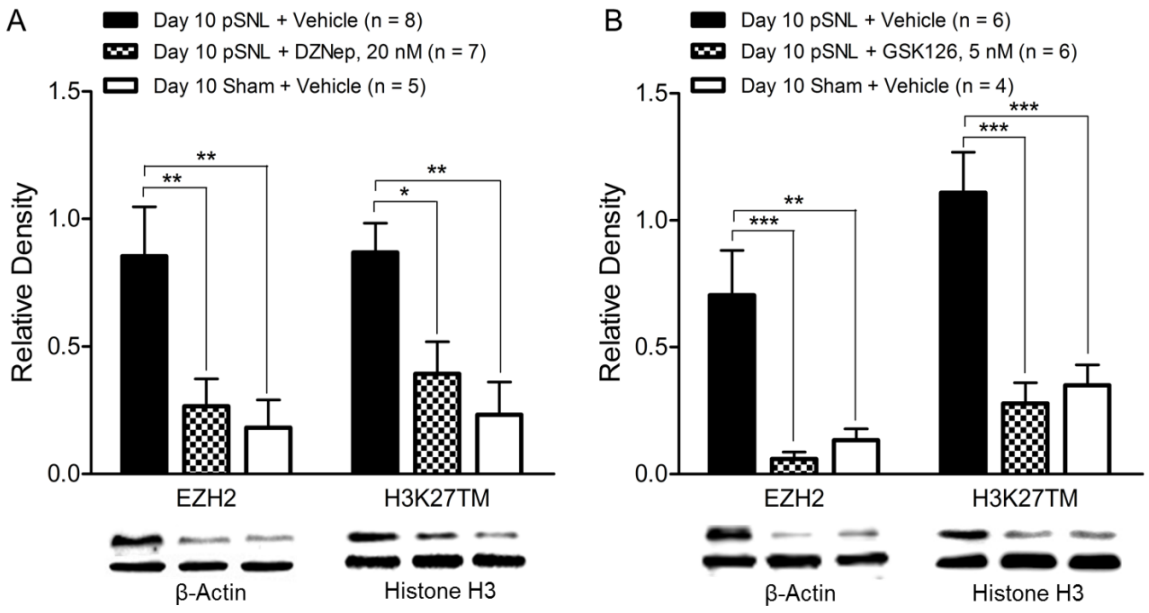

Figure 3.6: **Pre-emptive DZNep or GSK126 treatment significantly attenuates the increased levels of EZH2 and H3K27TM in the spinal dorsal horn induced by nerve injury.** (A): Data were obtained from the spinal dorsal horn of animals treated with daily intrathecal injection of either 20 nM DZNep (in 10 μL) or vehicle (10 μL) for 9 days. (B): Data were obtained from the spinal dorsal horn of animals treated with daily intrathecal injection of either 5 nM GSK126 (in 10 μL) or vehicle (10 μL) for 9 days. Bar graphs show the mean (+ S.E.) relative density ratio of EZH2 over β-actin and H3K27TM over total-histone H3. Western blot samples of each molecular protein in each group are displayed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

