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TWO TYPES OF ROS ARE DIFFERENTIALLY INVOLVED IN SPINAL SYNAPTIC PLASTICITY AND MECHANICAL HYPERSENSITIVITY IN NEUROPATHIC PAIN

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TWO TYPES OF ROS ARE DIFFERENTIALLY INVOLVED IN SPINAL SYNAPTIC PLASTICITY AND MECHANICAL HYPERSENSITIVITY IN NEUROPATHIC PAIN

By

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Dedication

To my wonderful parents Hassan Bittar and Shirine Abou Melhem

who taught me to never give up.

To my younger sisters Yara and Jana

whom I look up to and learn from every day.

To my beloved fiancé Dr. Nima Shirafkan

who taught me that positivity is the secret for a happier being.

To my dear uncle, Ali Bittar, who believed in me.

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Two types of ROS are differentially involved in spinal synaptic plasticity and mechanical hypersensitivity in neuropathic pain

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Central sensitization, characterized by an increase in dorsal horn synaptic efficacy, is believed to underlie the chronic nature of neuropathic pain. It has been shown that pain-relaying synapses in the dorsal horn express two forms of long term changes in excitability—long term potentiation (LTP) and long term depression (LTD)—which contribute towards increased pain signaling to higher brain regions. Molecular mechanisms involved in the induction and maintenance of spinal LTP and LTD are still unclear. This study investigates the type of synaptic plasticity expressed by two major types of pain-relaying neurons in the dorsal horn, excitatory spinothalamic tract neurons (STTn) and inhibitory gamma-amino-butyric acid interneurons (GABAn). In addition, reactive oxygen species (ROS) have been recently shown to play an indispensable role in pain related synaptic plasticity. Therefore, this study further investigates the role of ROS in STTn and GABAn synaptic plasticity. First, synaptic plasticity in STTn and GABAn was induced using different conditioned stimulation (CS) paradigms (high frequency and low frequency). Second, calcium influx following CS was imaged in STTn and GABAn. Third, the role of two types of ROS (superoxide and hydroxyl radicals) in pain behavior as well as in CS-induced STTn and GABAn synaptic plasticity was investigated. The role of superoxide and hydroxyl radicals was also investigated in spinal nerve ligation (SNL)-induced synaptic plasticity. Results indicate that synaptic plasticity in the dorsal horn is cell-type specific; STTn express LTP whereas GABAn express LTD consistently independent of CS frequency. Calcium influx was found to be similar in dynamics during the induction of both STTn-LTP and GABAn-LTD. In addition, it was found that

superoxide and hydroxyl radicals induce different levels of hyperalgesia and play a differential role in CS-induced STTn-LTP and GABAn-LTD as well as in SNL-induced changes in STTn and GABAn cellular excitability. Results hold key findings that might be helpful in better directing potential treatment methods for neuropathic pain.

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ACSF – Artificial Cerebral Spinal Fluid

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP – Adenosine Tri-Phosphate

BAPTA – 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid

CamKII – Ca^{2+}/Calmodulin Dependent Kinase II

CNS – Central Nervous System

CS – Conditioned Stimulus

CTb – Cholera Toxin B

DH – Dorsal Horn

DMTU – Dimethylthiourea (Hydroxyl radical scavenger)

EPSC – Excitatory Post-Synaptic Current

ERK – Extracellular-signal Regulated Kinase

fEPSP – Field Excitatory Post-Synaptic Potential

GABA – Gamma-Amino-Butyric Acid

GAD – GABA-synthesizing protein

GFP – Green Fluorescence Protein

GlyT2 – Neuronal glycine transporter

GPX – Glutathione peroxidase

GSH – Reduced Glutathione

H_2O_2 – Hydrogen Peroxide

HFS – High Frequency Stimulation

IASP – International Association for the Study of Pain

IL-1β – Interleukin 1 Beta

JNK – c-Jun N-terminal kinases

KO_2 – Potassium Superoxide (Superoxide radical donor)

LFS – Low Frequency Stimulation

LTD – Long Term Depression

LTP – Long Term Potentiation

MAPK – Mitogen

mEPSC – Miniature Excitatory Post-Synaptic Current

mGluR – Metabotropic Glutamate Receptor

mIPSC – Miniature Inhibitory Post-Synaptic Current

NADH – Nicotinamide adenine dinucleotide

NF-κB – Nuclear Factor-κB

NK1R – Neurokinin 1 Receptor

NMDA – N-Methyl-D-Aspartate

$O_2^{-\cdot}$ – Superoxide Radical

$OH^{\cdot}$ – Hydroxyl Radical

PBN – Phenyl-N-tert-butylnitrone (Non-specific ROS scavenger)

PKA – Protein Kinase A

PKC – Protein Kinase C

ROS – Reactive Oxygen Species

SG – Substantia Gelatinosa

SNL – Spinal Nerve Ligation

SOD – Superoxide Dismutase

STT – Spinothalamic Tract

STTn – Spinothalamic Tract Neurons

tBOOH – Tert-Butyl-Hydroperoxide (Hydroxyl radical donor)

TEMPOL – 4-Hydroxy-TEMPO (Superoxide radical scavenger)

TNFα– Necrosis Neurotrophic Factor Alpha

TRPV1– Transient Receptor Potential Vanilloid 1 channel

Trx – Thioredoxin Peroxidase

TTX-R Na^+ – TTX-resistant sodium channels

VGAT – GABA transporter proteins

VGLUT – Vesicular Glutamate Transporter

CHAPTER 1

INTRODUCTION

1.1 NEUROPATHIC PAIN

1.1.1 DESCRIPTION:

Treating the underlying pathophysiology of a disease is generally the way to reach optimum healing. However, this is not the case with neuropathic pain. Defined by the International Association for the Study of Pain (IASP) as "a disease or lesion affecting the somatosensory system", neuropathic pain is a debilitating disease with symptoms that extend beyond a lesion that heals with time (Rasmussen et al., 2004; Cohen and Mao, 2014). In principle, it can be the by-product of many diseases affecting the nervous system like diabetes, cancer, herpes-zoster, HIV, multiple sclerosis, not to mention neurodegenerative diseases such as Parkinson's diseases (Beiske et al., 2009; Posso et al., 2016). Due to its heterogeneous etiology, neuropathic pain affects approximately 67.5 million people worldwide (Global Industry Analysts, 2011). To date, the few treatment options commercially available, such as gabapentin and pregabalin, are inefficient in completely eradicating patients' pain, and the numbers of patients living with neuropathic pain are continuously increasing.

Neuropathic pain is characterized by evoked and/or spontaneous pain (Schaible, 2006; Costigan et al., 2009; Cohen and Mao, 2014). The clinically reported forms of evoked pain are primary hyperalgesia, secondary hyperalgesia and allodynia. Primary and secondary hyperalgesia are described as an increase in sensitivity to normally painful

stimulus at the site or at an adjacent site to the damaged tissue, respectively. Allodynia is characterized as a pain response to normally innocuous stimulus (Marchettini et al, 2006; Cohen and Mao, 2014). While evoked pain occurs only in response to a stimulation, spontaneous pain occurs for no apparent reason. Spontaneous pain is clinically reported as a continuous or intermittent sense of tingling, shooting, stabbing, sharp, sudden, burning, pins and needles. In addition, patients also suffer from several comorbid conditions such sleep disorders, depression and anxiety resulting at times in suicides (Marchettini et al., 2006; Bannister et al., 2009).

The lack of treatment options, medications and therapeutics to treat this disease is due to the fact that the underlying mechanisms remain ill-defined. To date, studies have shown that ongoing aberrant nociceptive signals after peripheral nerve injury induce central sensitization at the level of the dorsal horn. Central sensitization, manifested as long term changes in the efficacy of pain-relaying synapses, underlies the chronic nature of pain (Cohen and Mao, 2014). However, signaling mechanisms responsible for central sensitization remain unclear. Therefore, it is of paramount importance to investigate the molecular mechanisms involved in central sensitization and synaptic plastic changes. This investigation may hold promising implications for novel therapeutics that treat neuropathic pain efficiently and efficaciously.

1.1.2 NEUROANATOMICAL VIEW OF SENSORY TRANSMISSION

Primary Afferents:

Highly specialized primary nerve fibers carry sensory information from cutaneous and subcutaneous tissue to the central nervous system for higher order processing. These fibers first terminate at different laminae of the dorsal horn of the spinal cord, and then

synapse on a network of interneurons and projection neurons for relay to corresponding areas of the brain (Todd, 2010) (**FIG 1**).

Different features classify sensory nerve fibers into three types (Aβ, Aδ, and C fibers). These features include the sensory modality transmitted (mechanical, thermal), intensity of stimulus, size and conduction velocity (Almeida et al., 2004; Wooten et al., 2014). Aβ fibers are myelinated, large in diameter (> 10 μm), fast in conduction (30-100 m/s) and usually respond to low threshold mechano-stimulation, like light touch. These fibers innervate deep laminae of the dorsal horn (Iii-VI). Aδ fibers, on the other hand, are lightly myelinated, with a diameter ranging from 2.0 to 6.0 μm and a velocity of conduction up to 30 m/s. Last but not least, C fibers are unmyelinated with a diameter ranging between 0.4 and 1.2 μm and a conduction velocity of 0.5-2.0 m/s (Millan, 1999; Almeida et al., 2004). Aδ and C fibers terminate in laminae I and most of lamina II of the dorsal horn, and are the ones responsible for higher threshold nociception and thermo-reception (both heat and cold).

Further classification subcategorizes C fibers into peptidergic and non-peptidergic. Peptidergic C fibers release neurochemicals such as neuropeptide substance P (Lawson et al., 1997) and innervate deep layers of the skin, whereas non-peptidergic C fibers lack neuropeptides and innervate only the epidermal layers of the skin (Snider et al., 1998; Taylor et al., 2009; Todd, 2010). These two subgroups terminate in different layers of the superficial laminae of the dorsal horn and account for different nociceptive functions. For example, a population of non-peptidergic fibers was recently shown to selectively express Mas-related G-protein coupled receptor (MRGPRD) in mouse and mediate only noxious mechanical but not thermal stimuli (Cavanaugh et al., 2009).

Characterizing primary afferent fibers has been the focus of many studies over the past decades due to their significance in sensory and pain conduction. Damage along these fibers have been shown to result in various types of inflammatory and chronic pain disorders in humans. Researchers have developed several peripheral nerve injury models that reproduce clinical pathological pain behaviors. **In my aims**, I utilize a peripheral nerve injury model, spinal nerve ligation (SNL), that induces ligation injury in primary afferent nerve fibers, to investigate molecular mechanism underlying neuropathic pain.

Primary afferents share a common excitatory neurotransmitter, glutamate, as the main neurotransmitter to propagate action potentials into the central nervous system (CNS). When the action potential reaches primary afferent terminals, calcium influx, through voltage dependent calcium channels, induces the release of glutamate that binds to ionotropic receptors on the post-synaptic membrane. These receptors are ubiquitously expressed in the central nervous system and include N-Methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptors. NMDA receptors are tetramers composed of a dimer of NR1 glycine binding subunits, and a dimer of $NR2_{A-D}$ or $NR3_{A/B}$ (Stephenson et al., 2008). These heteromers form a channel like structure that is blocked by a magnesium ion under basal cellular conditions (Mayer et al., 1984). AMPA receptors are also channel-like tetramers that are composed of a combination of GluR1- 4 subunits. All AMPA subunits are calcium permeable except GluR2 (Polgar at al., 2008).

Glutamate also binds to Metabotropic Glutamate Receptors (mGluRs) on the primary afferent post-synaptic membrane. These are G-protein coupled receptors that also contribute to the stimulation-induced calcium influx (Latremoliere and Woolf, 2009).

While mGluRs were shown to mostly occupy the extremities of the post-synaptic membrane, NMDA and AMPA receptors were shown to spread over a wider area making them more important in sensory transmission. In addition, AMPA receptors were shown to be involved in basal nociceptive and pathological pain transmission, whereas mGluR and NMDA receptors were shown to be crucial only for pathological pain transmission (Young et al., 1997; Latremoliere and Woolf, 2009).

After glutamate binding, a network of interneurons and projection neurons receive the primary sensory input directly from afferent nociceptors. This network occupies the superficial laminae of the dorsal horn and serves as the first station at which sensory signals are managed before being transmitted to the brain (Todd, 2010). In other words, interneurons and projection neurons fire action potentials at a certain intensity of stimulation, below-which sensory signals are not conducted through. This property makes this network imperative in pain transmission; for any change in neuronal excitability at this level can lead to abnormal pain manifestations such as neuropathic pain. **In my aims,** I investigate peripheral nerve injury-induced changes in the neuronal excitability in the dorsal horn interneurons and projection neurons that lead to neuropathic pain. Therefore, it is important to understand the functions and characteristics of the neurons of interest.

Interneurons:

Interneurons are defined as neurons with axons that remain in the spinal cord, and they occupy most of laminae I, II and III. (Polgar et al., 2013) **(FIG 1)**. They are of excitatory or inhibitory nature. Excitatory interneurons are all glutamatergic and are identified by their expression of the vesicular glutamate transporters, specifically VGLUT2

(Todd et al., 2003; Maxwell et al., 2007). On the other hand, inhibitory interneurons are mainly either GABAergic alone or containing both GABA and glycine (Todd and Sullivan, 1990). They are usually identified via the expression of vesicular GABA transporter proteins (VGAT), GABA-synthesizing protein (GAD) or neuronal glycine transporter (GlyT2). Transgenic labeling techniques have been used, so far, to detect GABA-only expressing interneurons (Dougherty et al., 2005; Todd, 2010). These neurons occupy around 30% of lamina II where they were shown to preferentially express GluR1 Ca^{2+} permeable subunit of AMPA receptors under basal cellular conditions (Kerr et al., 1998). This suggests their importance in basal inhibition of subthreshold pain signaling. On the other hand, they were also found be fundamental for the pain disinhibition phenomenon in neuropathic pain models (Moore et al., 2002). **Aims 1 and 2 of my study** utilize genetically labeled GAD67-GFP tagged GABAergic interneurons to investigate the role of GABAergic disinhibition and neuropathic pain.

