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REACTIVE OXYGEN SPECIES INVOLVEMENT IN GABAERGIC DYSFUNCTION IN NEUROPATHIC PAIN

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REACTIVE OXYGEN SPECIES INVOLVEMENT IN GABAERGIC DYSFUNCTION IN NEUROPATHIC PAIN

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Dedication

To my parents, Mr. Somchai and Mrs. Laddaval Yowtak who have taught me about hard work and sacrifice and have supported me throughout my education

To my dear brother, Kris, who has endured many long phone calls from me and has dispensed much brotherly wisdom and encouragement

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Reactive Oxygen Species Involvement in GABAergic Dysfunction in Neuropathic Pain

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Neuropathic pain caused by peripheral nerve damage results in ectopic neuronal excitability, primary sensory neuron degeneration, loss of inhibition by spinal GABAergic neurons and more importantly, the development of central sensitization—increased sensitivity of dorsal horn neurons to stimuli. Oxidative stress due to excessive levels of reactive oxygen species (ROS) has been implicated in the development and maintenance of neuropathic pain. However, it is not known whether oxidative stress is related to the loss of GABAergic tone in the spinal cord. Therefore, the major goal of this work was to elucidate the effects of ROS on GABAergic neuron function and expression.

The spinal nerve ligation model (SNL) was useful to study chronic neuropathic pain. SNL mice were produced by tight ligation of the L5 spinal nerve, resulting in increased pain behaviors lasting for many weeks. The paw withdrawal response rates to von Frey filaments measured pain behaviors in the form of mechanical allodynia. Scavenging ROS or increasing spinal GABA neurotransmission produced analgesia in the SNL model. On the other hand, increasing spinal ROS levels or reducing GABA neurotransmission temporarily induced pain behaviors in normal mice. Field recordings demonstrated that the spinal cord dorsal horn neurons were sensitized in SNL mice, and scavenging ROS reduced central sensitization. Blocking GABA neurotransmission significantly reduced this desensitization, indicating that ROS acted mainly upstream to postsynaptic, spinal GABA receptors. Whole cell recordings revealed that elevated levels of ROS increased dorsal horn neuronal excitability but also reduced GABA neuronal excitability. This suggested that ROS may directly contribute to reduced GABA function. Stereological analysis demonstrated that the number of fluorescently tagged GAD67-containing (GABA) neurons is reduced after SNL in the affected spinal dorsal horn. Furthermore, treatment with a ROS scavenger significantly reduced the magnitude of the allodynic behaviors and the SNL-induced loss of GAD67 expression. Therefore, the loss of spinal GABAergic inhibition seen in neuropathic pain may be partly attributed to oxidative stress reducing GABA neuron excitability and promoting the loss of GAD67-producing neurons or down-regulating GAD67 expression. Overall, these

studies suggest that ROS play an important role in GABAergic dysfunction that contributes to neuropathic pain.

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CHAPTER 1

INTRODUCTION

1.1 CHRONIC NEUROPATHIC PAIN

1.1.1 DEFINITION AND FEATURES

The International Association for the Study of Pain defines neuropathic pain as pain that is "initiated or caused by a primary lesion or dysfunction in the nervous system," and it is considered chronic if the pain lasts longer than the time required for healing or longer than three months after injury. This definition of neuropathic pain has recently been criticized as too vague since the term "dysfunction" does not clearly distinguish neuropathic pain from other types of pain, such as inflammatory pain that also has components of neurological dysfunction (Backonja 2003). A more current definition of neuropathic pain has been proposed, one that is based on the type of clinical evidence presented in the patient. Therefore, the characteristics that would define neuropathic pain include (1) pain and sensory symptoms that lasts longer than the time required for healing, (2) the presence of neurological sensory signs, and (3) the presence of other neurological signs, including motor and autonomic symptoms (Backonja 2003).

Spontaneous pain may occur in neuropathic pain patients, and the pain has been described as electric shock-like, burning, cold, pricking, tingling or itching (Merskey 1994; Dworkin 2002). Examples of positive neurological sensory signs many patients experience include thermal or mechanical stimulus-dependent pain. The term *allodynia* refers to pain felt

following a stimulus that normally does not cause pain, and the term *hyperalgesia* describes the experience of feeling more intense pain from a stimulus that normally does cause pain (Merskey 1994; Dworkin 2002). Patients may also experience negative neurological sensory signs such as sensory loss. Furthermore, other neurological signs associated with neuropathic pain include weakness and muscle atrophy, referred sensation, swelling, skin flare and discoloration, hyperhydrosis and hypohydrosis and trophic changes.

There are many different causes of neuropathic pain, including cancer, trauma, autoimmune diseases such as multiple sclerosis, metabolic diseases such as diabetic neuropathy, infections such as post-herpetic neuralgia, and vascular diseases such as stroke (Campbell and Meyer 2006). While neuropathic pain can be distinguished by central or peripheral causes, this study will concentrate on neuropathic pain resulting from a peripheral nerve lesion.

For this study, behavioral experiments attempt to quantify an animal's level of pain through its withdrawal responses to the pressure applied by thin filaments of various forces. It is arguable whether these responses should be called allodynia or hyperalgesia, but the animal's responses to these filaments under normal conditions are either none or small, and for the sake of convenience, such responses in neuropathic animals will be termed "allodynia".

Neuropathic pain, resulting from a lesion in the peripheral nervous system currently affects roughly 4 million people in the United States (Eisenberg et al. 2005). Pain is a major public health problem, accounting for over $200 billion dollars annually in direct medical costs and indirect costs with neuropathic pain contributing a substantial burden (McCarberg and Billington 2006). It remains a challenging clinical problem since conventional analgesics

do not always provide relief. Therefore, understanding the mechanisms behind the pathogenesis of neuropathic pain is important for developing more effective treatment options.

1.1.2 THE ANATOMY OF SENSORY TRANSMISSION

Three main types of sensory fibers in the peripheral nervous system convey information from skin to the spinal cord – the large diameter, fast conducting, myelinated Aβ fibers, the smaller diameter, thinly myelinated Aδ fibers and the smallest diameter, slowest conducting, unmyelinated C fibers. The majority of Aβ fibers respond to light touch due to low activation thresholds and convey tactile information. However, some Aβ fibers convey nociceptive information (Djouhri and Lawson 2004). Furthermore, some Aδ and C fibers which generally have higher activation thresholds than the Aβ fibers and are thought to convey nociceptive information, may also serve as mechanoreceptors (Willis and Coggeshall 2004). Cutaneous nociceptors may respond to thermal, chemical or mechanical stimuli or a combination of these stimuli (Devor and Seltzer 1999; Willis and Coggeshall 2004).

Many different types of Aδ nociceptors exist, such as mechanical nociceptors that respond well to mechanical stimuli that damage skin, mechano-heat nociceptors that respond well to high rates of heat stimulation and intense cold temperatures, and mechanically insensitive nociceptors that respond to chemicals and heat (Willis and Coggeshall 2004). Aδ cold, mechano-heat-cold and mechano-cold receptors have the ability to respond to extremely cold temperatures (Willis and Coggeshall 2004).

Likewise, many different C nociceptors exist, which include mechano-thermal receptors that respond best to noxious stimuli of all modalities, mechanical nociceptors that respond to mechanical damage and not heat, mechanically insensitive nociceptors that are activated by heat, and cold or mechano-heat-cold nociceptors that respond best to severely cold temperatures (Willis and Coggeshall 2004).

Nociceptors are well equipped to respond to different forms of mechanical, thermal or chemical stimuli since they contain many different receptors and ion channels at their peripheral terminals in the skin. For instance, molecules that detect mechanical sensations include the stretch inactivated (SIC), degenerin (DEG), dorsal root acid-sensing ion channel (DRASIC) and TWIK-related K^+ channel-1 ion channels (TREK-1) (Ji and Strichartz 2004; Willis and Coggeshall 2004). Temperature detectors include the transient receptor potential (TRP) ion channels – TRPV1 (also known as the vanilloid receptor, VR1), TRPV2, TRPV3 and TRPV4 that are sensitive to heat while TRPM8 and TRPA1 are responsive to the cold (Willis and Coggeshall 2004; Wang and Woolf 2005). Chemical sensors include TRPV1, DRASIC and acid sensing (ASIC) channels that detect protons or acid and the purinergic P2X3 receptors that sense adenosine triphosphate (Ji and Strichartz 2004; Willis and Coggeshall 2004). If a sufficient number of these different channels are activated, action potentials are generated in the nociceptor, and this process depends on different ion channels that include voltage-gated sodium channels and tetrodotoxin-resistant sodium channels, such as Na_v 1.7, 1.8 and 1.9 (Willis and Coggeshall 2004; Wang and Woolf 2005). Signal transduction from the skin to the spinal cord occurs through the A and C fibers mentioned above.

These primary afferent fibers synapse onto their respective second order projection neurons and interneurons in the dorsal horn of the spinal cord, which is organized into different laminae. Information about the intensity and duration of the noxious stimuli is transferred from the primary afferent's presynaptic terminal to the postsynaptic neuron in the dorsal horn through action potentials inducing the activation of voltage-gated Ca^{2+} channels, such as Ca_v 2.2 and the subsequent release of neurotransmitters, such as glutamate, substance P, and BDNF, from the presynaptic terminal. The events occurring at the synapse between the primary afferent terminal and the second order dorsal horn neuron is described later on.

The majority of nociceptive Aδ and C fibers terminate in laminae I and II while Aβ fibers may send a collateral rostrally through the dorsal column which in turn projects to the contralateral VPL thalamic nucleus through the medial lemniscus and/or synapse with second order projection neurons in laminae III–V (reviewed in (D'Mello and Dickenson 2008)). From here, the projection neurons transmit information to the brain and brain stem areas including the thalamus (through the spinothalamic tract), the periaqueductal gray, and the rostroventromedial medulla through the spinoreticular and spinomesencephalic tracts (reviewed in (D'Mello and Dickenson 2008)). The cortical brain areas that are activated by painful stimuli include the primary and secondary somatosensory, insular, anterior cingulate and prefrontal cortices (Tracey and Mantyh 2007).

1.1.3 MECHANISMS BEHIND NEUROPATHIC PAIN

The mechanisms behind the development of neuropathic pain are not yet fully understood. However, much research has shown that there are both peripheral and central

changes following peripheral nerve injury that contribute to the progression and maintenance of neuropathic pain.

Peripheral nerve damage leads to the production of different inflammatory mediators, and these may include bradykinin, prostaglandin E2, tumor-necrosis factor-α, ATP, protons, histamine, serotonin, catecholamines, adenosine, excitatory amino acids, nerve growth factor and neurokinins (Willis and Coggeshall 2004; Cheng and Ji 2008). These compounds may be released at the site of injury by damaged axons as well as by non-neuronal cells, such as neutrophils, fibroblasts, mast cells, monocytes and Schwann cells (Cheng and Ji 2008). G-protein coupled receptors for bradykinin and prostaglandin E2, tyrosine kinase receptors for NGF and cytokines and ionotropic receptors for protons and ATP are located on the peripheral terminals, axons and somas of primary afferent neurons.

The activation of the various receptors may lead to the activation of second messenger cascades, such as protein kinase A, protein kinase C, protein kinase G, mitogen activated protein kinase, and phosphatidylinositol 3-kinase pathways (Willis and Coggeshall 2004; Cheng and Ji 2008). The end result of these cascades is peripheral sensitization, which refers to the increased responses of the primary afferents to noxious stimuli and their decreased thresholds for thermal and mechanical stimuli. The effectors of peripheral sensitization mainly include the phosphorylation of TRP and voltage-gated sodium channels which modulates their threshold and kinetics (Woolf and Ma 2007).

Changes in gene expression also contribute to peripheral sensitization. Activation of the second messenger cascades results in transcriptional regulation by the transcription factors, CREB, ELK-1, Jun, and ATF along with translational regulation (Cheng and Ji 2008). There is increased gene expression of different ion channels, such as TRPV1, TRPA1,

the tetrodotoxin resistant sodium channels, P2X3, and the Ca^{2+} channel α2δ subunit as well as neuromodulators, such as brain derived neurotrophic factor (BDNF), substance P, calcitonin gene related peptide, and TNF-α (Cheng and Ji 2008). Mechanoreceptive fibers may also undergo a phenotypic switch due to changes in the levels of transcripts and start expressing BDNF and substance P, which are usually only expressed in nociceptive fibers (Woolf 2004). The maintenance of peripheral sensitization depends on the persistent production of these proteins.

Furthermore, peripheral nerve injury, such as axotomy, may produce sprouting of primary afferent fiber axons and changes in the wiring of dorsal horn circuits as well as the down-regulation of different receptor proteins due to a reduced availability of neurotrophic factors (reviewed in (Willis and Coggeshall 2004)).

As mentioned previously, nociceptors transmit nociceptive signals from their peripheral terminals in the skin to their terminals in the spinal dorsal horn. Usually, action potentials are generated in the nociceptor's peripheral terminal. After peripheral nerve damage, action potentials may also start originating from unusual places, such as the axon or the dorsal root ganglion cell body (Woolf and Ma 2007). These action potentials constitute ectopic firing and may occur in the absence of ongoing peripheral inflammation or sensory stimuli. Ectopic activity caused by spontaneous depolarizations is the result of alterations in ion channel expression and trafficking that causes membrane hyperexcitability (reviewed in (Woolf and Ma 2007)). Not only do injured neurons in the dorsal root ganglion exhibit ectopic activity, it has been shown that neighboring intact fibers also contribute to this increase in spontaneous activity (Woolf and Ma 2007). One explanation for this is that the intact fibers are exposed to signal molecules produced by deafferented Schwann cells, such

as TNF. Potassium, sodium, and hyperpolarization-activated, cation-nonselective, cyclic nucleotide-modulated channels as well as Ca-activated chloride currents are thought to play a role in ectopic action potential generation (reviewed in (Woolf and Ma 2007)).

The ectopic discharges of axotomized sensory neurons as well as the spontaneous discharges of sensitized intact sensory neurons may cause spontaneous action potential discharges in dorsal horn neurons in the absence of obvious stimuli. This may explain the phenomenon of spontaneous pain and the development of central sensitization (Woolf 2004).

Central sensitization often occurs in dorsal horn neurons of the spinal cord and results in decreased excitatory thresholds (increased sensitivity) to stimuli, similar to the situation found in peripheral sensitization (Devor and Seltzer 1999). Peripheral sensitization can lead to central sensitization by increasing short-term synaptic efficacy which is dependent on nociceptive afferent activity. One important feature of this increased synaptic efficacy is that while it includes the synapses activated by the stimuli (homosynaptic facilitation), it also is associated with synapses made by Aβ fibers that normally do not respond to nociceptive stimuli (Ji et al. 2003). This heterosynaptic facilitation, where low threshold Aβ fibers can now activate high threshold nociceptive neurons due to a reduction in their activation threshold, helps explain the development of allodynia (Ji et al. 2003).

Central sensitization results from the convergence of many different intracellular signaling pathways in dorsal horn neurons. The initiating factors are neurotransmitters activating their receptors, including glutamate on its ionotropic and metabotropic receptors, substance P on the neurokinin-1 receptors, and brain-derived neurotrophic factor on the tyrosine kinase B receptors (Willis 2002; Ji et al. 2003). Once the receptors are activated, this results in the activation of different protein kinases, such as protein kinase A, protein kinase

C, extracellular-signal-related kinase, phospholipase C, and Ca^{2+}/calmodulin dependent kinase II (Ji et al. 2003). Downstream effects of these kinases include the phosphorylation of ionotropic glutamate receptors, the modulation of channels in order to increase synaptic efficacy, and trafficking of receptors to the synaptic membrane (Ji et al. 2003). The simplified diagram summarizes the signaling cascades found in central sensitization (**Fig 1.1**).

Another explanation for the enhancement of excitation in the spinal cord neurons is the loss of inhibitory mechanisms in the spinal cord. The main inhibitory influences in the nervous system are produced by the inhibitory neurotransmitters, glycine and gamma aminobutyric acid (GABA). The spinal inhibitory systems also appear to be affected in neuropathic pain. For instance, many investigators report a decreased inhibitory GABAergic tone in the spinal cord which contributes to the increased excitability of dorsal horn neurons (central sensitization) and thus, the maintenance of neuropathic pain (Castro-Lopes et al. 1993; Ibuki et al. 1996; Eaton et al. 1998; Moore et al. 2002). However, the cause of the decreased GABAergic function is not known. One possible cause of decreased GABAergic tone may be through GABA neuron death in the spinal cord which would affect nociceptive processing and transmission, although this has not yet been proven.

Numerous studies show that peripheral nerve injury does result in apoptosis and spinal cord neuron loss. Of note, Whiteside et al. found that chronic constriction injury of the sciatic nerve resulted in apoptosis of superficial dorsal horn neurons as early as 8 days after injury (Whiteside and Munglani 2001). Conversely, Polgar et al. reported that although some dorsal horn neuronal death does occur, significant loss of dorsal horn neurons after peripheral nerve injury is not necessary for development of neuropathic pain in the spared nerve injury

model (Polgar et al. 2005). Therefore, there is still much controversy regarding the fate of GABA neurons after an insult to the nervous system.

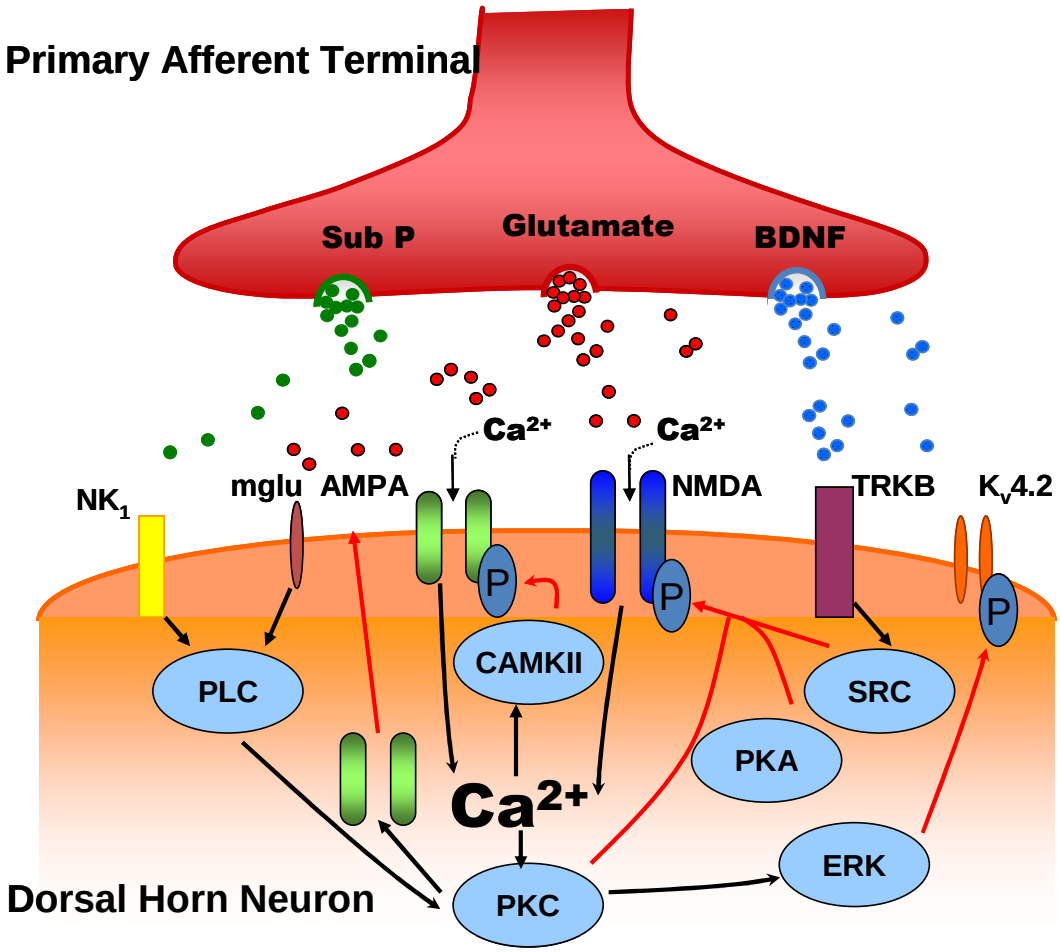


FIG. 1.1. THE INDUCTION OF CENTRAL SENSITIZATION IN DORSAL HORN NEURONS. The primary nociceptor afferent terminals release the neurotransmitter, glutamate (Glu) and neuromodulators substance P (SP) and brain-derived neurotrophic factor (BDNF). Glutamate binds to the ionotropic AMPA and NMDA receptors and metabotropic glutamate receptors (mglu). SP binds to the neurokinin 1 (NK_1) receptor, while BDNF binds to the tyrosine kinase receptor TrkB. The Ca^{2+} influx caused by the activation of the NMDA and AMPA receptors initiates the activation of protein kinases, including cAMP-dependent protein kinase (PKA), Ca^{2+} /phospholipid-dependent protein kinase C (PKC) and Ca^{2+} /calmodulin dependent protein kinase II (CAMKII). These kinases and the tyrosine kinase Src phosphorylate NMDA and AMPA receptors, leading to increased sensitivity of the postsynaptic neuron. Extracellular-signal-related kinase (ERK) phosphorylates the Kv4.2 K^+ channel. PKC recruits AMPA receptors to the synapse. Red arrows mark end points in central sensitization. Figure adapted from (Ji et al. 2003).

1.1.4 SPINAL NERVE LIGATION MODEL OF NEUROPATHIC PAIN

Peripheral nerve injury results from a variety of causes, including trauma, disease or toxins. In order to discover new treatment options for neuropathic pain, it is important to study the mechanisms involved in neuropathic pain. Therefore, animal models of peripheral neuropathy were developed to simulate what was seen in the clinical situation.

The first model of peripheral neuropathy, the chronic constriction injury (CCI) model, was developed in the laboratories of the Neurobiology and Anesthesiology Branch of the National Institute of Dental Research at the National Institutes of Health (Bennett and Xie 1988). In this model, the common sciatic nerve is exposed at the mid-thigh level. Four ligatures are loosely tied around the nerve, proximal to the sciatic's trifurcation, producing a partial denervation of the sciatic nerve that affects myelinated afferent axons much more severely than unmyelinated axons (Kim and Chung 1992; Bennett et al. 2003). This was the first model that allowed for the analysis of pain behaviors evoked by stimulation of the nerve's target—the hind paw.

The spinal nerve ligation (SNL) model, usually referred to as the "Chung model", was developed at the University of Texas Medical Branch (Kim and Chung 1992). In this model, originally developed in the rat, the spinal nerves L5/L6 together or L5 only are completely ligated where the dorsal and ventral roots join distal to the dorsal root ganglia, but proximal to the lumbar plexus where the spinal nerves sort themselves into the various peripheral nerves (Kim and Chung 1992). When the time courses and the magnitudes of the mechanical allodynia after L5 or L5/L6 SNL, were compared, there were no significant differences (Kim and Chung 1992). The L5 SNL model is more desirable to use than the L5/L6 SNL because the procedure is easier to perform and involves less peripheral nerve

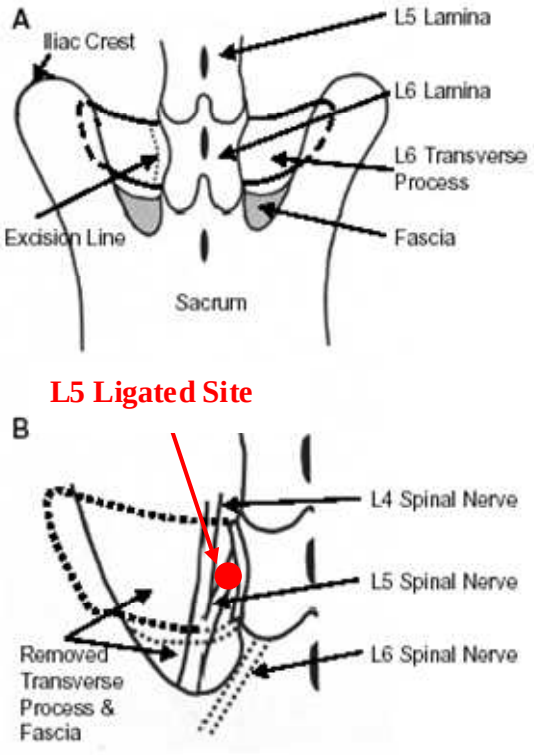


FIG. 1.2. THE ANATOMICAL LANDMARKS IN THE SPINAL NERVE LIGATION PROCEDURE. (A) The surgical site is depicted with the borders of the iliac crest, the spinous processes of L5 and L6 and the sacrum. The L6 transverse process is removed during surgery as indicated by the excision line. (B) An enlargement of the area beneath the L6 transverse process reveals the L4 and L5 spinal nerves running adjacent to each other before they merge. L5 runs more medially and superficially than the L4 spinal nerve. Figure is adapted from (Chung et al. 2004).

injury with a similar behavioral phenotype. A diagram of the surgical area in the rat and the location of the L4 and L5 spinal nerves are shown (**Fig 1.2**).

Like the CCI model, the SNL model results in a partial denervation of the sciatic nerve, but the main difference is that it affects afferent axons of all sizes equally due to complete, tight ligation of the spinal nerves (Kim and Chung 1992; Kim and Chung 1997; Bennett et al. 2003; Chung et al. 2004). One of the distinct advantages of the SNL model is that uninjured and injured afferent axons of the sciatic nerve can be accessed separately by

the 4th and 5th lumbar dorsal roots, respectively, since most of the afferent axons that travel in the sciatic nerve have their central branches in the 4th and 5th lumbar dorsal roots (Kim and Chung 1992; Bennett et al. 2003). Therefore, the only potential variability between the experimental animals would be differences in the proportion of the sciatic nerve that receives afferent contributions by its three spinal segments, which is a normal biological (not experimental) variability (Kim and Chung 1992).

Since its development, the validity and usefulness of the SNL model as a good model for neuropathic pain has been proven extensively. For example, many commonly used analgesics, such as gabapentin, antidepressants and cannabinoid agonists were found to reverse the behavioral signs of pain significantly, such as tactile allodynia in the SNL model (Abdi et al. 1998; LaBuda and Little 2005).

SNL is currently one of the most widely used neuropathic pain models, and it has been applied to other animals than rats as well, including monkeys and sheep. Our lab, using the spinal nerve ligation model (SNL), has discovered an important role of reactive oxygen species (ROS) in the neuropathic pain state and central sensitization (Kim et al. 2004; Kim et al. 2006). However, to uncover further the mechanisms behind ROS involvement in neuropathic pain, it is advantageous to use genetically manipulated animals, such as transgenic mice, to answer many unsolved questions, such as the means by which ROS contribute to pain. Therefore, we have applied the SNL model to the mouse.

1.2 REACTIVE OXYGEN SPECIES

1.2.1 ROS PHYSIOLOGY IN THE CNS

Reactive oxygen species (ROS) are derivatives of molecular oxygen and consist of both free radicals, such as superoxide and the hydroxyl radical, as well as non-radicals, such as hydrogen peroxide and peroxynitrite (Maher and Schubert 2000). These molecules are ubiquitously present in the body and participate in many normal cellular processes. ROS, which include both reactive oxygen species and reactive nitrogen species, are involved in processes ranging from hormone action and secretion, ion transport, transcription, neuromodulation to apoptosis (Lander 1997). In some cases, free radicals initiate cellular responses themselves and in other instances, they are required for ligand-stimulated gene expression (Lander 1997). For instance, hydrogen peroxide has been shown to activate different isoforms of protein kinase C through tyrosine phosphorylation of the catalytic domain (Genestra 2007). Given their ubiquitous nature and the widespread involvement of free radicals in many aspects of cellular function, the regulation of the levels of ROS is important, requiring a static balance between production and removal.

Sources of ROS in the nervous system include mitochondrial oxidative metabolism as well as enzymatic reactions that immune cells like macrophages and microglia use to target invading, foreign pathogens (Maher and Schubert 2000). For example, superoxide is formed by both cytosolic and membrane bound enzymes, such as xanthine oxidase, phopholipase A_2, and cytochrome P450, as well as by the leakage of high energy electrons along the mitochondrial electron transport chain during oxidative phosphorylation and the formation of adenosine triphosphate (ATP) (Simonian and Coyle 1996). Hydrogen peroxide is produced

as a byproduct of the enzymatic reactions by monoamine oxidase, tyrosine hydroxylase, and L-amino oxidase or by auto-oxidation of ascorbic acid and catecholamines (Coyle and Puttfarcken 1993).

The cell is equipped with many mechanisms that counterbalance the production of ROS and repair oxidative damage. Each of the three forms of the superoxide dismutases – extracellular, intracellular copper/zinc and mitochondrial manganese forms – catalyzes superoxide into hydrogen peroxide (Lewen et al. 2000). The majority of hydrogen peroxide in the brain is removed by glutathione peroxidase, and is also broken down into water and molecular oxygen by catalase (Simonian and Coyle 1996). Other antioxidants in the nervous system include glutathione, melatonin, NADPH: Quinone reductase, vitamin E, ceruloplasmin, uric acid, thioredoxin, bcl-2, metallothionein and heme-oxygenase (Simonian and Coyle 1996; Lewen et al. 2000; Kishida and Klann 2007). Therefore, ROS are normally important for cellular functions, and their production and removal are regulated by enzymes.

1.2.2 ROS PATHOPHYSIOLOGY IN THE CNS

In pathological conditions, the levels of ROS may increase, either due to decreased removal or increased production. The increase in ROS, termed oxidative stress, can lead to activation of specific signaling pathways by altering the activity of enzymes, including protein tyrosine kinases or serine/threonine kinases (Lewen et al. 2000).