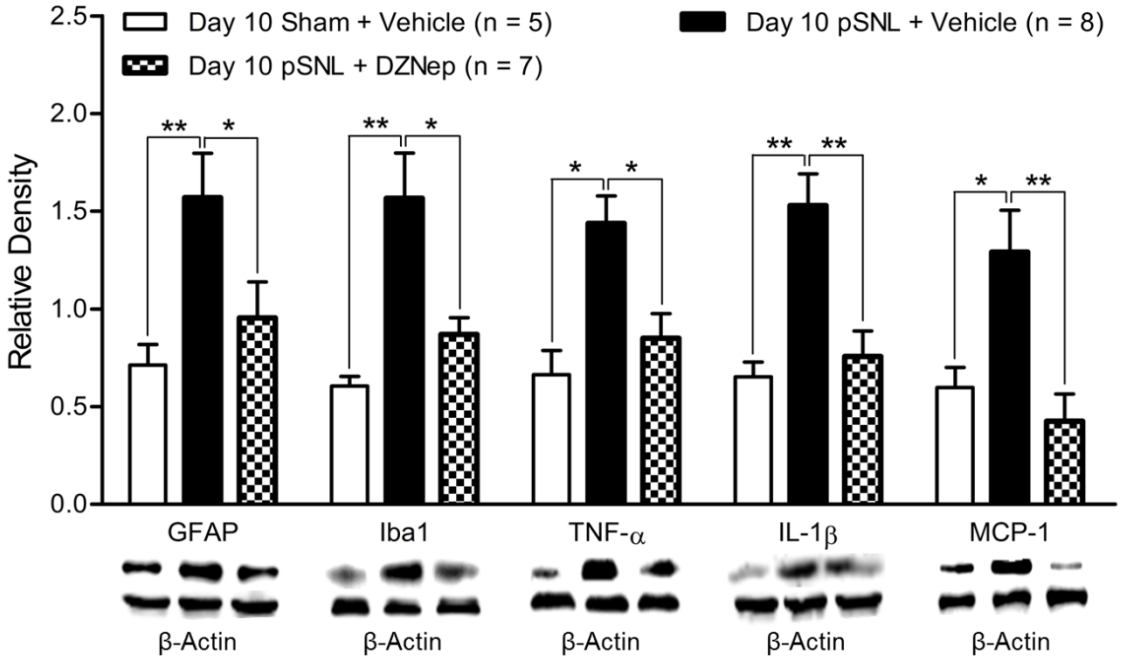

Figure 3.7: **Pre-emptive treatment of the EZH2 inhibitor attenuates the increased expressions of Iba1, GFAP, TNF-α, IL-1β and MCP-1 in the spinal dorsal horn of rats with nerve injury.** Data were obtained from the spinal dorsal horn of animals treated with daily intrathecal injection of either 20 nM DZNep (in 10 μL) or vehicle (10 μL) for 9 days. Bar graphs show the mean (+ S.E.) density of GFAP, Iba1, TNF-α, IL-1β and MCP-1 relative to β-actin in the spinal dorsal horn of sham operated rats treated vehicle, pSNL rats treated with DEZNep, and pSNL rats treated vehicle. Samples of each molecular protein in each group are displayed. *$p < 0.05$; **$p < 0.01$.

CHAPTER 4

PHARMACOLOGICAL MAMANGEMENT OF PAIN AND CURRENT CHALLENGES IN THE PAIN THERAPUETICS FILED

Introduction

The search for novel therapeutics with minimum or no side effects to treat and manage pathological pain conditions represents a largely unmet medical need: a lack of potent drugs to alleviate pain. Through failed clinical trials, we have learned that pathological pain is a multifaceted and complex condition. Several factors, such as complex etiology, genetic variation, environmental factors, lack of better understanding of complex pain mechanisms, limitations of basic biological research and tools for investigation, and lack of availability of good disease models representative of human-relevant pathological pain conditions are among the culprits that are hampering our progress in translational medicine to bring relief to patients suffering from chronic pain condition.

In the past, pain has been viewed as a symptom associated with another disease. Therefore, all the past drug development strategies were geared towards symptom reduction. Pain starts as a maladaptation in the peripheral or central nervous system nociceptive sensory processing, which can slowly transform all, one, or a combination of multiple networking systems including cognitive, motivational, reward/pleasure,

physiological rhythmic cycles, etc. (Baliki et al 2011, Cauda et al 2010, May 2008). Studies have shown that patients suffering from chronic pain condition often show lack of interest (Marbach et al 1983) and suffer from depression, posttraumatic stress disorder (Moeller-Bertram et al 2012), migraine (Maleki et al 2012) and irritable bowel syndrome (Blankstein et al 2010). Thus, identifying a drug target molecule(s) or a combination of drug target agents that can modulate multiple target proteins may have the potential to improve clinical outcome.

Our understanding of pain as a disease has opened doors for potential new therapeutic approaches, which can be divided into three basic strategies. The first approach includes finding novel ways of reformulation and/or drug delivery in a cost-effective manner to increase the efficacy and safety of existing drugs. Examples of novel reformulations include an oxycodone controlled-release formulation (OxyContin; Purdue Pharma) and a combination of morphine and naltrexone (Embeda; King Pharma), which are long-acting agents indicated for mild to moderate pain treatment. Drug delivery is also addressed by this approach. For example, the administration of NSAIDs for a prolonged period of time can cause serious/fatal gastrointestinal bleeding (Woodcock 2009). To overcome these side effects, a combination of histamine receptor H_2 antagonist famotidine with ibuprofen (Duvexa; Horizon Pharma) was approved by the FDA in 2011. This combination, when given to patients who are suffering from mild to moderate arthritis pain, has shown significant reduction in the number of NSAID-induced gastrointestinal ulcers.

The second approach includes finding new indications for known drugs. For example, duloxetine was previously used as an antidepressant and pregabalin as an

anticonvulsant; both got approval from the FDA to treat various types of neuropathic pain and fibromyalgia under the trade names Cymbalta (from Eli Lilly) and Lyrica (from Pfizer), respectively. The third approach to pain therapeutics includes the development of pharmaceutical agents with novel mechanisms of action. For examples, Ralfinamide, from Newron, is an inhibitor of sodium channels, including Nav 1.7 channel, N-type calcium channels and NMDA receptor blocker, and is under investigation for the treatment of neuropathic and other various types of chronic pain condition.

Below, I have summarized the current pain therapeutics being used for the treatment of neuropathic pain, their side effects and limitations. Additionally, I have provided a brief overview of the latest mechanistic-based therapeutics in preclinical and clinical trials, challenges to translational medicine and future directions in the pain therapeutics field.