In order to understand the functions of different types of interneurons, studies have focused on categorizing these neurons based on lamina localization and morphology. However, due to the heterogeneity of the dorsal horn neuronal population, clear classification schemes could not be established. Perl et al. (2002) classified lamina II interneurons into four classes mainly based on morphology of dendrites into islet, central, vertical and radial (Grudt and Perl, 2002). Other studies then adopted this classification scheme and tried to link morphology to function. These findings have shown that islet cells, with dense and long horizontal dendritic extensions, are mainly GABAergic. Radial and vertical interneurons, with round and vertical dendrites, respectively, are mainly glutamatergic. However, the central interneurons, along with a remaining of 30%

unidentified interneurons fell under either excitatory or inhibitory natures, rendering the scheme inaccurate.

When morphology was linked to firing patterns, tonic firing was observed in islet, vertical, and central interneurons. Delayed firing was observed in radial and vertical interneurons, whereas initial bursting was observed in central and islet neurons (Heinke et al., 2004; Graham et al., 2007; Yasaka et al., 2010). Therefore, firing patterns could not be clearly correlated to morphology either. This demonstrates the high complexity and heterogeneity of lamina II.

A similar situation was encountered when lamina I neurons were attempted to be classified. Morphological classification did not conform with those based on electrophysiological or functional properties, and no clear-cut schemes could be established (Todd, 2010). Characterizing dorsal horn neurons remains to be the focus of many studies due to the importance of such characterization in understanding the different functions of this region and to attain better understanding of pain signaling. **In my study**, I chose to focus on lamina II GABAergic interneurons, for these neurons are the most implied in neuropathic pain development.

Projection neurons:

Concentrated mainly in lamina I and dispersed throughout deeper laminae (III-VI), projection neurons receive primary input and relay sensory information to various supra-spinal regions **(FIG 1)**. To name a few, the most highlighted relay regions in pain signaling are the medulla, the lateral parabrachial, the periaqueductal gray, and the thalamus (Al-Khater and Todd, 2009). Using retrograde transported dyes such as Cholera Toxin B (CTb) and fluorogold, studies revealed that projection neurons represent only 5% of lamina I

neurons. Furthermore, studies using immuno-cytochemistry focused on characterizing projection neurons by relating receptor expression to function. Results showed that around 80% of lamina I projection neurons express neurokinin 1 receptor (NK1R); substance P's docking site, and respond to noxious stimuli (Salter and Henry, 1991; Todd et al., 2000). Ablation of NK1R in the lamina I neurons blocks hyperalgesia development in some inflammatory and neuropathic pain models (Mantyh et al., 1997; Nichols et al., 1999). Very few excitatory interneurons express NK1R as well (Littlewood et al., 1995), however in much lower expression levels than projection neurons (Al Ghamdi et al., 2009). In addition, most of NK1R-positive projection neurons express Ca^{2+}-impermeable GluR2 subunit under cellular basal conditions. Under pathological pain conditions, however, GluR1/GluR2 ratio increases significantly highlighting GluR1 prominent role in abnormal pain signaling. Therefore, hyperalgesia development is believed to be mostly mediated by lamina I projection neurons.

The majority of lamina I neurons project to the lateral parabrachial nucleus, whereas the minority projects to the thalamus (Burstein et al., 1990; Lima and Coimbra, 1988; Spike et al., 2003). Knowing that the spinothalamic tract pathway is indispensable in pain signaling, the low representation of spinothalamic tract (STT) projection neurons among lamina I neurons is not expected. Nevertheless, it was shown that most of the larger NK1R-expressing neurons and the "giant cells" that lack NK1R, are STT neurons. The large soma and dendrites of these neurons therefore accommodate a sizable and dense network of inhibitory and excitatory interneurons, making them highly regulated and imperative in pain signaling. Furthermore, it becomes worthy to note that few lamina I projection neurons receive direct monosynaptic input from primary afferents. Instead, the

majority of primary input is mediated via excitatory and inhibitory interneurons (Todd, 2010). Therefore, what becomes more important than the number of STT neurons, is their interaction with the interneuronal population that finely regulates pain transmission. **In my aims**, I propose a study that investigates neuronal excitability in a circuit of STT neurons and GABAergic interneurons, and its role in neuropathic pain signaling.

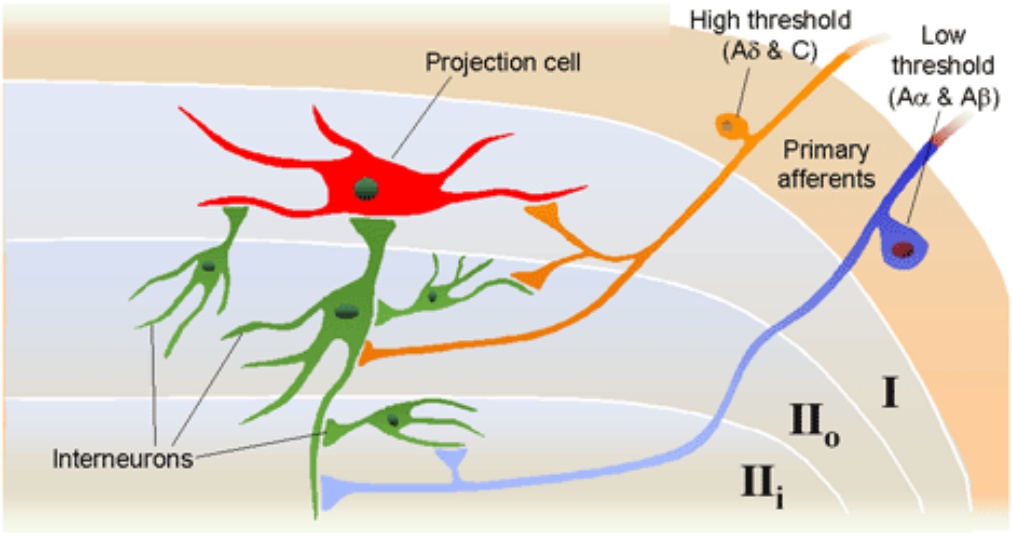


FIG 1: Primary afferent input onto projection neurons and interneurons of the superficial laminae of the dorsal horn. Projection neurons, that project to several brain regions (Thalamus, parabrachial nucleus, periaqueductal gray, medulla) receive direct input from high threshold Aδ and C fibers, and populate lamina I of the dorsal horn. Interneurons receive direct and indirect input from Aδ and C fibers in laminae I and II, as well as from low threshold Aβ fibers at deeper laminae. Excitatory interneurons further excite projection neurons whereas inhibitory interneurons deliver an inhibitory feedforward control over pain transmission through projection neurons. (Figure adapted from Wilkie et al., 2001).

1.1.3 NEUROPATHIC PAIN MECHANISMS:

Extensive research has been done over the past decades to uncover neuropathic pain mechanisms and utilize findings in clinical settings to reach optimal therapeutics. Despite the abundance of novel discoveries, the mechanisms of neuropathic pain are still unclear, and available therapeutic options have proved to be ineffective in most patients. It is established that both peripheral and central sensitization underlie the initiation and maintenance of neuropathic pain involving a wide multitude of pathophysiological changes at different levels of the peripheral and central nervous systems.

Peripheral sensitization and ectopic discharges:

The activation of the inflammatory system following peripheral nerve damage creates a state of hyperexcitability in the peripheral nervous system. This state is believed to disappear after the injury has healed. However, constant and repetitive peripheral nociceptive signaling creates a prolonged state of hyperexcitability called peripheral sensitization.

During the inflammatory response, many inflammatory mediators and neuropeptides leak from the bloodstream due to injury induced edema and bind to their corresponding receptors on peripheral nociceptors. The inflammatory "soup" then in turn, sensitizes nociceptors, i.e., increases the magnitude of response to noxious stimulation and lowers their firing thresholds, leading to augmented nociceptive output and hyperalgesia. Nociceptor sensitization also leads to ectopic discharge that may generate spontaneous action potentials and manifest as spontaneous pain (Baba et al., 1999; Campbell and Meyer, 2005; Schaible, 2006; Costigan et al., 2009). Furthermore, spontaneous pain can originate

from neighboring undamaged nerve fibers via ephaptic transmission, a non-synaptic communication between nerve fibers (Cohen and Mao, 2014).

A myriad of cellular signaling molecules, receptors, and mechanisms are involved in nociceptor sensitization, which explains why targeting one or a couple of these pathways reliefs pain in a partial manner. The inflammatory "soup" is composed of several cytokines (TNFα, IL6, IL10), growth factors (NGF), cannabinoids, bradykinins, serotonin, substance P and other molecules that are shown to be involved in peripheral mechanical and heat hypersensitivity (Opree and Kress, 2000; Campbell and Meyer, 2006; Cohen and Mao, 2014).

Studies have also shown that after nerve injury, several sodium channels sub-types, on primary afferent fibers, proliferate and undergo functional changes that lead to nociceptor sensitization (Campbell and Meyer, 2006; Levinson et al., 2012, Cohen and Mao, 2014). For example, a decrease in activation threshold and an upregulation of Na_V(s) 1.3, 1.7 channels trigger spontaneous pain and contribute to hypersensitivity. However, blocking these channels with carbamazepine produced a series of side effects due to low drug selectivity (Cohen and Mao, 2014). On the other hand, another type of ion channels, α2δ calcium channels, were shown to be upregulated in peripheral nerve fibers and dorsal root ganglion after nerve injury leading to hyperalgesia (Freynhagen et al., 2005; Bian et al., 2006). These channels are the target of gabapentin, the first line of treatment clinically used, which provides up to 50% of pain relief.

Other very important contributors to peripheral inflammation and sensitization are the transient receptor potential vanilloid 1 channel (TRPV1) (Schaible, 2006; Kim et al., 2009) and the TTX-resistant sodium (TTX-R Na^+) channels ($Na_V1.8$ and $Na_V1.9$)

(Ossipov, 2012). TRPV1 is expressed in C fibers and was shown to be important for heat hyperalgesia. Prolonged inflammation due to substance P, bradykinin, TNFα, or NGF was shown to upregulate TRPV1 in DRG and peripheral nerve fibers (Cheng and Ji, 2008) . In addition, TRPV1 specific knock-out mice fail to develop heat hyperalgesia (Caterina et al. 2000; Davis et al. 2000) whereas TRPV1 upregulation causes long-lasting hypersensitivity (Ji et al. 2002; Wilson-Gering et al. 2005). TTX-R Na^+ and TTX-sensitive Na^+ channels, on the other hand, are imperative in mechanical hyperalgesia. The slow activation/inactivation kinetics of TTX-R Na^+ 1.8 and 1.9, and the following transcriptional regulation of these channels, greatly contribute to the on-going peripheral pain signaling (Ossipov, 2012).

Following peripheral input, gene transcriptional and post-translational modifications require days to manifest, and therefore sustain pro-inflammatory and pro-nociceptive states after nerve damage. Protein kinase A (PKA) is one of the most prominent signaling molecules required for peripheral sensitization and the post translational modification of TRPV1 and TTX-R Na^+ channels. PKA was shown to directly phosphorylate and sensitize TRPV1 channels. In addition, it regulates PGE_2-meidated enhancement of TRPV1 currents and TTX-R Na^+ currents (Lopshire and Nicol, 1998; Gold et al., 1998). Moreover, PKA was shown to be critical for spontaneous neuronal activity in peripheral and dorsal root ganglion (DGR) neurons as well as in TNFα controlled C-fibers ectopic discharges. PKA mainly acts via cAMP signaling cascade and blocking PKA was shown to reverse peripheral inflammation and hyperalgesia (Hu et al., 2001; Zhang et al., 2002).

Another protein kinase, Protein Kinase C (PKC) is also indicated to be involved in TRPV1 and TTX-R Na^+ regulation. A specific isoform of PKC, PKC-epsilon, which is calcium independent, was shown to respond to inflammatory mediators and sensitize TRPV1 and TTX-R Na^+ channels (Cheng and Ji, 2008).

In addition, mitogen-activated protein kinases (MAPKs) family, such as ERK, were shown to be crucial for peripheral sensitization. These proteins help translate extracellular inflammatory signals into intracellular responses by genetic modulation. The MAPKs family were shown to be activated by TNF-α and IL-1β inflammatory cytokines, and in turn activate several signaling cascades that lead to protein production (Ji and Woolf, 2001; Cheng et al., 2008). With relatively specific inhibitors available, inhibition of three MAPKs together (ERK, p38, and JNK) was shown to alleviate chronic pain without modulating normal pain nociception (Ji et al., 2002). Peripheral targeting of the more than one MAP-Kinases at the same time could represent a better therapeutic approach than targeting a single molecule. (Cheng and Ji, 2008).

Inflammatory induced peripheral sensitization plays a major role in neuropathic pain initiation, however, the question remains whether it is important for the chronic phase of the disease. Peripheral sensitization and on-going ectopic discharge set the stage for a central sensitization, a longer lasting sensitization of pain-relaying neurons in the spinal cord. In addition to mechanical and thermal hyperalgesia mediated by both sensitizations, neuropathic pain patients suffer from allodynia and secondary hyperalgesia that can be explained only by the central sensitization. **In my study**, I focus on the chronic phase of neuropathic pain mainly mediated by central sensitization.

Central sensitization

Central sensitization is defined as an activity-dependent change in the efficacy of the dorsal horn synapses. It entails an increase in neuronal excitability, a decrease in excitatory neurons' firing threshold, and a decrease in the dorsal horn's inhibitory function (Schaible, 2006; Cohen and Moa, 2014). Synaptic transmission is mainly based on action potential firing that requires a certain strength of peripheral input. Increased synaptic excitability after nerve injury allows the recruitment of subthreshold synaptic input leading to changes in the functional properties of the dorsal horn neuronal network. These functional changes are summarized below **(FIG 2)**.

Upon intense peripheral input, central sensitization is triggered by the release of abnormal increased amounts of neurotransmitters and their binding to postsynaptic membranes. As mentioned earlier, glutamate is the major neurotransmitter released in the dorsal horn and it activates NMDA, AMPA and mGluRs receptors. Substance P and brain-derived neurotrophic factors are also released at the peripheral terminals to activate Neurokinin kinase 1 (NK1) and Tropomyosin receptor kinase B (TrkB) receptors, respectively (Latremoliere and Woolf, 2009) **(FIG 2)**.