ROS, especially the hydroxyl radical and peroxynitrite, can cause nonspecific cell damage, such as protein nitration, lipid peroxidation and deoxyribonucleic acid (DNA) oxidation (Lewen et al. 2000; Maher and Schubert 2000). Protein nitration usually inactivates proteins and increases their susceptibility to proteolysis. Lipid peroxidation leads to a chain

reaction of more ROS formation and decreases membrane stability (Simonian and Coyle 1996). The hydroxyl radical may break DNA strands by modifying the ribose phosphates, pyrimidine nucleosides and nucleotides, and sugar phosphate backbones (Simonian and Coyle 1996). The end result of overwhelming oxidative stress and damage is either necrosis or apoptosis.

Necrosis results from ATP depletion and the failure of the Na^+/K^+ ion pumps. The subsequent cell swelling, and finally, cell lysis stimulates inflammatory responses and edema. On the other hand, apoptosis results from mitochondrial cytochrome c release and the activation of caspases and nucleases that break up DNA. Nuclear condensation and cell shrinkage ensues, leading to the eventual disintegration of the cell (Lewen et al. 2000). The main factor dictating whether a cell will undergo apoptosis or necrosis is the level of ATP in the cell – low levels (less than 15%) results in necrosis and higher levels (between 25 – 70% of the normal level) results in apoptosis (reviewed in (Lewen et al. 2000)).

Nervous tissue is quite susceptible to oxidative stress, due to many different factors. Of importance, the brain utilizes a disproportionate amount of the body's oxygen. During resting conditions, the brain consumes about 20% of the body's total oxygen even though it comprises only 2% of the total body weight (Coyle and Puttfarcken 1993; Contestabile 2001). Therefore, in order to sustain this high level of metabolism, neurons contain many mitochondria, which are one of the main sources of free radical production in the brain (Contestabile 2001). Mitochondria are found not only in the cell body, but also throughout the axons, dendrites and synaptic boutons which use adenosine triphosphatases to maintain transmembrane gradients (Coyle and Puttfarcken 1993). In addition to possessing a high level of cellular activity, nervous tissue also contains low levels of antioxidant enzymes and

has high levels of iron (Contestabile 2001). Iron can participate in different Fenton reactions to generate free radicals, such as the very reactive hydroxyl radical. Furthermore, the brain is rich in polyunsaturated fatty acids that are vulnerable to attacks by the hydroxyl radical and subsequent lipid peroxidation under aerobic conditions (Coyle and Puttfarcken 1993). Therefore, the nervous system is especially vulnerable to attacks by free radicals, and specifically, ROS.

Besides the general process of aging, ROS have been implicated in pathogenesis of neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and ALS. ROS have also been implicated in traumatic brain injury, spinal cord injury and ischemia-induced injury (Lewen et al. 2000). The initial factor behind the oxidative damage seen in these neurodegenerative conditions is the excitatory neurotransmitter, glutamate (Lewen et al. 2000; Maher and Schubert 2000). Glutamate acting on its ionotropic receptors, the N-methyl-D-aspartate (NMDA), quisqualate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid (KA) receptors, leads to the influx of Ca^{2+} and Na^{+}. A number of pathways are activated that produce oxidative stress, including the release of arachidonic acid, the activation of nitric oxide synthase, and the activation of peptidases like calpain I. These generate ROS, such as the superoxide radical and nitric oxide (Coyle and Puttfarcken 1993; Simonian and Coyle 1996; Lewen et al. 2000).

In addition, some studies have discovered a role for ROS in the production of neuropathic and inflammatory pain (Kim et al. 2004; Muscoli et al. 2004; Wang et al. 2004). Wagner et al. found that in the chronic constriction injury model, dietary supplementation with a precursor for glutathione helped reduce hyperalgesia and neuropathological consequences (Wagner et al. 1998). This supports the idea that increased ROS levels due to

impaired removal in peripheral nerve injury are important in the development of pain (Wagner et al. 1998). Likewise, Wang and colleagues described superoxide as an agent mediating hyperalgesia in carrageenan-induced inflammatory pain and found that manganese superoxide dismutase was nitrated and unable to function (Wang et al. 2004). It has been reported that various pain models have increased nitric oxide synthase activity, which produces nitric oxide, an important signaling molecule; however, this also suggests there is an increase in free radicals in the spinal cord (Meller and Gebhart 1993; Dolan et al. 2000; Wu et al. 2001).

Our group has found that the systemic injection of free radical scavengers (spin trap agents), such as phenyl-*N*-tert-butylnitrone (PBN) and 5,5-dimethylpyrroline-*N*-oxide (DMPO), significantly decreases pain behavior in SNL rats (Kim et al. 2004). Another study found that a well known antioxidant, Vitamin E, was also effective in reducing pain behavior in the SNL model (Kim et al. 2006). These data strongly argue for the presence of increased spinal ROS with peripheral nerve injury. Furthermore, increased levels of mitochondrial ROS were demonstrated in the dorsal horns of SNL rats using a mitochondrial marker, Mito-tracker Red, which fluoresces when oxidized (Park et al. 2006). Therefore, much evidence suggests that there indeed is an increase in ROS in the spinal cord that may contribute to neuropathic pain.

However, there is a gap in the knowledge regarding the effecter mechanism by which ROS contributes to neuropathic pain. Since under normal conditions, ROS are known to interact with a number of enzymes, such as PKC, tyrosine phosphatases and tyrosine kinases and these are modulated in the state of neuropathic pain, ROS might also be involved in pain with respect to these molecules. Furthermore, since ROS are involved in the processes

leading to apoptosis and the apoptosis of GABA neurons has been observed in neuropathic pain, it seems logical that ROS may be involved in the apoptosis seen here. In this respect, ROS may very well influence the balance between the actions of the excitatory and inhibitory neurotransmitters in the development of neuropathic pain.

1.3 GABA

GABA is a major inhibitory neurotransmitter in the central nervous system of both vertebrates and non-vertebrates (Malcangio and Bowery 1996; Bowery et al. 2002). GABA is thought to be responsible for more than 40% of all inhibitory processes (Malcangio and Bowery 1996).

In the spinal dorsal horn, GABA occurs in a large percentage (~30 %) of the interneuron population in laminae I-IV as well as lamina X (reviewed in (Willis and Coggeshall 2004)). The morphology of many GABAergic interneurons in lamina II is that of the islet cell, which has a dendritic tree more elongated in the rostrocaudal direction rather than the dorsoventral direction (Heinke et al. 2004; Willis and Coggeshall 2004). Also, the GABA containing neurons may be postsynaptic to nociceptive primary afferents that contain calcitonin gene related peptide, substance P and glutamate (reviewed in (Willis and Coggeshall 2004)). It has been reported that a noxious stimulus, such as capsaicin application, results in glutamate release that activates metabotropic glutamate receptors located on GABA interneurons that in turn activates them and causes them to release GABA to modulate nociception (Willis and Coggeshall 2004; Zhou et al. 2007). The population of GABA neurons is heterogeneous, containing a variety of substances in addition to GABA, such as glycine, acetycholine, enkephalin, galanin, neuropeptide Y, nitric oxide synthase and

parvalbumin in different combinations (Willis and Coggeshall 2004). In fact, evidence suggest that GABA and glycine may act as inhibitory co-transmitters at spinal cord synapses since many synapses in the rat spinal cord contain postsynaptic receptors that are immunoreactive for both $GABA_A$ and glycine receptor subunits and presynaptic sites that are enriched with GABA and glycine (Todd et al. 1996). In laminae I-III all glycinergic cells are GABAergic, but the converse is not true. In the deeper laminae, GABA and glycine are found in separate neurons as well as are co-localized (reviewed in (Willis and Coggeshall 2004)).

1.3.1 GAD65 AND GAD67

GABA is synthesized from glutamate in the neuronal cytosol by the rate-limiting enzyme, GAD, which, unlike other synthetic enzymes for neurotransmitters, exists as two major gene products or isoforms. These two isoforms are named GAD65 and GAD67 because of their approximate molecular weights— 65,400 Daltons and 66,600 Daltons, respectively (Soghomonian and Martin 1998). GAD67 is expressed more than GAD65 in the spinal cord dorsal horn (Willis and Coggeshall 2004). Most GAD molecules exist as homodimers of 120,000 Daltons of either isoform, although there are also heterodimers (Pinal and Tobin 1998). Each of the proteins contains two domains, a unique N-terminal domain with 23% identity between them and a more highly conserved (73% identity) C-terminal, catalytic domain (reviewed in (Soghomonian and Martin 1998)). The N-terminal domain is responsible for the subcellular distribution of GAD and contains the phosphorylation and palmitoylation sites that participate in GAD's interaction with other

proteins. The C-terminal domain regulates the interaction with the cofactor, pyridoxal-phosphate (PLP) (reviewed in (Martin and Barke 1998; Soghomonian and Martin 1998)).

The importance of having two gene products that synthesize GABA has been extensively studied since Dr. Eugene Roberts first identified the second GAD extracted from chick embryos in 1970 (Erlander and Tobin 1991). In addition to its classical role as a neurotransmitter, GABA also has a metabolic role, being a part of the GABA shunt and the tricarboxylic acid cycle (Martin and Barke 1998; Soghomonian and Martin 1998). Therefore, it was expected that GAD65 and GAD67 would have two distinct, independent roles, one for synaptic transmission and one for non-synaptic, cellular metabolism.

Indeed, GAD65 and GAD67 differ from one another in many ways, including intracellular distribution and membrane association, interaction with and regulation by its cofactor, post-transcriptional processing, and involvement in the mode of GABA release and neuronal firing patterns (Erlander and Tobin 1991; Martin and Barke 1998; Pinal and Tobin 1998; Soghomonian and Martin 1998). The major differences are highlighted in **Table 1.1**.

First, many studies using subcellular fractionation and immunohistochemistry have shown that GAD67 is mostly concentrated in the soluble fraction and in the cell bodies and dendrites, suggesting it is responsible for cellular metabolism; in contrast, the majority of GAD65 is associated with the membrane fraction and at the nerve terminals, suggesting that it is responsible for the GABA pool at the synapses (reviewed in (Erlander and Tobin 1991)). However, both isoforms can be found in both the soluble and membrane fractions of the brain (Martin and Barke 1998).

	GAD65	GAD67
Chromosome location	2	10
Membrane associated	More	Less
Dependence on cofactor PYRIDOXAL PHOSPHATE	More Accounts for 83% of total apoGAD in synaptosomes	Less
Distribution in neurons	1. Major isotype in most regions of RAT brain 2. synaptic vesicles at axon terminals 3. membranes 4. neurons whose activation is highly dependent on synaptic inputs	1. Cytoplasm but also 2. in axon terminals 3. interneurons and neurons that fire tonically
Main Role in Brain	Synaptic transmission	General metabolic activity; responsible for most of GABA synthesis in brain; also for synaptic transmission
Transgenic Animals	Knockout has normal GABA levels	Knockout has reduced GABA levels

TABLE 1.1. A comparison of the various characteristics of GAD65 and GAD67.

The difference in cellular localization can be explained by the isoforms' divergent N-terminal domains. Specifically, the propensity of GAD65 to associate with membranes may be linked to four serine residues near the N-terminus which are able to be phosphorylated, and possibly, to the cysteine residues in the same region that can be palmitoylated (reviewed in (Martin and Barke 1998; Soghomonian and Martin 1998)). GAD67 must form a heterodimer with GAD65 by binding to the N-terminal domain of GAD65 in order to accompany it to the membrane (reviewed in (Martin and Barke 1998; Soghomonian and Martin 1998)).

Furthermore, the two isoforms differ in their prevalence for being in the active or inactive form. GAD exists in two forms – an inactive form that is not bound to PLP, known

as apoGAD, and an active form, known as holoGAD that is bound to PLP (Erlander and Tobin 1991). More than half of the GADs in the brain and synaptosomes are the inactive apoGAD, and interestingly, the majority of apoGAD consists of GAD65 (Pinal and Tobin 1998). In fact, studies have found that roughly 80% of the apoGAD in synaptosomes is GAD65; on the contrary, GAD67 is almost entirely found to be in the active form (holoGAD), saturated with PLP (reviewed in (Pinal and Tobin 1998)).

Conversion of apoGAD to the active holoGAD occurs through a complex, highly regulated process, and is influenced by neuronal activity (Erlander and Tobin 1991; Martin and Barke 1998; Pinal and Tobin 1998). Erlander and his group using recombinant GAD found that both GAD65 and GAD67 synthesized GABA at different specific activities, and that the activity of GAD65 was significantly increased with the addition of PLP compared to the activity of GAD67 (Erlander et al. 1991). Therefore, it seems that PLP plays a more important role in the regulation of GAD65's activity than that of GAD67, although this is still under debate (Martin and Barke 1998).

A corollary to this is that the regulation of GAD65 may depend more on neuronal activity than GAD67. Conditions during high neuronal activity favor the activation of GAD65 and its association with PLP, which may account for observed increases in GABA synthesis and argue for its role in GABA neurotransmission (Pinal and Tobin 1998).

Furthermore, GAD67 appears to undergo more post-transcriptional regulation than GAD65. Some studies have shown that GAD67 protein levels but not mRNA levels are influenced by changes in intracellular GABA levels in the cortex (reviewed in (Martin and Barke 1998)). Expression levels of GAD67 and its mRNA, but not GAD65, are affected by denervation or neuronal injury; moreover, animal models of spinal cord injury demonstrate

that spinal cord transection causes increased GAD67 mRNA levels with no changes in GAD65 mRNA (Pinal and Tobin 1998).

Finally, the two GAD isoforms are thought to be involved with different modes of GABA release and are present in neurons with different neuronal firing patterns. Some propose that due to GAD65's preferential localization to membranes and association with microvesicles, it may be involved with the release of vesicular GABA, while GAD67 synthesizes GABA primarily for non-vesicular release, which is reasonable due to its localization in the cytosol where GABA is used for metabolic functions (Soghomonian and Martin 1998). Tonically firing neurons and interneurons express higher levels of GAD67, while phasically active neurons dependent on synaptic inputs express higher levels of GAD65 (Pinal and Tobin 1998).

Interestingly, while both isoforms display many differences in the N-terminal sequence, subcellular localization, interaction with the cofactor PLP, post-transcriptional processing and regulation of expression, each isoform has the ability to and does synthesize GABA for both synaptic and non-synaptic functions, and the majority of GABA neurons contain both isoforms (Martin and Barke 1998; Mackie et al. 2003).

The results of knockout animal studies stress the importance of the GAD67 isoform and support the idea that both isoforms can participate in the different functions of GABA. For instance, GAD65 knockout mice have normal GAD67 and GABA levels in the brain, suggesting that GAD67 is the isoform responsible for most of the GABA synthesis; on the contrary, GAD67 knockout mice have normal GAD65 but reduced GABA levels, suggesting that GAD65 is not able to compensate for the partial reduction in GABA levels due to the absence of GAD67 (Soghomonian and Martin 1998).

For this study, the expression levels of both GAD65 and GAD67 enzymes were investigated since both are found in GABA neurons. Because GAD65 and GAD67 are co-localized in virtually every GABA neuron (Martin and Barke 1998; Mackie et al. 2003), using either GAD as a marker for GABA neurons should presumably label almost all GABA neurons. Due to its commercial availability and the fact that GAD67 is more abundantly expressed than GAD65 in the dorsal horn, the GAD67-EGFP transgenic mouse line was used because the GAD67-containing (GABA) neurons are labeled with the enhanced green fluorescent protein. This label helps to clearly identify the GABA neurons from the surrounding cells, which is very useful for our experiments.

1.3.2 GABA PHYSIOLOGY IN CNS

GABA is degraded by two enzymes, GABA-transaminase (GABA-T) and succinic acid semialdehyde (SSA) dehydrogenase (SSADH), which convert GABA to the SSA intermediate and finally to succinate, an intermediate of the tricarboxylic acid cycle (TCA) cycle (reviewed in (Schousboe and Waagepetersen 2006)). This cycle of GABA synthesis and breakdown is referred to as the GABA shunt of the TCA cycle. GABA may be released from neurons by two mechanisms: GABA is stored in synaptic vesicles and released in a Ca^{2+} dependent manner into the synaptic cleft (Malcangio and Bowery 1996) or in a Ca^{2+} independent manner, through the reversal of the GABA transporter to activate extrasynaptic receptors (Soghomonian and Martin 1998). The effects of GABA are mediated by the activation of either ionotropic or metabotropic receptors that may be located either pre- or postsynaptically (reviewed in (Owens and Kriegstein 2002)). GABA is inactivated by uptake into the GABAergic presynaptic endings or surrounding glia, such as astrocytes through

GABA transporters, four of which have been characterized (reviewed in (Schousboe and Waagepetersen 2006)).

In the astrocyte, GABA is either degraded into CO_2 by GABA-T, the TCA cycle, and pyruvate recycling (Schousboe et al. 2004) or converted to glutamine by the TCA cycle. The glutamine can then be transferred back to the GABA neuron where it is once again converted to glutamate by phosphate activated glutaminase (PAG) and finally back to GABA by GAD (Schousboe and Waagepetersen 2006). The processes involved in GABA synthesis and recycling are shown (**Fig 1.3**).

1.3.3 $GABA_A$ RECEPTORS

GABA can exert its effects on three different GABA receptors, $GABA_A$, $GABA_B$ and $GABA_C$, but only the first two will be briefly described here. The $GABA_A$ receptor is a pentameric molecule and is a member of the superfamily of ligand-gated ion channels that mediate the transmembrane flow of Cl^- and to a lesser degree, HCO_3^- (reviewed in (Michels 2007)). Five subunits comprise the receptor, and to date, 19 different subunits have been isolated: α 1-6, β 1-3, γ 1-3, δ, ε, π, ρ 1-3, and θ. The majority of $GABA_A$ receptors in the brain contain two α, two β and one γ subunit (reviewed in (Enna and McCarson 2006)). The subunit composition of the receptor is very important, dictating the receptor's affinity and sensitivity for modulators, agonists, and antagonists (Soghomonian and Martin 1998). Each subunit consists of an N-terminal extracellular domain, four α- helical transmembrane domains (TM1 – TM4) and a large intracellular loop between TM3 and TM4 (reviewed in (Sieghart 2006; Michels 2007)). This cytoplasmic loop contains most of the protein-protein interaction sites and phosphorylation sites for serine, threonine and tyrosine kinases (Michels 2007).

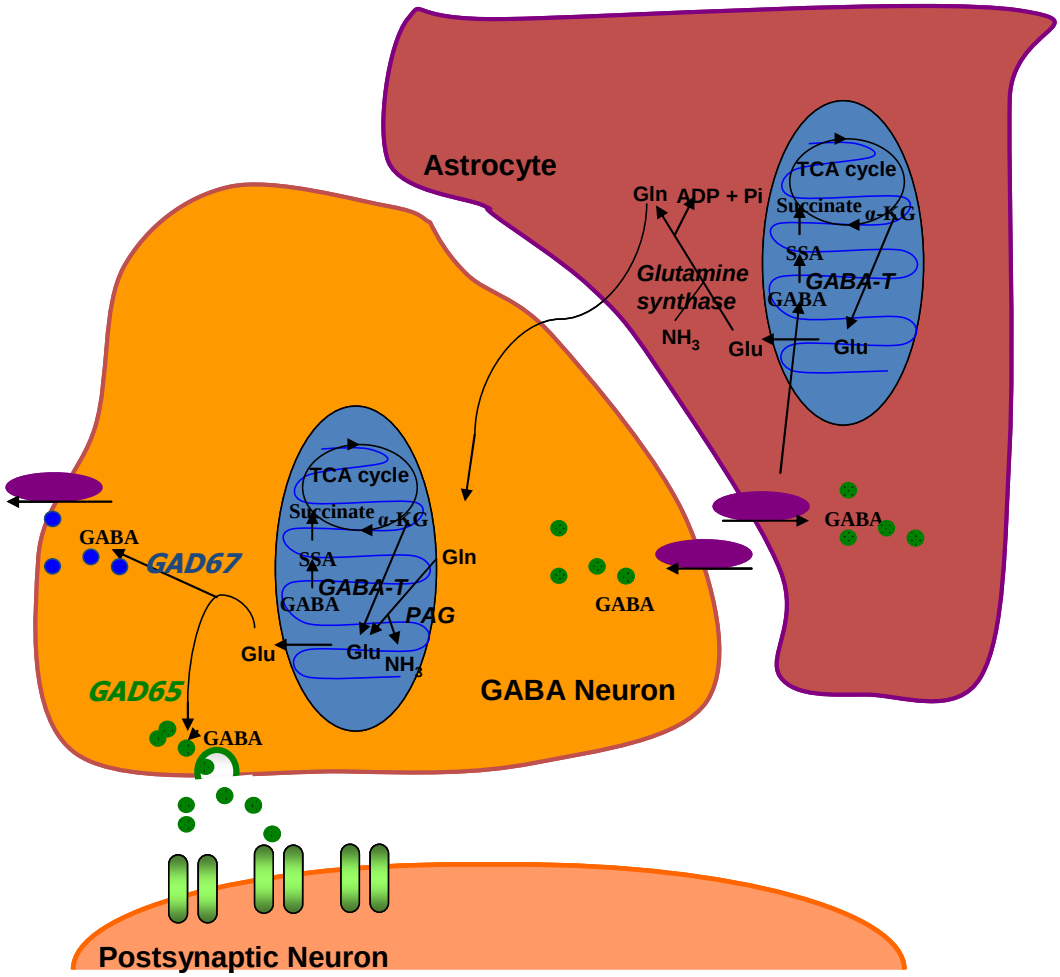


FIG. 1.3. GABA PHYSIOLOGY IN THE NERVOUS SYSTEM. Two glutamate decarboxylases (GADs) convert glutamate into GABA in the cytosol. GAD65 is predominantly associated with vesicles and is localized at the nerve terminal while GAD67 is more homogeneously distributed in the neuron. Synaptic vesicles containing GABA may be released from the synaptic cleft. Cytosolic GABA may be released by the reversal of the GABA transporter (purple oval with arrow) to activate extrasynaptic receptors. The different pools of GABA, vesicular and cytoplasmic, are shown in green and blue, respectively. Glutamine (Gln) is converted by phosphate-activated glutaminase (PAG) to form glutamate (Glu). Glutamate is also made from α-ketoglutarate (α-KG) by several enzymes, including GABA transaminase (GABA-T). GABA-T in the mitochondria of neurons and astrocytes degrades GABA into succinic semialdehyde (SSA); the SSA is then converted to succinate, a tricarboxylic acid (TCA) cycle intermediate. Glutamine is produced in astrocytes and exported to neurons. Figure adapted from (Soghomonian and Martin 1998).

The binding of GABA to an area in between the α and β subunits causes conformational changes in the receptor, increasing the permeability of the central ion pore to Cl^-. Usually, there is an influx of Cl^- which hyperpolarizes the cell and decreases the probability of the neuron to fire an action potential (Michels 2007). GABA may be taken up back into the presynaptic terminal or an astrocyte by a GABA transporter, causing the channel to close.

The $GABA_A$ receptor also has a binding site for benzodiazepines, found between the α and γ subunits in the extracellular N-terminal portion of the receptor subunits. Their binding leads to the allosteric modulation of the $GABA_A$ receptor, increasing the affinity of GABA for the Cl^- channel opening and increasing the probability of the frequency of opening of the Cl^- channels (Michels 2007). Unlike benzodiazepines, barbituates can also bind to the $GABA_A$ receptor, but instead of increasing the opening frequency of the Cl^- channel, they enhance the actions of GABA by increasing the duration of channel opening (Sieghart 2006). Besides these two binding sites, other distinct binding sites on the $GABA_A$ receptor exist for the convulsants, t-butyl-bicyclophosphorothionate and picrotoxinin (Sieghart 2006). Both t-butyl-bicyclophosphorothionate and picrotoxinin noncompetitively block the GABA-gated Cl^- movement by binding to sites within or close to the Cl^- channel (Sieghart 2006).

In the CNS, the $GABA_A$ receptor has been found on both the presynaptic primary afferent terminals and on postsynaptic dorsal horn neurons (Willis and Coggeshall 2004). Activation of the $GABA_A$ receptors on the primary afferent terminals is responsible for primary afferent depolarization (PAD) (Willis and Coggeshall 2004). This phenomenon occurs due to an uncharacteristically high intracellular Cl^- concentration in the primary afferent neuron, pumped into the cell by the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) (Willis Jr

1999; Stein and Nicoll 2003). When GABA binds to the $GABA_A$ receptor, the chloride channel opens and Cl^- leaves the cell, causing depolarization of the primary afferent neuron. Although PAD has been associated with presynaptic inhibition (a decrease in neurotransmitter release from the primary afferent terminal), it is not entirely clear how this comes about. Two hypotheses have been proposed regarding the affect of PAD on presynaptic inhibition. First, PAD might affect neurotransmitter release by affecting or inactivating other ion channels near the synapse, such as Na^+ or Ca^{2+} channels and thereby reducing the amplitude of the action potential or decreasing Ca^{2+} influx needed to induce exocytosis of the synaptic vesicles (Kullmann et al. 2005). On the other hand, the opening of the $GABA_A$ receptor further from the synaptic bouton might interfere with action potential propagation into the terminal by decreasing membrane resistivity (reviewed in (Kullmann et al. 2005)).

1.3.4 $GABA_B$ RECEPTORS

The $GABA_B$ receptor is a Class III, metabotropic, G-protein coupled heterodimer usually consisting of two subunits, a $GABA_{B1}$ and a $GABA_{B2}$ protein (Bowery et al. 2002). While it is now known that a fully functional $GABA_B$ receptor requires the coupling of the $GABA_{B1}$ and $GABA_{B2}$ proteins, recent studies suggest that $GABA_{B1}$ alone or $GABA_{B1}$ homodimers may also display some activity (Enna and McCarson 2006). The $GABA_{B1}$ protein is responsible for activation of the receptor and contains the recognition and binding site for GABA while the $GABA_{B2}$ protein transports the $GABA_{B1}$ protein to the plasma membrane and contains the G-protein coupled site (Bowery et al. 2002). The two proteins share many similarities, including seven transmembrane domains and an N-terminal

extracellular chain (Bowery et al. 2002). Numerous splice variants have been found for the $GABA_{B1}$ protein, and the two major variants, $GABA_{B1(a)}$ and $GABA_{B1(b)}$, are transcription start site variants (Bowery et al. 2002). On the other hand, splice variants for the $GABA_{B2}$ protein have not yet been found (Bowery et al. 2002).

The $GABA_B$ receptor is associated with Ca^{2+} or K^+ ion channels and is coupled mostly to the pertussis toxin-sensitive family of G proteins, $G_{i\alpha}/G_{o\alpha}$, especially $G_{i2\alpha}$ (Bowery et al. 2002). When GABA or $GABA_B$ agonists bind to the receptor on neurons, this usually reduces the intracellular levels of cyclic AMP (cAMP), although increases in cAMP may occur, depending on the types of adenylyl cyclase in the cell and the presence of the Gαs subunits (Enna and McCarson 2006). $GABA_B$ receptor activation also results in a decrease in Ca^{2+} conductance usually at presynaptic sites, associated with presynaptic P/Q- and N-type currents (Bowery et al. 2002). Also, there is an increase in K^+ conductance mostly at postsynaptic sites that may involve many types of K^+ channels (Bowery et al. 2002). $GABA_B$ receptor activation in lamina II neurons of the spinal dorsal horn leads to increased K^+ conductance through a G-protein-coupled inwardly rectifying K^+ channel (reviewed in (Pan et al. 2008)). Unlike $GABA_A$ receptors, the activation of $GABA_B$ receptors does not induce PAD even though it also causes presynaptic inhibition by a reduced neurotransmitter release from the primary afferent terminals (Willis Jr 1999).

The localization of both $GABA_A$ and $GABA_B$ receptors in the spinal cord has been well studied. While there are many more $GABA_A$ receptors in the spinal cord, there is more $GABA_B$ ligand binding in the spinal dorsal horn (Coggeshall and Carlton 1997). Responsible for presynaptic inhibition, both types of GABA receptors are located on DRG neurons and on primary afferent terminals, with an especially high concentration in lamina II of the spinal

dorsal horn, the main area for nociceptive, C fiber input (Coggeshall and Carlton 1997; Bowery et al. 2002). $GABA_A$ receptor immunoreactivity has been found primarily in laminae II, III and X (Willis and Coggeshall 2004). $GABA_B$ receptors have been reported to be concentrated in laminae I, III and IV which support their involvement in presynaptic control of both Aδ and Aβ primary afferents (Coggeshall and Carlton 1997). Both receptors are also located on intrinsic dorsal horn neurons, and $GABA_A$ receptors are also found on extrasynaptic sites (Coggeshall and Carlton 1997).

1.3.5 GABA'S INVOLVEMENT IN NEUROPATHIC PAIN

Nerve injury that affects the GABAergic system and its involvement in pain processing may play an important role in the development of chronic pain. For example, studies have reported that pharmacological antagonism of spinal GABA receptors results in tactile allodynia in both rats and mice with characteristics analogous to those found in chronic pain states (Yaksh 1989; Minami et al. 1994; Hwang and Yaksh 1997). Moreover, administration of GABA and GABA receptor agonists has been shown to alleviate pain behaviors in different models of peripheral neuropathy (Kendall et al. 1982; Hwang and Yaksh 1997; Patel et al. 2001; Malan et al. 2002; Franek et al. 2004). Electrophysiological studies found that in two peripheral neuropathic pain models, there are decreased $GABA_A$-receptor-mediated inhibitory postsynaptic currents with concomitant decreases in dorsal horn levels of GABA synthesizing enzymes and increased neuronal apoptosis (Moore et al. 2002). The same group demonstrated that antagonizing spinal $GABA_A$-receptors resulted in increased A fiber-evoked excitatory polysynaptic inputs to the superficial dorsal horn, or to put it simply, facilitation of excitatory synaptic transmission (Baba et al. 2003).