Pharmacological Management of Chronic Pain

Current pharmacological approaches available to treat chronic pain generally can be categorized into non-opioid analgesics, opioid analgesics and nonsteroidal anti-inflammatory (NSAIDs) classes of drugs. Combination therapy has shown little superiority in pain-relief outcomes to monotherapy. In most patients, these medications are ineffective or only moderately effective in treating various types of neuropathic pain, such as diabetic polyneuropathic pain, post-herpetic neuralgia and post-traumatic peripheral neuropathies, etc. For the treatment of acute to chronic pain, patients are usually prescribed COX-1 and COX-2 inhibitors, which reduce inflammation and relieve painful sensations by preventing the synthesis of prostaglandins and thromboxane. These

classes of drugs are known as nonsteroidal anti-inflammatory drugs (NSAIDs) and include aspirin, ibuprofen, ketoprofen and naproxen-like pharmacological agents. However, these drugs have proven questionable efficacy in most patients and bring moderate to no relief in neuropathic pain condition. Long-term usage of NSAIDs causes side effects such as heartburn, stomach ulcer and high blood pressure, which sometimes result in severe to fatal gastrointestinal bleeding (Graham et al 2013, Ligon et al 2016b).

"Antidepressant and antiepileptic drugs have been used to treat neuropathic pain (NP). Despite their estimated pain relief of no more than 40-50%, these non-opioid drugs have been used in clinics for the pharmacological treatment of NP (Schestatsky et al 2014). Opioids are narcotic pain medicines recommended for moderate to severe pain treatment. These drugs relieve pain by interacting with opioid receptors present in the brain, spinal cord and other organs. The opioid class of drugs includes both legally available prescription drugs (oxycodone, hydrocodone, codeine, morphine, and fentanyl) and the illegally available class of drugs such as heroin. Prescription opioid drugs are effective in relieving certain types of pain, but not all. Most often, opioid drugs are misused due to their euphoric effects in addition to the pain relief. Longer-term usage of these drugs causes serious side effects, such as addiction and tolerance, and is associated with drug overdose fatalities.

According to International Association for the Study of Pain (IASP) Neuropathic Pain Special Interest Group (NeuroPSIG), the recommended treatment for neuropathic pain syndrome is summarized in Table 1 (Dworkin et al 2010).

Table 4.1 Recommended prescribed medication for treatment of neuropathic pain syndrome by IASP NeuroPSIG

First–Line Treatment	**Second-Line Treatment**	**Third-Line Treatment**
1. **Tricyclic Antidepressants** Amitriptyline, Nortriptyline, Desipramine 2. **Norepinephrine and Serotonin reuptake inhibitors** Duloxetine Venlafaxine 3. **Anticonvulsants** Gabapentin, Pregabalin 4. **Topical Lidocaine**	1. **Tramadol** 2. **Controlled-release Opioids** Morphine Oxycodone Fentanyl Hydromorphone	1. **Antidepressants** Bupropion Citalopram Paroxetine 2. **Anticonvulsants** Carbamazepine Lamotrigine, Oxcarbazepine Topiramate Valproic acid 3. **Topical low-concentration Capsain** 4. **Dextromethorphan** 5. **Memeantine** 6. **Mexiletine**

There are a few drugs that are approved to treat neuropathic pain and share a significant pharmaceutical market across the major world markets including United States, Japan, France, Germany, Italy, Spain, and the United Kingdom. Due to their limited efficacy in treating neuropathic pain, off-label prescriptions are widespread (Nightingale 2012). In the table below, I have briefly summarized seven major drugs that have been approved to treat neuropathic pain across global major markets (Nightingale 2012).

Table 4.2 Drugs approved across the United States, Japan, France, Germany, Italy, Spain, and the United Kingdom for neuropathic pain treatment

Drug	Mode of action	Formulation	Manufacturer
1) Lyrica (controlled-release Pregabalin)	GABA α2δ subunit agonist, calcium channel modulator	Oral	Pfizer

2) Cymbalta (antidepressant Duloxetine)	Selective serotonin & norepinephrine reuptake inhibitor	Oral	Eli Lilly
3) Lidoderm (5% lidocaine patch)	Voltage –gated Na^+ channels blocker	Transdermal Patch	Endo/Grunenthal/Teikoku
4) Qutenza (8% capsaicin patch)	Transient receptor potential vanilloid 1 (TRPV1) agonist	Transdermal Patch	NeurogesX/Acorda Therapy.
5) Nucynta ER (extended release tapentadol)	Opioid receptor agonist, noradrenaline reuptake inhibitor	Oral	Grunenthal (Johnson &Johnson/Janssen)
6) Gralise (Extended-release gabapentin)	GABA modulator	Oral	Depomed
7) Horizant (gabapentin enacarbil)	GABA modulator, voltage-gated calcium channel modulator	Oral	XenoPort (GlaxoSmith Kline)

Anticonvulsant pregabalin (Lyrica)

Pregabalin, marketed under the brand name Lyrica, is an antiepileptic drug (anticonvulsant) used in the treatment of seizures, fibromyalgia, diabetic neuropathy, herpes zoster neuralgia and neuropathic pain associated with spinal cord injury in adults (Attal et al 2010). It was approved in the US by the FDA in 2005. Gabapentin and pregabalin demonstrate similar efficacy and tolerability. However, in comparison to gabapentin, pregabalin shows more dose-responsive effects. Lyrica capsules are orally

administered in a dosage range from 300 mg to 600 mg for herpes zoster neuralgia. However, pregabalin is ineffective in the treatment of trigeminal neuralgia (Bennett et al 2013) and migraines (Linde et al 2013), and its effectiveness in treating post-surgical chronic pain and cancer pain is controversial (Bennett et al 2013, Clarke et al 2012).