Following excessive receptor activation, intracellular calcium influx through AMPA and mGluRs increases significantly and activates downstream serine and threonine protein kinases such as PKA, PKC, and calcium/calmodulin-dependent kinase II (CamKII) (Latremoliere and Woolf, 2009). Excessive neurotransmitter binding also depolarizes the postsynaptic membrane and leads to the removal of NMDA-receptors magnesium block. NMDA-induced calcium influx then induces the insertion of more AMPA receptors to the membrane leading to increased membrane excitability and pain signaling up to the brain

(Latremoliere and Woolf, 2009). Moreover, PKA, PKC and ERK provoke further posttranslational and transcriptional modifications leading to protein expression, thus maintaining the sensitization state for many hours (Carvahlo et al., 2000; Lau and Zukin, 2007) **(FIG 2)**. Pharmacological manipulations and conditional knock-outs that inhibit NR1 subunit of NMDA receptors was shown to inhibit both NMDA currents and the subsequent development of mechanical hyperalgesia (South et al., 2003). The mechanisms of the long lasting sensitization described, resemble those of long term potentiation (LTP) in the brain, the phenomenon underlying hippocampal memory formation. LTP, a form of synaptic plasticity, is a prolonged increase in synaptic efficacy leading to increased transmission between two neurons. LTP in spinothalamic pain transmitting neurons was shown by our lab to be dependent on NMDA receptors. **It is one of the goals of aim 2 of my study** to further investigate the mechanisms of spinal LTP downstream of NMDA receptors.

There is a debate in the field around whether or not long term changes in the spinal cord are a form of LTP for two reasons. First, LTP in the hippocampus is of a homosynaptic nature, i.e. occurring only in the stimulated/conditioned synapses. However, spinal-LTP was shown to be of both homosynaptic and heterosynaptic nature, thus recruiting stimulated and close-by unstimulated synapses (Latremoliere and Woolf, 2009; Sandkuhler, 2010). Homosynaptic spinal-LTP, alongside with peripheral sensitization, underlie mechanical and thermal primary hyperalgesia observed in neuropathic pain. However, heterosynaptic LTP helps to explain secondary hyperalgesia and allodynia by involving neighboring synapses and Aβ-fibers' synapses on dorsal horn neurons, respectively (Ji et al., 2003).

Second, unlike hippocampal LTP, evidence of a non-reversible LTP in the spinal cord neurons is lacking. Spinal-LTP has been shown to last not more than 6 hours both *in vitro* and *in vivo* (Liu and Sandkuhler, 1998). Nevertheless, it becomes worthy to note that spinal-LTP is seldom investigated in neuropathic pain model animals several days after injury (Ikeda et al., 2000). On the contrary, it is often induced in naïve animals, via conditioning stimulus (injury mimic) that is often far from representing physiological nerve injury. Therefore, it is hard to deduce that nerve injury-induced spinal LTP is not a long-term lasting LTP. **Later in this study**, we provide novel findings pointing towards the existence of a spinal potentiation lasting at least 7 days after peripheral nerve injury. For the sake of convenience, the term LTP will be used to describe synaptic plasticity in the dorsal horn presented throughout this study.

Another mechanism contributing to central sensitization is GABA-disinhibition (Sandkuhler, 2009; Todd, 2010). Lamina II is rich with GABAergic and glycinergic inhibitory interneurons that synapse on projection pain relaying neurons, and attenuate pain transmission. Nerve injury was shown to induce a loss of inhibitory currents as well as death of GABAergic but not glycinergic interneurons in the dorsal horn (Moore et al., 2002). Mechanisms underlying these changes are still unclear. Some studies suggest that, after injury, diminished activity of potassium-chloride transporter selectively in GABA interneurons, causes an increase in intracellular chloride concentration. This increase then leads to the death of GABA interneurons and the subsequent facilitation of excitatory pain signaling (Moore et al., 2002; Drew et al., 2004; Janssen et al., 2011). GABAergic neuronal loss has been documented in several neuropathic pain models. In addition, loss of GABAergic inhibition directly correlates with the development of tactile allodynia and

hyperalgesia, whereas the inhibition of GABA neurons reverses hyperalgesia (Malan et al., 2002; Yowtak et al., 2011).

Controversy, however, surrounds the manifestation of GABA-disinhibition after nerve injury and its role in neuropathic pain behaviors. Some studies utilizing the spared nerve injury model (SNI) show that the development of tactile allodynia and hyperalgesia is rather independent of GABA-interneurons loss in dorsal horn laminae I and II (Polgar et al., 2005). Further experimentation is required to resolve this controversy. **My dissertation study** provides additional insight on the role of GABAergic disinhibition in spinal cord ligation (SNL) model.

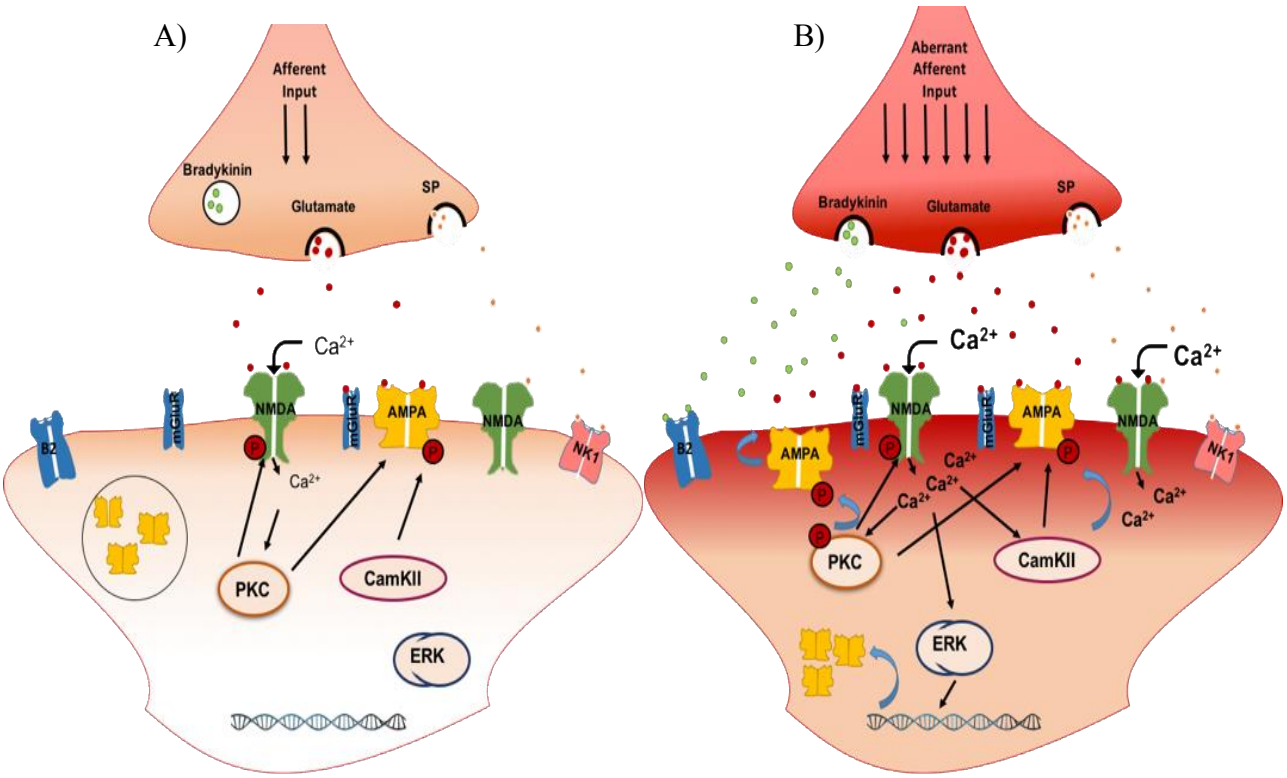


FIG 2: A comparison between physiologic and pathologic pain transmission (Central Sensitization: In response to normal afferent nociceptive input (A), neurotransmitters like glutamate and substance P (SP) get released from the central terminal of primary afferent fibers. Neurotransmitters then activate mainly AMPA and NMDA receptors leading to calcium influx, the generation of an action potential, and the propagation of an acute pain signal. However, in case of ongoing aberrant afferent nociceptive input (B), large amounts of neurotransmitters as well as inflammatory mediators (like bradykinin) will be released at the pre-synaptic terminal. This will result in the activation of more glutamatergic receptors, as well as SP-activated NK1 receptors and bradykinin-activated B2 receptors. NMDA mediated large calcium influx will flow into the cytoplasm activating calcium dependent protein kinases like PKC, PKA, CamKII, which in turn will activate ERK. ERK will then induce gene transcription and the production of new AMPA receptors to be inserted into the membrane. Consequently, the change in the membrane receptor composition will increase its excitability and the synapse's efficacy thus increasing pain signaling to second order neurons and higher order brain structures. (Figure created by Alice Bittar).

1.2 REACTIVE OXYGEN SPECIES (ROS)

1.2.1 ROS ROLE IN NEURONAL PHYSIOLOGY

Reactive oxygen species (ROS) are molecules containing at least one oxygen atom, and are more reactive than molecular oxygen. They include free radicals such as superoxide and hydroxyl radicals, as well as non-radicals such as hydrogen peroxide (Beckhauser et al., 2016). ROS were initially viewed as part of the defense mechanism against pathogens and bacteria. However, it has become well established that ROS also act as second messengers in cellular signaling pathways crucial for cell survival, differentiation, proliferation, and gene expression (Schieber and Chandel, 2014).

In the nervous system, redox signaling by free radicals was shown to be required for neurogenesis by activating tyrosine phosphorylated protein kinases, such PKC, and transcription factors, such as nuclear factor κB (NF-κB) (Giorgi et al., 2010). In addition, the redox balance was shown to regulate neuronal differentiation from precursor cells, and induce neurite and axonal growth. Anti-oxidants were shown to inhibit neurite outgrowth in a PC12 cell line (Suzukawa et al., 2000; Chao et al. 2013; Topchiy et al., 2013).

Recently, a great focus has been allocated to the role of ROS in neuronal transmission and excitability. Moreover, due to the recent association between ROS production and calcium signaling, ROS have been shown to take part in synaptic plasticity. In specific, oxidation by free radicals was found to increase the activity of protein kinases and decrease that of their counterpart phosphatases (Beckhauser et al., 2016). In addition, oxidation was also shown to differentially affect NMDA and AMPA receptors in terms of activation kinetics. Potential targets for redox modifications on signaling proteins are

cysteine, serine and threonine residues. Usually, these residues are susceptible for oxidation due to their low pK_a values (Rao et al., 2002; Rhee et al., 2005). These findings imply that the highly reactive nature of ROS can still be specific in action, especially in regulating synaptic plasticity mechanisms. **Specific aims 2 and 3 of my study** present a novel view of the level of ROS specificity in neuronal signaling mechanisms.

Sources of neuronal ROS:

ROS are produced by the mitochondria as a byproduct of oxidative phosphorylation and the subsequent production of adenosine triphosphate (ATP). During this process, nicotinamide adenine dinucleotide hydrogen (NADH) is oxidized by complex I of the electron transport chain. This oxidation results in the transfer of one electron from NADH to an oxygen molecule and the creation of the free radical superoxide anion ($O_2^{-\cdot}$). In a similar reaction, complex III as well contributes to the production of $O_2^{-\cdot}$. Neurons have relatively high metabolic rates in comparison to other types of cells and thus produce higher ROS levels as part of their normal cellular functions (Halliwell, 1992). ROS of mitochondrial origin are regulated by cytoplasmic calcium levels. It has been shown that treatment with high concentrations of calcium leads to increased generation of ROS (Dykens, 1994). In addition, NMDA receptor dependent calcium influx has been shown to regulate NADH gene expression which in turn controls ROS production. This suggests a strong correlation between cellular excitability and mitochondrial ROS production (Beckhauser et al., 2016).

ROS are also produced via NADPH oxidases which are transmembrane enzymes that mainly act as part of the immune system. NADPH oxidases produce superoxide

radicals in neutrophils in order to kill bacteria and pathogens swallowed. Other than in phagocytic cells, NADPH was also recently found on neuronal postsynaptic terminals in the brain. It is believed to play a role in cellular mechanisms necessary for neuronal communication under physiological conditions, and is activated by calcium influx into the cell (Beckhauser et al., 2016).

ROS homeostasis

Due to the high reactivity of ROS molecules and their involvement in a wide range of cellular functions, maintaining the physiologic levels of ROS in a cell is very important. Excess ROS is termed "oxidative stress" and can lead to deleterious effects damaging signaling molecules, proteins, DNA, lipids, and eventually leading to mutagenesis (Massad and Klann, 2010). For these reasons, cells, including neurons, possess ROS scavenging systems that help regulate ROS amounts and prevent oxidative stress-induced damage.

One of the most important ROS scavenging molecules is superoxide dismutase 2 (SOD2). It is located in the mitochondrial matrix and it transforms superoxide radicals ($O_2^{-\cdot}$) into hydrogen peroxide (H_2O_2), a less reactive and more stable form of ROS (Fridovich, 1995). Another superoxide dismutase, SOD1, is located in the mitochondrial membrane and the cytosol of the cell, and helps to convert cytosolic ($O_2^{-\cdot}$) to H_2O_2. H_2O_2 then undergoes detoxification via peroxidase enzymes such as glutathione peroxidase (GPX), reduced glutathione (GSH), and thioredoxin peroxidase (Trx) (Massad and Klann, 2010). These peroxidases also subsequently protect from the H_2O_2 elevation-induced production of hydroxyl radicals ($OH^{\cdot}$), the most reactive and vigorous ROS (Stowe and

Camara, 2009). $OH^{\cdot}$ are produced as a result of a reaction between H_2O_2 or $O_2^{-\cdot}$ with metal ions such as ferric iron (Cohen, 1994; Lipinski, 2011).

Understanding the cellular ROS production and scavenging mechanisms is very instrumental in managing oxidative stress related diseases. The three mentioned types of ROS are shown to contribute to synaptic plasticity and neuropathic pain. Several animal models utilize genetic manipulation techniques to knock-down or upregulate SOD2 or NADPH in an attempt to study oxidative stress's effects on pain behavior. Other methods of more direct and transient ROS levels manipulation include the use of pharmacological ROS scavengers such as phenyl N-t-butylnitrone (PBN), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), and N,N-Dimethyltryptamine (DMTU) (Kim et al., 2004). **Aims 2 and 3 of my study** utilize the mentioned ROS scavengers in order to study ROS role in spinal dorsal horn synaptic plasticity.