Thus, many agree that one of the mechanisms behind the development of chronic neuropathic pain is disinhibition, particularly from the disruption of the spinal GABAergic system; however, questions remain about how this dysfunction occurs and where exactly does it happen (i.e. GABA neurons, postsynaptic receptors, transporters). Some believe that the decreased GABAergic tone is due to the loss of spinal cord dorsal horn GABAergic interneurons, and this is supported by the finding of reduced GABA immunoreactivity in the injured spinal cord following sciatic nerve transection (Castro-Lopes et al. 1993). Others argue it is due to inefficient uptake or recycling of GABA by down-regulated or decreased levels of GABA transporter genes such as GAT-1 (Miletic et al. 2003). Others suggest there is a loss in activity of the GABA synthesizing enzymes, glutamic acid decarboxylase 65 and glutamic acid decarboxylase 67 (GAD65 and GAD67), which can explain the down-regulation of GABA transmission (Eaton et al. 1998; Moore et al. 2002). We hypothesize that increased spinal levels of ROS cause GABA neuron dysfunction since GABA neurons have been found to be more sensitive to oxidative stress than other neurons (Bickford et al. 1999).

1.3.6 GABA TRANSGENIC MOUSE

Until very recently, it was very technically challenging to study the functional properties of GABA neurons since they cannot be easily identified in an acute slice preparation (Jonas et al. 1998; Monyer and Markram 2004). Besides this, although antisera have been developed that bind to GABA and its synthesizing enzymes, GAD65 and GAD67, there are many difficulties with this approach. For instance, the use of GAD antisera to label neurons required treatment of the tissue with colchicine (Ribak et al. 1979) that would

possibly have cross-reactivity with cysteine sulfinic acid decarboxylase II (Hodgson et al. 1985; Kaduri et al. 1987). The development of GABA antibodies to circumvent this problem necessitated strong glutaraldehyde fixation to improve the cytological preservation of the tissue and limit the diffusion of GABA; however, this resulted in poorer antibody penetration into the tissue and reduced the sensitivity of detection of GABA immunoreactivity (Kaduri et al. 1987). Therefore, the advent of single-cell reverse transcriptase polymerase chain reaction, labeling neurons with an *in vivo* marker along with the creation of transgenic mice to identify and study GABA neurons has greatly aided in their study (Monyer and Markram 2004).

Likewise, in order to facilitate the study of GABAergic neurons in the spinal cord, we have used a transgenic mouse line, *FVB-Tg(GadGFP)45704Swn/J,* which selectively and consistently expresses enhanced green fluorescent protein (EGFP) in a certain population of GABAergic neurons. The upstream regulatory sequence from the murine *Gad1* (coding for GAD67) gene was used to control EGFP expression (Oliva Jr et al. 2000). Only a small fragment of the *Gad1* gene was used in order to confer GABAergic specificity to the transgene expression and allow for the temporal and spatial variability of the transgene to be governed by the site of transgene integration into the mouse genome (Oliva Jr et al. 2000). EGFP expression can be seen throughout the neuron since it could diffuse easily into the dendrites and axons. Pronuclear injections of transgene DNA were performed, and founder mice were produced and bred with wild-type FVB mice so that subsequent generations became homozygotic for the transgene (Oliva Jr et al. 2000).

Two studies have characterized the properties of these EGFP-expressing GABAergic interneurons in laminae I and II of the spinal cord dorsal horn (Heinke et al. 2004; Dougherty et al. 2005).

1.4 RATIONALE FOR THE STUDY

1.4.1 BACKGROUND OF PROBLEM

The mechanisms behind the development of neuropathic pain resulting from peripheral nerve injury are complex and still remain mostly unsolved. An emerging field in the study of neuropathic pain involves the role played by oxidative stress and its mediators, especially ROS. We were interested in this question of whether increased levels of spinal ROS play an important role in the development of neuropathic pain in mice. Specifically, we wanted to determine whether ROS were important in attenuating spinal GABAergic function, contributing to the loss of the intrinsic GABAergic tone and thus the disinhibition of dorsal horn neurons involved with nociceptive signaling.

1.4.2 CENTRAL HYPOTHESIS

Our overall hypothesis is that ROS and oxidative stress cause spinal GABA dysfunction which plays a role in central sensitization. Specifically, we propose that: 1) SNL causes an increase in ROS in the spinal cord that contributes to neuropathic pain; 2) one mechanism is that elevated spinal ROS levels modulate GABA function that contribute to the development and maintenance of neuropathic pain; and 3) ROS accumulation results in the

loss of GABA expression in the spinal cord. The central hypothesis was tested in the three following specific aims.

1.4.3 SPECIFIC AIMS

Specific Aim 1 tests hypothesis 1: SNL causes an increase in ROS in the spinal cord that contributes to neuropathic pain (CHAPTER 3)

Exp. 1 – to determine whether SNL causes pain behavior in FVB/NJ mice and the time course of pain behavior

Exp. 2 – to determine whether free radical scavengers attenuate the pain behavior caused by SNL and whether ROS donors cause the development of pain behavior

Exp. 2a – by determining the effect of systemic (intraperitoneal) administration of free radical scavengers, PBN and TEMPOL, using behavioral testing for mechanical allodynia in SNL mice

Exp. 2b – by determining the effect of intrathecal administration of PBN using behavioral testing for mechanical allodynia in SNL mice

Exp. 2c – by intrathecal administration of ROS donor, t-BOOH, and behavioral testing for mechanical allodynia in non-ligated, wild-type mice

Exp. 3 – To determine whether ROS play a role in central sensitization by recording field excitatory postsynaptic potentials (fEPSPs) in the spinal dorsal horn of SNL mice and testing whether superfusion with the ROS scavenger, PBN, can attenuate the changes

Specific Aim 2 tests hypothesis 2: An increase in spinal ROS modulates GABA function that contributes to the development of neuropathic pain (CHAPTER 4)

Exp. 1 – To determine whether GABA antagonists cause the development of pain behavior and whether GABA agonists attenuate pain behavior caused by SNL

Exp. 1a – to test whether an intrathecal injection of a GABA antagonist induce pain behavior in non-ligated, wild-type mice

Exp. 1b – to test whether an intrathecal injection of a GABA agonist attenuates pain behavior in SNL mice

Exp. 2 – to determine whether an exogenously applied ROS donor, t-BOOH, alters the function of non-tagged and EGFP-tagged dorsal horn neurons in non-ligated GAD67-EGFP mice

Exp. 3 – To examine whether SNL causes an alteration of the spinal GABA system, and whether this alteration is mediated by ROS by testing whether GABA antagonists can prevent the effect of ROS scavengers on the sensitized fEPSPs in SNL mouse spinal cord slices

Specific Aim 3 tests hypothesis 3: Elevated ROS levels result in a loss of GABA expression in the spinal cord (CHAPTER 5)

Exp. 1 – to test whether SNL causes a loss of spinal GABA expression

Exp. 1a – by immunoblotting for GABA synthesizing enzyme expression (GAD65 and GAD67)

Exp. 1b – by stereological counts of GAD67-EGFP+ neurons

Exp. 2 – To examine whether repetitive treatment with a ROS scavenger prevents the development of pain behavior in SNL mice and reduces the loss of GAD67 expression after SNL

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS: Young adult male *FVB/NJ* (20-30 g) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were housed in groups of four or five in plastic cages with standard bedding and were provided with free access to food and water under a 12/12 hour light-dark cycle (light cycle: 7:00 A.M. – 7:00 P.M.). All animals were acclimated for 7 days before each experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and are in accordance with NIH guidelines.

2.2. BREEDING: Two breeding pairs of *FVB-Tg(GadGFP)45704Swn/J* mice (20-30 g) were purchased from the Jackson Laboratory and were interbred. The mice are homozygous for the TgN(GadGFP)45704Swn transgene that expresses Enhanced Green Fluorescent Protein (EGFP) under the control of the mouse *Gad1* gene promoter. One male and one female were housed in a plastic cage with standard bedding and were provided with free access to food and water under a 12/12 hour light-dark cycle (light cycle: 7:00 A.M. – 7:00 P.M.). Weaning took place 21 days after a new litter was born.

2.3. NEUROPATHIC PAIN MODEL: Peripheral nerve injury, by tightly ligating the L5 spinal nerve (SNL), was done in mice 5 - 7 weeks of age under isoflurane anesthesia (2% induction and 1.5% maintenance). After the first incision, the paraspinal muscles over the L5/6

vertebrae were removed, and forceps were used to remove the L6 transverse process to expose the left L5 spinal nerve. The L5 spinal nerve was gently freed from adjacent structures and tightly ligated with 7-0 silk thread. The surgical site was closed, and the anesthesia was discontinued. Mice were returned to their cages to recover. Sham surgery was performed using the same procedure described above, but the L5 nerve was not touched or ligated.

2.4. DRUG TREATMENT FOR BEHAVIORAL TESTING OF ROS SCAVENGERS AND DONORS: Seven days after SNL, mice were randomly divided into a treatment group and a control group. Mice in one treatment group were injected by one of two different routes with the ROS scavenger, phenyl-*N*-tert-butylnitrone, PBN (Sigma, St. Louis, MO). Doses of PBN were 150 mg/kg (i.p.) or 100 µg (i.t.), which were dissolved in saline at a concentration of 20 mg/ml for both modes of administration. Mice in another treatment group were injected intraperitoneally with 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, TEMPOL (Sigma). TEMPOL was dissolved in saline at a concentration of 75 mg/ml. Mice in the control groups were treated with the same volume of saline. Non-ligated, wild-type mice were divided into four groups, three being injected intrathecally with 0.05, 0.10, or 0.25 µg of the ROS donor, an organic hydroperoxide, tert-butyl hydroperoxide (t-BOOH) (Sigma) dissolved in 5 µl saline and the other group being injected intrathecally with 5 µl saline alone to serve as controls.

2.5. DRUG TREATMENT FOR BEHAVIORAL TESTING OF GABA ANTAGONISTS AND AGONISTS: Four days after SNL, mice were randomly divided into four treatment and one

control groups. Mice in two of the four treatment groups were intrathecally injected with either 0.05 or 0.1 µg of the $GABA_A$ receptor agonist, muscimol (Sigma). Mice in the other set of treatment groups were intrathecally injected with either 0.03 or 0.06 µg of the $GABA_B$ receptor agonist, baclofen (Sigma). All drugs were dissolved in 5 µl saline. Mice in the control groups were treated with 5 µl saline alone. Non-ligated, wild-type mice were also divided into four treatment and one control groups. Two of the four treatment groups were intrathecally injected with either 0.5 or 1 µg of the $GABA_A$ receptor antagonist, (-)-bicuculline methiodide (Sigma). Mice in the other treatment groups received 0.25 or 0.5 µg (i.t.) of the $GABA_B$ receptor antagonist, CGP46381 (Tocris, Ellisville, MO). All drugs were dissolved in 5 µl saline. Mice in the control groups were treated with 5 µl saline alone.

2.6. INTRATHECAL ADMINISTRATION OF DRUGS: For intrathecal injection, mice were anesthetized with isoflurane (2% induction and 1.5% maintenance) and placed in the prone position. Hair of the caudal back was clipped, and the injection was performed with a modified method of direct transcutaneous intrathecal injection (Lee et al. 2006). The experimenter's thumb and middle finger held the lumbar region just cranial to both iliac crests, and the index finger palpated the highest spinous process to guide a 30 gauge hypodermic needle connected to a 10 µl Hamilton syringe. The needle was inserted caudally to the sixth lumbar spinous process at a 45° angle with respect to the vertebral column, facing the cranial direction. Penetration of the needle tip into the intervertebral space between the fifth and sixth lumbar vertebrae was signified by a sudden lateral movement of the tail. The agent (dissolved in 5 µl saline) or 5 µl of saline alone was injected slowly for 5 seconds.

Then, the syringe was held in place for 5 more seconds before removal in order to avoid drug spillage into the epidural space.

2.7. BEHAVIORAL TESTING: Testing for pain behavior consisted of assessing for mechanical allodynia. All experiments were conducted by a person blinded to the treatment groups. Mechanical sensitivity of the hind paw was measured by determining the frequency of foot withdrawals to 10 stimuli produced with a von Frey (vF) filament. Mice were placed in a plastic box (4 X 4 X 12 cm) on a metal grid floor and acclimated to the box for 8 minutes prior to testing. The vF filament was applied from underneath to the skin on the left hind-paw between the 3rd and 4th digits, which was found to be the most sensitive area for the FVB mouse. The hind-paw was stimulated with the filament vF 2.48, equivalent to 0.03 grams, vF 3.0, equivalent to 0.1 grams or vF 3.61, equivalent to 0.41 grams. The vF filament was applied perpendicularly for 2-3 seconds with enough force to bend it slightly. A positive response consisted of an abrupt withdrawal of the foot during or immediately after stimulation. Response rates were calculated as a percentage of the number of positive responses/10 stimuli.

2.8. TESTS FOR PHARMACOLOGICAL SIDE EFFECTS: To determine if ROS scavengers produced the side effect of sedation, which would influence the posture and righting reflexes, the following assessments were made (adapted for mice from (Kim et al. 2004))

Five-point scale for posture:

0 normal posture, rearing and grooming;

1 moderate atonia and ataxia. Weight support, but no rearing;

2 weight support, but severe ataxia;

3 muscle tone, but no weight support and only small, purposive movements;

4 flaccid atonia, fully immobilized with no attempts at movement.

Five-point scale for righting reflexes:

0 the mouse struggles when placed on its side, followed by rapid forceful righting;

1 moderate resistance when the mouse is placed on its side, with rapid but not forceful righting;

2 no resistance to the mouse being placed on its side, with effortful but ultimately successful righting;

3 unsuccessful righting;

4 no movements.

2.9. IN-VITRO FIELD EXCITATORY POSTSYNAPTIC POTENTIAL (FEPSP) RECORDING FROM THE SPINAL CORD SLICE WITH ATTACHED DORSAL ROOT: Adult male mice (5 – 8 weeks of age) were used. Under urethane (1.5mg/kg i.p.) anesthesia, the lumbar spinal cord was removed from the mouse and placed in pre-oxygenated, cold (< 4°C) artificial cerebrospinal fluid (ACSF) (composition in mM: NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose 11), saturated with 95% O_2 and 5% CO_2 mixed gas. The dura and ventral roots were removed. A 3% agar block of the lumbar spinal cord was made, and 450 µm thick transverse slices, leaving the dorsal roots attached, were cut with a

VT1000S vibratome (Leica, Bannockburn, IL). The slices were transferred to warm (30˚C) ACSF and oxygenated for one hour before recording. The slice was then placed in a recording chamber on the stage of an inverted microscope equipped with an ACSF superfusion system. During the recording, the slice was continuously perfused with oxygenated, 30˚C ACSF at a rate of 0.5-1 ml/min. A glass pipette (ACSF internal solution, 2MΩ) was placed on the superficial dorsal horn for recording the field excitatory postsynaptic potentials (fEPSPs) induced by electrical current stimulation in a population of superficial dorsal horn neurons.

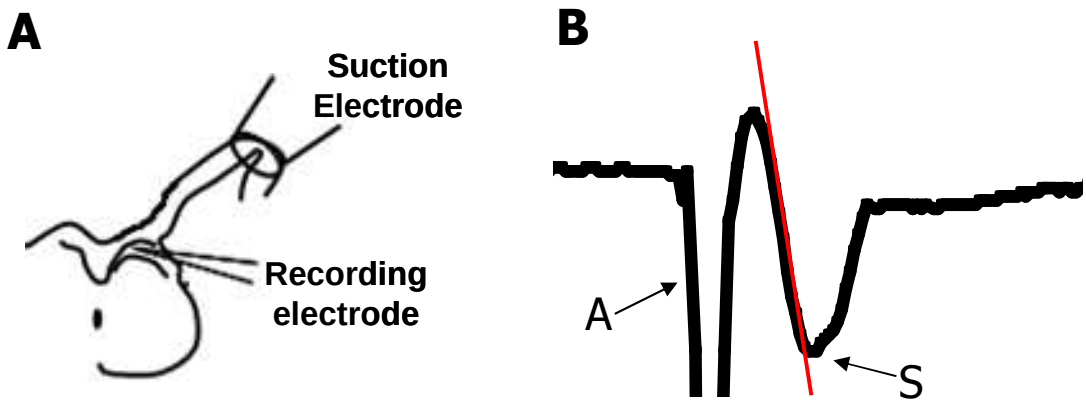


Fig. 2.1 A diagram of the set-up for the recording and a raw trace of a fEPSP. (A) The slice is placed in the recording chamber and is continuously perfused with ACSF. The dorsal root is attached to the suction electrode which also delivers the test stimuli. A recording electrode is placed in the superficial dorsal horn, particularly in laminae I-III. (B) The raw trace consists of a stimulation artifact (A) and the fEPSP (S) with a negative going slope. Changes in the slope (red line) of this fEPSP are analyzed.

Test stimuli, whose strengths ranged from 30-60 μA (0.5 ms), were delivered through a suction electrode attached to the end of the dorsal root. The strength of the test stimuli excited A fibers and produced a half-maximal initial slope of the extracellular field excitatory postsynaptic potential (fEPSP). Responses to test stimuli were measured once every 2 min. Each response was an average of four individual traces delivered at 0.3 Hz. The slope values

were measured from the initial to the peak value of the field potential. After baseline recording of 20 min, drugs were administered using a superfusion system. PBN and H7-dihydrochloride (Tocris), a nonspecific protein kinase inhibitor, were measured and dissolved in ACSF immediately before use. 100 mM bicuculline was prepared in distilled H_2O and stored at -70°C. Frozen aliquots were diluted in ACSF before use. Recordings were made using a patch-clamp amplifier, Multiclamp 700B, and the CLAMPEX 9 acquisition software (Axon Instruments, Union City, CA). Statistical analyses were done using one-way analysis of variance (ANOVA), followed by the Holm-Sidak post hoc test.

2.10. IN-VITRO WHOLE CELL PATCH RECORDING FROM THE SPINAL CORD SLICE: Young transgenic male mice (2 – 3 weeks of age) were used for recording. Under 2% isoflurane and urethane (1.5mg/kg) anesthesia, the lumbar spinal cord was removed from the mouse and placed in preoxygenated, cold (< 4°C) artificial cerebrospinal fluid (ACSF) (composition in mM: NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose 11), saturated with 95% O_2 and 5% CO_2 mixed gas. The dura, afferents and ventral roots were removed. A 3% agar block of the L5 spinal cord was made, and 350 µm thick transverse slices were cut with a vibratome. The slices were transferred to warm (30˚C) ACSF and oxygenated for one hour before recording. A slice was then placed in a recording chamber on the stage of an inverted microscope equipped with an ACSF superfusion system and a Nomarski differential interference contrast (DIC) prism as well as an epifluorescent attachment. The slice was continuously perfused with oxygenated, room temperature ACSF at a rate of 0.5-1 ml/min. Whole cell recording was performed using patch pipettes of 5-10 MΩ resistance filled with an internal solution (composition in mM: potassium gluconate 120,

KCl 20, $MgCl_2$ 2, Na_2ATP 2, NaGTP 0.5, HEPES 20, EGTA 0.5, pH 7.28 with KOH, measured osmolality 300 mosmol kg^{-1}). Epifluorescence was used to locate the EGFP-tagged neurons in lamina II, which was identified as a translucent band in the superficial dorsal horn. Dorsal horn neurons were visualized with DIC, and epifluorescence was used to confirm they were not tagged with the EGFP. Briefly, once whole cell conditions were established, the neuron was held at a sustained holding potential of -50 mV. This holding potential was sustained by a current injection with a magnitude of usually 1.5x to 2x the activation threshold of the neuron. The action potentials generated during the sustained holding potential were recorded and are called “sustained action potentials”. The ROS donor, tert-butyl hydroperoxide (t-BOOH) (Sigma) was dissolved in ACSF immediately before use. Recordings were made using a patch-clamp amplifier, Multiclamp 700B, and the CLAMPEX 9 acquisition software (Axon Instruments). Statistical analyses were done using the paired t-test to compare changes to the control period.

2.11. IMMUNOBLOTTING: Mice were deeply anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg i.p.). When anesthesia was deep, the chest was opened and perfused through the aorta with ice cold saline. The L4/L5 spinal cord segments were divided into separate ipsilateral and contralateral halves, frozen immediately on dry ice, and stored at -70°C until use. Samples were thawed and homogenized in 100 µl ice cold lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 2 µl protease inhibitor (Sigma) and 4 µl phosphatase inhibitor (Sigma) along with 0.1 mm Zirconia/Silica beads (Biospec Products, Bartlesville, OK). Samples were placed in Mini bead-beater 1 (Biospec Products) at 4800 oscillations per

minute for 20 seconds, twice. After centrifugation at 13,500 rpms for 25 minutes at 4°C, ~90 µl of the supernatant was collected. A BCA assay (Pierce Technologies, Rockford, IL) was performed to determine total protein concentration of each sample. 7.5% (w/v) Tris-HCl gels (BioRad, Hercules, CA) were loaded with 20 µg protein per well, and the proteins were fractionated according to size. After SDS-PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes that were probed overnight at 4°C with the mouse monoclonal anti-GAD67 antibody (1:5000, Chemicon, Temecula, CA), the rabbit monoclonal anti-GAD65 antibody (1:5000, Sigma) or the mouse polyclonal β-actin antibody (1:10,000, Sigma). Anti-mouse (1:3000) and anti-rabbit (1:10,000) secondary antibodies are coupled to an ECL substrate (Amersham, Piscataway, NJ) which provided for chemiluminescent detection of the desired protein.

2.12. PERFUSION FIXATION OF TISSUE FOR STEREOLOGY: Mice were deeply anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg i.p.). When anesthesia was deep, the chest was opened and perfused through the aorta with ice cold saline, followed by ice cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 for 30 minutes. The L4-L5 segments of spinal cord were isolated and removed. In order to distinguish the two sides, a notch in the right ventral horn was made using a razor blade. The tissues were stored in fixative for 4 hours to overnight and then placed into 30% sucrose until equilibration. The tissues were embedded in Tissue-Tek® embedding media (Sakura Finetek, Torrance, CA) and stored at -70˚C until use. A cryostat was used to section L4-L5 spinal cord tissue serially into 80 µm thick transverse slices which were then mounted on gelatin coated Superfrost Plus® slides (Fisher Scientific), preserving the correct serial order. Only L5 sections were

selected by a systemic random sampling method from each animal and then analyzed for GABA neuron number.

2.13 STEREOLOGICAL COUNTS FOR NUMBERS OF DORSAL HORN GABAERGIC NEURONS: For all experiments, the person conducting the stereological analysis was blind to the treatment group. Stereological analyses were done with a stereology workstation consisting of an Olympus BX51 microscope (Olympus; Tokyo, Japan) with UV fluorescence (using the UPlanApo objective 10 x [numerical aperture = 0.4] and UPlanApo 20 x [numerical aperture = 0.7]), a motorized z-axis and X and Y stage, and a CX900 color video camera (Microbrightfield, Williston, VT). Using the StereoInvestigator software, (Microbrightfield, Williston, VT), the optical fractionator method (Schmitz and Hof 2005) was used to estimate the total number of GAD67 EGFP+ neurons in the L5 spinal dorsal horn in three areas: the medial and lateral halves of laminae I-II as well as the deeper laminae III-V. A total of 4 sections from the L5 segment of each animal were analyzed.

Briefly, three different contours were traced for each ipsilateral and contralateral side of a section under epifluorescence in the software program, using the cytoarchitectonic organization (Rexed 1952; Molander et al. 1984; Coggeshall et al. 2001) and some visual landmarks, such as the translucent band delineating the substantia gelatinosa. Then, a 10,000 μm^2 counting frame was created in the program and placed randomly within the sampling grids distributed over each traced contour. The optical dissector height was set to 25 µm in the z- direction since the average measured section thickness was usually around 30 µm, leaving guard zones around the dissector height that excluded the distances closest to the slide and the cover slip. Fluorescent green cells were marked positive as their nuclei came

into focus within the counting frame when scrolling in the z-direction. Neurons were not counted if they intersected the "forbidden lines" of the counting frame. Once counting in a particular sampling grid was completed, stepwise movements in the x- and y- directions automatically brought another area into view, and the steps were repeated systemically until all areas were counted.

The total number of EGFP+ neurons (N) for the ipsilateral and contralateral sides of L5 were estimated using the formula N = Q x V where V (volume fraction) equals 1/*hsf* x 1/*asf* x 1/*ssf* and where Q (sum of counts) equals the total count of EGFP+ neurons (West et al. 1991). The *hsf* equals the tissue height of the sampling fraction, *asf* equals the area of the sampling fraction, and *ssf* equals the section sampling fraction. The *hsf* is the optical dissector height relative to the average mounted section thickness. The *asf* equals the area of the counting frame relative to the grid size area. The *ssf* equals ¼ since every fourth section was sampled. The coefficient of error of the population size estimate according to Schmitz-Hof equation provided in the software was used, and CE < 0.05 for each estimate.

2.14 STATISTICAL ANALYSIS: All data are expressed as the mean ± standard error of the mean (SEM). For the behavioral tests for mechanical allodynia for the ROS donor and ROS scavengers, changes from the sham or vehicle controls were compared using the two-way repeated-measures ANOVA followed by Duncan's post hoc tests. For the behavioral tests for mechanical allodynia for the GABA agonists and GABA antagonists, changes from the sham or vehicle controls were compared using the two-way repeated-measures ANOVA followed by Holm-Sidak post hoc tests. The changes in the fEPSP recordings compared to the control period were done using one-way repeated measures ANOVA, followed by the Holm-Sidak

post hoc tests. The changes in the whole cell recordings compared to the control period were analyzed using the paired t-test. The behavioral changes in the test for mechanical allodynia in sham vs. SNL and sham vs. SNL vs. PBN were all compared using the two-way repeated-measures ANOVA followed by Duncan's post hoc tests. For the stereological counts, the difference in the number of GAD67 EGFP+ neurons between sham vs. SNL was compared using the t-test. The difference in the number of GAD67 EGFP+ neurons between sham vs. SNL vs. PBN was compared using one-way repeated measures ANOVA followed by the Holm-Sidak test. In all tests, $p < 0.05$ was considered significant. The SigmaStat program (Version 3.1, Systat Software, San Jose, CA) was used to analyze all the data.

CHAPTER 3

SPINAL NERVE LIGATION CAUSES AN INCREASE IN REACTIVE OXYGEN SPECIES IN THE SPINAL CORD THAT CONTRIBUTES TO NEUROPATHIC PAIN

3.1 ABSTRACT

Oxidative stress due to the overproduction of ROS or impaired removal by the cell's defense systems may disrupt normal cellular processes and ultimately result in cell death. Previously, we found that ROS contribute to the development and maintenance of neuropathic pain in the spinal nerve ligation (SNL) model in the rat. To extend these studies to transgenic mice, we first established the SNL model in the mouse. The purpose of this study was to determine the role of ROS in the SNL model in mice. **METHODS:** SNL mice were produced by tight ligation of the L5 spinal nerve in FVB/NJ adult male mice. Mechanical allodynia was assessed by testing the paw withdrawal response rates to von Frey filaments 2.48 (0.03 g), 3.0 (0.1 g) and 3.61 (0.41 g). At one week post-SNL, the effects of systemic or intrathecal administration of the ROS scavengers, phenyl-*N*-tert-butylnitrone (PBN) at 150 mg/kg (i.p.) or at 100 µg/5 µl (i.t.), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) at 300 mg/kg (i.p.), on pain behavior were tested. To examine further the role of ROS in inducing pain behavior, the effects of an intrathecally administered ROS donor, tert-butyl hydroperoxide (t-BOOH) at 0.05, 0.10, and 0.25 µg/5 µl (i.t.) in normal mice were tested. To determine ROS involvement in central sensitization, the effects of PBN (5 mM) or a positive control, the protein kinase inhibitor, H7-dihydrochloride

(H7, 300 µM), on field excitatory postsynaptic potentials (fEPSPs) generated in the superficial dorsal horn of both sham and SNL mice were studied. **RESULTS:** Mechanical allodynia, measured as the frequency of responses to von Frey stimuli, was consistently increased in the majority of SNL mice (70%-100%) compared to baseline (0-13%). The time course of pain behavior lasts over eight weeks. Systemic PBN (150 mg/kg i.p.) or TEMPOL (300 mg/kg i.p.) temporarily reversed mechanical allodynia up to 2 hours one week after SNL. Intrathecal PBN (100 µg/5 µl) also decreased mechanical allodynia with a similar time course. Intrathecal t-BOOH dose-dependently induced mechanical allodynia for 2 hours after injection. PBN (5 mM) and the protein kinase inhibitor H7 (300 µM) significantly reduced the slope values of the fEPSPs in SNL mice. **CONCLUSIONS:** These data show that SNL produces neuropathic pain in the mouse. Scavenging ROS with systemic PBN, systemic TEMPOL, or intrathecal PBN has analgesic effects in the SNL model in mice. On the other hand, increasing the levels of spinal ROS with intrathecal t-BOOH temporarily induced pain behavior similar to that found in the SNL pain model. Intrathecal data suggest ROS act primarily in the spinal cord to modulate pain processing. The spinal cord dorsal horn neurons are sensitized in SNL mice, and scavenging ROS can reduce central sensitization. The results suggest that ROS play an important role in neuropathic pain and central sensitization in mice.

3.2 INTRODUCTION

Reactive oxygen species (ROS) have been implicated in the development of persistent pain states that result from nerve injury or inflammatory insult (Tal 1996; Kim et al. 2004; Wang et al. 2004; Khattab 2006; Kim et al. 2006; Park et al. 2006; Gao et al. 2007; Lee et al. 2007). The administration of ROS scavengers and antioxidants that supplement endogenous defense systems against free radicals produces temporary analgesia in both neuropathic pain (Tal 1996; Kim et al. 2004; Kim et al. 2006; Siniscalco et al. 2007) and inflammatory pain models (Wang et al. 2004; Khattab 2006; Lee et al. 2007). In addition, morphological observations of the spinal cords of neuropathic rats reveal that the dorsal horn neurons express increased levels of mitochondrial ROS, pointing to a major source of the aberrant ROS production (Park et al. 2006).