Pregabalin, a gabapentenoid molecule, is a GABA analog. Pregabalin is a potent and selective ligand for alpha(2)delta-1 and alpha(2)delta-2 calcium channel subunits and exerts its analgesic effects by modulating the GABA α2δ subunit containing voltage – gated calcium channels (VDCC) (Bian et al 2006, Li et al 2011b). Pregabalin is rapidly absorbed on an empty stomach, and peak concentration is reached in plasma within one hour of oral administration. It undergoes negligible metabolism in human and is primarily eliminated by renal excretion. Relative to benzodiazepines, pregabalin has less potential for abuse but still is associated with physical dependence (2005) and fatal overdose risks (Miljevic et al 2012). Adverse effects such as dizziness, sedation, peripheral edema, weight gain, dry mouth, and headache have been observed with pregabalin administration.

Gralise (extended-release gabapentin)

An immediate-release formulation of Neurontin was approved by the FDA in 2011 for post-herpetic neuralgia. Gabapentin is an anticonvulsant drug initially tested for psychiatric along with conjunctive anticonvulsant therapies (Chouinard et al 1998, Pande et al 1999, Pande et al 2000). Other therapeutic uses of gabapentin include painful diabetic neuropathy (Backonja et al 1998) and treatment of migraine (Mathew et al 2001). It is an analog of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Satzinger 1994) but has not shown any agonist-like effects on GABA receptors (Jensen

et al 2002, Lanneau et al 2001). Although the mechanism of analgesic action of gabapentin is unknown, it has been shown to prevent allodynia and hyperalgesia in neuropathic pain models (Rose & Kam 2002). In vitro studies suggest that gabapentin binds with high affinity to the α2δ subunits of voltage-activated calcium channels (Gee et al 1996) and proposed to activate descending noradrenergic inhibition via disinhibition of Locus coeruleus (LC) neurons(Yoshizumi et al 2012).

The total dosage of 1800mg/day of gabapentin, administered three times a day, has been proven to be efficacious for post-herpetic neuralgia. Gralise is a gastro-retentive (GR) formulation of gabapentin with an effective dosage of 1800mg once a day with the evening meal and was approved by the FDA in 2011 (Argoff et al 2012, Chen et al 2013b).

Swellable polymer-based technology applied to Gralise has optimized its drug delivery to the upper small intestine, where maximum gabapentin absorption takes place. Due to this modification, after oral administration Gralise can be retained in the stomach for approximately 8-10 h if taken with a meal (Abrahamsson et al 1993, Chen et al 2013a). This specific formulation does not alter the area under the concentration curve (AUC). The most frequent side effects associated with Gralice administration are dizziness, somnolence, nausea and headache (Anderson & Saneto 2015). With similar efficacy and tolerability to the immediate-release formulation of gabapentin, the primary advantage of taking Gralise is the once-daily dose instead of thrice-daily doses for the immediate-release formulation of gabapentin (Leppik & Hovinga 2013).

Horizant (gabapentin enacarbil)

Gabapentin encarbil is an oral prodrug of gabapentin under brand name Horizant. Horizant was approved in the USA by the FDA in 2011 for the treatment of moderate-to-severe primary restless leg syndrome and for the management of postherpetic neuralgia in adults. The recommended dosage for post-herpetic neuralgia patients taking Horizant extended-release tablets starts at 600 mg daily and ranges up to 600 mg twice/day orally with food (Backonja et al 2011). Horizant is not approved outside of the USA. This formulation was discovered and manufactured by XenoPort/GSK. After oral administration, the prodrug gabapentin enacarbil undergoes extensive first pass metabolism by carboxylesterases and releases the byproducts of gabapentin, carbon dioxide, acetaldehyde and isobutyric acid. Gabapentin itself is not metabolized and is excreted by the kidneys via an organic cation transporter (OCT2). The elimination half-life of gabapentin ranges from 5.1 to 6.0 hours. Side effects such as dizziness, drowsiness, dry mouth, headache, weight gain, etc., have been reported (Backonja et al 2011). Gabapentin encarbil has moderate efficacy and its long-term usage side effects for patients suggest that there is further need to improve this pharmacological agent.

Lidoderm (5% Lidocaine patch)

Lidocaine, also known as xylocaine, is primarily used as a local nerve block to numb tissue in a specific area (local anesthesia). For direct intravenous injection, either lidocaine hydrochloride or lidocaine sodium hydroxide sterile nonpyrogenic solution is administered intravenously. By inhibiting the ionic fluxes, lidocaine application interferes with the initiation and conduction of the action potential in nerves. However, intravenous administration of lidocaine (5mg/kg) shows adverse cardiac antiarrhythmic side effects. The first lidocaine patch was approved by the FDA in 1999 for the treatment of

postherpetic neuralgia. Since then, several formulations of patches have been used effectively without the risk of development of tolerance, physical dependence or addiction that other analgesics such as opioids carry (Galer & Gammaitoni 2003). Part of the reason these patches were effective is due to their ability to intercept the initiation and transmission of nociceptive information from the peripheral nervous system to the central nervous system (Argoff 2000).