ROS in neuropathic pain

Specifically, in the spinal cord, oxidative stress has been implicated in dorsal horn synaptic plasticity mechanisms that drive central sensitization and neuropathic pain development. For instance, ROS levels are shown to be significantly increased in spinal dorsal horn after nerve injury, and this increase is directly correlated with hyperalgesia. Moreover, SOD2 knockout mice were shown to develop stronger secondary hyperalgesia than wild type litter mates in a capsaicin model. This hyperalgesia was reversed by TEMPOL, a superoxide radical scavenger. In addition, the same study reports that SOD2 transgenic (overexpression) mice failed to develop secondary hyperalgesia (Schwartz et al., 2009). These findings propose a central role of mitochondrial ROS in neuropathic pain

development (Kim et al., 2011). NADPH-induced oxidative stress has also been implicated in neuropathic pain, however, not in the central nervous system. In the peripheral nervous system, NADPH-induced oxidative stress has been linked to the inflammatory response preceding peripheral sensitization. Nox2, a subunit of NADPH, was shown to generate ROS right after spinal nerve injury. Nox2 deficient mice had attenuated allodynia and hyperalgesia levels after L5 spinal nerve transection (Kim et al., 2010). **Aim 2 of my study** provides additional insight about the role of mitochondrial ROS in neuropathic pain behavior.

Cellular mechanisms underlying the role of oxidative stress in neuropathic pain recently caught the attention of pain researchers due to the promising analgesic effects produced by ROS scavengers in several animal models. Despite the well-defined role of ROS in synaptic plasticity in the brain, the role of ROS in dorsal horn pain-related synaptic plasticity is still unclear. In the spinal cord **(FIG 3)**, ROS leads to the activation of NMDA and AMPA receptors following conditioning stimulus (CS) in naïve animals, spinal nerve ligation (neuropathic pain model) and capsaicin pain model (inflammatory pain model) (Gao et al., 2007; Lee et al., 2010; Lee et al., 2012). ROS scavengers were shown to decrease AMPA receptors GluR1-subunit phosphorylation and membrane localization in a capsaicin model and after the intrathecal injection of NMDA (Lee et al. 2012). Moreover, ROS donors (tBOOH) induce LTP of field excitatory post synaptic potentials (fEPSP) in the dorsal horn, and ROS scavengers block the induction of CS-induced fEPSP-LTP. Furthermore, CS-induced and tBOOH-induced LTP occlude each other (Lee et al., 2010). These findings suggest that oxidative stress is necessary for LTP induction in the spinal cord.

In the process of LTP induction, ROS have been shown to facilitate the phosphorylation and activation of several protein kinases such as PKC, PKA, ERK, CamKII. Kinase activation will induce AMPA receptor phosphorylation and upregulation on the membrane and the subsequent increase in synaptic efficacy **(FIG3)**. ROS have been shown to enhance PKC-induced AMPA GluR1 phosphorylation at Ser831 residue (Lee et al., 2010). The same site was also found to be phosphorylated by ROS induced CamKII activation (Jenkins et al., 2012). Phosphatases such as calcineurin and phosphatase A1 and 2 have also been shown to be regulated by ROS (Kamsler and Segal, 2003; Kim et al., 2015; Beckhauser et al., 2016). The activation of phosphatases has been linked to the development of long term depression (LTD) phenomenon; the opposite of LTP (Mulky et al., 1992). LTD results in a permanent reduction in membrane excitability, and it has been shown to play a major role in GABAergic disinhibition process underlying neuropathic pain (Kim et al., 2012).

ROS have been shown to activate phosphatases in the process of LTD formation and to inactivate phosphatases in the process of LTP in a concentration dependent manner. For instance, a concentration of 20 μM of hydrogen peroxide was shown to activate calcineurin and mediate LTD in the hippocampus, whereas lower concentrations (1 μM) were shown to induce LTP and inhibit LTD (Kamsler and Segal, 2003). These findings indicate that oxidative balance finely and specifically regulates synaptic plasticity mechanisms and is, as a result, imperative for the process of neuropathic pain signaling and expression. In the spinal cord, little is known about the mechanisms of ROS induced regulation of the kinase/ phosphatase balance in synaptic plasticity. **Aim 2 of my study**

focuses on better understanding the relationship between oxidative stress and spinal synaptic plasticity (LTP and LTD).

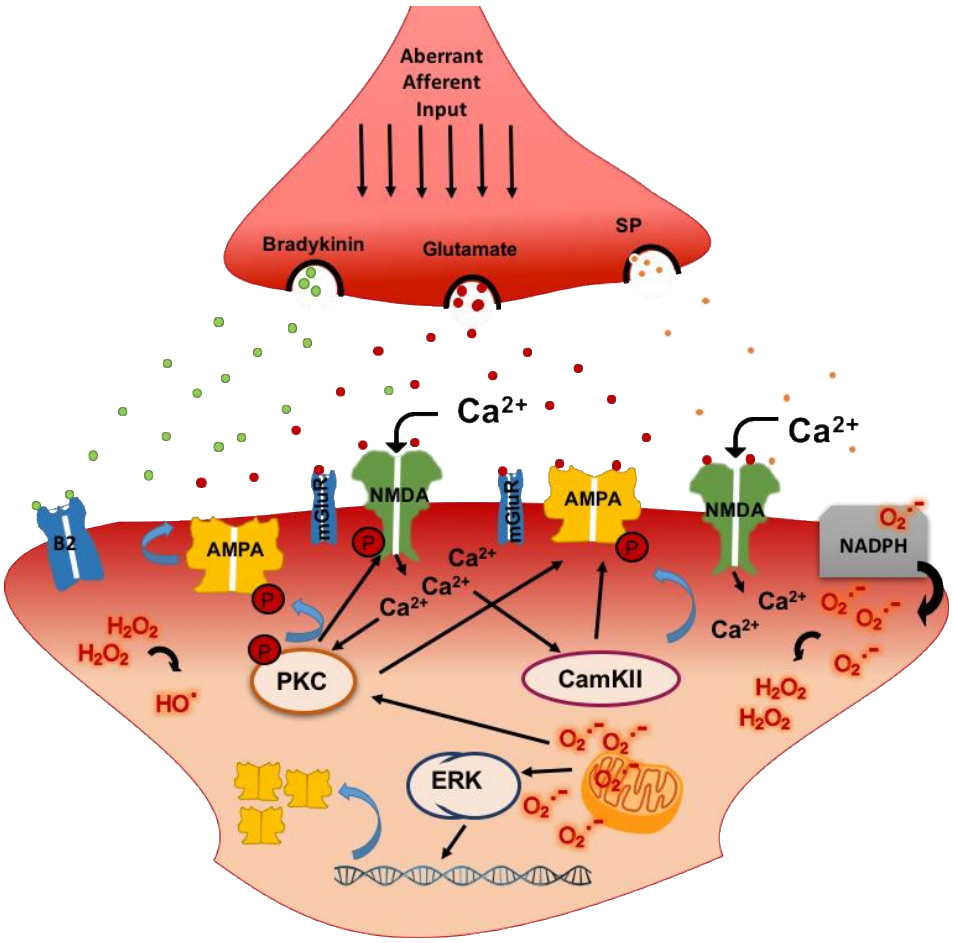


FIG 3: Oxidative stress in central sensitization: During physiologic pain transmission, minimal amounts of reactive oxygens species (ROS) are produced during pain signal transduction. Neurons are well-equipped to scavenge ROS using SOD proteins and peroxidases. However, during on-going pathologic pain transmission, large calcium influx leads to increased production and accumulation of ROS and thus oxidative stress. When this oxidative stress is not large enough to induce apoptosis, ROS interfere downstream of calcium and activate calcium-dependent kinases leading to increased synaptic efficacy. On-going nociceptive input is believed to induce an on-going ROS production. Superoxide radicals are the main type of ROS produced from the mitochondria and NADPH oxidases. Superoxide radicals then are transformed into hydrogen peroxide and hydroxyl radicals that further contribute to central sensitization. (Figure was created by Alice Bittar).

1.3 RATIONALE FOR THE STUDY

1.3.1 BACKGROUND

Spinal synaptic plasticity in the dorsal horn is a crucial contributor to central sensitization resulting in the development and maintenance of neuropathic pain (Koltzenburg et al., 1992; Campbell and Meyer, 2006; Zhou et al., 2011). Those synaptic plastic changes lead to increased excitatory but decreased inhibitory tones of Laminae I-II of the dorsal horn (Ashmawi and Freire, 2016). However, synaptic plasticity mechanisms underlying neuropathic pain are not clear. In addition, reactive oxygen species (ROS) have been shown to be crucial for neuropathic pain signaling (Klann et al., 1993; Tal, 1996). Therefore, this study investigates neuropathic pain related synaptic plastic changes after peripheral nerve injury and focuses on the role of ROS in synaptic plasticity on excitatory spinothalamic tract neurons and inhibitory GABAergic interneurons in the dorsal horn in neuropathic pain.

1.3.2 CENTRAL HYPOTHESIS

The overall hypothesis of this study states that reactive oxygen species are crucial for neuropathic pain behavior development and maintenance as well as for the induction and maintenance of synaptic plasticity in the spinothalamic tract neurons and GABA-interneurons of the dorsal horn of the spinal cord.

1.3.3 SPECIFIC AIMS

SA1: To determine the form of synaptic plasticity expressed by STTn and GABAn of the spinal cord dorsal horn in response to different stimulation paradigms:

Exp 1: Test the effect of low frequency stimulation on STTn and GABAn excitability in spinal cord tissue of normal mice.

Exp 2: Test the effect of high frequency stimulation on STTn and GABAn excitability in spinal cord tissue of normal mice.

Exp 3: Test the change in intracellular Ca^{2+} levels in STTn and GABAn after low frequency stimulation.

Exp4: Test the effect of Ca^{2+} chelator on induction of STTn-LTP and GABAn-LTD.

SA2: To determine whether different types of ROS are specific to STTn-LTP and GABAn-LTD induction and maintenance

Exp 1: Test the effect of spinal nerve ligation (SNL) on mechanical hypersensitivity for two weeks after surgery

Exp 2: Test the effect of specific ROS donors (KO_2 for $O_2^{\cdot-}$ and tBOOH for $OH^{\cdot}$) on pain behavior in naïve mice

Exp 3: Test the effect of specific ROS scavengers (PBN, TEMPOL and DMSO/DMTU) on pain behaviors in SNL mice.

Exp 4: Test the effect of specific ROS donors (KO_2 for $O_2^{\cdot-}$ and tBOOH for $OH^{\cdot}$) on STTn and GABAn excitability.

Exp 5: Test the effect of a non-specific ROS scavenger (PBN) on spinal LTP (LTP) and LTD (LTD) induction and maintenance phases in STTn and GABAn respectively.

Exp 6: Test the effect of specific ROS scavengers (TEMPOL for $O_2^{\cdot-}$ and DMSO for $OH^{\cdot}$ and DMTU for $OH^{\cdot}$) on CS-induced synaptic plasticity induction and maintenance phases in STTn and GABAn respectively.

SA3: To determine whether cell type specific synaptic plasticity is established in neuropathic mice and whether ROS subtype specificity is conserved in SNL model

Exp1: Test the presence of established spinal synaptic plastic changes in the dorsal horn neurons in neuropathic animals. The levels of EPSC amplitude from LTP and LTD recordings in normal mice will be compared to that in neuropathic mice.

Exp2: Test the presence of established spinal synaptic plasticity in the dorsal horn neurons in neuropathic animals after applying afferent conditioning stimulus to STTn and GABAn in neuropathic animals.

Exp 3: Test the effect of TEMPOL on evoked EPSCs recorded from STTn and GABAn of SNL mice.

Exp 4: Test the effect of DMTU on evoked EPSCs recorded from STTn and GABAn of SNL mice.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS:

Experimental protocols and procedures were approved by the Animal Care and Use Committee at the University of Texas Medical Branch and are in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

2.1.1. Behavioral experiments: Ten-week-old C57BL/6 male mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed on a 12/12 hour light/dark cycle in groups of four in plastic cages with standard bedding and free access to food and water. All behavioral measurements started at 1 pm. All animals were given the chance to acclimate for at least 4 days, including 3 days of handling before any experimental procedures were performed.

2.1.2. Electrophysiology experiments: Three to four-week-old male mice (FVB-Tg(GAD67-GFP) 45704Swn/J, Jackson Laboratory) were used for spinal cord slicing and patch clamp recordings. These mice express GFP exclusively in GABAn, which makes identifying and recording from these cells possible.

2.2. BEHAVIORAL TESTING:

Mice were placed on an elevated metal grid and left in plastic chambers to acclimate for 10-15 min. Mechanical sensitivity was assessed by recording the frequency of pain behavior over 10 consecutive von Frey filament (VFF) stimuli, at 20-30 sec intervals.

Percent (%) frequency of pain-like behaviors was calculated and presented as data in the graphs.

After handling the mice for 3 days prior to spinal nerve ligation (SNL), baseline measurements were taken. SNL surgery was then done on Day 0 and the mice were allowed 5 to 7 days to develop mechanical hyperalgesia. On the day of drug administration, pre-drug baseline was measured. Drugs were then administered, and behavioral tests were done 20, 30, 40, 60, and 120 min after the drug administration.

2.3. SPINAL NERVE LIGATION (SNL):

Mice were anesthetized with isoflurane (1.5% during induction and 1% during maintenance). A 2 cm incision was made 1 mm to the left of the midline, parallel to the vertebral spines, and the muscles covering the transverse process of the L6 vertebra were removed. A part of the L6 transverse process was removed to expose the L4 and L5 spinal nerves. Without damaging the L4 spinal nerve, L5 spinal nerve was ligated using a 7-0 sterile silk thread. The wound was closed by suturing the incision using a 5-0 sterile silk thread and cleaned with alcohol wipes. The mice were then treated once with the analgesic buprenorphine (0.03 mg; i.p.) immediately after the surgery. Complete recovery was monitored closely before they were transferred to their corresponding cages (Chung et. al., 2004).