ROS are known to cause nonspecific damage to DNA, proteins, and lipids as well as interact with several cell signaling pathways (reviewed in (Lewen et al. 2000; Maher and Schubert 2000)). With respect to persistent pain, several protein kinase cascades, such as protein kinase A (PKA), protein kinase C (PKC), extracellular signal-related kinases (ERKs), calcium/calmodulin-dependent kinase II (CAMKII), and Akt contribute to the increased excitability of spinal cord neurons involved in pain transmission, a phenomenon known as central sensitization (Woolf and Thompson 1991; Dubner and Ruda 1992; Willis 2002; Guedes et al. 2008). Not only was the ROS scavenger, PBN, effective at reducing the enhanced responsiveness of dorsal horn neurons in neuropathic (Kim et al. 2004) or capsaicin-treated rats (Lee et al. 2007), it also blocked the phosphorylation of spinal NMDA receptors, a critical process in central sensitization (Gao et al. 2007). Therefore, much

evidence suggests ROS are important for the development of pain through central sensitization.

Nevertheless, many questions still remain about how ROS are involved in the development of neuropathic pain. For instance, are ROS associated with a diminished inhibitory tone in the neuropathic spinal cord? In order to answer some of these questions, the use of genetically manipulated animals, especially mice, offers many advantages since one can focus on a gene of interest. However, the SNL model must first be established in the mouse. Therefore, the purpose of this study was to confirm and extend previous observations that ROS contribute to neuropathic pain in rats using the SNL mouse model of peripheral neuropathy.

3.3 MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

3.4 RESULTS

3.4.1 SPINAL NERVE LIGATION LEADS TO THE DEVELOPMENT OF MECHANICAL ALLODYNIA

To determine the time course of pain behavior initiated by neuropathic injury, the response rates of mice to von Frey filaments were examined before and after either sham or L5 spinal nerve ligation surgery. The responses to von Frey filaments 3.0 and 3.61 are shown in **Fig. 3.1A** and **Fig. 3.1B**, respectively. Before SNL, the response rates of the left hind paw

to von Frey filaments 3.0 and 3.61 were 2 ± 1% and 1 ± 5% (mean ± SEM), respectively. Following SNL, the response rates increased significantly 1 day after surgery, reached 90 ± 4% and 91 ± 3% by 3 d, and were maintained at high levels for 8 weeks. On the other hand, the sham-treated mice did not develop allodynic behaviors after surgery. Therefore, SNL in mouse leads to the development of long-lasting, mechanical allodynia which is a hallmark of chronic neuropathic pain in human patients.

3.4.2 SYSTEMICALLY ADMINISTERED REACTIVE OXYGEN SPECIES SCAVENGERS, PBN AND TEMPOL, REDUCE PAIN BEHAVIORS IN NEUROPATHIC PAIN

Next, to determine whether ROS play a role in mechanical allodynia in the SNL mouse model, the effects of ROS scavengers on pain behavior were examined. The effects of a single, systemic phenyl-*N*-tert-butylnitrone (PBN) injection on pain behaviors induced by von Frey filaments 3.0 and 3.61 are shown in **Fig. 3.2A** and **Fig 3.2B**. Seven days after SNL, the response rates were 82 ± 7% and 89 ± 3%, respectively. PBN (150 mg/kg, i.p. injection) significantly decreased the response rates up to 2 h with the peak PBN response at 0.5 h (24 ± 11% and 37 ± 13%) after injection. On the other hand, the same volume of saline (i.p. injection) had little effect on the response rates. For von Frey filament 3.0, pre-injection values of 78 ± 4% changed to 71 ± 7% at 0.5 h after injection. For von Frey filament 3.61, pre-injection values changed from 88 ± 4% to 84 ± 4% at 0.5 h after injection. Therefore, systemic administration of a ROS scavenger can temporarily reduce mechanical allodynia in mice.

To confirm that PBN's analgesic effect was due to its ROS scavenging abilities, another antioxidant with a different mechanism of action was tested. 4-hydroxy-2,2,6,6-

tetramethylpiperidine-1-oxyl (TEMPOL) is a piperidine nitroxide that acts as a superoxide dismutase mimetic (Thiemermann 2003), while PBN is a nitrone spin-trapping agent that non-specifically targets free radicals (Kotake et al. 1999). Seven days after SNL, a single systemic treatment with TEMPOL (300 mg/kg, i.p.) also significantly reduced response rates at 1 h after injection compared to vehicle (from 80 ± 5% to 48 ± 12% and 93 ± 3% to 53 ± 8%, respectively) (**Figs. 3.3A** and **3.3B**). Together, the data show that systemic administration of the two different ROS scavengers, PBN and TEMPOL, each produced analgesic effects in the SNL model of neuropathic pain in mice. This suggests that ROS play a contributory role in neuropathic pain.

3.4.3 INTRATHECALLY ADMINISTERED REACTIVE OXYGEN SPECIES SCAVENGER, PBN, REDUCES PAIN BEHAVIORS IN NEUROPATHIC PAIN

To determine whether the spinal cord is a major site of action for PBN's analgesic properties, the effects of PBN in the spinal cord were examined by intrathecal injection and behavioral testing for mechanical allodynia. Seven days after SNL, intrathecal PBN (100 µg) significantly reduced response rates to von Frey filaments 3.0 and 3.61 for up to 2 h. Relative to vehicle controls, the response rates decreased from pre-injection levels of 89 ± 4% to post-injection levels of 46 ± 11% for filament 3.0. Similarly, response rates decreased from pre-injection levels of 95 ± 3% to post-injection levels of 63 ± 8% for filament 3.61 **(Fig. 3.4)**. Although not shown here, a higher dose of PBN (200 µg in 5 µl) (n = 6) did not result in a further reduction in response rates; therefore, the maximal effective dose of PBN was less than 200 µg. Interestingly, the maximum effect of intrathecal PBN was smaller than that of the systemic PBN, suggesting that PBN has additional sites of action besides the spinal cord. Nevertheless, intrathecal PBN's effect was greater than 60% of systemic PBN's effect,

indicating that the spinal cord is one of the main sites of action for PBN. In summary, scavenging ROS in the spinal cord can temporarily relieve pain behaviors in neuropathic mice, suggesting that spinal ROS is a necessary component for the maintenance of neuropathic pain.

3.4.4 EXAMINATION OF A POSSIBLE SIDE EFFECT OF SEDATION OR ANESTHESIA IN MICE WITH PBN OR TEMPOL ADMINISTRATION

Since sedation or anesthesia could influence the behavioral responses to mechanical stimulation that may be interpreted incorrectly as analgesia, it was important to determine whether the doses of PBN and TEMPOL given resulted in these side effects. All mice used in (**Figs. 3.2 – 3.4**) were examined for the posture and righting reflexes based on the five-point scales described in CHAPTER 2. The results are shown in **Table 3.1**. All mice that received either an intraperitoneal injection of 300 mg/kg TEMPOL or an intrathecal injection of 100 µg PBN scored 0 at all time points for both posture and righting reflexes, indicating that only analgesic effects, rather than sedative effects, were responsible for the behavioral changes.

During preliminary experiments intraperitoneal injection of 350 mg/kg TEMPOL caused obvious behavioral abnormalities, particularly sedation after injection, and this effect lasted for 1 h. Therefore, the concentration of 300 mg/kg i.p. TEMPOL was considered the highest appropriate amount to use for the behavioral experiments.

Intraperitoneal injection of 150 mg/kg PBN resulted in a score of 1 on the posture scale 0.5 hr after injection in six of eight mice, since they remained stationary and did not rear or groom normally. This data indicated that PBN produced a slight sedative effect once it reached supraspinal levels, although this effect was diminished by 1 hr, and activity of the mice returned to normal, scoring 0 at all time points afterwards. Also, the mice scored 0 on

the righting reflex scale at all time points. Therefore, while this dose of intraperitoneal PBN had a sedative effect that reduced rearing incidence, it did not impair weight support or righting reflexes. In summary, PBN's overall effectiveness in reducing mechanical allodynia is not due to its sedative effects.

3.4.5 REACTIVE OXYGEN SPECIES DONOR, T-BOOH, INDUCES PAIN BEHAVIORS

To determine whether an increase in spinal ROS would be sufficient to induce pain behaviors in non-injured mice, the effects of intrathecal administration of an exogenous ROS donor were investigated. The ROS donor tert-butyl hydroperoxide (t-BOOH) was injected into the intrathecal space and the effects on paw-withdrawal response rates in non-ligated, wild-type mice are shown in **Fig 3.5**. Baseline responses to von Frey filaments 2.48 and 3.0 were recorded for four groups of mice prior to receiving a single intrathecal injection of either 0.05, 0.10, or 0.25 µg t-BOOH dissolved in 5 µl saline or an injection of 5 µl saline (vehicle control). t-BOOH (0.05, 0.10, or 0.25 µg, i.t.), dose-dependently increased paw-withdrawal response rates compared to vehicle injection, and the change lasted up to 2 h. With von Frey filament 3.0, 0.25 µg t-BOOH significantly increased response rates from 6 ± 2% to 80 ± 4% at 0.5 h after injection (**Fig. 3.5B**). This data show that increased levels of spinal ROS can induce mechanical allodynia in mice, which resemble the pain behaviors seen in the SNL mice.

3.4.6 PBN REDUCES CENTRAL SENSITIZATION IN THE SPINAL DORSAL HORN OF SNL MICE

In order to examine the effects of ROS scavengers on physiological properties of the spinal dorsal horn, field potential recordings were performed in spinal cord slice preparations of both sham and SNL mice at three to seven days after surgery. The field excitatory postsynaptic potentials (fEPSPs) evoked by electrical stimulation (30-60 μA, 0.5 ms) of the dorsal root were recorded. Examples of representative raw traces recorded during the experiments in SNL slices are shown (**Fig. 3.6a-e**), and the averaged slopes of fEPSPs for every 2 minutes are plotted (**Fig. 3.6A, 3.6C**) along with summary graphs for the average fEPSP slope values for each condition (**Fig. 3.6B, 3.6D**). When 5 mM PBN was administered to the recording chamber, the slopes of the fEPSPs were decreased, indicating a reduction in central sensitization in the SNL group (**Fig. 3.6C**). This effect was not observed in the sham group (**Fig. 3.6A**). On average, PBN significantly reduced the slopes of neuropathic fEPSPs to 72.49 ± 5.70% (mean ± SE) of the baseline control level of 100% (**Fig. 3.6D**). During the washout of PBN, the slopes recovered to 93.72 ± 1.10% of the control levels (**Fig. 3.6D**). This data indicate that ROS enhancement of central sensitization was reversible.

The activation of numerous protein kinases, including PKC and PKA, is the hallmark of the enhancement of dorsal horn neuron responses and has been used as an acceptable marker of central sensitization (Willis 2002). To confirm that central sensitization occurred in the SNL mouse model, the effect of PBN was compared to that of the nonspecific protein kinase inhibitor H7. Superfusion with 300 μM H7 significantly decreased the average fEPSP slope values to 78.61 ± 8.19% in the SNL group (**Fig 3.6D**). This result implies that central sensitization occurred in the SNL group. Recovery of the fEPSPs occurred during washout,

but recovery time was longer than that of PBN, reaching an average of 94.19 ± 5.13% only during the last ten minutes (**Fig. 3.6C, 3.6D**). Similar to PBN, H7 had no effect on the fEPSPs in the sham group (**Fig. 3.6A**), indicating that the dorsal horn neurons in the sham slices were not sensitized. In the SNL slices, PBN's effectiveness in reducing the fEPSP slope values were similar to that of H7, thus suggesting that ROS play a critical role in central sensitization.

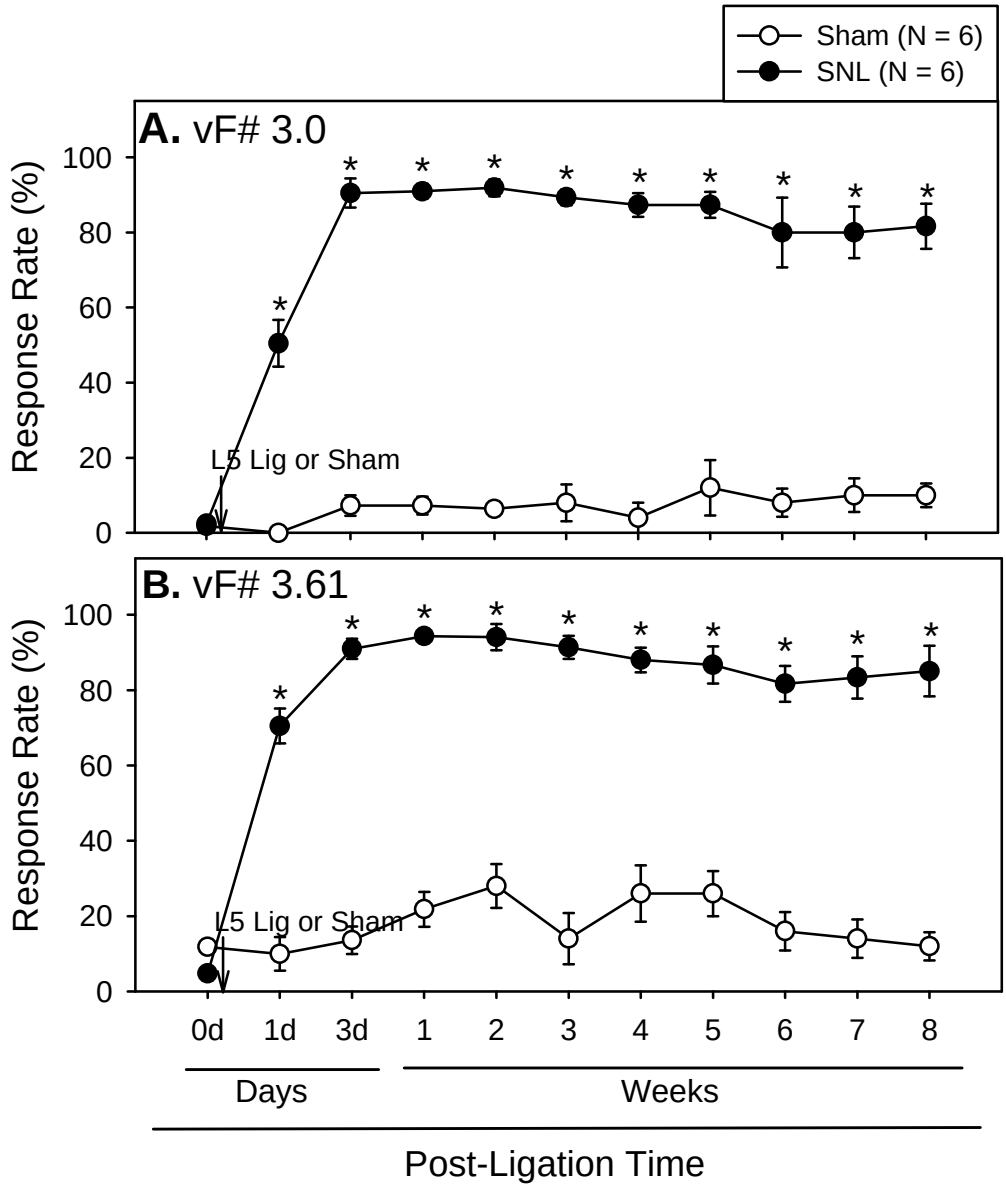


FIG. 3.1 **The time course of paw withdrawal response rates to mechanical stimulation in SNL and sham mice.** Response rates to von Frey filaments 3.0 (A) and 3.61 (B), corresponding to 0.1 g and 0.41 g, were measured at 0 d for pre-surgical levels and became gradually increased following SNL. The mice exhibited signs of mechanical allodynia at 1 d which peaked by 3 d and were stably maintained, lasting longer than 8 wks in all operated mice (n = 6). Sham operation (n = 6) did not result in any significant change in response rates to the stimuli throughout the testing period. Data are presented as means ± SEM. L5 Lig or Sham, time of L5 SNL or sham surgery; *, the value is significantly ($p < 0.05$) different from that of the sham control by two-way repeated-measures ANOVA.

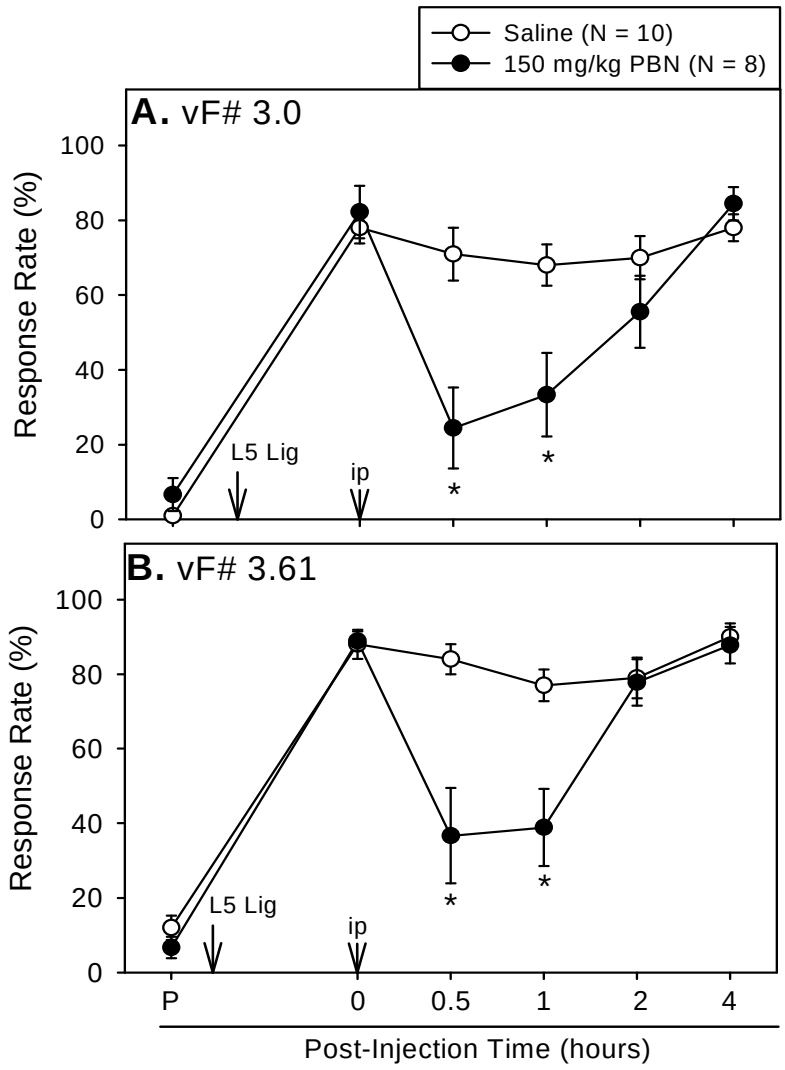
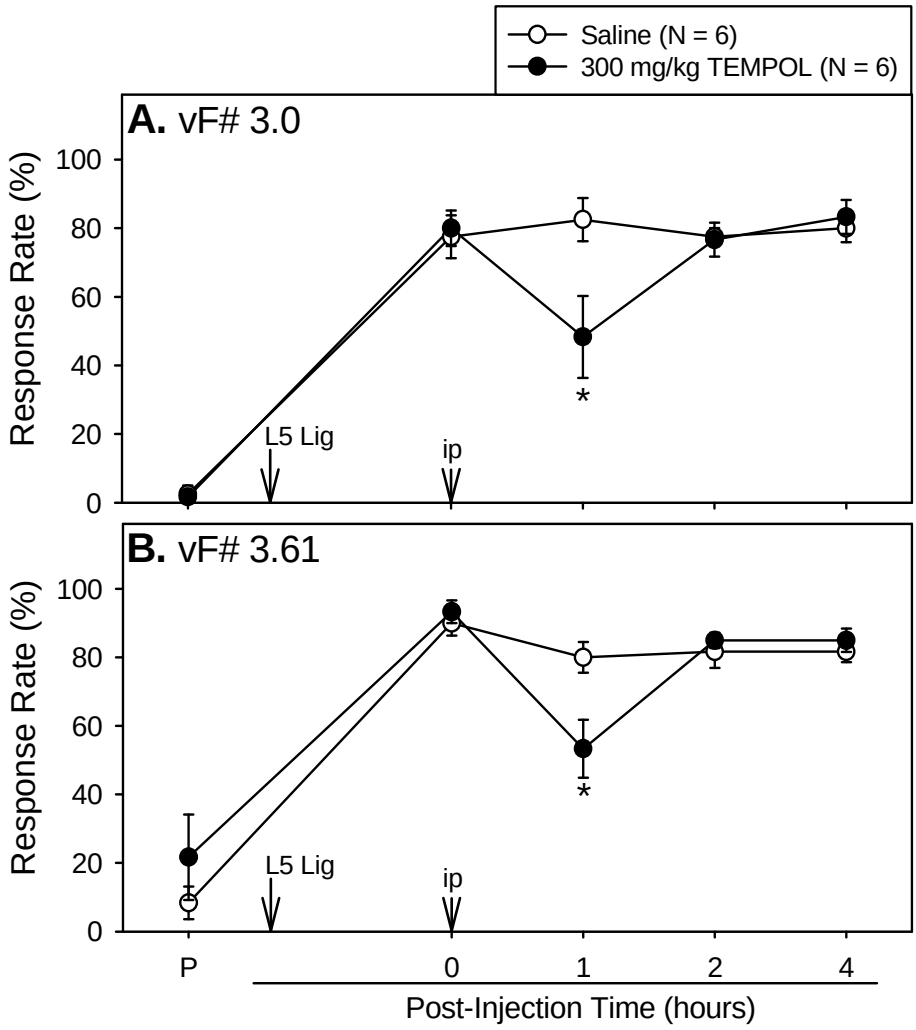


FIG. 3.2. **The effects of intraperitoneally administered ROS scavenger, PBN, on paw withdrawal response rates in SNL mice.** SNL resulted in significantly increased response rates to von Frey filaments 3.0 (A) and 3.61 (B) from pre-surgical levels (P) in all operated mice (n = 18). One week after surgery, a single systemic injection of 150 mg/kg PBN (n =8) alleviated mechanical allodynia up to 2 h. Vehicle treatment (n = 10) resulted in little change in response rates. Data are presented as means ± SEM. P, pre-surgical time; L5 Lig, time of L5 SNL; i.p., intraperitoneal injection; *, the value is significantly (p < 0.05) different from that of the vehicle control by two-way repeated-measures ANOVA followed by Duncan's post hoc tests.



* P < 0.05 significance by Two Way Repeated ANOVA

FIG. 3.3. **The effects of intraperitoneally administered ROS scavenger, TEMPOL, on paw withdrawal response rates in SNL mice.** SNL resulted in significantly increased response rates to von Frey filaments 3.0 (A) and 3.61 (B) from pre-surgical levels (P) in all operated mice (n = 12). One week after surgery, a single systemic injection of 300 mg/kg TEMPOL (n = 6) alleviated mechanical allodynia to von Frey filaments 3.0 (A) and 3.61 (B) at 1 h. Vehicle treatment did not affect response rates (n = 6). Data are presented as means ± SEM. P, pre-surgical time; L5 Lig, time of L5 SNL; i.p., intraperitoneal injection; *, the value is significantly (p < 0.05) different from that of the vehicle control by two-way repeated-measures ANOVA followed by Duncan's post hoc tests.

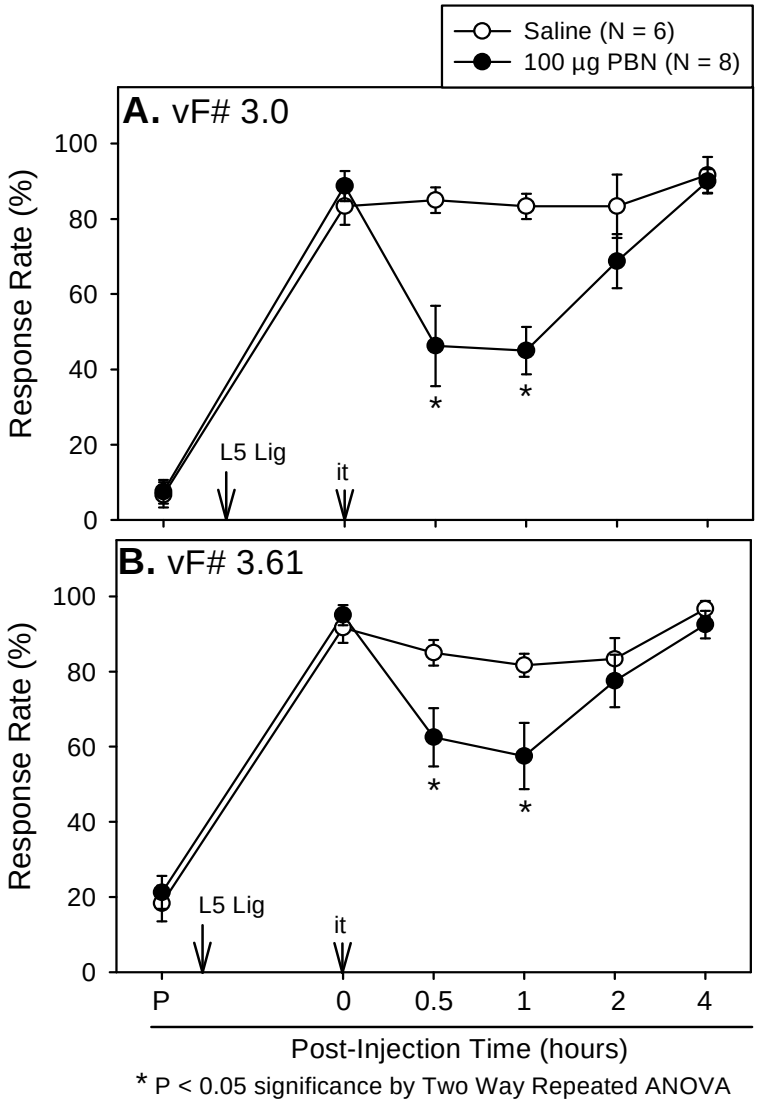
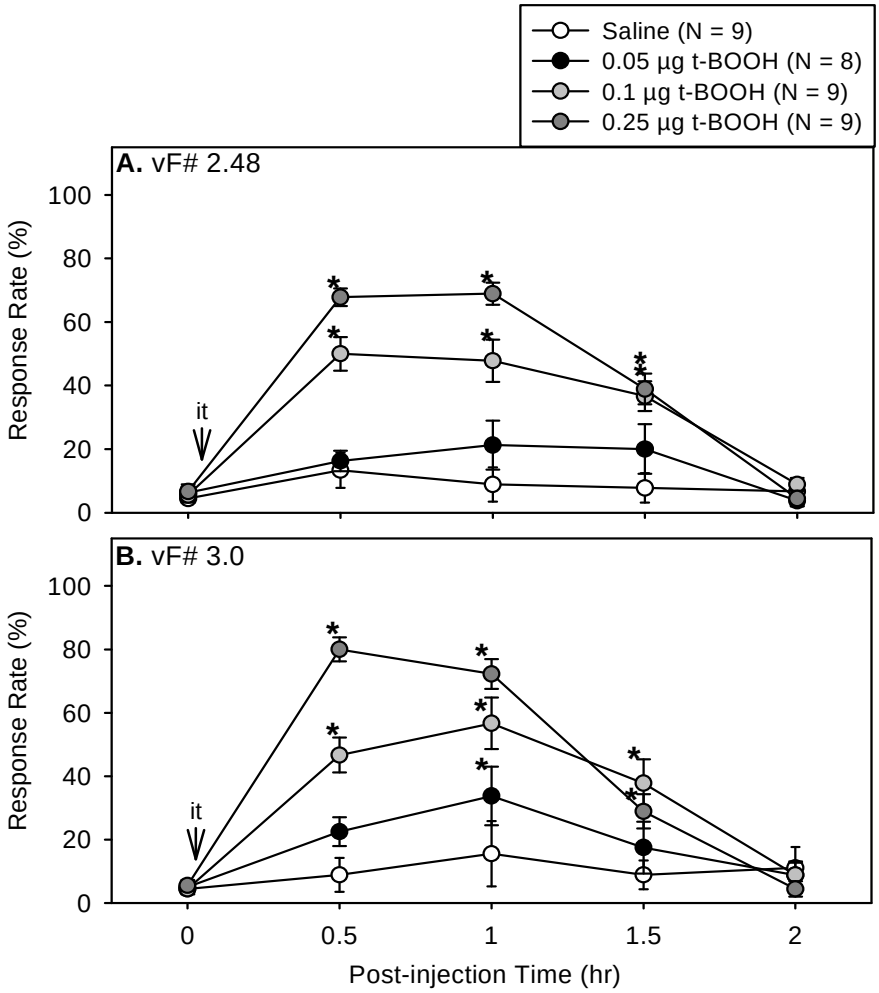


FIG. 3.4. **The effects of intrathecally administered ROS scavenger, PBN, on paw-withdrawal response rates in SNL mice.** SNL resulted in significantly increased response rates to von Frey filaments 3.0 (A) and 3.61 (B) from pre-surgical levels (P) in all operated mice (n = 14). One week after surgery, a single intrathecal injection of 100 µg PBN in 5 µl saline (n = 8) alleviated mechanical allodynia up to 2 h. Vehicle treatment did not affect response rates (n = 6). Data are presented as means ± SEM. P, pre-surgical time; L5 Lig, time of L5 SNL; i.t., intrathecal injection; *, the value is significantly (p < 0.05) different from that of the vehicle control by two-way repeated-measures ANOVA followed by Duncan's post hoc tests.