Lidoderm, 5% lidocaine patch, was approved by the FDA for the treatment of postherpetic neuralgia for targeted peripheral analgesic treatment and has been recommended as a first-line therapy for the treatment of neuropathic pain from post-herpetic neuralgia (Davies & Galer 2004). The Lidoderm patch (10x14 cm) is adhesive with 5% lidocaine (700mg/patch) along with other inactive ingredients enmeshed in a nonwoven polyethylene-backed patch (Comer & Lamb 2000). Up to three patches can be applied at once to effectively cover as much of the painful area as possible. These patches may be applied to intact skin for 12 hours, followed by an interval of 12 hours with no patch (Rowbotham et al 1996). Application of 5% lidocaine in combination with other analgesics has been shown to be effective in reducing the intensity of pain in patients suffering from postherpetic neuralgia, painful diabetic neuropathy, and lower back pain conditions (Argoff et al 2004). Mild side effects such as redness, swelling, irritation or numbness have been observed at the application site. However, there are some shortcomings of utilizing 5% lidocaine as an analgesic for pain relief, as in some patients it does not provide lasting 24-hour analgesic relief due to its currently approved dosing regimen, 12-hour application of patch followed by a 12-hour off period. Two clinical trials with patch application for 18 hours and 24 hours have shown their safety,

tolerability with minimum risk of systemic adverse side effects and drug-drug interaction (Gammaitoni et al 2002, Gammaitoni & Davis 2002). However, further studies and clinical trials need to be done to explore the effectiveness of 5% lidocaine patches as an analgesic, either alone or in combination with other treatment options for other different types of neuropathic conditions.

Quentza (8% capsaicin patch)

Capsaicin is an irritant, naturally found in hot peppers, and is a ligand for transient receptor potential channel vanilloid receptors (TRPV). Capsaicin receptors, TRPV1, are abundantly present in nociceptive nerve terminals (Aδ and C fibers) in the skin, dorsal root and trigeminal ganglia (Schumacher & Pasvankas 2014), and have been implicated in neuropathic pain pathology. In addition to capsaicin, TRPV1 can also be activated by acids, various endogenous mediators and high temperature (Alawi & Keeble 2010). Historically, the use of capsaicin as a balm for pain relief has been known for centuries, but only in the late 19th century did scientists begin to systematically explore its usage and function in the desensitization process of neurons (Alawi & Keeble 2010).

Quentza (8% capsaicin patch) is manufactured by NeurogesX/Astellas Pharma and was approved in the US by the FDA in 2010 for the treatment of postherpetic neuralgia (PHN). A single application of the 8% capsaicin patch for 60 minutes relieves pain in patients suffering from PHN condition. 90 days after the first application of the patch, the 8% capsaicin patch can be reapplied in case pain returns. At the beginning, topical application of capsaicin causes erythema and intense burning pain and discomfort followed by the desensitization of sensory neurons, resulting in the inhibition of pain transmission from the peripheral to central nervous system (Laklouk & Baranidharan

2016). This transient increase in pain usually persists for more than two days after capsaicin patch application before any analgesic effects begin for PHN patients (Irving et al 2012). The patches are usually applied in clinical settings. To reduce the painful side effects before application of the 8% capsaicin patch, the patient is given either a local lidocaine injection or a prescribed oral analgesic. High-dose capsaicin patches are safe and effective for PHN (Simpson et al 2010), however, due to their initial discomfort, the side effects of pain and poor tolerance to a local irritant associated with Quentza patches are expected to limit their future use.

Cymbalta (antidepressant duloxetine)

Cymbalta (duloxetine) is a selective serotonin and norepinephrine reuptake inhibitor (SSNRI) (Wong et al 1993). Duloxetine is used to treat depression and anxiety disorders, both in adult and children older than seven years of age. It is also prescribed to patients who are suffering from bone and muscle pain. It is one of the top-selling drugs, manufacture by Eli Lilly pharmaceutical company, and was approved by the FDA in 2004 to treat depression. Within a year of this approval, the FDA approved this drug for diabetic neuropathy treatment. In 2007, the FDA also gave approval for the treatment of fibromyalgia. Finally, in 2010, Cymbalta was also approved by the FDA for chronic musculoskeletal pain, including osteoarthritis, despite the many reports of serious side effects such as liver damage and skin diseases.

Duloxetine can bind with high affinity to serotonin (5-HT) and norepinephrine (NE) uptake transporters, subsequently increasing norepinephrine and 5-HT concentration and thereby suppressing neuronal firings (Bymaster et al 2001). A maximum plasma concentration is achieved six hours after oral administration of

duloxetine, (Zhao et al 2009). More than 90% of duloxetine binds to blood plasma proteins, such as albumin or α1-acid-glycoprotein. Following the absorption of duloxetine, it is quickly metabolized in the liver by cytochrome p-450 isoenzymes 2D6 and 1 A2 (Owens et al 1997, Skinner et al 2003). The half-life of duloxetine is 12 hours. 70% of duloxetine metabolites are excreted in urine and approximately 20% is eliminated via feces (Lantz et al 2003).