2.4. DRUG TREATMENTS:

All tested drugs were injected into the intrathecal space by a direct lumbar puncture method using a 10 μL Hamilton syringe. While mice were under anesthesia (1.5% isoflurane), the needle was introduced dorsally in between the L5 and L6 vertebrae until the tail flicking reflex was observed. Five microliters of drug were injected slowly and the needle was held

in place for an additional 4-5 sec to prevent drug leakage into the epidural space. The dorsal lower back was disinfected using alcohol wipes before and after injection. Behavioral results showed a complete recovery to the pre-drug treatment levels after 4 hr, with each drug treatment.

Behavioral experiments included testing the following drugs: potassium superoxide (KO_2), a superoxide radical donor, tert-Butyl hydroperoxide (tBOOH), a hydroxyl radical donor, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL), a superoxide radical scavenger (Lejeune et. al., 2006), dimethyl sulfoxide (DMSO) (Rosenblum and El-Sabban, 1982) and dimethylthiourea (DMTU) (Vander-Heide et. al., 1987), hydroxyl radical scavengers, and phenyl-N-tert-butylnitrone (PBN), a non-specific ROS scavenger. To test whether mechanical hyperalgesia is due to ROS-induced GABA dysfunction, the effects of $GABA_A$ and $GABA_B$ receptor antagonists (Bicuculline and CGP46381, respectively) (Philip et al., 2002; Yowtak et. al., 2013) in combination with ROS scavengers were also examined. All drugs were prepared in saline just prior to injection.

Electrophysiology experiments tested the ROS donors KO_2 (1 mM) and tBOOH (1 mM); and the ROS scavengers, TEMPOL (1 mM) and DMTU (20 mM). In both behavioral and electrophysiological experiments, a dose-response curve was built for each drug, from which the used doses were chosen.

2.5. IDENTIFICATION OF STTn AND GABAn:

Three to four-week-old mice were anesthetized (1.5 % isoflurane) and then placed on a rodent stereotactic apparatus supplied with a mouse adapter (Stoelting, Wood Dale, IL). To identify STTn, a retrograde tracer (1% FAST-DiI in 10% ethanol, Invitrogen, Carlsbad,

CA) was injected into the ventrobasal thalamus using a 5 µL Hamilton syringe needle. Coordinates of the injection site relative to bregma were: 1.48 mm posteriorly, 1.37 mm laterally, and 3.3 mm deep into the brain using the adjusted ratio of bregma-lambda distance specific for young mice (4.5 mm) (Kim et. al., 2015). Tracer was allowed 5 to 7 days post injection to be transported to the lumbar spinal cord, after which the mice were sacrificed for spinal cord slicing and patch clamp recording.

2.6. WHOLE-CELL RECORDINGS:

Whole-cell patch clamp recordings were made from acute spinal cord slices of mice. Spinal cord was removed while mice were under isoflurane anesthesia and transferred into cold high-magnesium artificial cerebrospinal fluid (high Mg ACSF) (in mM: 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 7 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 11 Glucose, aerated with 95% O_2 and 5% CO_2, 0-4°C). The lumbar part of the spinal cord was cut transversely at 350 µm thickness using a vibratome VT1000S (Leica Biosystems, Buffalo Grove, IL). Cord slices were first incubated in high Mg ACSF for 30 min then moved to standard ACSF (in mM: 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 11 Glucose) at 2 ml/min) for another 30 min at 34 °C. Slices were then kept at room temperature for the rest of the experimental time. The recording chamber was superfused with room temperature standard ACSF and an Olympus fluorescence microscope (BX51W1) was used to visualize STTn and GABAn. For establishing whole-cell patch clamp, we used a glass patch pipette (4-6 MΩ) pulled by Flaming Brown Micropipette Puller, model P-97 (Sutter Instrument, Novato, CA). The pipette was filled with internal solution containing (in mM): 120 K-gluconate, 10 KCl, 2 Mg-ATP, 0.5 Na-GTP, 0.5 EGTA, 20 HEPES, and 10

phosphocreatine. All chemicals were from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Evoked EPSCs were recorded in a voltage-clamp mode using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and pCLAMP 10 data acquisition software (Molecular Devices). To evoke EPSCs, the dorsal root entry zone (DREZ) was electrically stimulated with a bipolar glass micropipette electrode (2-4 MΩ). STTn and GABAn in the laminae I-Il were identified under a fluorescence microscope for recording. Holding potential was -70 mV. Test pulses were given at a 20 sec interval (0.5 ms, 100-170 mA). Recording was made only when the EPSCs were monosynaptic, based on EPSC waveforms with a short latency, single peak, and stable responses to repeated stimuli (Arancio et al., 1996). All recordings showing polysynaptic response were disregarded.

To induce synaptic plasticity via electrical stimulation, conditioning stimulation (CS) was applied in the DREZ (at 2 Hz for 40 sec = a total of 80 pulses with 0.5 ms pulse width, at the same intensity as the test stimulus [range: 100-170 mA]). Synaptic plasticity was also induced via chemical stimulation; patched neurons were exposed to the chemicals for 10 min right after obtaining baseline recordings (3 min), and then chemicals were washed out with standard ACSF for the rest of the 55 min. The amplitudes of 3 consecutive EPSCs were averaged and normalized to the baseline recorded before the application of the CS or chemical stimulation for each cell. Plasticity was defined as a greater than 20% change in EPSCs amplitude from baseline over a period of 15 to 25 minutes after CS (Randic et al., 1993).

2.7. DATA ANALYSES:

Data are expressed as mean ± SEM. Time-dependent behavioral/electrophysiological responses were analyzed by two-way repeated-measures ANOVA with Bonferroni post hoc comparisons to pre-drug/baseline responses within a treatment and between treatments at a given time point. $P < 0.05$ was considered significant. The amplitudes of EPSCs after CS or drug treatments were normalized to the mean amplitude of baseline EPSCs recorded for 3 min. Three EPSCs were obtained per minute and their amplitudes were averaged to represent EPSC amplitudes at 1-min intervals.

CHAPTER 3

SYNAPTIC PLASTICITY IN THE DORSAL HORN OF SPINAL CORD IS CELL-TYPE SPECIFIC

3.1 ABSTRACT:

The underlying mechanism of chronic pain is believed to involve changes in excitability in spinal dorsal horn (DH) neurons receiving abnormal peripheral input. Increased excitability in pain transmission neurons, and depression of inhibitory neurons, are widely recognized mechanisms in the spinal cord of animal models of chronic pain. The possible occurrence of two parallel but opposing forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) was tested in two types of identified DH neurons using whole- cell patch-clamp recordings in mouse spinal cord slices. The test stimulus was applied to the dorsal root entry zone to evoke excitatory postsynaptic currents in identified spinothalamic tract neurons (STTn) and GABAergic neurons (GABAn). Conditioning stimulation (CS) applied to primary afferent fibers with various frequency stimulation parameters induced LTP in STTn but LTD in GABAn in a consistent manner. Both the LTP in STTn and the LTD in GABAn were blocked by an intracellular Ca^{2+} chelator, BAPTA. Both the pattern and magnitude of intracellular Ca^{2+} after CS were almost identical between STTn and GABAn based on live-cell calcium imaging. The results suggest that the intense sensory input induces similar intracellular Ca^{2+} increase in both STTn and GABAn, but produces opposing synaptic plasticity. This aim shows that

there is cell type–specific synaptic plasticity in the spinal DH.

3.2 INTRODUCTION:

The lack in understanding neuropathic pain underlining mechanisms has rendered it one of the most debilitating pathological conditions around the globe (Woolf and Mannion, 1999). Intense afferent inputs due to tissue injuries often lead to amplified pain sensation underlined by plastic changes in the spinal dorsal horn (DH) (Ji and Woolf, 2001; Ji et al., 2003). Abnormal pain sensation can be manifested as hyperalgesia (elevated nociception to noxious sensory inputs) and/or allodynia (nociception to innocuous sensory inputs). Plastic changes are believed to drive increased excitability of pain transmission neurons and depression of inhibitory functions in the spinal DH (Woolf and Salter, 2000). These plastic changes are thus considered a fundamentally important mechanism underlying pain chronicity (Sandkuhler, 2000; Woolf and Salter, 2000).

Two forms of synaptic plasticity, LTP and LTD, were recorded in the spinal DH neurons after high-frequency stimulation of primary afferent fibers about two decades ago by Randic and his colleagues (1993). In this study, LTP was recorded in some spinal DH neurons, whereas LTD was recorded in some others, in response to the same stimulus. This indicates that both LTP and LTD can be induced in the spinal DH neurons in response to the same stimulation. Because the neuronal population in the superficial DH is highly heterogeneous and responds differently to primary afferent stimulation (Yezierski and Broton, 1991; Pan and Pan; 2004), it is difficult to identify the critical factors that determine the direction of plasticity, LTP vs LTD. It is also difficult to identify the overall effects of these changes to pain, without knowing the identity of recorded neurons in the spinal cord.

This aim of our study is to test the effects of intense afferent inputs on synaptic plasticity in two identified groups of neurons of the spinal cord DH: spinothalamic tract neurons (STTn) and GABAergic interneurons (GABAn). These neurons were shown to be important for pain transmission along the spinothalamic tract (STT) pathway (Kim et al., 2011; Kim et al., 1997; Latremoliere and Woolf, 2009; Todd, 2010).

Spinothalamic tract neurons were identified by retrograde labeling via injected dye (DiI) in the thalamus, whereas GABAn were recognized by using a transgenic mice line of which GAD-67 (a synthetic enzyme of GABA) was tagged with green fluorescent protein (GFP). Evoked excitatory postsynaptic currents (EPSCs) were recorded from these neurons by using whole-cell patch-clamp techniques in spinal cord slices. The results show that various means of intense peripheral stimulation consistently induces LTP in STTn but LTD in GABAn, with similar induction mechanisms. The data suggest that spinal LTP and LTD are induced in a cell-type dependent manner.

3.3 METHODS:

Please refer to the materials and methods section (Chapter 2).

3.4 RESULTS:

3.4.1 High frequency stimulation (HFS) induces LTP in excitatory STT neurons and LTD in inhibitory GABA neurons

Knowing that both LTP and LTD develop in the DH (Randic et. al., 1992), this experiment investigates the form of synaptic plasticity developed in two identified types of neurons involved in the excitatory/inhibitory balance of pain transmission via the spinothalamic tract pathway after conditioning stimulus. These types of neurons are the

excitatory spinothalamic tract neurons (STTn) and the inhibitory GABA interneurons (GABAn). STTn shown in **Fig. 3.1B** were identified via retrograde labeling using DiI injected in the ventro-basal thalamus region (**Fig. 3.1A**). GABAn express GFP protein (**Fig. 3.1C**). Induction of either LTP or LTD was defined as a greater than 20% change in EPSCs amplitude from baseline over a period of 25-30 minutes after CS (Randic et al., 1993). By applying the same HFS applied in Randic et al. (1993); bursts of 100 Hz (1 second long) repeated 3 times at a 10- second interval (total of 300 pulses for 30 seconds) with a holding potential of -50 mV (Ikeda et al., 2006), 4 out of 7 identified STTn developed a gradual and prolonged increase (>20% change) in EPSC amplitudes in comparison to baseline.

Another high frequency stimulation paradigm was tested by applying continuous 10-Hz pulses for 10 seconds (100 pulses) with a holding potential of 110 mV (Giese et al., 1998), 7 out 10 STTn successfully developed a greater than 20% increase in EPSC amplitudes in comparison to baseline. In total, 11 out of 17 tested STT neurons developed long term increase in EPSC amplitudes whereas no change (<20% increase) was detected in the remaining 6 (**Fig. 3.1D**). On the other hand, GABAn produced a prolonged decrease (>20% decrease) in the amplitude of EPSCs recorded after HFS (7 of 8 with 100 Hz and 4 of 7 with 10 Hz; **Fig. 3.1E**). All responses are plotted in **Fig. 3.1**.

3.4.2 Low frequency stimulation (LFS) induces LTP in STTn and LTD in GABAn

To see whether the responses reported by the previous experiment are dependent on stimulation frequency or cell type, we induced synaptic plasticity with low frequency application. Two low frequency stimulation paradigms were used: 1) continuous 1 Hz for

100 seconds with a holding potential of 240 mV (Bortolotto et al., 2010), and 2) continuous 2-Hz pulses for 40 seconds (80 pulses) with a holding potential of 130 mV (Tsvetkov et al., 2011). 14 of 18 STTn tested (4 of 5 with 1 Hz and 10 of 13 with 2 Hz; **Fig. 3.2A**) showed a gradual and prolonged increase (>20%) in EPSC amplitude after LFS, whereas 8 of 15 GABAn exhibited a rapidly developed and sustained decrease (>20%) in EPSC amplitude (3 of 7 with 1 Hz and 5 of 8 with 2 Hz; **Fig. 3.2B**). One GABAn showed LTP with 1 Hz stimulation and insignificant changes (>20%) were detected from all remaining recorded GABAn (n=6).

When both HFS and LFS data are combined, conditioning stimulus (CS) induced LTP in the majority of the STTn (28 of 38; 74%), but LTD in the majority of GABAn (19 of 31; 61%). The present data thus suggest that induction of LTP and LTD in the spinal cord is cell-type specific, and not stimulation parameter dependent.

Among 4 tested electrical conditioning stimulations, a 2-Hz continuous CS (for 40 seconds with a holding potential of 130 mV) most reliably produced LTP in STTn (10 of 13; 77%) and LTD in GABAn (5 of 8; 63%). Therefore, this CS protocol was used in all the subsequent experiments.

3.4.3 Intracellular Ca^{2+} levels change similarly during the induction of STTn-LTP and GABAn-LTD

It is well known that a transient increase of intracellular Ca^{2+} ($[Ca^{2+}]i$ transients) after NMDAR activation is a determining factor for neuronal plasticity. It has also been proposed that the magnitude and pattern of intracellular Ca^{2+} increase determine the

direction of neuronal plastic changes as to LTP or LTD (Malenka, 1994; Mizuno et al., 2001; Ismailove et al., 2004). To determine whether these findings also hold true in the spinal cord (LTP in STTn and LTD in GABAn), intracellular Ca^{2+} levels were measured before, during, and after the induction of LTP in STTn and LTD in GABAn by using a live-cell calcium imaging technique. As shown in figures **3.3 A** and **B**, both STTn and GABAn show a rapid increase in cytosolic Ca^{2+} to peak levels during the application of CS (2 Hz, 40 seconds, +30 mV holding potential), which was followed by a slow decline to baseline levels after CS termination. The magnitude and pattern of the intracellular Ca^{2+} transients were almost the same in both STTn and GABAn, in response to the same conditioning stimulation (**Fig. 3.3A** and **B**).