SCALE	150 mg/kg PBN (i.p.)			300 mg/kg TEMPOL (i.p.)			100 µg PBN (i.t.)		
	Time (hr)	Score (#/5)	Ratio (mice/total)	Time (hr)	Score (#/5)	Ratio (mice/total)	Time (hr)	Score (#/5)	Ratio (mice/total)
Posture	0	0/5	(8/8)	0	0/5	(6/6)	0	0/5	(8/8)
	0.5	**0/5 1/5**	**(2/8) (6/8)**	0.5	0/5	(6/6)	0.5	0/5	(8/8)
	1	0/5	(8/8)	1	0/5	(6/6)	1	0/5	(8/8)
	2	0/5	(8/8)	2	0/5	(6/6)	2	0/5	(8/8)
	4	0/5	(8/8)	4	0/5	(6/6)	4	0/5	(8/8)
Righting Reflex	0	0/5	(8/8)	0	0/5	(6/6)	0	0/5	(8/8)
	0.5	0/5	(8/8)	0.5	0/5	(6/6)	0.5	0/5	(8/8)
	1	0/5	(8/8)	1	0/5	(6/6)	1	0/5	(8/8)
	2	0/5	(8/8)	2	0/5	(6/6)	2	0/5	(8/8)
	4	0/5	(8/8)	4	0/5	(6/6)	4	0/5	(8/8)

TABLE 3.1. The scores for the assessment of posture and righting reflexes in mice receiving intraperitoneal PBN and TEMPOL and intrathecal PBN. Mice were observed immediately before injection, 0, and at 0.5, 1, 2, and 4 hr after injection for both posture and righting reflexes. The descriptions for the scores in each scale are described in Chapter 2. Each score was based on a five-point scale, 0 showing no impairment and 5 showing extreme impairment. The ratio indicates the number of mice from each group that received a certain score out of a total number of mice.



* P < 0.05 significance by Two Way Repeated ANOVA

FIG. 3.5. **The effects of an intrathecally administered ROS donor, t-BOOH, on paw withdrawal response rates in non-ligated, wild-type mice.** In non-ligated, wild-type mice, response rates to von Frey filaments 2.48 (A) and 3.0 (B) were measured prior to and after injection. A single intrathecal injection of 0.05, 0.10, or 0.25 µg t-BOOH dissolved in 5 µl saline (n = 8, 9, 9 respectively) dose-dependently increased paw withdrawals, which lasted up to 2 h. Vehicle injection did not affect response rates (n = 9). Data are presented as means ± SEM. i.t., intrathecal injection; *, the value is significantly ($p < 0.05$) different from that of the vehicle control by two-way repeated-measures ANOVA followed by Duncan's post hoc tests.

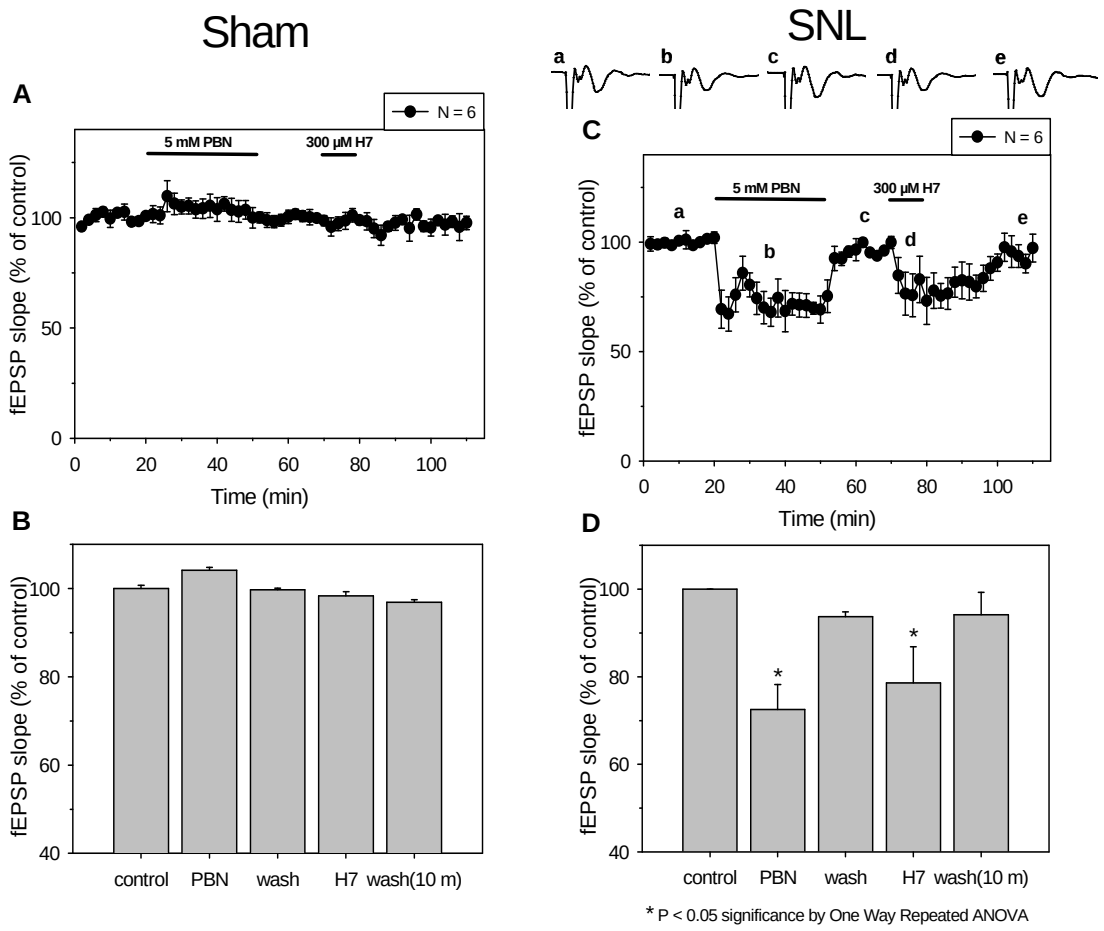


FIG. 3.6. **The effects of the ROS scavenger, PBN, on fEPSPs in sham and SNL mice.** (A and C) fEPSP slope values, generated by test stimuli (30-60 µA , 0.5 ms duration), are averaged in 2 minute intervals and are plotted as a percentage of the control against time. The average of current-evoked, baseline fEPSP slope values recorded for the initial 20 min of the experiment was used as the control. (C) 5 mM PBN application for 30 min (indicated by the horizontal bar) significantly decreased the fEPSP slopes in SNL slices (n = 6). Washout of PBN for 20 min led to the recovery of the slope values. Superfusion of the slices with 300 µM H7 for 10 min (indicated by the horizontal bar) significantly decreased slope values. Washout of H7 led to the recovery of fEPSPs. (A) PBN and H7 did not affect fEPSP slope values in sham control slices (n = 6). **a – e** are representative single fEPSP traces recorded during the times indicated in (C). (B and D) The summary graph of the averaged fEPSP slopes at various conditions: control baseline (control), during PBN superfusion (PBN), during washout of PBN (wash), during H7 superfusion (H7), and during the last 10 min of washout of H7 (wash (10 m)). Note that PBN and H7 significantly reduced the slopes of the fEPSPs in SNL slices in (D), suggesting that ROS are important for the maintenance of central sensitization. Data are presented as means ± SEM. *, the value is significantly (p < 0.05) different from the control period by one-way repeated measures ANOVA, followed by the Holm-Sidak post hoc test.

3.5 DISCUSSION

This study examined the modulation of pain in mice by ROS. SNL produced mechanical allodynia lasting longer than 8 weeks in FVB/NJ mice, and removal of ROS with a single, systemic injection of either PBN or TEMPOL transiently reduced pain behaviors. An intrathecal injection of PBN also produced a significant analgesic effect, suggesting that ROS in the spinal cord was necessary for the maintenance of pain. Elevated levels of ROS in the spinal cord were also sufficient to induce pain, which was demonstrated by the intrathecal injection of the ROS donor, t-BOOH. t-BOOH induced transient pain behaviors similar to those found in the chronic SNL model. Finally, this study shows for the first time that SNL enhanced the evoked responses in a population of dorsal horn neurons, and this enhancement was reduced by PBN application. Overall, these data demonstrate the importance of ROS involvement in the spinal cord for the development of pain and central sensitization.

Two different ROS scavengers with different mechanisms of action were used to demonstrate that removing ROS can have analgesic effects. PBN was chosen because it is the most widely-studied scavenger and has very few side effects (Kotake et al. 1999). In fact, PBN has been used successfully in the SNL model in the rat (Kim et al. 2004). DMPO, a water-soluble nitrone compound similar to PBN was also utilized, but proved less efficacious than PBN in preliminary studies. While it has been documented that PBN can also inhibit the gene induction of iNOS and can activate the transcription factor NFκB, the rapid onset of PBN's effects (within 30 minutes after administration) and the duration of PBN's effects (lasting only 2 hours) are not consistent with gene induction. Since other free radical scavengers (i.e. Vitamin E, DMPO) produce similar effects on mechanical allodynia as PBN, it is likely that PBN's analgesic effect is due to its ability to sequester ROS (Kim et al. 2004).

TEMPOL was chosen because it is a stable nitroxide radical that mimics the action of superoxide dismutase (Krishna et al. 1996), acting independently of PBN to remove ROS

(Tal 1996). The data show that the magnitude of TEMPOL's maximum effect was smaller than that of PBN, probably because PBN removes ROS non-discriminately, while TEMPOL acts more selectively to remove the superoxides. The dose of TEMPOL used was the highest level achieved without producing significant sedative effects; however, the dose of PBN used did produce a sedative effect. Since this dose of PBN resulted in impairment of normal rearing activity in 75% of mice up to 1 h after systemic injection, one cannot rule out the possibility that sedation may explain this greater reduction in response rates. Despite this result, intrathecal administration of PBN did not produce any obvious behavioral signs of sedation and yet had an analgesic effect comparable to that of TEMPOL. Therefore, the data suggest that superoxides may be important for the development of pain.

In this study, t-BOOH was shown to increase pain behaviors dose-dependently in non-ligated mice. This pain behavior was a transient response that lasted for approximately 90 minutes after intrathecal injection. The transient pain behavior elicited by t-BOOH was different from the pain behavior seen in the SNL model which lasts for weeks, not minutes. One explanation for the short duration of t-BOOH's action is that once it entered the intrathecal space, it was quickly decomposed by the spinal cord's antioxidant defense systems, such as glutathione peroxidase. On the other hand, a contrasting situation may be found in the SNL model where the neurons may overproduce ROS due to injury and overload the cellular machinery that disposes of ROS. However, the source of excessive ROS in the spinal cord, whether endogenous or exogenous, may have the same end result of initiating pain by affecting the same cellular mechanisms.

The electrophysiological experiment in this study recorded the summative changes in the synaptic function of a population of dorsal horn neurons in neuropathic, dorsal root-evoked fEPSPs due to the ROS scavenger, PBN. Previous studies were limited to only single cell recordings (Kim et al. 2006; Lee et al. 2007). Therefore, the finding that PBN reduced

the slopes of the sensitized fEPSPs as effectively as a protein kinase inhibitor provides convincing evidence that ROS contributes to the development of central sensitization. Furthermore, the reversible effects of PBN in the electrophysiological experiments support the transient analgesia seen in the behavioral experiments and suggest that removal of ROS can reverse the oxidative damage responsible for the pain production.

While it seems clear that ROS are involved in pain, the types and sources of ROS have not yet been fully identified. Studies have reported that various pain models demonstrate increased nitric oxide synthase activity, which produces nitric oxide, an important signaling molecule (Levy et al. 1999; Dolan et al. 2000; Guhring et al. 2000; Wu et al. 2001). Others working on inflammatory pain have shown that superoxides, normal by-products of cellular metabolism, are involved in pain (Wang et al. 2004; Khattab 2006). Likewise, our group has previously shown that superoxides are involved in neuropathic pain since there are increased levels of mitochondrial ROS in the dorsal horns of SNL rats using a mitochondrial marker, MitoTracker-Red, which fluoresces when oxidized (Park et al. 2006). Very recently, a study demonstrated that hydrogen peroxide concentrations in the spinal cord increased after sciatic nerve transection (Guedes et al. 2008). Other possible sources of ROS that may be involved in pain in the central nervous system include monoamine oxidase, cyclooxygenase, and NADPH oxidase (Kishida and Klann 2007).

Another critical question to be answered is how increased spinal ROS levels act to initiate or maintain pain. As stated earlier, a few studies show that ROS play a role in NMDA receptor activation in the development of pain (Woolf and Thompson 1991; Gao et al. 2007). Further studies must be done to delineate the roles that ROS serve in the development of central sensitization and pain. One important issue in this study of neuropathic pain involves the loss of inhibitory influences in the spinal cord by the GABA interneurons. This will be

looked at more closely in the next chapters where the influence of ROS on the function and expression of GABA neurons in the spinal cord dorsal horn will be examined.

In conclusion, this study demonstrated that ROS scavengers effectively produced analgesia in the mouse model of SNL-induced peripheral neuropathy and reduced the enhancement of the evoked responses from a population of “sensitized” dorsal horn neurons. Conversely, an injection of a ROS donor directly onto the spinal cord temporarily initiated pain behaviors in non-ligated mice. Therefore, ROS play an important role in the development of peripheral neuropathic pain.

CHAPTER 4

AN INCREASE IN SPINAL REACTIVE OXYGEN SPECIES CAUSES DECREASES IN GABA FUNCTION THAT CONTRIBUTE TO THE DEVELOPMENT OF NEUROPATHIC PAIN

4.1 ABSTRACT

Oxidative stress due to the overproduction of ROS or impaired removal by the cell's defense systems may disrupt normal cellular processes and ultimately result in cell death. Previously, we found that ROS scavengers attenuate pain behavior in the murine spinal nerve ligation (SNL) model of neuropathic pain. One feature of neuropathic pain is the loss of spinal GABAergic inhibition in the pain processing pathway. We hypothesize that ROS directly contribute to this loss of GABAergic inhibition since previous studies have demonstrated that GABA neurons are particularly vulnerable to oxidative stress. The purpose of this study was to assess the role of GABA in the development of neuropathic pain in mice and to study the effects of ROS accumulation on the function of the spinal GABAergic system and on the development of pain. **METHODS:** SNL mice were produced by tight ligation of the L5 spinal nerve in FVB/NJ adult male mice. Mechanical allodynia was assessed by measuring the paw withdrawal response rates to von Frey filaments 3.0 (0.1 g) or 3.61 (0.41 g). At four days post SNL, the effects of the $GABA_A$ agonist, muscimol, at 0.05 and 0.1 µg in 5 µl (i.t.) and the $GABA_B$ agonist, baclofen, at 0.03 and 0.06 µg in 5 µl (i.t) on pain behavior were tested. Furthermore, to examine the role of GABA in modulating pain behavior, the effects of the intrathecally administered $GABA_A$ antagonist, bicuculline, at 0.5

or 1 µg in 5 µl (i.t.) and the $GABA_B$ antagonist, CGP43681, at 0.25 or 0.5 µg in 5 µl (i.t.) in non-ligated mice were tested. In addition, to determine the effect of the ROS donor, t-BOOH, on neuronal function, *in vitro* whole cell recordings were performed on lumbar spinal cord slices from non-ligated, transgenic GAD67-EGFP mice expressing enhanced green fluorescent protein-tagged (EGFP+) GABA neurons. t-BOOH (2 mM) was applied to both EGFP+ GABA neurons and non-labeled neurons in lamina II of the spinal cord dorsal horn, and changes in the number of sustained action potentials elicited by a long current injection pulse were recorded. To determine whether SNL causes an alteration in the spinal GABAergic system through the actions of ROS, field excitatory postsynaptic potentials (fEPSPs) were studied in the superficial dorsal horn of both sham and SNL mice. The $GABA_A$ receptor antagonist, bicuculline, was used to determine whether PBN's ability to reduce central sensitization was mediated by GABA neurotransmission. **RESULTS:** A single intrathecal injection of either muscimol or baclofen dose-dependently and transiently reversed mechanical allodynia up to 1.5 – 2 h four days after SNL. Conversely, intrathecally administered bicuculline or CGP43681 dose-dependently induced mechanical allodynia for more than 1.5 h after injection in normal mice. t-BOOH decreased the excitability of the majority of EFGP-labeled GABA neurons and increased the excitability of the majority of non-EGFP+ dorsal horn neurons in lamina II of the spinal cord *in vitro*. Field potential recordings show that bicuculline attenuated PBN's effectiveness in reducing central sensitization in the neuropathic dorsal horn. **CONCLUSIONS:** Overall, these data suggest that ROS may contribute to neuropathic pain by directly decreasing GABAergic function, which may include changes in the excitability of the GABA interneuron itself.

4.2 INTRODUCTION

Gamma-aminobutyric acid (GABA) is one of the main inhibitory neurotransmitters in the mammalian nervous system, especially in the spinal dorsal horn. The role of the GABAergic system in pain perception has been extensively studied. Pharmacological antagonism of spinal GABA receptors results in tactile allodynia in rodents with characteristics analogous to those found in chronic pain states (Yaksh 1989; Minami et al. 1994; Sivilotti and Woolf 1994; Malan et al. 2002; Gwak et al. 2006). Moreover, administration of GABA and GABA receptor agonists has been shown to alleviate pain behaviors in different models of peripheral neuropathy (Hwang and Yaksh 1997; Eaton et al. 1999; Patel et al. 2001; Malan et al. 2002; Franek et al. 2004). Electrophysiological studies found that in two peripheral neuropathic pain models, there are decreased $GABA_A$-receptor-mediated inhibitory postsynaptic currents with concomitant decreases in dorsal horn levels of GABA synthesizing enzymes and increased neuronal apoptosis (Moore et al. 2002). The same group demonstrated that antagonizing spinal $GABA_A$-receptors resulted in facilitation of excitatory synaptic transmission (Baba et al. 2003).

On the other hand, contradictory findings have shown no significant change in the number of GABA-IR neurons (Polgar et al. 2003) or GABA content in synaptosome preparations (Somers and Clemente 2002) in the ipsilateral dorsal horn of the CCI rats when compared to the contralateral side or sham animals. Thus, many agree that one of the mechanisms behind the development of chronic neuropathic pain is disinhibition, particularly from the disruption of the spinal GABAergic system; however, questions remain about how this dysfunction occurs and where exactly does it happen (i.e. GABA neurons, postsynaptic receptors, transporters).

Several studies have proposed a unique vulnerability of GABA neurons to oxidative stress and the effects of ROS. For instance, hyperoxia reduced GABAergic inhibition of Purkinje neurons *in vivo* (Bickford et al. 1999). Similarly, resveratrol, an antioxidant found in red wine, imparted neuroprotective effects *in vivo* against kainate-induced excitotoxicity (oxidative stress) since it selectively attenuated the decrease in GABA synthesizing enzyme levels in the rat striatum, which were used as markers of GABA neurons (Virgili and Contestabile 2000). Furthermore, in isolated sheep brain synaptosomes, Fe^{2}+/ascorbate-induced lipid peroxidation caused a significant reduction in both Ca^{2+} dependent and Ca^{2+} independent release of GABA stores, as well as increased free ionic calcium levels (Palmeira et al. 1993). The data implied that oxidative stress may lead to impaired GABA synaptic transmission along with calcium-induced excitotoxicity and cell damage (Palmeira et al. 1993). Recently, the ROS, hydrogen peroxide (H_2O_2), was found to modulate the presynaptic activity of spinal GABAergic interneurons through the IP_3R-mediated-release of calcium, indicating the susceptibility of GABA neurons in the spinal cord to oxidative stress (Minami et al. 1994; Takahashi et al. 2007). Therefore, we hypothesize that ROS could directly alter the spinal GABA system, disrupting normal GABA transmission by targeting the function of GABA neurons, which may contribute to the maintenance of neuropathic pain.

In Chapter 3, the importance of ROS for the development of central sensitization and neuropathic pain was demonstrated in the murine SNL model. Now, the issue of how ROS influences the sensitivity of pain-transducing, dorsal horn neurons will be addressed. Thus, the present study examines the role of ROS in neuropathic pain with respect to their interaction with the spinal GABA system. First, the importance of GABA's inhibitory role in pain transmission is confirmed using behavioral testing for mechanical allodynia after

pharmacological manipulation of the spinal GABA system. Secondly, in order to study directly the properties of GABA neurons, a transgenic mouse line is used that expresses the glutamic acid decarboxylase 67-enhanced green fluorescent protein (GAD67-EGFP) transgene, which unequivocally labels a subset of GABA neurons in the spinal cord (Oliva Jr et al. 2000; Heinke et al. 2004). The effects of the ROS donor, t-BOOH, is determined on the excitability of fluorescent, GABAergic interneurons in lamina II of the spinal dorsal horn using whole cell recordings. Finally, to determine whether the removal of ROS reduces central sensitization by augmenting GABA neurotransmission, the effects of the ROS scavenger, PBN, and the $GABA_A$ receptor antagonist, bicuculline, are examined on field excitatory postsynaptic potential (fEPSP) recordings in the superficial dorsal horn of neuropathic mice.

4.3 MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

4.4 RESULTS

4.4.1 INTRATHECALLY ADMINISTERED GABA RECEPTOR AGONISTS TRANSIENTLY ATTENUATE PAIN BEHAVIORS AFTER SNL

In order to investigate the role of ROS in modulating spinal GABAergic function, GABA's inhibitory role in pain processing was first confirmed. The effects of a single intrathecal injection of the $GABA_A$ agonist, muscimol (0.05 or 0.1 µg, i.t.), on pain behaviors for von Frey filaments 3.0 and 3.61 are shown in **Fig. 4.1A** and **Fig 4.1B**, respectively. The

doses of muscimol used in this study did not impair the animal's mobility or produce any noticeable side effects, such as hyperexcitability or seizures. Mice resumed normal activity immediately after these injections. In preliminary studies, muscimol doses of 0.5 µg and higher produced hind-limb paralysis up to 3 hours after intrathecal injection into SNL mice. Four days after SNL, the foot withdrawal response rates to filaments 3.0 and 3.61 were 81 ± 2% (mean ± SEM) and 95 ± 2%, respectively. After muscimol injection (0.1 µg, i.t.), the responses were maximally reduced to 35 ± 13% and 40 ± 13% at 0.5 h. Intrathecal muscimol significantly decreased the response rates up to 1.5 h. in a dose dependent manner. On the other hand, the same volume of saline (i.t.) had little effect on the response rates, which changed from a pre-injection value of 96 ± 3% to 79 ± 6% 0.5 h after injection for von Frey filament 3.0 and from a pre-injection value of 98 ± 3% to 88 ± 4% 0.5 h after injection for von Frey filament 3.61.

Furthermore, the effects of a single intrathecal injection of the $GABA_B$ agonist, baclofen (0.03 or 0.06 µg, i.t.), on pain behaviors for von Frey filaments 3.0 and 3.61 are shown in **Fig. 4.2A** and **Fig 4.2B**, respectively. The concentrations of baclofen used in this study did not impair the animal's mobility or produce any noticeable side effects, such as hyperexcitability or seizures. Mice resumed normal activity immediately after these injections. Doses of baclofen 0.2 µg and higher produced hind-limb paralysis lasting up to 3 hours after injection. Four days after SNL, the response rates to filaments 3.0 and 3.61 were 86 ± 4% (mean ± SEM) and 91 ± 3%, respectively. After baclofen injection (0.06 µl, i.t.), the responses were maximally reduced to 25 ± 10% and 28 ± 14% at 0.5 h after injection. Intrathecal baclofen dose-dependently and significantly decreased the response rates up to 1.5 - 2 h. On the other hand, the same volume of saline (i.t.) had little effect on the response

rates, changing from a pre-injection value of 97 ± 2% to 76 ± 6% 0.5 h after injection for von Frey filament 3.0 and from a pre-injection value of 97 ± 3% to 80 ± 7% 0.5 h after injection for von Frey filament 3.61. Therefore, supplementation with GABA receptor agonists in the spinal cord alleviates pain behaviors in the SNL model in mice, confirming the importance of GABA's inhibitory influence in pain processing.

4.4.2 INTRATHECALLY ADMINISTERED GABA RECEPTOR ANTAGONISTS TRANSIENTLY INDUCE PAIN BEHAVIORS IN NON-LIGATED MICE

Since augmenting GABA transmission in the spinal cord has been shown to temporarily relieve pain behaviors in the SNL model of neuropathic mice, the data suggest that decreased GABAergic inhibitory function contributes to neuropathic pain. Conversely to determine whether suppressing GABA inhibition in the spinal cord would initiate pain behaviors in non-injured mice, the effects of intrathecally administered GABA antagonists were investigated. Bicuculline was used for the $GABA_A$ antagonist and CGP43681 was used for the $GABA_B$ antagonist. The effects of a single intrathecal injection of bicuculline (0.5 or 1 µg) on paw withdrawal response rates to von Frey filament 3.0 are shown in **Fig. 4.3A**. Baseline responses were recorded for three groups of mice prior to receiving a single intrathecal injection of either 0.5 or 1 µg bicuculline dissolved in 5 µl saline or an injection of 5 µl saline (vehicle control). Bicuculline dose-dependently increased paw withdrawal response rates compared to vehicle injection, and the increases lasted over 1.5 h. For example, 1 µg bicuculline changed response rates from 3 ± 2% to 64 ± 7% at 0.5 h after injection, which was significantly different from the pre-injection value.

Likewise, the effects of a single intrathecal injection of CGP43681 (0.25 and 0.5 µg, i.t.) on paw withdrawal response rates to von Frey filament 3.0 are shown in **Fig. 4.3B**. Baseline responses were recorded for three groups of mice prior to receiving a single intrathecal injection of either 0.25 or 0.5 µg CGP46381 dissolved in 5 µl saline or an injection of 5 µl saline (vehicle control). CGP46381 dose-dependently increased paw withdrawal response rates compared to vehicle injection, which lasted over 1.5 h. For example, 0.5 µg CGP46381 changed response rates from 8 ± 3% to 80 ± 7% at 0.5 h after injection, which was significantly different from the pre-injection value. Therefore, the data show that antagonism of the $GABA_A$ and $GABA_B$ receptors in the spinal cord resulted in mechanical allodynia in normal mice, indicating that decreasing GABAergic inhibitory tone in the spinal cord may be important for the maintenance of pain behaviors.

4.4.3 A ROS DONOR DIFFERENTIALLY MODULATES THE EXCITABILITY OF EGFP-TAGGED GABA NEURONS VERSUS NON-TAGGED, DORSAL HORN NEURONS IN LAMINA II OF THE SPINAL DORSAL HORN *IN VITRO*

Once the importance of GABA for the generation of neuropathic pain had been confirmed in the murine model of SNL, the effects of elevated levels of ROS on the electrophysiological properties of EGFP-tagged GABA neurons and non-tagged neurons were studied. The transgenic mouse line which expresses EGFP-tagged GABA neurons was used to distinguish them easily from other neurons. The effects of a ROS donor, tert-butyl hydroperoxide (t-BOOH), on the responses of superficial dorsal horn neurons located in laminae I-III of the dorsal horn in the L5 spinal cord of transgenic, GAD67-EGFP mice are shown in **Fig. 4.4**.

Sustained action potentials were recorded from each neuron held at a depolarizing potential (-50 mV) during whole cell recording. Current injection on the order of 1.5x to 2x the magnitude of the activation threshold of the neurons was used to maintain the holding potential. Representative traces for the activity of a non-tagged dorsal horn neuron (n = 15) and an EGFP-tagged GABA neuron (n = 10) are displayed in **Fig 4.4A** and **Fig. 4.4B**, respectively. Superfusion with 2 mM t-BOOH for 5 minutes resulted in an increase in the frequency of sustained action potentials in 10 of the 15 non-tagged dorsal horn neurons (**Fig. 4.4C**). The frequency of sustained action potentials was significantly increased, from 1.3 ± 0.5 Hz to 2.8 ± 1.3 Hz, which is plotted in **Fig. 4.4D**. In contrast, t-BOOH attenuated the frequency of sustained action potentials observed in 9 of the 10 EGFP-tagged GABA neurons (**Fig. 4.4E**). The average frequency of sustained action potentials was significantly reduced, from 4.0 ± 1.3 Hz to 2.1 ± 0.8 Hz as summarized in **Fig. 4.4F**. Therefore, the data demonstrate that oxidative stress induces differential changes in the dorsal horn neurons and interneurons that may be involved in central sensitization. Namely, it reduces excitability of the GABA neurons and increases the excitability of other dorsal horn neurons, which possibly include second order pain transmission neurons and excitatory glutamatergic interneurons in the spinal cord.

4.4.4 THE SUPPRESSION OF CENTRAL SENSITIZATION BY THE ROS SCAVENGER, PBN, DEPENDS ON THE INHIBITORY INFLUENCES OF THE SPINAL GABA SYSTEM

In order to more closely examine the relationship between the effects of ROS scavengers and the spinal GABAergic system, field potential recordings were performed in spinal cord slice preparations of both sham and SNL mice at three days to one week after surgery. The field excitatory postsynaptic potentials (fEPSPs) evoked by current stimulation

(30-60 μA, 0.5 ms) of the dorsal root were recorded. The averaged slopes of fEPSPs for every 2 minutes are plotted (**Fig. 4.5A, 4.5C**) along with summary graphs for the average fEPSP slope values for each condition (**Fig. 4.5B, 4.5D**). When 5 mM PBN was administered in the recording chamber, the slopes of the fEPSPs decreased in the SNL group (**Fig. 4.5C**) but not in the sham group (**Fig. 4.5A**). On average, PBN significantly reduced the slopes of neuropathic fEPSPs to 72.57 ± 14.91% (mean ± SE) of the baseline control levels (**Fig. 4.5D**). During the washout of PBN, the slopes recovered to 99.86 ± 4.59% of the control levels (**Fig. 4.5D**).

Superfusion with both 5 mM PBN and 10 μM bicuculline brought the average fEPSP slope values to 92.18 ± 11.19%, which was not significantly different from the baseline levels (**Fig 4.5D**), suggesting that PBN's effect is diminished by bicuculline. Recovery of the fEPSPs occurred during wash out, reaching an average of 104.42 ± 3.06% (**Fig. 4.5C, 4.5D**). Similar to PBN, co-administration of PBN and bicuculline had no effect on the fEPSPs in the sham group (**Fig. 4.5A**), suggesting that the dorsal horn neurons in the sham slices are not sensitized.