Duloxetine doses from 60mg-120mg have been shown to be efficacious for treating pain in diabetic peripheral neuropathy (Lunn et al 2014). Side effects such as nausea, dry mouth, and somnolence were the most common (Detke et al 2002). Following its patent expiration in June 2013, there are now generic versions of the drug available in the US market.

Nucynta ER (Tapentadol)

In 2008, tapentadol hydrochloride's immediate release was approved by the FDA in the United States to treat moderate to severe acute pain (Wade & Spruill 2009). Opioids, such as morphine, are very effective in treating acute pain but are only moderately efficacious in treating chronic pain condition induced by nerve injury or inflammation. Additionally, the development of tolerance, addiction, nausea, dizziness and emesis associated with opioid intake makes them unpopular choices to treat chronic pain conditions (Raffa et al 2012). Tapentadol is a novel class of drug with dual mechanisms of action. It combines the action of the μ-opioid receptor (MOR) agonist and a noradrenaline reuptake inhibitor and thus offers a broad therapeutic spectrum for nociceptive and neuropathic pain. In comparison to opioid analgesics, tapentadol interacts weakly with MOR.

Tramadol (Ultram), which showed side effects similar to opioids, was later sequentially modified to develop tapentadol in the late 1980s (Chang et al 2016). Tapentadol (100-250 mg twice daily) is effective for the management of moderate to severe chronic pain (Afilalo et al 2010, Buynak et al 2015). It is rapidly absorbed; a mean maximum plasma concentration is reached within 1.25-1.5 hours after oral ingestion. 95% of the tapentadol is excreted by the kidneys within 24 hours (Terlinden et al 2007). Tapentadol, a long-acting opioid with reduced classical opioid-associated side effects and less potential for abuse, provides key clinical advantages and is most likely to be prescribed for the long-term treatment of neuropathic and nociceptive pain. However, the risk of addiction, abuse, life-threatening respiratory depression, overdose and death are still associated with this formulation. Other side effects, such as hypotension, sedation, and coma, are associated with tapentadol ingestion. Therefore, it is indicated to use as needed where alternative options are ineffective, not tolerated or otherwise have failed to provide adequate pain management.

Evidence of Mechanistic-based Therapeutics in Preclinical and Clinical Studies of Pain

Advances in understanding the pathophysiology of pain helps to identify target discovery for pain treatment. In recent decades, biomedical research in chronic pain has greatly improved our understanding of pain mechanisms. We already know that the hyperactivation of nociceptor cells transforms nociceptive inputs and consequently affects the experience of pain in humans. Genetic mutations that alter pain sensations, such as calcium channel and sodium channels, found in specific patient populations have

exposed new therapeutic targets for the development of new medication for the treatment of pain, for example, mutation in the P/Q type of calcium channels alter Ca^{2+} influx and neurotransmitter release in familial hemiplegic migraine (Barrett et al 2008). Ca^{+2} ions play an important role in membrane potential and activate several pathways by acting as secondary messengers. Identification of N-type calcium channels in nociceptive pain and selectively targeting them using ziconotide peptide is one example where a mechanistic study was translated in the clinic for the treatment of pain (Zamponi 2016).

A genome wide meta-analysis in more than 300,000 individuals has identified 38 susceptible loci for migraine (Gormley et al 2016). Peripheral loss or gain of function in the sodium channels Nav1.7 encoding gene can result in congenital insensitivity to pain or abnormal pain sensation, respectively (Eberhardt et al 2014). Similarly, gain of function in Nav1.8, Nav1.9 and Nav1.6 are associated with abnormal high-frequency firing patterns in peripheral neurons and pain (Dib-Hajj et al 2015, Tanaka et al 2016, Yang et al 2016). Inhibition of sodium channels could prove to be a viable approach for pain therapy, but requires further testing in human trails.

Nerve growth factor (NGF) play role in the development and maintenance of sympathetic and sensory neurons, and has been found in association with increased sensitivity to pain sensation (Rukwied et al 2010). Several clinical trials are in the process of evaluating the therapeutic effects of anti-nerve growth factor in various diseases, including pain. For treating knee osteoarthritic chronic pain, human anti-nerve growth factor monoclonal antibody AMG 403 is undergoing in a phase 1 clinical trial to evaluate its safety tolerability, and pharmacokinetic profile in human subjects.

Calcitonin gene-related peptide and angiotensin pathways have been implicated in pain conditions. A randomized double-blind, placebo-controlled phase 2 clinical trial is underway to determine the effectiveness of AMG 334, a fully human monoclonal antibody, against the CGRP receptor. Results from this trial suggest that a 70 mg subcutaneous injection of AMG 334 might be a potential therapy for migraine prevention in patients with episodic migraine (Sun et al 2016). In an animal model, the angiotensin II type 2 receptor inhibitor neutralizes capsaicin responses (Anand et al 2013) and hyperalgesia condition in neuropathic pain model (Sun et al 2016). The angiotensin II type 2 receptor exerts its analgesic effects through inhibiting the p38 mitogen activated protein kinase and p44/42 MAPK activation pathway (Sun et al 2016).