3.4.4 Calcium chelator BAPTA abolishes the induction of STTn-LTP and GABAn-LTD

We tested the effect of a Ca^{2+} chelator, BAPTA (5 mM; bis-[aminophenoxy] ethane-tetraacetic acid; applied into the cytoplasm of the cell by loading it in a patch pipette), on the induction of LTP in STTn and LTD in GABAn. Neither LTP in STTn nor LTD in GABAn could be elicited by CS when the intracellular Ca^{2+} transients were buffered by loading a Ca^{2+} chelator, BAPTA (5 mM), into STTn or GABAn (**Fig. 3.4 A** and **B**). The results indicate that the neuronal plasticity in STTn and GABAn share similar mechanisms of intracellular Ca^{2+} transients.

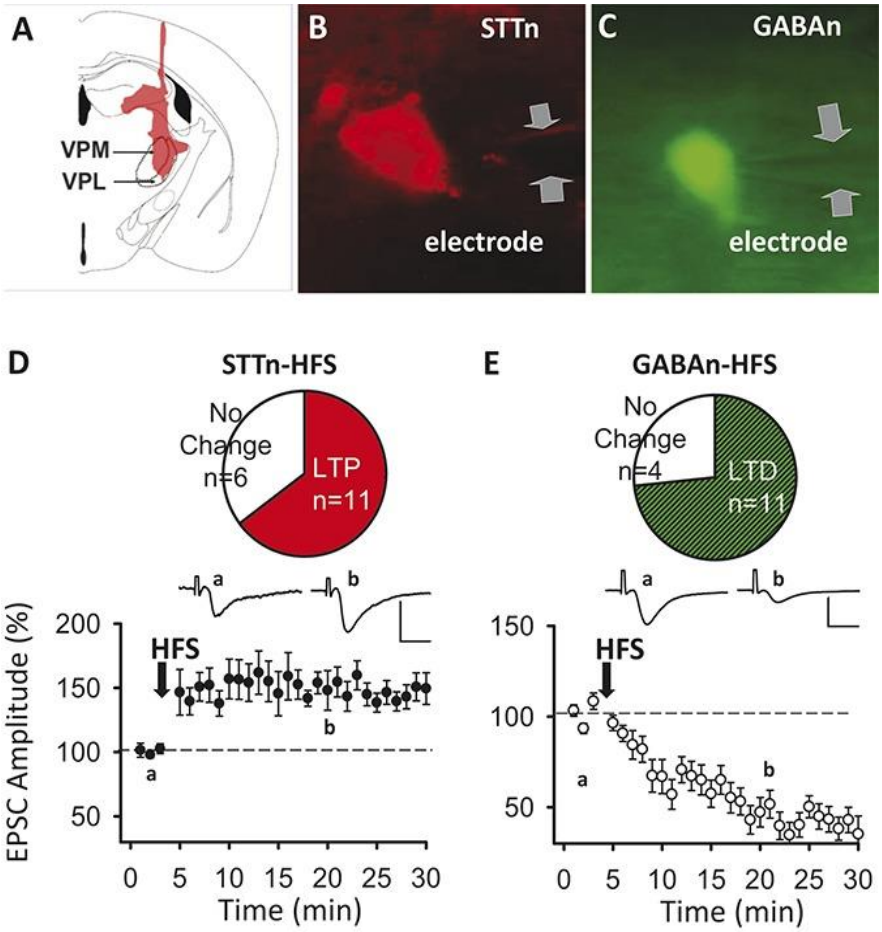


FIG 3.1: High-frequency conditioning stimulation [HFS] evokes long-term potentiation (LTP) in STTn but long-term depression (LTD) in GABAn. (A) Frozen sections of the brain were made from mice injected with a tracer dye (DiI) and a composite drawing of DiI marking was made from 1 mouse as an example. DiI (red) spread in many areas of the brain but included the ventral posterior lateral (VPL) and ventral posterior medial (VPM) nuclei of the thalamus. (B and C) Examples of STTn identified by retrogradely labeled DiI (B) and GFP+ GABAn (C) that were patch-clamped for excitatory postsynaptic currents (EPSC) recordings (recording electrode edges are indicated by paired enclosing arrows). (D and E) Effects of a HFS (either 100 Hz for 1 second, repeated 3 times at a 10-second interval, holding potential at −50 mV or continuous 10-Hz pulses for 10 seconds with a holding potential of +10 mV) on EPSC amplitudes averaged from n=11 STTn (D) and n=11GABAn (E). The recording traces (shown above each plot) are the averages of 6 consecutive EPSC recordings before (a) and 15 minutes after (b) HFS. Calibration: 100 pA, 10 milliseconds. Pie charts (shown above the plots in D and E) show the numbers of STT or GABA neurons that showed a significant change (>20%) after HFS.

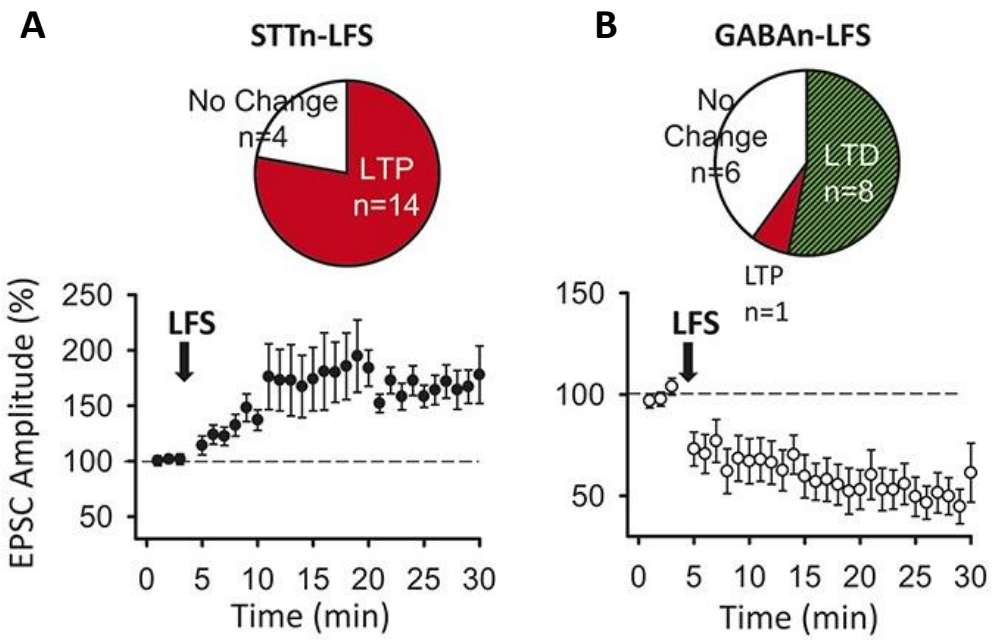


FIG 3.2: low-frequency conditioning stimulation [LFS] evokes long-term potentiation (LTP) in STTn but long-term depression (LTD) in GABAn. (A and B) Effects of an LFS (1 Hz for 100 seconds, holding potential −40 mV or 2-Hz pulses for 40 seconds, holding potential of +30 mV) on EPSC amplitudes averaged from 14 STTn (A) and 8 GABAn (B). Pie charts (shown above the plots in A and B) show the numbers of STT or GABA neurons that showed a significant change (>20%) after LFS.

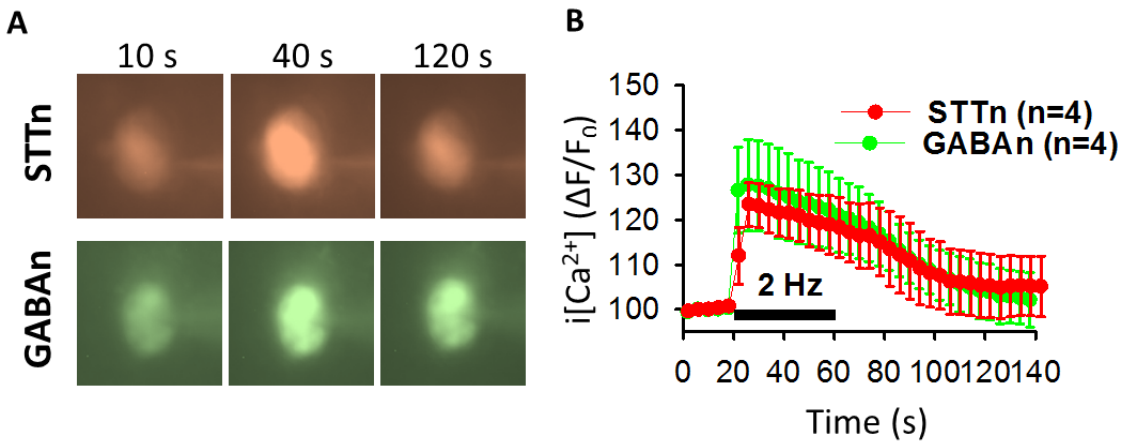


FIG 3.3: Intracellular Ca^{2+} ($[Ca^{2+}]i$) increase following LTP and LTD induction is similar in STTn and GABAn, respectively. (A and B) $[Ca^{2+}]i$ levels were visualized with Oregon Green 488 BAPTA-1 (0.2 mM, administered intracellularly by preloading it into the patch pipette) before, during, and after 2-Hz CSin an STTn and a GABAn (A). The averaged changes of $[Ca^{2+}]i$ levels after 2-Hz CS (B) show that intracellular free-Ca^{2+} levels in both STTn and GABAn increase at a similar rate and amplitude (n = 4 each).

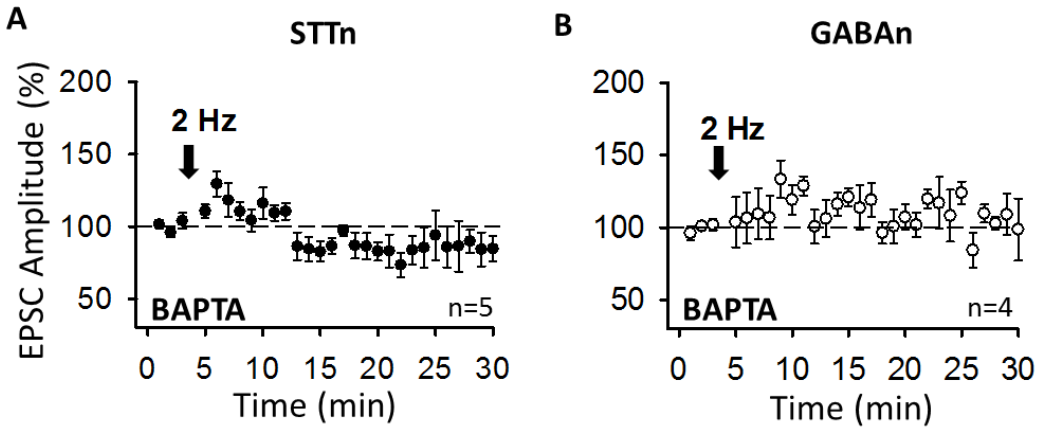


FIG 3.4 Calcium chelator inhibits both STTn-LTP and GABAn-LTD. Conditioning stimulation with 2 Hz failed to induce either LTP in spinothalamic tract neurons (STTn) (A) or LTD in GABAn (B) when Ca^{2+} chelator, BAPTA (5 mM), was administered intracellularly by preloading it into the patch pipette.

3.5 DISCUSSION:

There has been an unresolved issue about what determines the direction of synaptic plasticity after the two opposing forms of synaptic plasticity, LTP and LTD, were first observed in spinal DH neurons two decades ago (Randic et al., 1993). We now show that these two opposing synaptic plastic changes depend on neuronal types: LTP in STTn and LTD in GABAn. Because STTn are pain transmission neurons and GABAn are inhibitory interneurons, the observed phenomenon is consistent with the neuronal changes in chronic pain conditions where pain transmission neurons show hyperexcitability while inhibitory function of the spinal cord is depressed (Sandkuhler, 2000; Woolf and Salter, 2000; Ji and Woolf, 2001; Ji et al., 2003). After peripheral nerve injury, the sensitized spinal cord is known to induce decreased GABA immunoreactivity and GABAergic inhibitory transmission (Castro-lopes et al., 1993; Moore et al., 2002; Jansen et al., 2011). Data from this aim of our study show that LTD in GABAn further depresses inhibitory function, thus enhancing pain transmission in the sensitized spinal cord.

Many different types of CS have been used to mimic afferent inputs that cause synaptic plasticity. The most widely used CS is the high-frequency stimulation consisting of bursts of 100 Hz (1 second long) repeated 3 times at a 10-second interval (total of 300 pulses for 30 seconds) with a holding potential of 250 mV (Ikeda et al., 2006). Our data show that his high-frequency CS consistently elicited plastic changes in the spinal cord in this aim. However, such high- frequency paradigm may not represent natural nociceptive afferent input to the spinal cord because the normal firing rate of nociceptors is much lower (Beitel et al., 1976; Torbjork et al., 1984; Slugg et al., 200). This study also used three other LTP-producing CS protocols that were used in other brain areas: (1) continuous 10 Hz for

10 seconds (100 pulses) with a holding potential of 110 mV (Giese et al., 1998), (2) continuous 2 Hz for 40 seconds (80 pulses) with a holding potential of 130 mV (Tsvetkov et al., 2002), and (3) continuous 1 Hz for 100 seconds with a holding potential of 240 mV (Bortolotto et al., 2011). All of these CSs also produced LTP in STTn and LTD in GABAn, and certainly fall within the natural firing rate of nociceptors (Beitel et al., 1976; Torebjork et al., 1984; SLugg et al., 2000).