In contrast, in the SNL slices, PBN's effectiveness in reducing the fEPSP slope values was dramatically attenuated by bicuculline's postsynaptic blockade of the $GABA_A$ receptor. This suggests that the removal of ROS was reducing central sensitization by a modification of events upstream to the $GABA_A$ receptor, possibly by acting on the GABAergic interneurons to augment the inhibitory influence of ROS. This evidence, in addition to the whole cell recordings, suggests that increased spinal ROS levels may act primarily on GABA interneurons to facilitate central sensitization while causing GABA dysfunction.

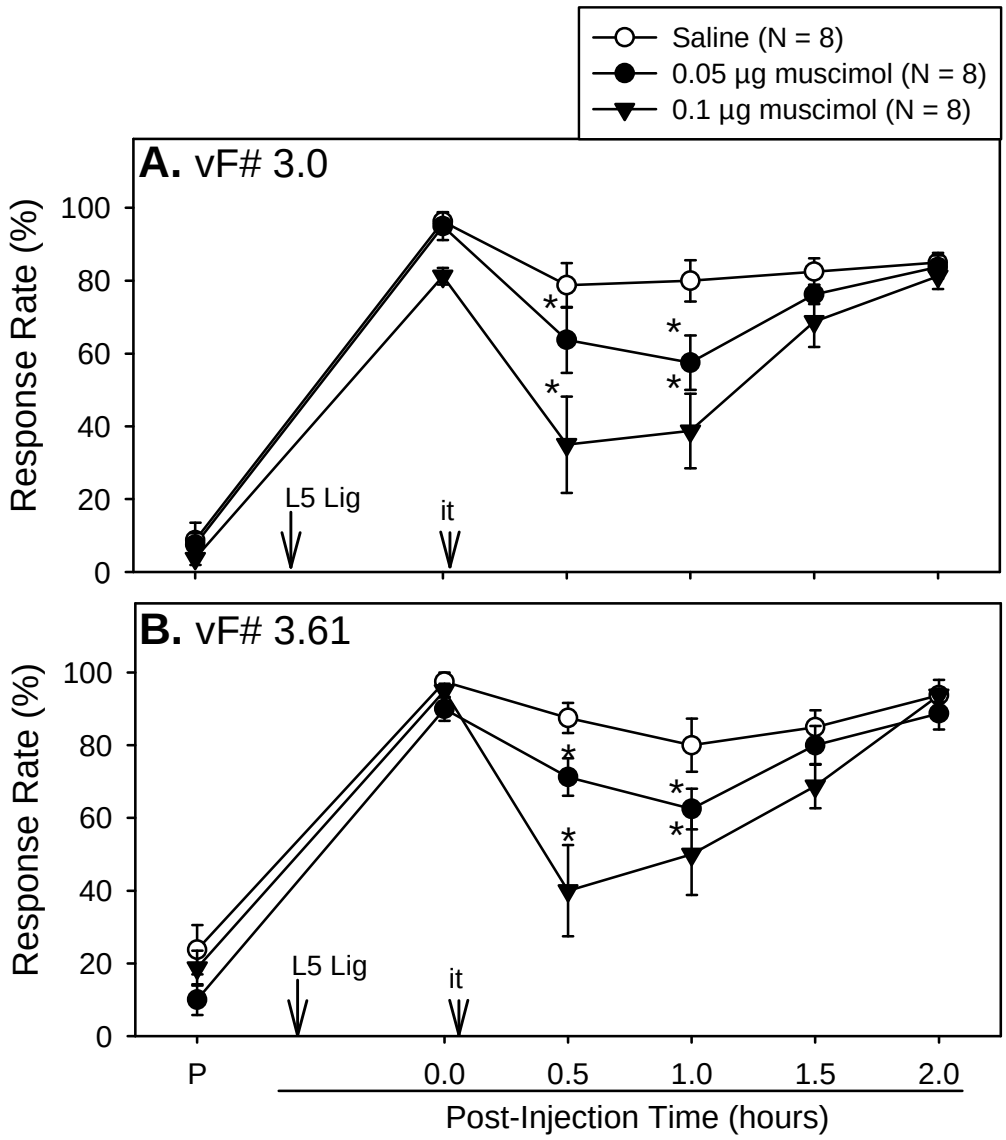


FIG. 4.1 **The effects of the intrathecally administered $GABA_A$ receptor agonist, muscimol, on paw withdrawal responses in SNL mice.** SNL resulted in significantly increased response rates to von Frey filaments 3.0 (A) and 3.61 (B) from pre-surgical levels (P) in all operated mice (n = 24). Four days after surgery, intrathecal injection of 0.05 and 0.1 µg muscimol dissolved in 5 µl saline (n = 8 for each group) dose-dependently decreased nociceptive responses up to 1.5 h. Vehicle treatment did not affect response rates (n = 8). Data are presented as means ± SEM. P, pre-surgical time; L5 Lig, time of L5 SNL; i.t., intrathecal injection; *, the value is significantly ($p < 0.05$) different from that of the vehicle control by two-way repeated-measures ANOVA followed by the Holm-Sidak post hoc tests.

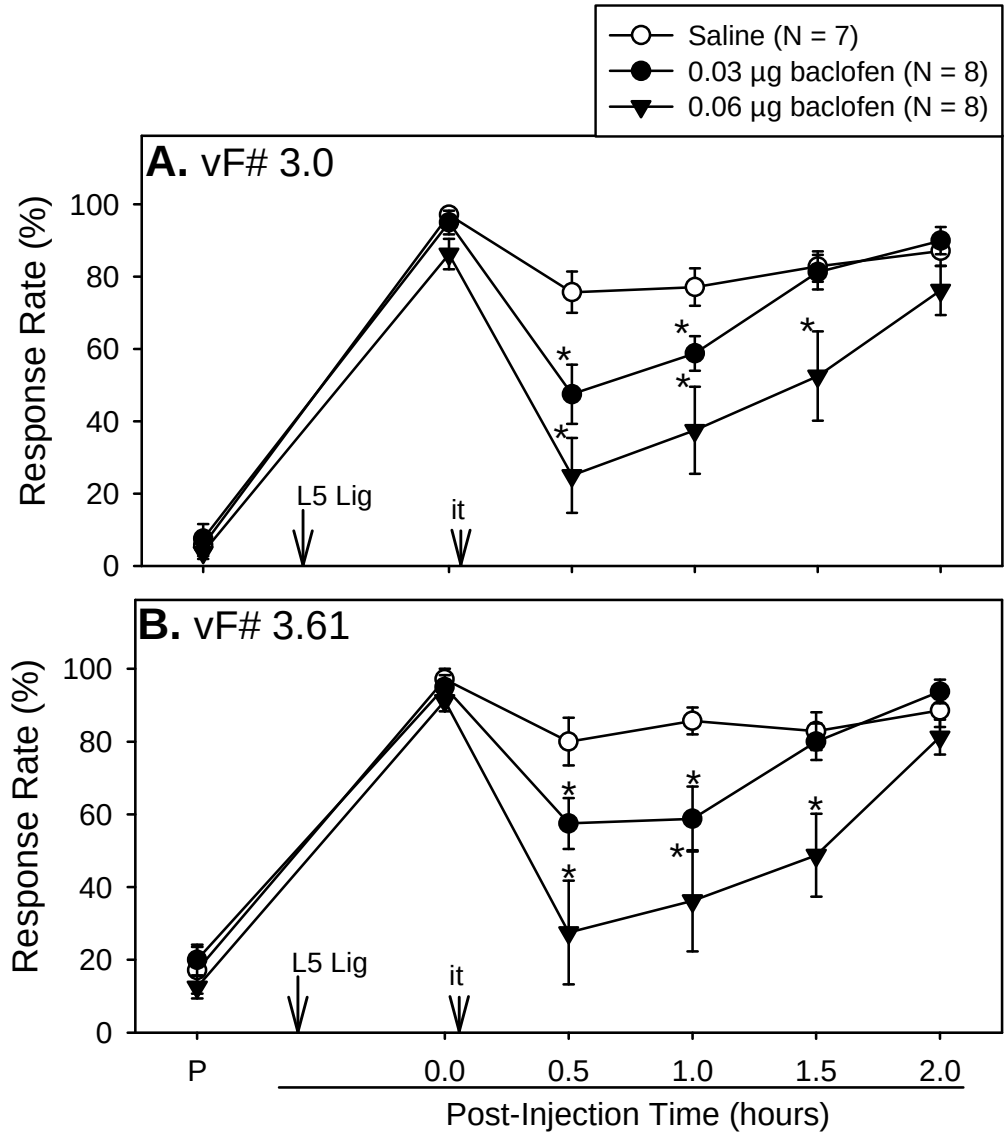


FIG. 4.2. **The effects of the intrathecally administered $GABA_B$ receptor agonist, baclofen, on paw withdrawal responses in SNL mice.** SNL resulted in significantly increased response rates to von Frey filaments 3.0 (A) and 3.61 (B) from pre-surgical levels (P) in all operated mice (n = 23). Four days after surgery, intrathecal injection of 0.03 and 0.06 µg baclofen dissolved in 5 µl saline (n = 8 for each group) dose-dependently decreased nociceptive responses up to 1.5 - 2 h. Vehicle treatment did not affect response rates (n = 7). Data are presented as means ± SEM. P, pre-surgical time; L5 Lig, time of L5 SNL; i.t., intrathecal injection; *, the value is significantly ($p < 0.05$) different from that of the vehicle control by two-way repeated-measures ANOVA followed by the Holm-Sidak post hoc tests.

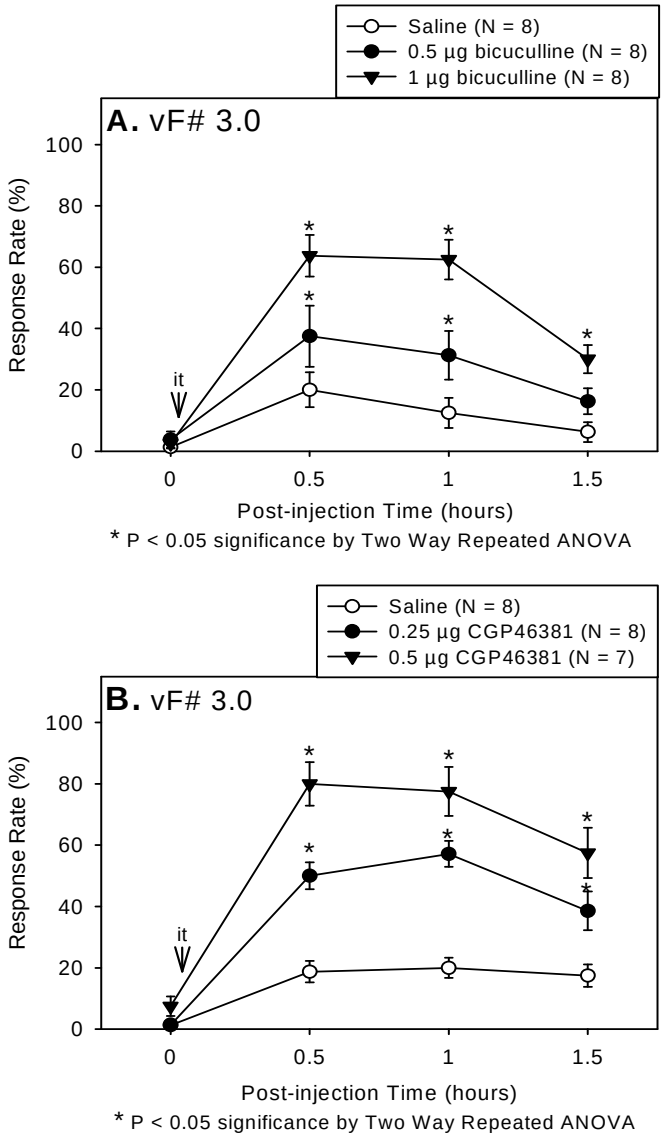


FIG. 4.3. **The effects of intrathecally administered GABA receptor antagonists on paw withdrawal responses in non-ligated, wild-type mice.** (A) In non-ligated, wild-type mice, a single intrathecal injection of 0.5 or 1 µg bicuculline dissolved in 5 µl saline (n = 8 for each group) dose-dependently increased paw withdrawals to von Frey filament 3.0, which lasted longer than 1.5 h. Vehicle injection did not affect response rates (n = 8). (B) In non-ligated, wild-type mice, a single intrathecal injection of 0.25 or 0.5 µg CGP43681 dissolved in 5 µl saline (n = 8, 7 respectively) dose-dependently increased paw withdrawals to von Frey filament 3.0, which lasted longer than 1.5 h. Vehicle injection did not affect response rates (n = 8). Data are presented as means ± SEM. i.t., intrathecal injection; *, the value is significantly ($p < 0.05$) different from that of the vehicle control by two-way repeated-measures ANOVA followed by the Holm-Sidak post hoc tests.

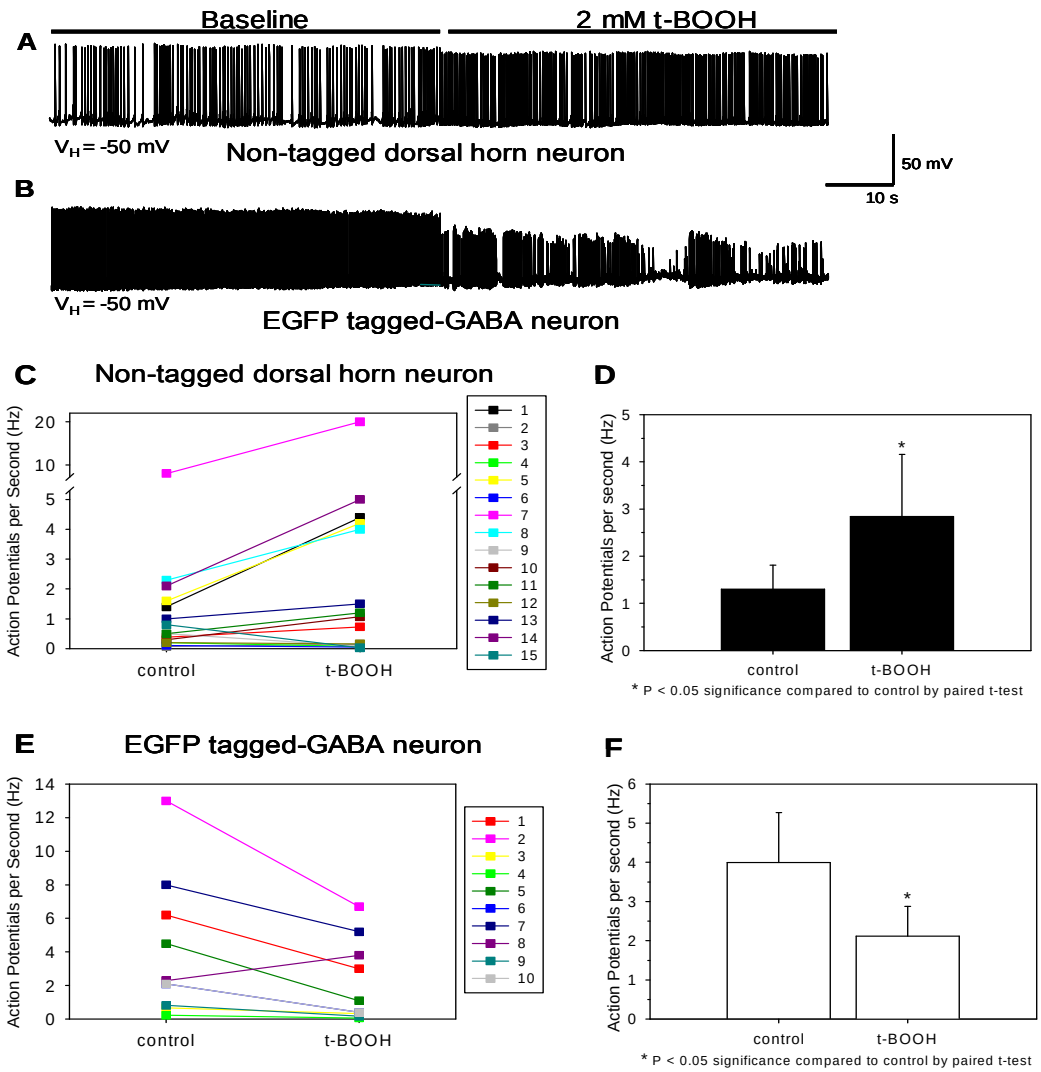


FIG. 4.4. **The effects of a ROS donor on EGFP-tagged GABA neurons and non-tagged dorsal horn neurons in laminae II of the spinal cord.** Under whole cell conditions, the neuron was held at a depolarizing potential, (-50 mV) to generate sustained action potentials (traces shown in A and B). After one minute of baseline recording, 2 mM t-BOOH was superfused in the chamber, increasing the frequency of action potentials in non-GABA neurons and decreasing the frequency of action potentials in GABA neurons. (C and E) The average number of action potentials generated every one second are plotted for non-tagged neurons (n = 15) and EGFP-tagged GABA neurons (n = 10). (D and F) The frequency means for each group of neurons during the control and t-BOOH perfusion periods are plotted. t-BOOH significantly increases the frequency of action potentials (excitability) of non-tagged, dorsal horn neurons and significantly decreases the number of action potentials or excitability of GABA neurons. Data are presented as means ± SEM. *, the value is significantly ($p < 0.05$) different from that of the vehicle control by paired t-test.

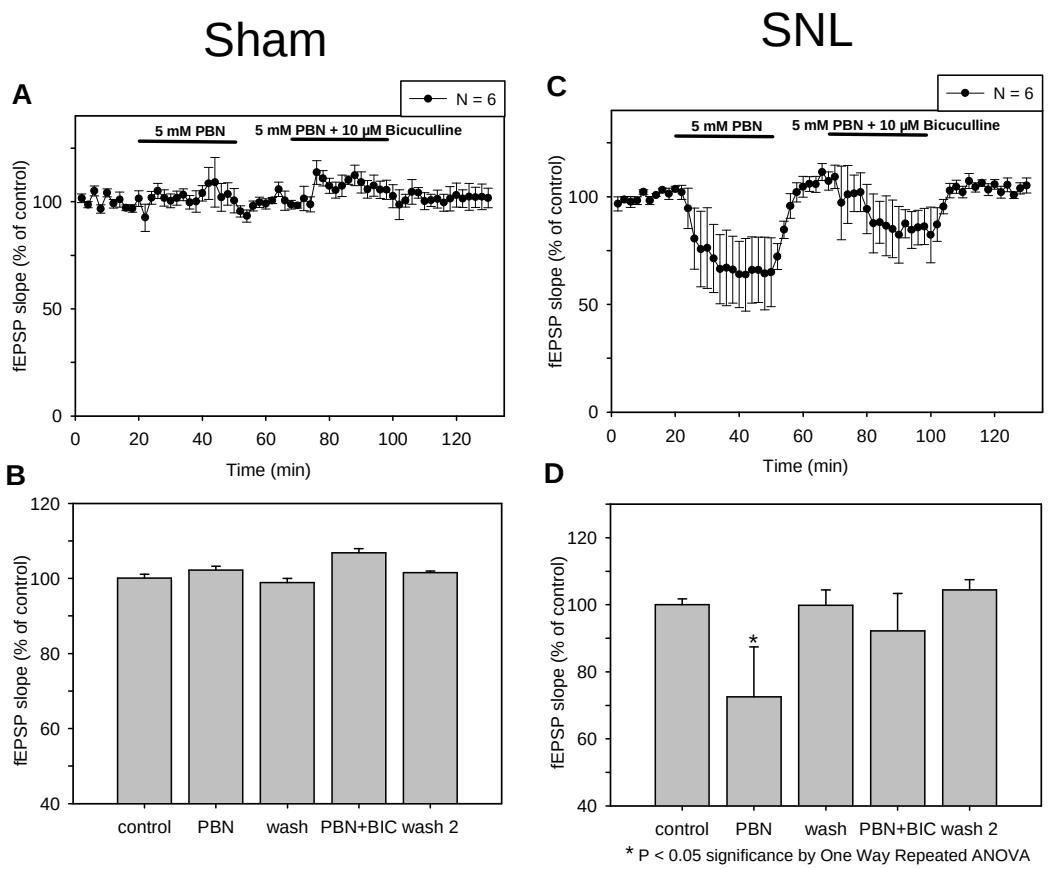


FIG. 4.5. **The effect of the ROS scavenger, PBN, and the $GABA_A$ receptor antagonist, bicuculline, on fEPSPs in sham and SNL mice.** (A and C) fEPSP slope values, generated by test stimuli (30-60 µA , 0.5 ms duration), are averaged in 2 minute intervals and are plotted as a percentage of the control against time. The average of current-evoked, baseline fEPSP slope values recorded for the initial 20 min of the experiment was used as the control. (C) 5mM PBN application for 30 min (indicated by the horizontal bar) significantly decreased the fEPSP slopes in SNL slices (n = 4). Washout of PBN for 20 min led to the recovery of the slope values. Superfusion of the slices with 5 mM PBN + 10 µM bicuculline for 30 min (indicated by the horizontal bar) lessened PBN's effect on the slope values. Washout of 5 mM PBN + 10 µM bicuculline led to the recovery of the fEPSPs. (A) PBN and PBN + bicuculline did not affect fEPSP slope values in sham control slices (n = 6). (B and D) The summary graph of the averaged fEPSP slopes under various conditions: control baseline (control), during PBN superfusion (PBN), during washout of PBN (wash), during PBN + bicuculline superfusion (PBN+BIC), and during the washout of PBN + bicuculline (wash 2). Note that PBN + bicuculline did not reduce the slopes of the fEPSPs in SNL slices as much as PBN alone in (D), suggesting that PBN acts through the spinal $GABA_A$ receptors to reduce central sensitization. *, the value is significantly ($p < 0.05$) different from the control period by one-way repeated measures ANOVA, followed by the Holm-Sidak post hoc test.

4.5 DISCUSSION

The present study examined the modulation of pain in mice by GABA. Supplementation of spinal GABA receptor agonists with a single, intrathecal injection of either muscimol or baclofen transiently reduced pain behaviors produced by SNL. The converse was also demonstrated, that spinal antagonism of the GABA receptors by either bicuculline or CGP 43681 alone could initiate transient pain behaviors similar to those found in the chronic SNL model. These data support previous studies which show the importance of GABA's inhibitory role in pain transmission. Also, the data confirm previous reports regarding the presence of an inhibitory spinal GABAergic tone under normal conditions and its loss in neuropathic pain. Finally, this study shows the direct effects of increased ROS levels on the excitability of GABAergic interneurons and non-labeled dorsal horn neurons in the superficial dorsal horn, reducing spontaneous responses and enhancing responses, respectively. These data were supported by fEPSP recordings that demonstrated that central sensitization could be potentiated by the blockade of postsynaptic $GABA_A$ receptors on the dorsal horn neurons which curtail the suppressive effects of a ROS scavenger acting directly on GABA neurons. A simplified diagram of the proposed mechanism of action by ROS is displayed in **Fig. 4.6**. Overall, these data demonstrate the importance of GABA dysfunction in the spinal cord for the development of pain and central sensitization and the possible role of oxidative stress in initiating this dysfunction.

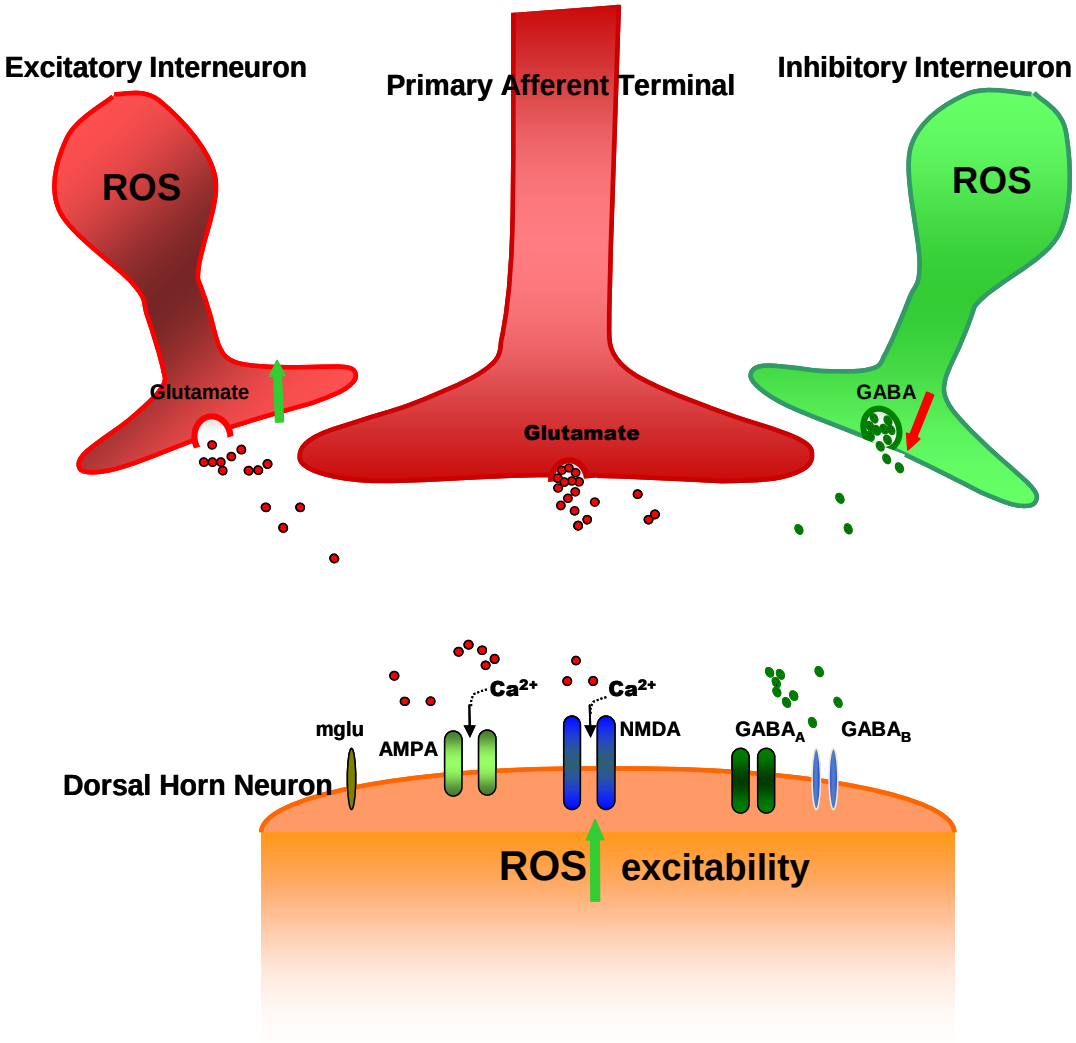


FIG. 4.6. The proposed role of ROS in promoting central sensitization in the spinal cord. The GABA neuron exerts its inhibitory influence through the release of GABA onto the $GABA_A$ and $GABA_B$ receptors located postsynaptically on the dorsal horn neuron. There is a resultant decrease in the responses of the postsynaptic neuron. However, ROS decrease the excitability of the GABA neuron, probably causing a reduction in GABA release from its synaptic terminals while comcomitantly increasing the excitability of the postsynaptic neuron and reducing its activation threshold. ROS may also increase the excitability of excitatory interneurons to enhance further nociceptive signaling. Removal of ROS ameliorates this process, preventing the block on normal GABA transmission. Antagonism of the postsynaptic GABA receptors reduces the effect of PBN because they are downstream of PBN's action. Therefore, even with increased GABA release, GABA cannot exert its actions by binding to its receptors on the postsynaptic neuron.

The pharmacological data show that both the $GABA_A$ and $GABA_B$ receptors mediate nociceptive processing in the spinal cord that is important for the maintenance of pain. The activation of each receptor set into motion very different pathways. Activation of a spinal $GABA_A$ receptor leads to the movement of chloride ions and depolarization of the primary afferent terminal along with presynaptic inhibition, resulting in reduced neurotransmitter release. In the postsynaptic dorsal horn neuron, $GABA_A$ activation usually causes hyperpolarization of the cell and decreased excitability. Likewise, $GABA_B$ receptor activation results in reduced neurotransmitter release from the primary afferent terminal and thus, presynaptic inhibition. Also, increased K^+ conductance in the postsynaptic neuron leads to suppression of nociceptive signaling.

Recently, a novel mechanism was proposed to explain the loss of GABAergic inhibition in the neuropathic spinal cord. In lamina I neurons, peripheral nerve injury resulted in a decrease in the expression of the potassium-chloride co-transporter 2 (KCC2) and consequently, a pathologically high intracellular concentration of chloride. This shifted the anion reversal potential so that activation of the $GABA_A$ receptor caused chloride efflux and depolarization, increasing lamina I neuronal excitability (Coull et al. 2003). This may be mediated in part by the release of brain-derived neurotrophic factor (BDNF) from activated microglia (Coull et al. 2005) and is dependent on BDNF's actions on the TrkB receptors (Miletic and Miletic 2007). However, this was demonstrated only in lamina I neurons, which are important for nociceptive input from C fibers. Neurons in lamina II and deeper laminae are also important for pain processing from C fibers as well as Aδ fibers. Furthermore, it seems the consequences after nerve injury on the GABA system are complex, functionally. While a study concluded there was an increased endogenous GABAergic tone in the spinal

cord after peripheral nerve injury (Kontinen et al. 2001), the contrary has also been demonstrated. Decreased $GABA_A$-mediated currents in lamina II neurons are seen after peripheral nerve injury in various neuropathic models (Moore et al. 2002). Recently, nociceptive input that causes the release of glutamate from the primary afferent terminal was reported to heterosynaptically activate group II and group III metabotropic glutamate receptors located on spinal GABAergic interneurons, leading to a suppression of GABA transmission (Zhou et al. 2007). Besides this, the α2 and α3 subunits of the $GABA_A$ receptor, which are concentrated in neurons of laminae I and II, were found to be responsible for the antinociceptive effect of the benzodiazepine, diazepam, in the CCI model; this indicates that activation of the $GABA_A$ receptor in lamina I does not necessarily produce excitation, but also inhibits pain (Knabl et al. 2008). Although GABA receptor activation results in variable effects in localized regions of the brain and spinal cord, generally, their activation leads to antinociceptive responses (Hwang and Yaksh 1997; Malan et al. 2002; Gwak et al. 2006). Also, the data presented here in the whole cell and field recordings support the notion of disrupted GABA inhibitory function after injury that can be restored by attenuating the effects of ROS. Therefore, many different mechanisms may be needed to explain the whole picture, given the multifaceted nature of neuropathic pain.