Anandamide (AEA) is a lyophilic endogenous molecule and an essential part of the endocannabinoids system. In response to stress and noxious stimuli, AEA molecules are released locally and relieve painful sensations by modulating calcium and potassium channel activity and suppressing the proinflammatory mediators (Piomelli & Sasso 2014). These endogenous cannabinoids are very short-lived and are quickly degraded by the fatty acid amide hydrolase (FAAH). Anandamide exerts its effects by binding to cannabinoids receptors (CB1, CB2) and partially by binding to transient receptor potential cation channel subfamily V membrane 1 (TRPV1) (Hohmann et al 2004, McDowell et al 2013). In animal models, inhibition of FAAH increased the endogenous level of cannabinoid and created analgesic effects. In clinical phase 1 & 2 trials on human subjects, when BIA 10-247 was administered orally twice a day for 10 days, severe neurological side effects occurred, such as headache, cerebellar syndrome, memory impairment, and altered consciousness. In this study, one patient died during the trial due

to the adverse side effects of the investigational new drug and the trial was aborted (Kerbrat et al 2016). This recent failure in clinical trials suggests that the identification of drug targets and mechanistic study done in preclinical model, like cell culture and animal models, do not always necessarily translate into the clinic.

With current analgesisc available in the market it is clear that alternate therapeutic approaches are required with new mechanisms of action. Recent findings demonstrate that epigenetic mechanisms are involved in chronic pain state and could be manipulated to develop new therapy for patients. However the epigenetic therapeutic are at their infancy stages. Due to lack of selective of epigenetic compounds and their limited side effects profile availability, epigenetic therapeutic compounds cannot be used in clinical settings now. A greater understudy of epigenetic and genetics mechanism in pain which could help us unravel novel therapeutic approaches for the treatment of chronic pain conditions.

Challenges in Chronic Pain Translational Research

Despite a large push and billions of dollars of spending on preclinical research and early clinical drug development for neuropathic pain, a huge translation gap exists. Several factors have been identified that impede the management and treatment of neuropathic pain conditions (Baron 2009, Borsook et al 2014, Melnikova 2010, Nascimento et al 2016). Some of these factors are listed below:

- Due to discrepancies in defining neuropathic pain among researchers and clinicians and lack of clear guidelines to classify different types of neuropathic pain often resulting into misdiagnosis and inadequate treatment care. According to the International Association for the Study of Pain (IASP), neuropathic pain

disorders occur after lesion or dysfunction to the nociceptive system in the peripheral and central nervous system. Based on the current criteria of diagnosis and outcome measurements, there is no standardized approach available in the neuropathic field. Therefore, in clinical practice it is difficult to distinguish neuropathic pain from other types of chronic pain conditions and effectively treat it. Additional there are no consensus among clinicians and researchers about measuring outcomes with respect to pain intensity.

- Lack of sensitive diagnostic tools to measure pain perception. As pain perception is very subjective and changes from one subject to another, there is significant variation in responses to noxious stimuli and pain treatments. Additionally, pain perception is a very complex and dynamic process that alters the processing of sensory information over a period of time and is very much affected by other environmental and social cues, which make it further challenging to diagnose patients suffering from various types of chronic pain conditions.
- In the preclinical research field the, lack of an animal model which can truly represent the patient's symptoms and mechanism of pain is truly a limiting factor in translating research findings in patient practice . Animal models are distinctively very different from human subjects, and it is always a challenge to how far we can extrapolate the animal research findings the in clinic.
- Limitation of resources due to cuts in federal funding in pain research area. Lack in data sharing/confidentiality vs. open access is posing hurdles in the progression of pain research field.

Concluding Remarks

Beside these challenges we need to re-evaluate the current strategies being used in translating findings from basic biomedical sciences to the clinic. We need to take hard look on why despite of increased in spending money on basic pharmaceutical research and investment in clinical translation are not producing new drug entities in the market to treat chronic pain condition. For example: We need to reevaluate our process and protocols on the clinical end on how to store patient samples, how to properly evaluate patient's phenotypic the clinical features with chronic pain conditions, how to improve clinical trial recruitment process, how to identify representative patient populations and how to establishes standardized protocols for acquisition of biological samples and data analysis. Most importantly we need to improve on the process of sharing negative and positive research findings in this area.

In general we need to build on efficient partnership between academia, industry and government (FDA, NIH) in order to identify novel candidates of drugs and to bring them in the market in a cost effective manner.

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APPENDICES

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Regulator of G Protein Signaling 10 (*Rgs10*) Expression Is Transcriptionally Silenced in Activated Microglia by Histone Deacetylase Activity