Studies have shown that induction of LTD in inhibitory interneurons is not consistent in different areas of the brain either. In the hippocampus, some GABAn show consistent LTD (McMahon and Kauer; 1997) or both LTP and LTD, depending on the cell type (Nissen et al., 2010). Therefore, LTD can be induced in a subpopulation of GABAn in the hippocampus. Although we were able to induce LTD consistently in GABAn in the spinal cord, we cannot conclude that LTD is induced in all spinal GABAn because only a subpopulation (35%) of GABAn show GFP in our GAD67-GFP transgenic mice (Heinke et al., 2004). Because not all GABAn are the same in their characteristics and enzyme contents (Qi et al., 2009), it is possible that the GFP negative spinal GABA neurons may respond differently from GFP positive GABAn (Abeliovich et al., 1993). Similarly, all our LTP recordings were done on STTn in the superficial lamina (I-IIa) of the DH, although many more labeled STT neurons were detected in deeper laminae (IV-V). Thus, it is possible that cell type–specific LTP shown in this study is limited only to STT neurons in the superficial lamina. Further studies are warranted to clarify this issue.

Historically, many aspects of LTP have been established from extensive studies in hippocampal neurons (Bliss et al., 1993). There are two well- known necessary conditions for synaptic plasticity: NMDA receptor activation and intracellular Ca^{2+} ($[Ca^{2+}]i$) increase

(Malenka and Bear, 2004). Spinal cord LTP, in many ways, is similar to hippocampal LTP. Both show an enhanced responsiveness of the affected neurons with a prolonged time course and dependency on NMDA receptors for the initiation (Randic et al., 1993; Lui and Sankuhler, 1995; Svendsen et al., 1998; Lee et al., 2010). Previous studies from our laboratory demonstrate that both NMDA receptor activation and subsequent $[Ca^{2+}]i$ increase are necessary conditions for the induction but not the maintenance of both LTP in STTn and LTD in GABAn (Kim et al., 2015). NMDA dependency on the induction of spinal LTP and LTD is most likely related to the NMDA dependency on the initiation of hyperalgesia and central sensitization after intense nociceptive inputs (Stanton et al., 1991; Woolf and Thompson, 1992; Gao et al., 2005)

The amplitude and duration of $[Ca^{2+}]i$ transients are suggested as the key element for determining the direction of synaptic plasticity (Malenka, 1994). Abrupt and large $[Ca^{2+}]i$ transients lead to the activation of kinases like PKC, PKA, CamKII, and ERK, and the induction of LTP (Elgersma et al., 1999; Boehm et al., 2005; Gwak et al., 2011; Kim et al., 2015). On the other hand, moderate and prolonged [Ca21]i transients activate phosphatases such as calcineurin and induce LTD (Mulkey et al., 1992; Mizuno et al., 2001; Ismailov et al., 2004). Furthermore, LTP can be depotentiated by lowering $[Ca^{2+}]i$ levels in hippocampal neurons (Cho et al., 2001) and cortical pyramidal neurons (Ismailov et al., 2004). The present data, however, show an almost identical abrupt and large $[Ca^{2+}]i$ transient in both STTn-LTP and GABAn-LTD. Therefore, the role of $[Ca^{2+}]i$ transients in synaptic plasticity in the spinal cord DH neurons seems to be different from other parts of the brain.

Other aspects of calcium influx, other than amplitude and duration, can contribute to the differential synaptic plasticity polarity. A recent study showed that high calcium influx in the cerebellum induces LTD. They explained this phenomenon by appealing to the different spatial and temporal aspects of calcium signaling in the cerebellum: timing, source, and location of calcium influx in the neuron can greatly influence the type of signaling molecules and thus the machinery activated (Bading et al., 2013). Correspondingly, similar differences in spatial distribution of calcium influx inside excitatory neurons (STTn) vs. inhibitory interneurons (GABAn), can lead to the opposite polarity of synaptic plasticity observed. However, further experiments are warranted to investigate more in depth the spatial and temporal aspects of calcium signaling in STTn vs. GABAn.

It is also likely that STTn and GABAn activate different downstream signaling pathways with the same [Ca^{2+}] transient. One can speculate that there may be differences in intracellular factors influencing the downstream signaling pathways. For example, excitatory projection neurons usually contain a high level of calbindin, whereas GABAn possess a high level of parvalbumin (Antal et al., 1991; Palestini et al., 1993). Long-term potentiation induction is facilitated by calbindin, while the excitability of interneurons is depressed by parvalbumin (Jouvenceau et al., 2002). In addition, CaMKII and calcineurin are deficient in GABAn (Sik et al., 1998) while PKC, which contributes to central sensitization (Martin et al., 2001) and LTP (Abeliovich et al., 1993) is found mainly in excitatory spinal pain transmission neurons (Polgar et al., 1999). However, how these differences potentially affect the direction of synaptic plasticity is a matter that needs to be explored in future studies.

Another suggested factor that controls the direction of synaptic plasticity is the level of postsynaptic depolarization (holding potential) during the CS: LTP and LTD would be induced when the depolarization is above and below the NMDAR activation threshold, respectively (Artola et al., 1990). However, this study shows an opposite synaptic plasticity in STTn and GABAn with the same holding potential (130 or 110 mV). The data in this study thus suggest that the direction of synaptic plasticity is not always determined by the extent of postsynaptic depolarization in the spinal DH neurons.

Additional factors concerning synaptic plasticity that need to be addressed include: (1) whether the synaptic plasticity is through presynaptic or postsynaptic mechanisms, and (2) whether [Ca^{2+}] transients are sufficiently established by Ca^{2+} entry through membrane channels (e.g., NMDAR) or require Ca^{2+} release from intracellular storage.

In summary, the whole-cell patch recordings from identified spinothalamic tract (STTn) and GABAn in the mouse spinal cord showed that the same CSs induced LTP in STTn but LTD in GABAn. The types of neurons rather than parameters of ACSs determined the direction of synaptic plasticity. Induction of both LTP in STTn and LTD in GABAn were accompanied by a similar rapid rise in intracellular Ca^{2+} transients.

CHAPTER 4

SUPEROXIDE AND HYDROXYL RADICALS DIFFERENTIALLY REGULATE CELL-TYPE SPECIFIC SYNAPTIC PLASTICITY

4.1 ABSTRACT:

Spinal synaptic plasticity drives central sensitization that underlies the persistent nature of neuropathic pain. Our recent data showed that synaptic plasticity in the dorsal horn is cell type-specific: intense afferent stimulation produced long-term potentiation (LTP) in excitatory spinothalamic tract neurons (STTn), whereas it produced long-term depression (LTD) in inhibitory GABAergic interneurons (GABAn). In addition, reactive oxygen species (ROS) were shown to be involved in LTP in STTn (STTn-LTP) as well as in LTD in GABAn (GABAn-LTD). This study examined the more specific roles of two biologically important ROS—superoxide [$O_2^{-\cdot}$] and hydroxyl radicals [$OH^{\cdot}$]—in neuropathic mechanical hyperalgesia and cell type-specific spinal synaptic plasticity. A [$O_2^{-\cdot}$] donor induced stronger mechanical hyperalgesia than a [$OH^{\cdot}$] donor in naïve mice. On the other hand, a [$O_2^{-\cdot}$] scavenger showed greater anti-hyperalgesic effect than [$OH^{\cdot}$] scavengers in the spinal nerve ligation (SNL) mouse model of neuropathic pain. Moreover, a [$O_2^{-\cdot}$] donor induced STTn-LTP and GABAn-LTD, but a [$OH^{\cdot}$] donor induced only GABAn-LTD. Confirming the same phenomena with a different approach, a [$O_2^{-\cdot}$] scavenger inhibited STTn-LTP and GABAn-LTD induction (via conditioning stimulus (CS)) in naïve mice and alleviated SNL-induced potentiation and depression, respectively.

Also, [$OH^{\cdot}$] scavenger selectively inhibited GABAn-LTD induction and maintenance as well as SNL-induced depression. These results indicate that mechanical hyperalgesia in SNL mice is the result of the combination of STTn-LTP and GABAn-LTD. Behavioral outcomes compliment electrophysiological results which suggest that [$O_2^{-\cdot}$] mediates both STTn-LTP and GABAn-LTD, whereas [$OH^{\cdot}$] is involved only in GABAn-LTD.

4.2 INTRODUCTION:

Aim 1 of this study showed that two opposing types of synaptic plasticity develop in the excitatory synapses of STT projection neurons (STTn) and GABAergic inhibitory interneurons (GABAn) in the spinal dorsal horn. These opposing synaptic plastic changes are cell type-specific: long-term potentiation (LTP) of excitatory postsynaptic current (EPSC) in STTn as opposed to long-term depression (LTD) of EPSC in GABAn in response to the same conditioning stimulus (CS) (Kim et al., 2015). LTP in STTn is speculated to contribute to increased pain transmission (Kim et al., 2015), while LTD in GABAn is speculated to result in pain disinhibition which further intensifies pain transmission to higher order pain processing regions (Yowtak et al., 2011).

Evidence has pointed to reactive oxygen species (ROS) being important molecular players in central sensitization and neuropathic pain mechanisms. It is becoming more accepted by pain researchers that increased ROS levels downregulate GABA transmission in the dorsal horn after spinal cord injury (Gwak et al., 2013; Yowtak et al., 2011), and that the resulting GABAergic disinhibition in turn controls the excitatory-inhibitory balance of the dorsal horn and drives enhanced excitatory transmission, leading to neuropathic pain

(Todd, 2010). It has also been shown that dorsal horn neurons produce significant amounts of superoxide radicals in a capsaicin model, and that ROS levels are correlated with the level of the secondary hyperalgesia observed (Schwartz et al., 2009). Additionally, superoxide radical scavengers were shown to reduce dorsal root stimulation-induced LTP of field excitatory post-synaptic potentials (fEPSP) in the superficial medial dorsal horn (Lee et al., 2010). Nevertheless, the causal relationship between ROS and synaptic plasticity in both excitatory and inhibitory circuits of the dorsal horn is yet to be determined.

At a behavioral level, many studies show a clear correlation between increased ROS levels and pathological pain development. For instance, ROS scavengers produce anti-hyperalgesic effects in animal models of neuropathic pain, such as the spinal nerve ligation (SNL) and chronic constriction injury of sciatic nerve models (Kallenborn-Gerhardt et al., 2012; Kim et al., 2008). Conversely, intrathecal administration of an ROS donor—t-butyl-hydroperoxide (tBOOH)—induces pain behaviors in naïve animals (Yowtak et al., 2011). Furthermore, anti-oxidant superoxide dismutase (SOD2) over-expressing transgenic mice show mitigated capsaicin-induced secondary hyperalgesia, whereas SOD2 knockout mice show significantly higher capsaicin-induced hyperalgesia (Schwartz et al., 2009).

Despite the studies showing the role of ROS in central sensitization and neuropathic pain, the key evidence linking ROS to cell type-specific synaptic plasticity in the spinal cord (i.e., LTP in STTn and LTD in GABAn) is lacking. Therefore, this study investigates the effects of donors and scavengers of two biologically important ROS—superoxide and hydroxyl radicals—on pain behaviors and synaptic plasticity of STTn and GABAn in normal and neuropathic (SNL) mice. We report that superoxide radicals are responsible for

LTP in STTn and LTD in GABAn as well as neuropathic pain behaviors. On the other hand, hydroxyl radicals are responsible only for the LTD in GABAn and contribute to pain partially through GABAergic disinhibition.

4.3 METHODS:

Please refer to materials and methods section (Chapter 2).

4.5 RESULTS:

4.5.1 Shaking behavior best represents neuropathic mechanical pain status:

Despite the common use of withdrawal response to Von Frey stimuli as a reliable method to detect mechanical allodynia and hyperalgesia in neuropathic pain models, the debate about the validity of the test, in terms of reflecting pain perception, continues to exist (Keizer, 2008). Researchers have thus developed several methods to improve the quality of the obtained results and reduce inter-experimenter variability (Pitcher et al., 1999; Bouhassira et al., 2004; Chung et al., 2004, Tena et al., 2012). However, the question remains whether the observed response represents complex pain perception or is just a simple spinal reflex.

Several types of behaviors have been reported throughout literature as signs of positive pain responses, such as paw licking, guarding, and shaking (Mogil et al., 2010). One of these responses is usually considered a positive pain response. In an attempt to refine the VFF method in this study, we categorized the withdrawal response into several different responses: flinch, shake, lick, and hold. Flinch is defined as a quick (< 1 sec) paw withdrawal after which the paw is replaced back on the metal mesh floor in its previous

position. Shake is defined as withdrawing the paw, shaking the paw at least twice while holding it up before replacing it back on the mesh floor. Lick is defined as licking the paw at least once after stimulation. Hold is defined as holding the paw towards the abdomen and maintaining the holding position for longer than 4 seconds before replacing it back on the mesh. Common pain behavior quantification methods combine all the explained behaviors under "paw withdrawal".

The mouse hind paw is illustrated in **Fig. 4.1A**. The most pain-sensitive spot after SNL surgery is marked under the shaded area, which represents the area where VFF stimulations were applied. Any responses due to stimulations outside the marked area were discarded. **Fig. 4.1B** shows a Venn diagram representing relative occurrence (%) and overlap of each finch, shake, lick and hold behavior in response to VFF (#3.00, 0.1 g force) stimulation 1 week following SNL surgery. Circle sizes are proportional to corresponding percentages.

Results in **Fig. 4.1B** show that shake behavior was the most prevalent, with a total occurrence of up to 78.6% of paw withdrawals (n=9). Flinch behavior alone, being the closest to a simple reflex, did not overlap with the other behaviors and was detected 15.1% of the time. Hold (21.4%) and lick (16.7%) often accompanied shake behavior, with few instances occurring alone (hold alone: 5.6%, lick alone: 0.8%). The significant overlap of shake with hold and lick, but not with flinch, suggests that shake is less likely to represent a simple spinal reflex. Accordingly, we chose shake behavior as the pain perception behavior to be used in the rest of our experiments.