Whole cell recordings of the EGFP-tagged GABA neurons revealed an interesting response to the oxidative stress induced by t-BOOH, a decrease in the generation of sustained action potentials. On the other hand, members of the non-tagged neuronal population exhibited the opposite effect, an increase in neuronal activity. This supports the hypothesis that oxidative stress promotes 1) an increased neuronal excitability and the development of central sensitization and also 2) the inhibition of the inhibitory influence of GABA neurons.

However, an important concern about these results is whether these alterations do affect the final output of the nociceptive signal transduction pathway. The whole cell recordings show the effects of elevated ROS levels on individual neurons, but they do not address whether these changes translate into enhanced pain transmission overall. Depending on synaptic organization of the nociceptive relaying pathway and the location of the affected neurons in this circuit, the modulatory effect of ROS may not necessarily strengthen central sensitization. Usually, nociceptive information travels down the primary afferent axon and is transmitted to the second order dorsal horn neuron. The primary afferent may also synapse with other intrinsic neurons, such as an inhibitory interneuron or an excitatory interneuron which then synapses onto a second order dorsal horn neuron. In this case, the changes in the excitability of any of these neurons by ROS would most likely result in an augmentation of the pain signal. However, in cases where the interneurons and dorsal horn neurons are not synaptically arranged in this manner, ROS may not produce the same effects. Therefore, the results of the whole cell recordings should be supported by studies that have examined the changes in neuron responses in the context of an intact, electrophysiological circuit.

The field recordings performed in this study have attempted to address this issue by examining broader changes associated with a population of neurons in the spinal dorsal horn rather than just individual cells. Still, this recording method does not fully address the problem of whether the output of the nociceptive circuit is increased since a disadvantage of the slice preparation used in this study is that the synaptic pathways between the periphery and the brain have been disrupted and the response properties of the neurons may be different than what is found in physiological conditions. The ideal experimental situation would be to record the activity from these different neurons (dorsal horn interneurons and each of the

pain transducing neurons) in an awake, behaving animal that is intrathecally injected with a ROS donor or a ROS scavenger in the case of a neuropathic animal.

Furthermore, the most important ROS for this effect and the mechanisms by which ROS exert their influence on GABA neurons and other spinal dorsal horn neurons remain to be identified. Therefore, further studies must be continued on the effects of oxidative stress on GABA neurons.

Moreover, it would be interesting to see how ROS mediates its effects, possibly by modulating certain ion channels or intracellular receptors. First, in a separate preliminary study using whole cell recording, perfusion of the spinal cord slice with t-BOOH changed the shape of the action potentials produced by electrical stimulation in the EGFP-tagged GABA neurons. Specifically, the action potential shape changed to include an after hyperpolarization component, which is thought to be mediated by Ca^{2+} dependent K^{+} channels. This observation is currently being studied. Furthermore, recently, H_2O_2 was found to increase the GABAergic mIPSC frequency by an IP_3R-mediated-release of calcium (Takahashi et al. 2007). The authors speculated that this prolonged activation of the IP3Rs would result in a long-term decrease in GABAergic inhibitory synaptic transmission due to the depletion of neurotransmitter vesicles and a resultant overall increase in dorsal horn neuron excitability (Takahashi et al. 2007).

It is also important to determine whether ROS affects GABA neurons during a chronic period after nerve injury. To date, only one study has reported on the functions of GABA neurons in neuropathic animals. In fact, using the same transgenic mouse line as this study, there were no significant changes found in the electrophysiological properties, such as membrane excitability, observed firing patterns, or synaptic input, of the lamina II EGFP-

tagged GABA neurons in CCI-operated mice compared to sham mice (Schoffnegger et al. 2006). Therefore, these data suggest that another mechanism as opposed to changes in GABA function would contribute to neuropathic pain development. The authors, however, acknowledged that different neuropathic pain models may demonstrate differences in spinal pain processing and conclusions about GABA neurons in the SNL model could not be made. The time frame in which their experiments were done was also not desirable, being ten or eleven days after surgery, since we are interested in early changes occurring during the development of pain behavior in the SNL model. Therefore, further studies must be done to determine if SNL produces aberrations in an individual GABA neuron's electrophysiological properties.

Finally, the field recordings showed that removal of ROS could desensitize the fEPSPs in the neuropathic mice, and the desensitization was critically dependent on GABA neurotransmission through the $GABA_A$ receptors. The data suggest that ROS act on the GABA neurons themselves to decrease their excitability and possibly the release of GABA. However, blocking the $GABA_A$ receptor alone did not entirely eliminate the ROS scavenger effect. This suggests several possibilities, such as that the effects of GABA were not entirely eliminated since the activation of $GABA_B$ receptors also mediate both presynaptic and postsynaptic inhibition.

In addition, ROS have been found to act also at different sites on the postsynaptic dorsal horn neurons. For example, ROS are importantly involved in the phosphorylation of spinal NMDA receptors by PKC, a critical process in central sensitization (Gao et al. 2007). It would be interesting to see whether ROS play a role in the phosphorylation of $GABA_A$ receptors by PKC as well, since phosphorylation of these receptors has been shown to

modulate their function (Saito and Shirai 2002). Evidence for oxidative stress compromising $GABA_A$ receptor function has also been reported in the hippocampus (Sah and Schwartz-Bloom 1999; Sah et al. 2002). This same group found that hydrogen peroxide bound non-competitively to the t-butylbicyclophosphorothionate binding site, inhibiting the movement of Cl^- into the cell (Sah et al. 2002). Therefore, the study of ROS effects on the $GABA_A$ receptor in the spinal cord is warranted. Finally, the contribution of C-fibers and not just the A fibers examined here should also be studied to come to a better understanding at the mechanisms behind ROS involvement in central sensitization and pain development.

CHAPTER 5

ELEVATED REACTIVE OXYGEN SPECIES LEVELS RESULT IN A LOSS OF GAD67 EXPRESSION IN THE SPINAL CORD

5.1 ABSTRACT

One feature of neuropathic pain is the loss of spinal GABAergic inhibition in the pain processing pathway. However, it remains to be elucidated at what level (neuron, transporter, postsynaptic receptor, etc.) and by what mechanism this loss occurs. We showed in a previous chapter that oxidative stress due to elevated levels of ROS was important for the development of pain and central sensitization in the SNL model of neuropathic pain. GABA neurons were found to be sensitive to oxidative stress, since the excitability of GABA neurons decreased in the presence of a ROS donor *in vitro*. We hypothesize that ROS also contribute to the loss of GABAergic inhibition by reducing the expression of GABA synthesizing enzymes in the spinal cord. The purpose of this study was to investigate the effects of peripheral nerve injury on the spinal GABA system and to assess the role of ROS on GABA in the development of neuropathic pain in mice. **METHODS:** SNL was produced by tight ligation of the L5 spinal nerve in FVB/NJ and transgenic GAD67-EGFP adult male mice. Mechanical allodynia was assessed by testing for the paw withdrawal response rates to von Frey filaments 3.0 (0.1 g) and 3.61 (0.41 g). To determine the effect of SNL on spinal GABA expression levels, at three and seven days after SNL, the FVB/NJ mice were perfused, and the L4-L5 spinal segments were homogenized. Immunoblotting was performed to determine the expression levels of the GABA synthesizing enzymes, GAD65 and GAD67,

and the loading control, β-actin. To investigate the effect of SNL on the number of EGFP+ GAD67-producing neurons in the L5 dorsal horn, the transgenic GAD67-EGFP mice were perfused one week after SNL, and the L5 spinal cords were sampled. Thick transverse sections (80 μM) were made throughout the entire length of the L5 cord and were collected in sequence. The number of EGFP-tagged, GAD67-containing neurons was counted by the Optical Fractionator technique using the Stereo Investigator program. Cell counting was done in the superficial and deep layers (laminae I-V) of the dorsal horn of four sections sampled by a systematic random sampling method from each animal. Finally, the effects of the ROS scavenger, PBN, on the number of EGFP-tagged neurons in the L5 dorsal horn after SNL were studied. Two daily injections of 150 mg/kg PBN (i.p.) or vehicle (i.p.) were administered for one week, the first of which was given immediately before the surgery. One week after the surgery, the transgenic GAD67-EGFP mice were perfused, and the L5 spinal cords were sampled as above to determine the number of EGFP-tagged neurons. **RESULTS:** Western blot analysis for spinal GAD65 and GAD67 showed no quantitative changes on either the ipsilateral or contralateral side at three days or one week after SNL when compared to sham animals. However, stereological analysis demonstrated that the number of EGFP+ GABA neurons in the superficial laminae of L5 on the ipsilateral side decreased significantly by one week after SNL as compared to sham controls. Furthermore, the course of treatment with PBN significantly reduced the magnitude of the allodynic behaviors and significantly reduced the loss of EGFP-tagged GABA neurons when compared to the untreated or saline treated SNL mice. **CONCLUSIONS:** These data show that while the overall expression of GABA synthesizing enzymes does not change, the number of EGFP-labeled GABA neurons is reduced after spinal nerve injury. Oxidative stress contributes to this loss of GAD67/EGFP

expression since ROS removal by PBN ameliorates pain behaviors and recovers the EGFP+ neuronal count in the L5 dorsal horn. Therefore, the loss of spinal GABAergic inhibition seen in neuropathic pain may be partly attributed to oxidative stress inducing a loss of GAD67 expression in the spinal cord.

5.2 INTRODUCTION

Gamma-aminobutyric acid (GABA) is one of the main inhibitory neurotransmitters in the mammalian nervous system, especially in the spinal dorsal horn. The role of the GABAergic system in pain perception has been extensively studied. Pharmacological antagonism of spinal GABA receptors results in tactile allodynia in both rats and mice with characteristics analogous to those found in chronic pain states (Yaksh 1989; Minami et al. 1994; Sivilotti and Woolf 1994; Malan et al. 2002). Moreover, administration of GABA and GABA receptor agonists has been shown to alleviate pain behaviors in different models of peripheral neuropathy (Hwang and Yaksh 1997; Eaton et al. 1999; Patel et al. 2001; Malan et al. 2002; Franek et al. 2004). Electrophysiological studies found that in two peripheral neuropathic pain models, there are decreased $GABA_A$-receptor-mediated inhibitory postsynaptic currents with concomitant decreases in dorsal horn levels of GABA synthesizing enzymes and increased neuronal apoptosis (Moore et al. 2002). The same group demonstrated that antagonizing spinal $GABA_A$-receptors resulted in facilitation of excitatory synaptic transmission (Baba et al. 2003).

Thus, many agree that one of the mechanisms behind the development of chronic neuropathic pain is disinhibition, particularly from the disruption of the spinal GABAergic

system; however, questions remain about how this dysfunction occurs and where exactly does it happen (i.e. GABA neurons, postsynaptic receptors, transporters). It has been reported that the decreased GABAergic tone is due to the loss of spinal dorsal horn GABAergic interneurons. Sciatic nerve transection resulted in reduced GABA-immunoreactivity (IR) and GABA content on the side of the spinal cord ipsilateral to the injured nerve (Bennett and Xie 1988; Castro-Lopes et al. 1993). In the chronic constriction injury (CCI) model (Bennett and Xie 1988), GABA-IR in the dorsal horn dramatically decreased on both sides of the spinal cord, with a more pronounced reduction ipsilateral to the injury; however, incomplete recovery did occur by eight weeks (Ibuki et al. 1996; Eaton et al. 1998). In the model of partial sciatic nerve injury, GABA-IR of cell bodies and axon terminals in the spinal dorsal horn also decreased (Ralston DD 1997). Likewise, one study comparing the spared nerve injury (SNI), CCI and SNL models demonstrated numerous apoptotic profiles in the dorsal horn ipsilateral to injury, which included GABAergic neurons (Scholz et al. 2005). The concomitant reduction in the inhibitory postsynaptic currents in lamina II neurons was attributed to the loss of GABAergic inhibition through cell death.

On the other hand, contradictory findings have shown no significant change in the number of GABA-IR neurons (Polgar et al. 2003) or GABA content in synaptosome preparations (Somers and Clemente 2002) in the ipsilateral dorsal horn of the CCI rats when compared to the contralateral side or sham animals. Even more disparate studies demonstrate increased GABA concentrations in the spinal dorsal horn ipsilateral to sciatic nerve ligation (Satoh and Omote 1996) as well as increased GABA inhibitory tone after SNL (Kontinen et al. 2001). Therefore, the fate of GABA neurons in the spinal cord after peripheral nerve

injury remains unresolved due to the difficulties in labeling GABA neurons and the inconsistent methods used across studies to analyze GABA content.

This lack of consensus can be overcome with a technique allowing the investigator to identify easily and consistently GABA neurons in the spinal cord. A transgenic mouse line that contains GABA neurons that express enhanced green fluorescent protein (EGFP) driven by the GAD67 regulatory element (Oliva Jr et al. 2000; Heinke et al. 2004) eliminates the obstacle of antibody specificity and the problems faced by previous studies. Since both GAD65 and GAD67 antibodies are co-localized in virtually all GAD-immunoreactive boutons in the spinal grey matter (Mackie et al. 2003), the expression of EGFP in the GAD67-producing neuron would identify it as a GABA neuron.

Finally, in the previous chapter, we found that, generally, in the presence of excessive free radicals, the excitability of the EGFP+ GABA neurons became reduced. The data suggest that oxidative stress may perturb GABA function in acute situations. What happens to GABA neurons under oxidative stress in a chronic situation, such as after peripheral nerve injury? A recent study suggests that ROS may influence apoptotic gene expression, since shortly after CCI injury, removal of ROS with PBN significantly reduced both pro-apoptotic gene over-expression and apoptotic neuronal profiles in the laminae I-III of the rodent spinal cord (Siniscalco et al. 2007). Thus, we propose that one of the mechanisms by which ROS contribute to central sensitization is by promoting the loss of GABA neurons and, consequently, the disinhibition of the spinal dorsal horn.

Therefore, the main purpose of this study was to determine the effect of SNL-induced neuropathy on spinal GABA expression levels and on the expression of EGFP+ GAD67-containing neurons and to investigate whether oxidative damage played a role.

5.3 MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

5.4 RESULTS

5.4.1 SPINAL NERVE LIGATION DOES NOT SIGNIFICANTLY ALTER SPINAL GABA SYNTHESIZING ENZYME EXPRESSION

To examine the total GABA content in the L4-L5 spinal cord, western blot analysis for the expression levels of the two GABA synthesizing enzyme isoforms, GAD65 and GAD67, was performed. The time points were chosen because the development of mechanical allodynia peaks three days after SNL, and by one week after SNL, the pain behavior is stably maintained. The blots show that at both three days and one week after SNL, GAD65 and GAD67 levels for both the contralateral and ipsilateral sides were not very different from the sham group (**Fig 5.1A**). The bands were quantified and normalized to β-actin expression, and statistical analysis of the results confirms that SNL does not significantly affect GAD65 or GAD67 expression (**Fig. 5.1B** and **5.1C**).

5.4.2 THE NUMBER OF EGFP+ GAD67-PRODUCING NEURONS DECREASES IN THE SPINAL DORSAL HORN ONE WEEK AFTER L5 SPINAL NERVE LIGATION

To determine whether reduced EGFP+ GAD67 containing neuron counts could account for the decreased GABAergic tone, stereological analysis was performed on the L5 spinal cord of SNL and sham mice one week after injury. The transgenic mouse line

containing GABA neurons that express EGFP under the control of the GAD67 promoter was used. One week after surgery, SNL resulted in the disappearance of green fluorescent GABA neurons (bright green dots) in the superficial dorsal horn, particularly in the lateral portion of the ipsilateral side; however, the contralateral side exhibited a more homogeneous distribution of these EGFP+ GABA neurons (**Fig. 5.2B**). When comparing the ipsilateral L5 spinal cord, SNL displays a pronounced reduction in the number of fluorescently tagged GABA neurons as opposed to sham (**Fig. 5.2A**).

The total number of EGFP+ neurons in the L5 spinal cord was estimated by stereological analysis for the medial (M) and lateral (L) halves of laminae I-II and the deeper laminae III-V (D) on both sides of the sham and SNL mice (**Fig. 5.2C**). On the side ipsilateral to the injury, SNL resulted in a significant decrease in the number of EGFP-labeled GABA neurons in L (641.20 ± 41.70) when compared with sham (835.64 ± 42.08). Therefore, the data suggest that SNL causes a loss in the number of EGFP-expressing GABA neurons in the spinal dorsal horn.

5.4.3 Repetitive treatment with PBN recovers the number of EGFP+ GAD67-producing neurons after SNL

Since the data show that scavenging ROS effectively alleviates pain behaviors as well as decreases central sensitization in the SNL model, PBN's ability to prevent or reduce the loss of EGFP+ GABA neurons was investigated. Immediately before SNL surgery and eight hours later, one group of mice received a systemic injection of PBN (150 mg/kg), and the other group received the vehicle. For the next six consecutive days, both groups of mice received two daily injections, eight hours apart of either PBN (150 mg/kg) or vehicle. Behavioral testing for mechanical allodynia was performed daily, prior to the first injection.

On day seven, after behavioral testing, all mice were sacrificed, and their spinal cord tissues were processed for stereological analysis. All SNL mice, regardless of treatment, developed significant mechanical allodynia to both von Frey filaments, vF 3.0 (**Fig. 5.3A**) and vF 3.61 (**Fig. 5.3B**), as opposed to the sham mice (n = 6). However, PBN treatment (n = 8) significantly decreased nociceptive responses starting at 3 d up to 1 wk after surgery, when compared to vehicle treated mice (n = 6), which produced similar responses to SNL alone (**Fig. 5.3A, B**). For example, on day 7, SNL mice exhibited a response rate of 85 ± 4% to filament 3.0 and 94 ± 4% to filament 3.61 while response rates of the PBN treated mice were 58 ± 11% and 66 ± 10%, respectively.

The estimated total number of EGFP-labeled neurons in the L5 spinal cord was calculated for the ipsilateral and contralateral sides and plotted for the three areas of the dorsal horn in the sham, SNL, and SNL + PBN-treated mice (**Fig. 5.3C**). The SNL mice had similar results to that of vehicle treated SNL mice. PBN treatment, when compared to SNL on the side ipsilateral to the injury, resulted in a significant increase in the number of EGFP-labeled GABA neurons in L (809.49 ± 69.39 for PBN vs. 641.20 ± 41.70 for SNL). The number of EGFP-expressing neurons in L of the PBN treated mice was not significantly different than the sham treatment (835.64 ± 42.08), indicating that PBN almost completely prevented the EGFP expression loss induced by SNL. Thus, repetitive treatment with PBN successfully recovers the number of EGFP+ GAD67-producing neurons in the spinal dorsal horn and provides long-lasting analgesia in SNL mice.

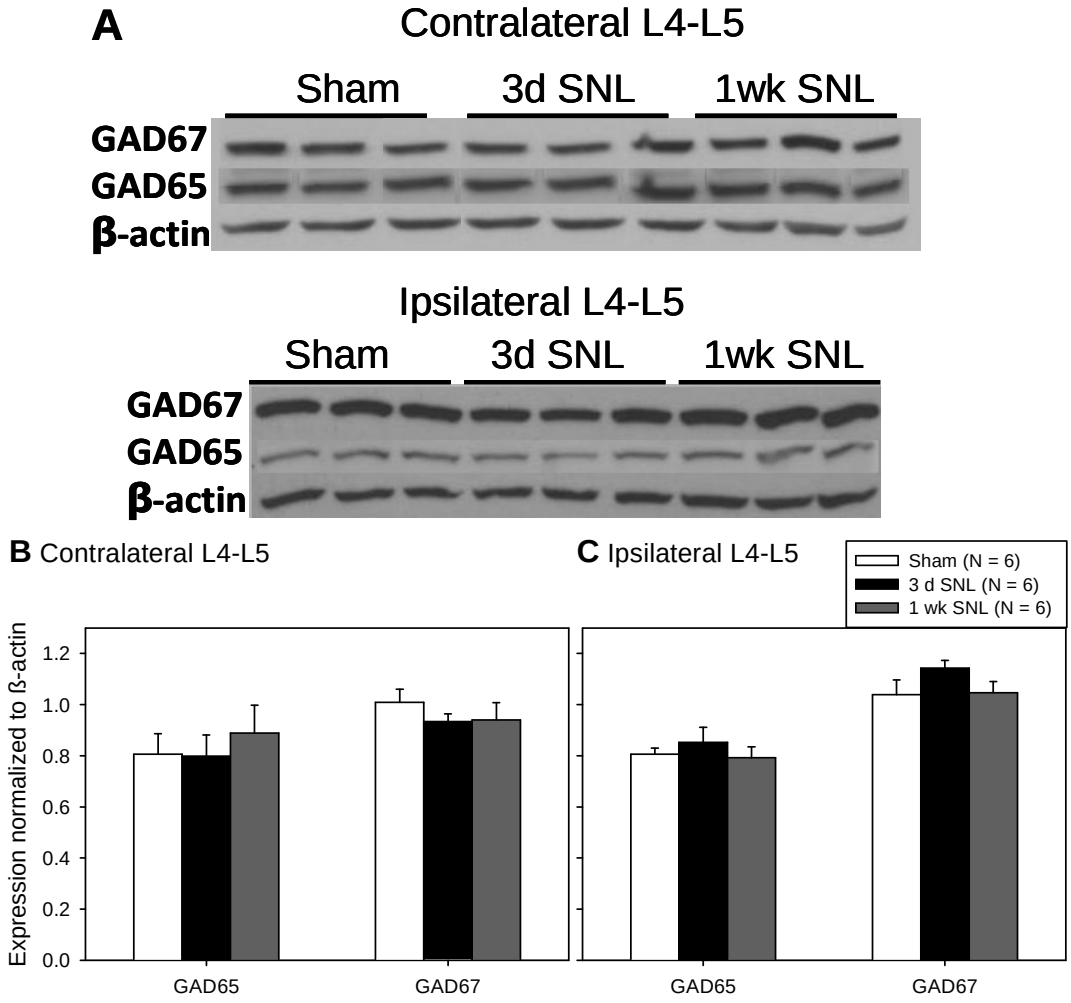


FIG. 5.1. **Immunoblotting and protein expression analysis of the GABA synthesizing enzymes GAD65 and GAD67 in the L4-L5 spinal cord.** Three days and one week after SNL (n = 6 for each group) or one week after sham surgery (n = 6), western blot analysis was done. (A) Representative blots are shown for the expression levels of the GABA synthesizing enzymes GAD65 and GAD67 and the loading control protein, β-actin. In the upper panel, a blot of the contralateral half of the L4-L5 spinal cord shows little difference between the different conditions of sham (n = 3), 3 d SNL (n = 3), and 1 wk SNL (n = 3). In the lower panel, a blot of the ipsilateral half of the L4-L5 spinal cord shows little difference between the different conditions of sham (n = 3), 3 d SNL (n = 3), and 1 wk SNL (n = 3). The protein expression levels are quantified, averaged and normalized to β-actin in (B) for the contralateral side (n = 6) and (C) for the ipsilateral side (n = 6). Compared to the sham mice, both GAD65 and GAD67 expression did not significantly change at three days or one week after SNL surgery in both the contralateral half and the ipsilateral half of the L4-L5 spinal cord. Data are presented as means ± SEM.

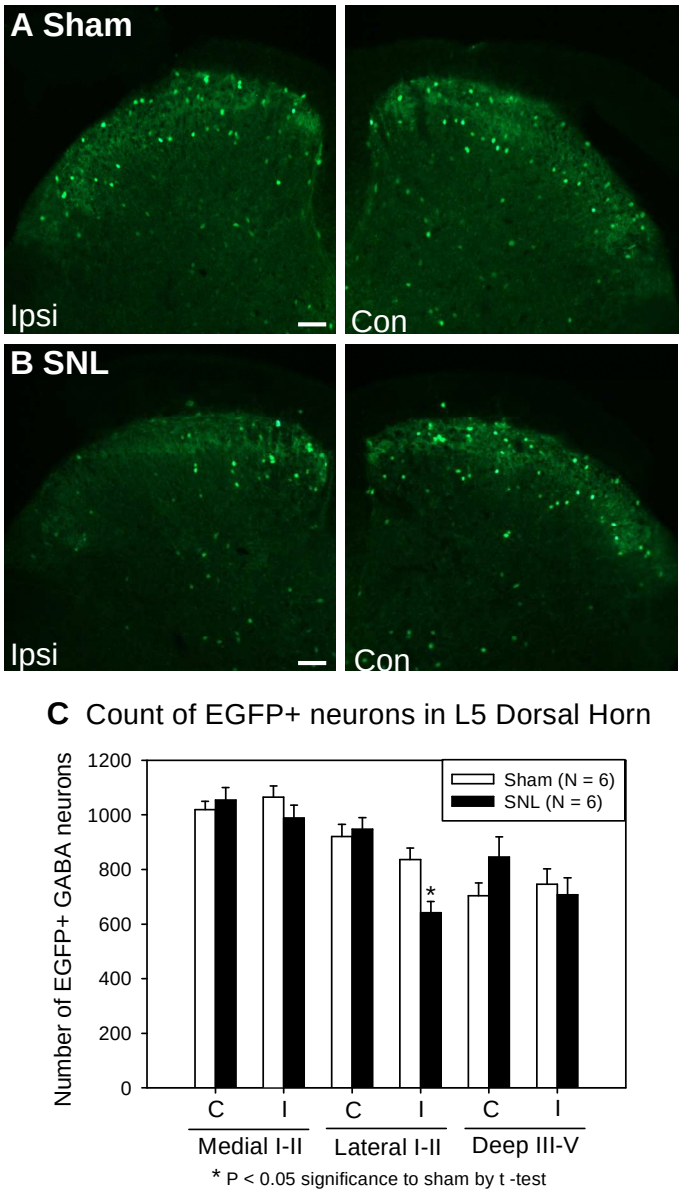


FIG 5.2. **Fluorescence micrograph and stereological analysis of EGFP+ neurons in the L5 dorsal horn.** (A and B) The L5 dorsal horn one week after sham (n = 6) or SNL surgery (n = 6) under 200X magnification. The bar denotes a distance of 60 µm. (A) Sham surgery did not affect the number of EGFP-tagged GABA neurons on the ipsilateral (Ipsi) side. (B) After SNL, the EGFP-tagged neurons that resided in the superficial dorsal horn on the ipsilateral side have decreased. (C) The number of EGFP+ neurons for the medial (M) and lateral halves (L) of laminae I-II and the deeper laminae III-V (D) are shown for sham or the SNL mice one week post surgery. Data are presented as means ± SEM. Ipsi or I, ipsilateral side; Con or C, contralateral side; *, the value is significantly (p < 0.05) different from the sham control by the student t-test.

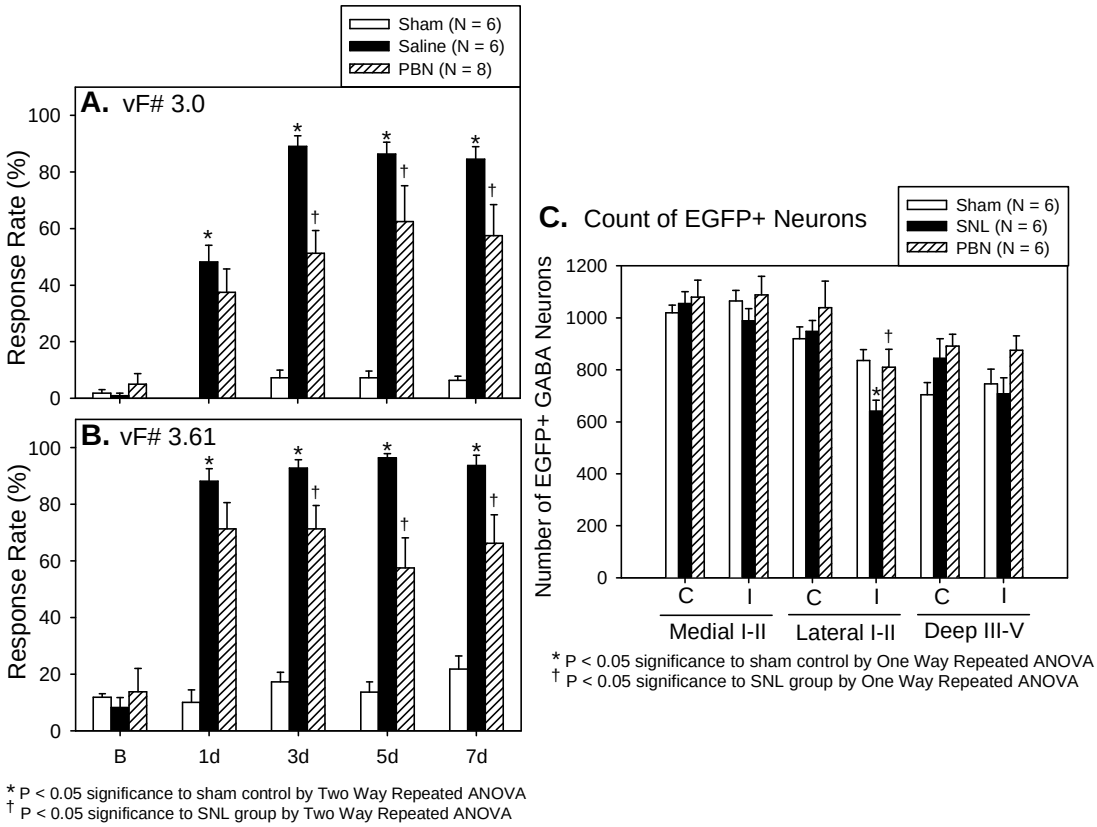


FIG 5.3. **The effect of repeated systemic PBN treatment on paw withdrawal response rates and stereological counts of EGFP+ GABA neurons in the L5 spinal dorsal horn.** Response rates to von Frey filament 3.0 **(A)** and 3.61 **(B)** were measured immediately before and on 1 d, 3 d, 5 d and 7 d following SNL (n = 6) and sham surgery (n = 6). PBN (150 mg/kg, i.p., n = 8) or vehicle was given twice a day, eight hours apart for one week with the first injection immediately before SNL. SNL significantly increased the response rates to both filaments, when compared to sham surgery. Sham surgery did not significantly affect response rates. Daily behavioral testing for the PBN and vehicle treated mice was done immediately before the first injection of the drug. PBN significantly reduced response rates at 3, 5 and 7 d post SNL when compared to vehicle treatment. **(C)** Stereological estimates for the number of EGFP+ neurons in the L5 spinal cord for the medial (M) and lateral halves (L) of laminae I-II and the deeper laminae III-V (D). PBN significantly increased the number of EGFP+ neurons in the lateral half of laminae I-II as compared to the SNL mice. Data are presented as means ± SEM. B, baseline, pre-surgical time; Ipsi or I, ipsilateral side; Con or C, contralateral side; *, value is significantly ($p < 0.05$) different from the sham control; †, value is significantly ($p < 0.05$) different from the SNL group.