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ABSTRACT

RGS10 has emerged as a key regulator of proinflammatory cytokine production in microglia, functioning as an important neuroprotective factor. Although RGS10 is normally expressed in microglia at high levels, expression is silenced in vitro following activation of TLR4 receptor. Given the ability of RGS10 to regulate inflammatory signaling, dynamic regulation of RGS10 levels in microglia may be an important mechanism to tune inflammatory responses. The goals of the current study were to confirm that RGS10 is suppressed in an in vivo inflammatory model of microglial activation and to determine the mechanism for activation-dependent silencing of *Rgs10* expression in microglia. We demonstrate that endogenous RGS10 is present in spinal cord microglia, and RGS10 protein levels are suppressed in the spinal cord in a nerve injury–induced neuropathic pain mouse model. We show that the histone deacetylase (HDAC) enzyme inhibitor trichostatin A blocks the ability of lipopolysaccharide (LPS) to suppress *Rgs10* transcription in BV-2 and primary microglia, demonstrating that HDAC enzymes are required for LPS silencing of *Rgs10*. Furthermore, we used chromatin immunoprecipitation to demonstrate that H3 histones at the *Rgs10* proximal promoter are deacetylated in BV-2 microglia following LPS activation, and HDAC1 association at the *Rgs10* promoter is enhanced following LPS stimulation. Finally, we have shown that sphingosine 1-phosphate, an endogenous microglial signaling mediator that inhibits HDAC activity, enhances basal *Rgs10* expression in BV-2 microglia, suggesting that *Rgs10* expression is dynamically regulated in microglia in response to multiple signals.

Introduction

Microglia are central nervous system (CNS)–resident macrophages that serve protective functions to combat infection and clear cellular debris, as well as developmental functions, including synaptic pruning (Gehrmann et al., 1995; Stevens et al., 2007; Trang et al., 2011). In addition to these normal physiologic functions, dysregulated microglial activation has been implicated in the initiation and progression of neurodegenerative disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Fu et al., 2014), and in neuropathic pain (Trang et al., 2011). Identifying signaling pathways regulating microglial functions bears significance in the development of strategies for the treatment of such neurologic disorders.

Regulator of G protein signaling (RGS) 10 has emerged as an important anti-inflammatory regulator in microglia. RGS10 is a member of the RGS superfamily of proteins that deactivate heterotrimeric G proteins, with profound effects on G protein–coupled receptor (GPCR) signaling in neural diseases (Zachariou et al., 2003; Nishiguchi et al., 2004; Hurst and Hooks, 2009; Okahisa et al., 2011; Vellano et al., 2011). RGS proteins are a highly diverse group of proteins that regulate signaling pathways downstream of GPCRs. The classic role of RGS proteins is to regulate the duration and amplitude of G protein signaling through their ability to function as GTPase-activating proteins to accelerate the deactivation of G proteins by increasing the rate of GTP hydrolysis (Posner et al., 1999). RGS10 selectively deactivates Gi family G proteins (Hunt et al., 1996), and it is expressed at high levels in the brain (Gold et al., 1997) and immune tissues (Haller et al., 2002), with specific enrichment in microglia (Waugh et al., 2005).

Recent studies suggest that RGS10 protein in microglia serves to suppress microglial activation, proliferation, and nuclear factor-κB (NF-κB) activity downstream of Toll-like receptor 4 (TLR4) receptors, and loss of RGS10 enhances microglial-mediated neuroinflammation and neurotoxicity.

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ABBREVIATIONS: 5-Aza, 5-Aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; CNS, central nervous system; DNMT, DNA methyl transferase; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein–coupled receptor; HDAC, histone deacetylase; IL, interleukin; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MS, multiple sclerosis; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pSNL, partial sciatic nerve ligation; RGS, regulator of G protein signaling; RT-PCR, reverse-transcription PCR; S1P, sphingosine 1-phosphate; TLR, Toll-like receptor; TNF, tumor necrosis factor; TSA, trichostatin A.

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Interleukin-1beta enhances endocytosis of glial glutamate transporters in the spinal dorsal horn through activating protein kinase C

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Abstract

Excessive activation of glutamate receptors in spinal dorsal horn neurons is a key mechanism leading to abnormal neuronal activation in pathological pain conditions. Previous studies have shown that activation of glutamate receptors in the spinal dorsal horn is enhanced by impaired glial glutamate transporter functions and pro-inflammatory cytokines including interleukin-1 beta (IL-1β). In this study, we for the first time revealed that spinal glial glutamate transporter activities in the neuropathic animals are attenuated by endogenous IL-1β. Specifically, we demonstrated that nerve injury results in an increased expression of IL-1β and activation of PKC in the spinal dorsal horn as well as suppression of glial glutamate uptake activities. We provided evidence that the nerve-injury induced suppression of glial glutamate uptake is at least in part ascribed to endogenous IL-1β and activation of PKC in the spinal dorsal horn. IL-1β reduces glial glutamate transporter activities through enhancing the endocytosis of both GLT-1 and GLAST glial glutamate transporters. The IL-1β induced trafficking of glial glutamate transporters is through the calcium/PKC signaling pathway, and the dynamin-dependent endocytosis, which is dependent on the integrity of actin filaments. The signaling pathway regulating glial glutamate transporters revealed in this study provides novel targets to attenuate aberrant activation of glutamate receptors in the spinal dorsal horn, which could ultimately help the development of analgesics.

Introduction

Interactions between neurons and glial cells are crucial mechanisms underlying synaptic plasticity in the spinal dorsal horn in pathological pain conditions (Ren and Dubner, 2010, Chen et al., 2012, Kanda et al., 2013, Tsuda et al., 2013, Grace et al., 2014). Excessive activation of glutamate receptors in spinal dorsal horn neurons is a key mechanism leading to abnormal neuronal activation in the pain signaling system (Moore et al., 2000, Salter,

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The authors declare that there are no conflicts of interest.