5.5 DISCUSSION

This study examined the consequences of peripheral nerve injury on GABA synthesizing enzyme expression and on the number of EGFP+ GAD67-containing neurons in the spinal dorsal horn; in addition, the effects of an ROS scavenger on the number of the EGFP-tagged neurons were studied. SNL produced little change in the total expression levels of the enzyme isoforms, GAD65 and GAD67, at three days and one week after injury on either the ipsilateral or contralateral sides. Despite this, one week after SNL, reduced numbers of EGFP-tagged neurons were found in the spinal dorsal horn on the side ipsilateral to the injury, particularly in the lateral half of laminae I-II. After repetitive treatment with PBN, the neuronal counts of this population of EGFP+ neurons increased, up to ~97% of the sham values. Therefore, this study shows that ROS and oxidative stress may contribute to the loss of EGFP+ GABA neurons. This result may be due to a downregulation of GAD67 or the death of these neurons. In either case, the loss of EGFP+ neurons may promote the disinhibition of spinal dorsal horn neurons, which may contribute to central sensitization and the symptoms of neuropathic pain.

According to the data in this study, the overall expression of GAD65 and GAD67 remains relatively unchanged after SNL. Presumably, since only GABA neurons contain these synthesizing enzymes, the data imply that the spinal GABAergic system is not affected after peripheral nerve injury. This apparent contradiction with the stereological analysis of the EGFP-labeled GABA neuron count can be explained by the differences in the techniques. Historically, other studies that have detected changes in GAD expression have used only the dorsal portion of the spinal cord halves of the affected spinal segment, which was possible since rats have greater amounts of tissue than mice. Immunoblotting in the mouse required

that certain steps were done to ensure sufficient tissue was obtained to allow for a reasonable amount of proteins to be collected from each mouse, eliminating the need for the pooling of tissue samples. Proteins were extracted from both the L4 and L5 spinal cord rather than just the L5 segment, since it has been reported that changes in adjacent spinal segments to the injury also occur (Scholz et al. 2005). Also, instead of isolating only the dorsal horns, the entire hemisections were used for the ipsilateral and contralateral halves. As a result, any differences in GABA synthesizing enzyme expression may be masked by the dilution of the tissue. The resultant data show that these measures obviously sacrificed the sensitivity of the method to detect the subtle differences observed by the stereological method. Therefore, the western blots performed here do not provide a satisfactory detection method for small differences in GABA expression.

The main question answered in this study was what happened to a population of EGFP-expressing, GAD67-containing neurons in the spinal cord after SNL-induced neuropathy. The transgenic mouse line FVB-Tg(GadGFP)45704Swn/J was chosen for these experiments, since previously antisera against GABA have produced inconsistent results in immunohistochemical and immunoblotting studies (Castro-Lopes et al. 1993; Ibuki et al. 1996; Eaton et al. 1998; Polgar et al. 2003). These mice expressing EGFP-labeled GABA neurons have been studied, and the properties of green-fluorescent GABA neurons in the hippocampus and spinal cord laminae I and II are well characterized (Oliva Jr et al. 2000; Heinke et al. 2004; Dougherty et al. 2005).

A potential caveat is that while 73% of the EGFP+ neurons in lamina I and 86% of the EGFP+ neurons in lamina II were also immunopositive for GABA, only ~75% of the GABA neurons were found to be EGFP+ in lamina I and only ~35% of GABA neurons were

found to be EGFP+ in lamina II (Heinke et al. 2004; Dougherty et al. 2005). Thus, roughly one third of all spinal dorsal horn GABAergic neurons are labeled with EGFP (Heinke et al. 2004). Although it is difficult to make conclusions about the other two-thirds of the GABA neuron population that is not EGFP+, greater (or fewer) GAD67/EGFP loss might have occurred than the level of loss observed in this study. However, an advantage of using the transgenic mouse with the EGFP+ GABA neurons is the avoidance of many previously encountered technical issues inherent with the use of GABA anti-sera, such as the extent of antibody penetration and reproducibility of the staining. Thus, this technique provides consistent labeling of the spinal GABA neurons, since nearly all the EGFP+ neurons have been shown to be GABA neurons (Heinke et al. 2004).

Another important concern with the results of this study is that a reduction in the number of EGFP+ neurons does not necessarily translate into the loss of GABA neurons. An alternate explanation for the disappearance of fluorescently labeled neurons could be that there is a down-regulation of GAD67 production and hence, a down-regulation in the production of the marker, EGFP. Decreased synthesis of EGFP in the GAD67-producing neuron could very likely lead to a low level of marker that is below the detection limits of the techniques used in this study. Hence, the GABA neuron expressing low levels of EGFP would not be counted even though it is still viable, leading to an error. Therefore, an additional study must be done to delineate whether the disappearance of these EGFP+ neurons is due to the cells undergoing apoptosis or whether the production of GAD67 in these neurons is being down-regulated.

If the reduction in the number of EGFP-labeled neurons is indeed the result of cell loss and not a down-regulation in the production of GAD67, then these results would conflict

with an earlier study that found that the death of GABA neurons did not contribute to pain development in the CCI model (Polgar et al. 2003). Besides the differences in the model used (which might also explain the different results), the previous study relied on GABA immunoreactivity to identify GABA neurons.

Unlike two previous studies that used the CCI model and found a dramatic loss of GABA-IR after two weeks (Ibuki et al. 1996; Eaton et al. 1998), no significant loss in the GABA neuron population was found at the same time after CCI (Polgar et al. 2003). However, these results may be correct; the total number of GABA neurons in the entire population may not be reduced after neuropathic injury. Our study used stereological analysis to determine carefully the number of EGFP-tagged GABA neurons distributed in laminae I-V of the spinal dorsal horn after SNL and found a loss of ~23% in the number of EGFP+ neurons whose population comprises roughly one-third of the entire population of GABA neurons. This reduction amounts to a modest 8% decrease in the entire GABA population overall, if none of the unlabeled GABA neurons were lost.

Therefore, it is reasonable to expect that the stereological analysis of immunostained neurons (Polgar et al. 2003) may not have the sensitivity required to detect such a small loss as was found in this study. In the immunostaining method, a loss of this magnitude might be deemed as standard, normal variability between subjects. Although the observed loss of EGFP+ GABAergic neurons in the present study may seem small, it is clearly not insignificant, since the sequestration of ROS led to improved behavioral outcomes and prevented the reduction in the EGFP+ counts.

The results presented here, however, supported a study that did not use immunostaining, but rather, *in situ* hybridization with GAD67 mRNA to label GABA

neurons (Scholz et al. 2005). Four weeks after spared nerve injury (SNI), the number of ipsilateral GABAergic interneurons was decreased by ~25% in lamina I-II and lamina III. TUNEL staining demonstrated an increase in the number of apoptotic profiles in the ipsilateral dorsal horn which appeared one day after injury, peaked on day 7 and remained elevated after 21 days (Scholz et al. 2005). Therefore, while that group did not look at the number of GABA neurons at earlier time points, it is possible that by one week after injury, a major portion of the GABA neurons may already have undergone apoptosis. We also observed a decrease in the number of EGFP+ GABA neurons in the superficial dorsal horn four weeks after SNL comparable to that seen after one week (Wang et al. 2007). Thus, the time course of GABA neuron loss may occur much earlier than four weeks since the majority of behavioral data confirming GABA's role in pain mediation have been studied in animals within a few weeks after injury.

The proportion of death in GABA neurons was not different than that of other neurons (Scholz et al. 2005), indicating that GABA neurons are not especially vulnerable to nerve-injury. While that claim cannot be made from the data in this study since we did not look at other neurons or measure cell death directly, we propose that the susceptibility of GABA neurons to oxidative stress may be more important than the vulnerability of other neurons for the maintenance of pain since GABA neurons are responsible for inhibition in the spinal pain processing pathways. Preventing the reduction in the number of EGFP+ GAD67-producing neurons or reversing the down-regulation of GAD67 synthesis due to oxidative stress has the same effect as transplanting GABA-differentiated human neural precursor cells into the spinal cord (Mukhida et al. 2007) — the increased number of GAD67-producing neurons or increased expression of GAD67 resulted in reduced pain

behavior in the respective animal model. Therefore, maintaining spinal GABA expression is very important, and understanding the mechanism behind ROS's actions in the development of neuropathic pain is crucial.

Another important question regarding the role of GABA in pain besides neuron viability after injury includes the question of GABA function after injury. To date, only one study has reported on the functions of GABA neurons in neuropathic animals. In fact, using the same transgenic mouse line as this study, there were no significant changes in the electrophysiological properties, such as membrane excitability, observed firing patterns, or synaptic input, of the lamina II EGFP-tagged GABA neurons in CCI-operated mice compared to sham mice (Schoffnegger et al. 2006). Therefore, their data suggest that another mechanism as opposed to changes in GABA function could contribute to neuropathic pain development. It would have been interesting if the authors performed stereological analysis in the study to determine if the number of EGFP-tagged GABA neurons decreased in the CCI model. The authors, however, acknowledged that different neuropathic pain models may demonstrate differences in spinal pain processing and conclusions about GABA neurons in the SNL model could not be made. The time frame in which their experiments were done was also not desirable, being ten or eleven days after surgery, since we are interested in early changes occurring before the loss of the EGFP-tagged neurons seen at one week in the SNL model. Therefore, further studies must be done to determine if SNL produces aberrations in an individual GABA neuron's electrophysiological properties.

Other possible contributory mechanisms that may explain the loss of GABAergic inhibition which were not explored here include an impairment of GABA reuptake into the presynaptic terminal or impaired vesicular release of GABA after injury. For instance,

GABA transporters located on the plasma membrane of the presynaptic terminal of GABA neurons and on glia are responsible for recycling the GABA released into the synaptic cleft (Soudijn and van Wijngaarden 2000). The expression levels of the GABA transporter, GAT-1, were significantly reduced one week after CCI, and this may have contributed to a depletion of GABA in the presynaptic terminals (Miletic et al. 2003).

On the postsynaptic side, a loss in the number or function of the $GABA_A$ or $GABA_B$ receptors or both may provide alternate explanations for diminished GABA tone. However, evidence that supports receptor dysfunction is lacking for both receptors. Immunohistochemistry and the analysis of $GABA_A$-mediated IPSCs demonstrate that $GABA_A$ receptors remain intact and functional after both CCI and SNI (Moore et al. 2002). Moreover, the density of $GABA_B$ receptor sites and receptor affinity for GABA remain unaltered after CCI (Smith et al. 1994). In the SNL model, the expression and function of the $GABA_B$ receptors in the spinal cord and dorsal root ganglia did not change up to 3 months after injury (Engle et al. 2006). Therefore, while receptor dysfunction or loss may not play a role in the disinhibition of the spinal cord, studies that look at the phosphorylation state of the receptors or of their trafficking to the membrane and availability of accessory proteins have not yet been done.

Finally, additional benefits of PBN, besides being a potent ROS scavenger, include the ability of PBN to inhibit gene induction of inducible nitric oxide synthase which produces another free radical species, nitric oxide, and the ability of PBN to inhibit the activation of the nuclear factor kappa B, which is responsible for mediating inflammatory processes, as well as other apoptosis-associated genes (Kotake et al. 1999). A recent study also demonstrated PBN's ability to induce over-expression of the anti-apoptotic gene, bcl-2,

which was thought to be partly responsible for reducing the number of apoptotic profiles seen a few days after surgery in the CCI model (Siniscalco et al. 2007). Therefore, PBN contains many desirable properties, making it an excellent therapeutic candidate.

In conclusion, this study demonstrates that ROS may be involved in the loss of inhibitory tone in the neuropathic pain state since reducing ROS levels can recover to a certain extent the loss of EGFP+ GABA neurons seen after SNL. Further studies must be done to determine whether this loss of EGFP/GAD67 expression is due to neuron death or a down-regulation in the synthesis of GAD67.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS: THE ROLE OF REACTIVE OXYGEN SPECIES IN GABAERGIC DYSFUNCTION IN THE DEVELOPMENT OF NEUROPATHIC PAIN

6.1 A MECHANISM OF OXIDATIVE STRESS-INDUCED GABAERGIC DYSFUNCTION IN THE DEVELOPMENT OF NEUROPATHIC PAIN

The major points of interest emerging from the studies are described here. The findings that lead to the conclusion that tight ligation of the L5 spinal nerve produces increased spinal ROS levels that contribute to neuropathic pain in mice are: (1) L5 SNL consistently produces a long-lasting, painful neuropathy in mice that is expressed as mechanical allodynia. (2) Increased levels of ROS, especially spinal ROS, contribute to the development and maintenance of the pain behaviors produced by L5 SNL in mice. (3) Increased levels of ROS are also important for the development of increased sensitivity of dorsal horn neurons to electrical stimulation, which is the key feature of central sensitization.

The findings that lead to the conclusion that increased spinal levels of ROS cause spinal GABA dysfunction in neuropathic pain in mice are: (1) Spinal GABAergic inhibitory tone is important to modulate nociceptive signaling under normal conditions and appears to be reduced in neuropathic conditions. (2) Acutely elevated ROS levels differentially influence the excitability of both GABAergic neurons in the superficial dorsal horn as well as other neurons, resulting in the enhancement of the nociceptive signal transduction pathway. (3) After L5 SNL, chronically elevated levels of ROS help maintain the state of central sensitization through actions on spinal GABA neurons.

The findings that lead to the conclusion that increased spinal levels of ROS cause a reduction in GAD67 expression that contribute to neuropathic pain in mice are: (1) The total expression levels of GABA synthesizing enzymes do not appear to change after L5 SNL; however, L5 SNL reduces the number of EGFP-tagged GAD67-containing neurons in the dorsal horn which can partly explain the disinhibition found in sensitized dorsal horn neurons. (2) ROS are important for inducing the loss of these EGFP-tagged neurons. Altogether, these findings indicate that ROS play a functionally important role in mediating the dysfunction of spinal GABA neurons during the progression of neuropathic pain. Here we propose a model whereby ROS act on GABA neurons and other neurons to promote the development of neuropathic pain after peripheral nerve injury (**Fig. 6.1**).

6.2 FUTURE DIRECTIONS

Several important questions remain concerning the contributory role of ROS toward the development of neuropathic pain through perturbations in the spinal GABAergic system. (1) What kinds of ROS are produced in SNL? (2) How does ROS act on the GABA neuron to inhibit its function? (3) Is the loss of GAD67 expression after SNL a result of apoptosis or a reversible down-regulation of the synthesis of GAD67? (4) Are the remaining GABA neurons fully functional or are they experiencing oxidative stress? (5) Is the glycinergic inhibitory system in the spinal dorsal horn also affected by oxidative stress? (6) At which point does an increase in ROS occur—during the development or maintenance of neuropathic pain or both? While this study has attempted to answer the last question and has implicated

ROS in the development and maintenance of neuropathic pain through disruption of the spinal GABA system, the answer is still incomplete.

First, it is not clear which type or types of ROS are overproduced or if there is decreased ROS turnover which is responsible for increased ROS levels after injury. The most likely culprits for the overproduction of ROS are probably the superoxides, due to the abundance of mitochondria in neurons that rely on aerobic metabolism and that produce superoxides as byproducts. Also, the dependence of central sensitization on the influx of Ca^{2+} through the activation of the ionotropic NMDA receptors may indirectly result in the overproduction of superoxides, by stimulating cellular metabolism.

Many studies have investigated the role of another ROS, nitric oxide, in the development and maintenance of central sensitization and neuropathic pain. Nitric oxide, which is produced by three different isoforms of nitric oxide synthase, is involved in many physiological and pathophysiological processes. Much evidence has shown that noxious stimuli lead to the activation of NMDA receptors that increases spinal nitric oxide production (primarily by neuronal nitric oxide synthase) which then increases the generation of the second messenger cyclic GMP and leads to the subsequent modification of ion channels, phosphodiesterases, and protein kinases (reviewed in (Meller and Gebhart 1993; Xu et al. 2007)).

Conversely, impaired removal by the cellular antioxidant mechanisms could also enhance ROS levels and contribute to oxidative stress. One of the main antioxidant systems in the cell could be impaired, such as the superoxide dismutases (SOD). Considering that one of the main sources of ROS in the neuron is the mitochondria, it is critical to determine whether the SODs are functioning normally under neuropathic conditions.

Another important antioxidant system in the neuron is glutathione peroxidase. Glutathione peroxidase, which is particularly abundant in mitochondria, functions in the removal of hydrogen peroxide and other peroxides (Maher and Schubert 2000). Therefore, it is important to measure the levels and activity of these two candidates, the SODs and glutathione peroxidase, in the SNL spinal cord to find the source of ROS overproduction or reduced ROS removal in neuropathic pain conditions.

In this study, we failed to measure directly or quantitatively the increased ROS levels by biochemical means. Rather, we relied on behavioral and electrophysiological experiments to conclude the presence of excessive ROS after SNL. Technical problems precluded biochemical measurement of ROS. These included the relatively small amount of tissues and fluids an individual mouse can provide as substrates for analysis, the poor sensitivity of the available methods for the detection of possibly subtle changes in the levels of ROS, and the uncertainty regarding the time course of increased ROS levels after injury. Therefore, it would be advantageous to develop new techniques that can quantitatively assess ROS levels. This could aid in proving whether increased ROS levels within GABA neurons directly contribute to cell death shortly after injury. Also, the use of transgenic mice either over-expressing or under-expressing antioxidant genes, such as the mitochondrial form of SOD, can help solve the question regarding the association of ROS with pain.

Furthermore, the precise mechanisms behind the inhibitory effects of ROS on GABAergic neuron excitability remain unknown. Recently, hydrogen peroxide was found to modulate the activity of GABAergic interneurons in lamina II of the dorsal horn by binding to the IP_3R receptor and releasing intracellular stores of Ca^{2+} (Takahashi et al. 2007). This led to an initial enhanced probability of presynaptic release of GABA, but was speculated to

result in an overall synaptic depression of GABA's inhibitory influence. ROS are known to interact with other ion channels, receptors, and effectors of different signaling cascades, and it would be worthwhile to investigate whether the interaction of ROS with other receptors in GABAergic interneurons may affect their function. As stated in CHAPTER 4, t-BOOH changed the shape of the action potentials produced by the EGFP-labeled GABA neurons. This after-hyperpolarization is currently being investigated since Ca^{2+}-gated K^{+} channels may be activated by ROS.

Did the EGFP+ GABA neurons undergo apoptosis due to oxidative stress? While the stereological counts demonstrated a reduction in the number of labeled GAD67-containing neurons after SNL, the question remains about what was responsible for the decreased counts. Future experiments will investigate the possibility that the GAD67-expressing neurons undergo apoptosis shortly after L5 SNL.

Also, what happens to the other GABA neurons after SNL? This study estimated that GAD67/EGFP expression in about 8% of the entire GABA neuron population in the spinal dorsal horn is lost due to nerve injury-induced oxidative stress. With ROS removal, GAD67/EGFP expression loss was prevented, and yet, the pain behaviors were not completely restored. Therefore, other mechanisms besides the loss of GAD67-producing neurons are involved in the maintenance of neuropathic pain. This study has shown that acutely elevated ROS levels can decrease GABA neuron excitability. One possible explanation is that oxidative stress may cause irreversible changes in the GABA neurons that might not be severe enough to induce cell death or a down-regulation of GAD67 expression, but the GABA neurons might not be functioning normally. Although no significant changes in the electrophysiological properties were found in CCI injured EGFP+ GABA neurons

(Schoffnegger et al. 2006), a similar study investigating the functions of EGFP+ GABA neurons at earlier time points after SNL should be done to answer this question.

Finally, is glycinergic inhibitory transmission also affected by oxidative stress in neuropathic conditions? Besides GABA, glycine is another important inhibitory neurotransmitter in the spinal dorsal horn (Yoshimura and Nishi 1995; Todd et al. 1996). The modulation of glycine neurotransmission also seems to be important for the development of pain (Yaksh 1989; Sivilotti and Woolf 1994; Cronin et al. 2004). An intrathecal injection of the glycine receptor antagonist, strychnine, in rats elicited touch-evoked agitation and spontaneous pain behaviors (Yaksh 1989). Strychine applied to trigeminal dorsal horn neurons facilitated postsynaptic discharges evoked by tactile stimulation of afferents (Yaksh 1989). Strychine applied to motor neurons enhanced flexor motor neuron responses to mechanical stimuli applied to the skin of the hind-paw of a decerebrate rat, indicating that loss of the glycinergic inhibitory tone may contribute to touch-evoked allodynia, particularly the nociceptive flexion withdrawal reflex (Sivilotti and Woolf 1994).

Inhibition of the spinal glycinergic system seems to occur both in inflammatory and neuropathic pain. In inflammatory pain, the prostaglandin, Prostaglandin E2 (PGE_2), has been shown to block the glycine receptor isoform that contains the α3 subunit (GlyRα3) (reviewed in (Zeilhofer and Zeilhofer 2008)). The inhibition of GlyRα3 is caused by the activation of the EP2 receptor for PGE_2 which leads to the downstream activation of PKA. PKA subsequently phosphorylates GlyRα3 and blocks glycine from binding to its receptor, contributing to the disinhibition of superficial dorsal horn neurons (Ahmadi et al. 2002; Harvey et al. 2004; Reinold et al. 2005). However, PGE_2–mediated inhibition of spinal glycinergic inhibition does not seem to occur in neuropathic pain, particularly, in the CCI

model (Hosl et al. 2006), suggesting that other mechanisms are involved in the disinhibition of the spinal dorsal horn neurons.

In neuropathic pain, after tibial nerve transection, nicotine was found to facilitate the activities of glycinergic neurons at the spinal level and reduce nociceptive transduction (Abdin et al. 2006). Furthermore, intrathecal injections with various inhibitors of the glycine transporters, GlyT1 and GlyT2, have been shown to alleviate mechanical allodynia in mice by enhancing spinal glycine transmission (Morita et al. 2008). Therefore, the disinhibition of neurons in central sensitization may involve the spinal glycinergic system; however, the mechanism behind this loss of inhibition remains largely unsolved.

One explanation, as mentioned previously, was that peripheral nerve injury resulted in a decrease in the expression of the potassium-chloride co-transporter 2 (KCC2) and consequently, a pathologically high intracellular concentration of chloride. This reduction shifted the anion reversal potential so that activation of both the glycine and $GABA_A$ receptor caused chloride efflux and depolarization, increasing lamina I neuronal excitability (Coull et al. 2003). Thus, the reduction in KCC2 may be one explanation for the loss of spinal glycinergic inhibition. However, other mechanisms may contribute to the reduction in glycinergic transmission in the spinal dorsal horn, and it would be interesting to examine whether oxidative stress also plays a role.

In summary, the results in this study have provided a mechanism by which ROS can influence the establishment of central sensitization through attenuation of GABA transmission in the spinal cord. The data show that a ROS-induced loss in GAD67 expression occurs relatively early after injury, within one week. However, many unanswered questions remain regarding the role of ROS in the development of neuropathic pain. Future studies

must be done also to determine whether cell death accounts for the loss of GAD67 expression.

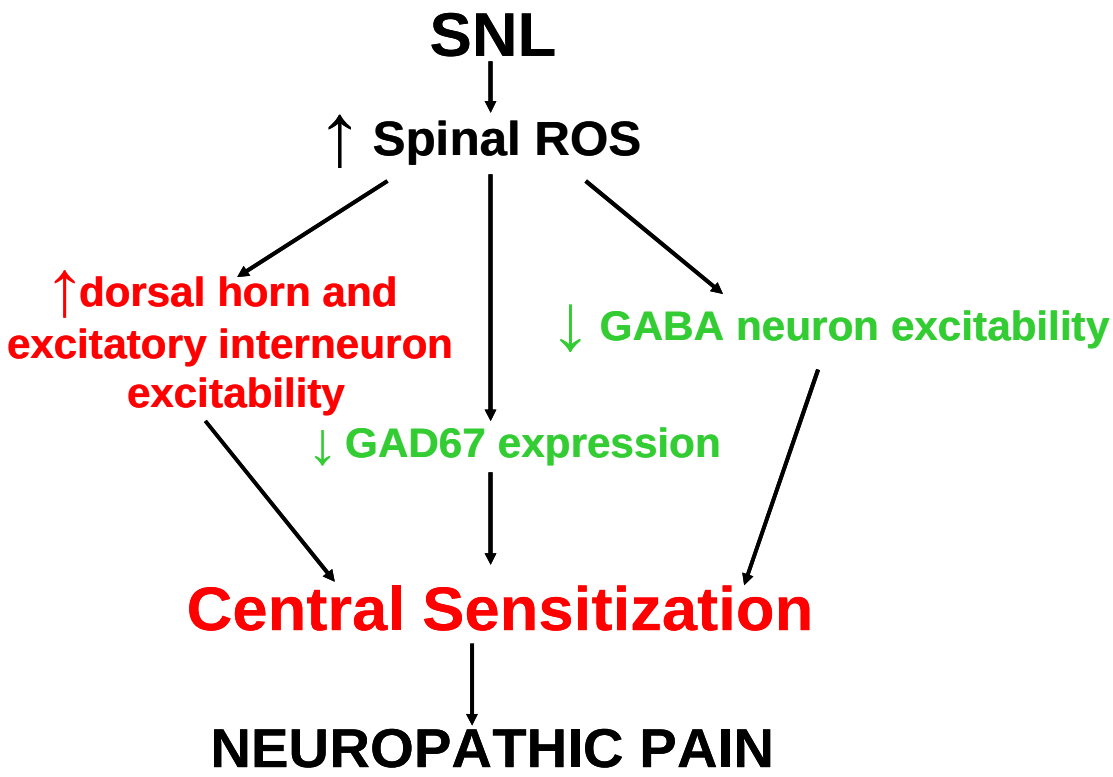


FIG. 6.1. The importance of ROS for the production of neuropathic pain through their effects on the spinal GABAergic system. Peripheral nerve injury as modeled by SNL produces an increase in spinal levels of ROS that contributes to the development of central sensitization by three mechanisms. ROS increase the excitability of dorsal horn neurons and excitatory interneurons and decrease the excitability of GABAergic interneurons. ROS also induce the loss of GAD67 expression in the spinal dorsal horn which also results in further disinhibition of spinal dorsal horn neurons. The net result is an enhancement of nociceptive processing, resulting in central sensitization – a key feature of neuropathic pain.

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VITA

June Yowtak was born on June 22, 1980 in New York City, New York to Mrs. Laddaval Yowtak and Mr. Somchai Yowtak. Moving to Irving, Texas, June graduated as valedictorian from MacArthur High School in 1998. In 1998, she was selected for the Science Teacher Access to Resources at Southwestern program at the University of Texas Southwestern Medical Center in Dallas. June participated in an Alzheimer disease research project under the direction of Dr. Lawrence Honig. She learned the basic biomolecular techniques and analyzed the effects of beta-amyloid protein on the synaptic integrity of cultured chick retinal neurons. In 2000, she participated in the Research Experience for Undergraduates program at the University of Florida in Gainesville, Florida. Working under the guidance of Dr. Mark Meisel, she studied the effects of homogeneous magnetic fields on genetically engineered Arabidopsis Thaliana. Her work on a second project where she modeled the forces the produce magnetic levitation on Arabidopsis Thaliana was presented in a poster at the annual meeting of the American Society for Gravitational and Space Biology in 2000. She received a Bachelor of Science in 2002, with a major in Biochemistry from the University of Dallas. Following graduation from the University of Dallas, June entered the M.D./Ph.D. Combined Degree Program at the University of Texas Medical Branch in Galveston, Texas in 2002. June joined the laboratory of Dr. Jin Mo Chung in November 2005. She conducted her dissertation research focusing on the role of oxidative stress on GABAergic dysfunction during the development of neuropathic pain. June presented posters on her research at the Society for Neuroscience meetings in 2006 and 2007, the American Pain Society meeting in 2007 and the International Conference on the Mechanisms and Treatment for Neuropathic Pain conference in 2007. Following the completion of her dissertation, June will finish the last two years of medical school to earn her M.D. and continue with residency training. She plans to continue her scientific training after residency.

Education

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Yowtak, J., Lee, K. Y., Kim, H. K., Wang, J., Kim, H. Y., Lee, I., Chung, K., Chung, J. M. "Reactive Oxygen Species are Important for Neuropathic Pain in Mice by Causing Spinal GABA Dysfunction." (in preparation).

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