4.5.2 Superoxide and hydroxyl radicals' donors produce hyperalgesic effect in naïve mice.

Several doses of each drug were tried in preliminary studies, and the dose with the maximum effect for each drug was chosen for our experiments. The effects of superoxide radical donor (KO_2, 71.1 μg) and hydroxyl radical donor (tBOOH, 0.25 μg) on shake pain behavior are shown in **Fig. 4.2**. Each ROS donor was injected intrathecally in normal C57BL/6 mice, and percent paw shaking frequency upon VFF (#3.0) stimulation was measured at pre-drug, 20, 40, 60, and 120 min post-drug injection. Both ROS donors increased the shaking responses transiently with peaking at 40 min. The peak response induced by KO_2 (75 ± 3.3%, n=6) in paw shake frequency was significantly higher than that induced by tBOOH (65 ± 3.6%, n=6).

4.5.3 Spinal nerve ligation (SNL) produces mechanical hypersensitivity for at least 2 weeks after surgery

Spinal neve ligation (SNL) model is one of the most commonly used neuropathic pain models and was developed in our laboratory in 1992. The Chung model applies a L5 nerve ligation affecting by the process primary afferent fibers of various diameters and modalities and resulting in long lasting mechanical and thermal hyperlagesia as well as allodynia. SNL model is advantageous over other models in that the ligation is applied just before the terminal of the spinal nerve and affects myelinated and unmyelinated fibers equally. This confines the injury-induced alterations to a small area and produces more specific behavioral manifestations. Therefore, SNL model was the model of choice in the following experiments.

Spinal nerve ligation procedure (explained in detail in chapter 2 methods section) was performed on 10 weeks old C57BL/6 mice (n=6). Sham SNL surgery was performed on another group of 6 mice. Paw shake frequency was measured at one day pre-SNL and post-SNL days 1, 3, 5, 7 and 14. Measurements always took place for all mice at a fixed time of the day for consistency. Results show that paw shake frequency increases up to 80% and peaks at 7 days after surgery in the SNL group and does not change in the sham group (**Fig. 4.3**).

4.5.4 Superoxide and hydroxyl radicals affect pain behaviors in different magnitudes, and hydroxyl radicals are specifically involved in GABA disinhibition

After showing that ROS donors produce a similar level of mechanical hyperalgesia to SNL, we decided to investigate the effects of different and specific ROS scavengers—PBN (non-specific scavenger), TEMPOL (superoxide scavenger), DMTU and DMSO (hydroxyl radical scavengers) —on pain behavior in SNL mice. Baseline pain behavior measurements were taken just before every drug injection, 7 days after SNL. As shown in **Fig. 4.4**, all SNL mice developed mechanical hyperalgesia, showing on average 70% shaking frequency 7 days after the surgery. PBN (100 mg/kg) (n=6), injected systemically, reduced initial pain behavior by 90 ± 3.9% (**Fig. 4.4A**). TEMPOL (100 μg) caused a 93 ± 4.0% (n=8) reduction in paw shaking frequency at 1 hour after injection. DMTU (1.04 mg) and DMSO (0.45 mg), however, caused a 32 ± 4.0% (n=8) and 70 ± 1.8% (n=8) reduction in paw shaking behavior, respectively. Two hours after TEMPOL, DMTU, or DMSO injection, the levels of hyperalgesia were returned to the pre-drug treatment levels. The level of anti-hyperalgesia produced by TEMPOL was statistically higher than that by DMTU or DMSO (**Fig. 4.4B**).

To isolate GABAergic contribution to the observed anti-hyperalgesic effects of ROS scavengers, the effect of GABA receptor (GABA-R) antagonists was tested in combination with each specific ROS scavenger using the following treatments: TEMPOL (100 μg), TEMPOL + GABA-R antagonists (Bicuculline, 1 μg and CGP46381, 0.5 μg), DMSO (0.45 mg), and DMSO + GABA-R antagonists. The effects of either TEMPOL and DMSO alone (**Fig. 4.4B**) are in comparison to the combined treatments. GABA-R antagonists completely reversed DMSO-induced analgesia (**Fig. 4.4D**) but only partially reversed TEMPOL-induced analgesia (**Fig. 4.4C**).

4.5.5 Superoxide and hydroxyl radicals are differentially involved in the synaptic plasticity in STTn vs. GABAn

To test free radicals' effects on synaptic plasticity, subtype-specific free radicals donors were applied to STTn and GABAn. Superoxide and hydroxyl radicals donors (KO_2, 1 mM; tBOOH, 1 mM respectively) were each perfused into the recording chamber after obtaining a 3 min baseline. Without any CS or electrical manipulation, KO_2 induced an increase in STTn EPSC amplitudes resembling CS-induced LTP (**Fig. 4.5A**), while GABAn showed LTD-like reduction in EPSC size (**Fig. 4.5B**). However, tBOOH failed to cause changes in the amplitude of EPSC in STTn (**Fig. 4.5C**) but induced LTD in GABAn (**Fig. 4.5D**). These results confirm that superoxide radicals are involved in both STTn-LTP and GABAn-LTD, whereas hydroxyl radicals are involved only in GABAn-LTD, introducing a notion that superoxide and hydroxyl radicals are differentially involved in synaptic plasticity.

4.5.6 Non-specific ROS scavenger (PBN) inhibits induction but fails to inhibit maintenance of STTN-LTP and GABAN-LTD

To investigate the importance of reactive oxygen species in synaptic plasticity in a more specific way, we tested the effect of PBN, a general and potent ROS scavenger, on both the induction and maintenance phases of CS-induced STTn-LTP and GABAn-LTD. Baseline recordings were obtained for 3 min after obtaining a whole cell configuration. PBN (1 mM) was superfused into the recording chamber simultaneously with CS application (at 2 Hz for 40 sec with a holding potential of +30 mV). PBN perfusion lasted for a total of 5 min and was followed by a 10 min washout. PBN completely blocked the induction of both STTn-LTP (**Fig. 4.6A**) and GABAn-LTD (**Fig. 4.6B**). Results so far indicate that reactive oxygen species are necessary for the induction of both STTn-LTP and GABAn-LTD in the DH of the spinal cord.

A second set of experiments investigated the importance of reactive oxygen species in maintaining STTn-LTP and GABAn-LTD. Here, PBN (1 mM, for 10 min duration) was applied at 16 min after CS, when STTn-LTP and GABAn-LTD were fully established. PBN (1 mM) failed to reverse the already established LTP in STTn and LTD in GABAn (**Fig. 4.6C and D**). Higher doses (5 mM, 10 mM) were also examined, however, these doses compromised the patch conditions and prevented us from obtaining valuable data.

4.5.7 Superoxide radical scavenger TEMPOL blocked CS-induced induction but not maintenance of STTn-LTP and GABAn-LTD

Keeping in mind that superoxide radicals are the most abundant type of ROS in a neuron in physiological and pathophysiological settings, this experiment investigates the

importance of superoxide radicals in cell-type specific synaptic plasticity. Therefore, we tested the effect of TEMPOL, a specific superoxide radical scavenger, on STT-LTP and GABA-LTD induction and maintenance phases. Baseline EPSC recordings were obtained for 3 min after obtaining a whole cell configuration. Then, TEMPOL (1 mM) superfusion into the recording chamber was started simultaneously with CS application (at 2 Hz for 40 sec with a holding potential +30 mV). TEMPOL perfusion lasted for a total of 5 min and was then washed out. TEMPOL completely blocked the induction of both STTn-LTP (**Fig. 4.7A**) and GABAn-LTD (**Fig. 4.7B**). This confirms that superoxide radicals are a critical component in inducing both types of synaptic plasticity in the spinal cord.

To investigate the possible role of superoxide radicals on the maintenance phase of LTP and LTD, TEMPOL (1 mM, for 10 min duration) was applied at 16 min after CS, when STTn-LTP and GABAn-LTD were established. TEMPOL (1 mM) failed to reverse the already established LTP in STTn and LTD in GABAn (**Fig. 4.7C and D**).

4.5.8 Hydroxyl radical scavenger blocked the induction and maintenance of GABAn-LTD but not STTn-LTP

We also tested the effects of scavenging hydroxyl radicals on the synaptic plasticity in STTn and GABAn. The underlying reasoning aimed at investigating the specificity of the observed effects of ROS on synaptic plasticity and on the cell types tested. Surprisingly, a hydroxyl radical scavenger, DMTU (20 mM), did not interfere with the induction of LTP in STTn (**Fig. 4.8A**) but it did impede the induction of LTD in GABAn (**Fig. 4.8B**). The same dose of DMTU failed to affect the maintenance phase of LTP in STTn (**Fig. 4.8C**) but fully reversed the maintenance phase of LTD in GABAn (**Fig. 4.8D**).

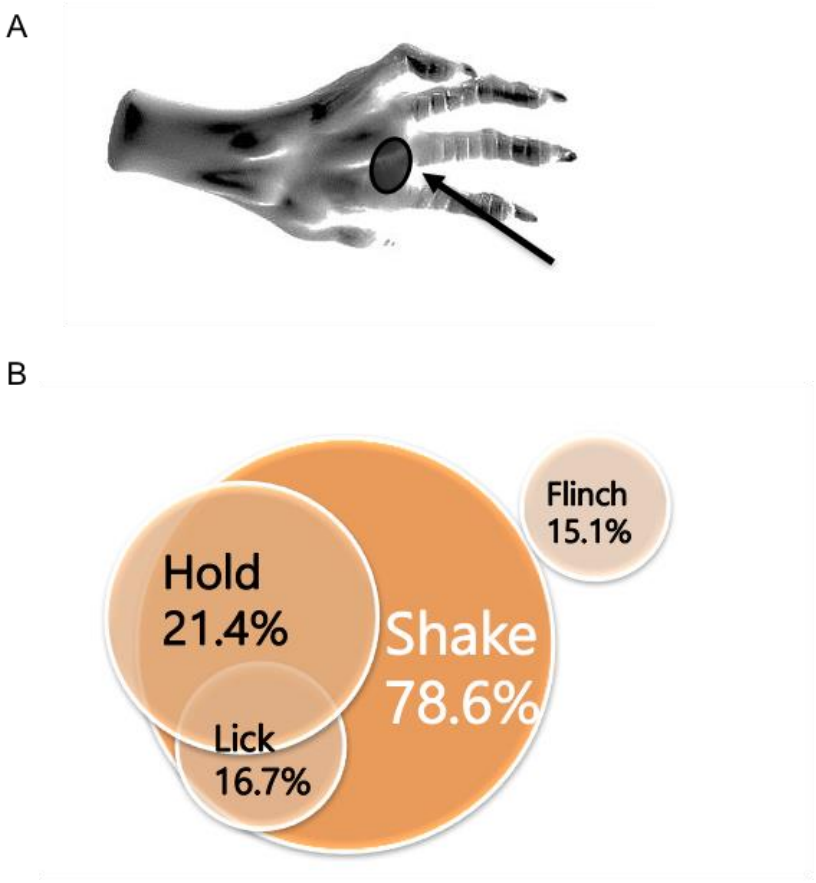


FIG 4.1: Shake behavior best represents mechanical pain in the mouse. (A) Graphic illustration of a hind paw showing the area stimulated (shaded area) by von Frey filaments (VFF) in pain testing (digital illustration of a rat paw: http://toxic929.deviantart.com/art/Rat-Paw-Sketch-213935890). (B) Venn diagram illustrating a subcategorization of the conventional "paw withdrawal" behavior to VFF stimulation in the SNL mouse model of neuropathic pain. Overlapping behaviors are superimposed and the size of the circles is proportional to the frequency of occurrence. Among the 4 types of paw withdrawal behaviors, shake behavior was the most consistent and common behavior, and thus was chosen to represent neuropathic mechanical pain in this study.

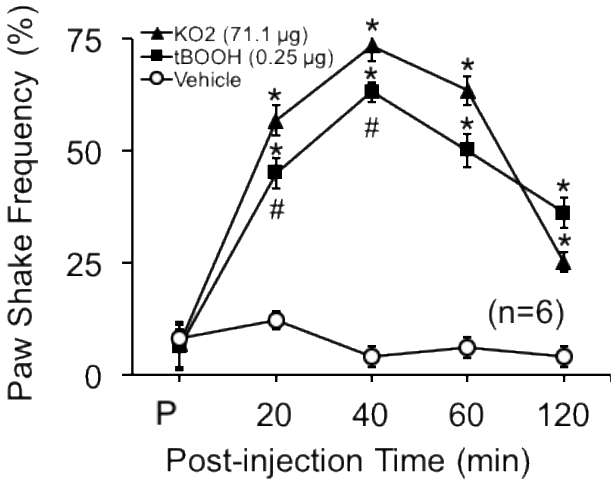


FIG 4.2: Specific ROS subtype differentially affect pain behaviors of naïve mice. Behavioral responses of C57BL/6 mice to VFF (# 3.00 = 0.1 g force) stimulations at pre- (P) and post-intrathecal drug injections (5 µL). (A) Superoxide radical donor (KO_2; 71.1 µg, n=6) induced greater hyperalgesia than hydroxyl radical donor (tBOOH; 0.25 µg, n=6) in naïve mice. Data were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test. Data were presented as mean ± SEM (*, different from pre-drug within a treatment; #, different between treatments, $P<0.05$).

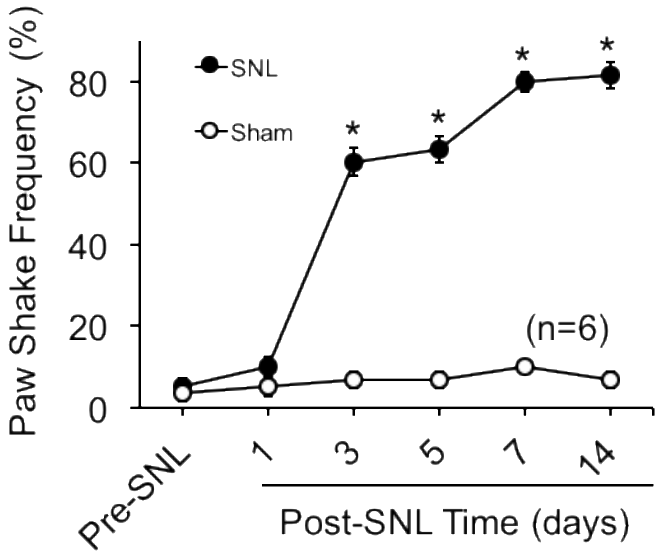


FIG 4.3: Shake behavior persists for at least two weeks following spinal nerve ligation surgery. Spinal nerve ligation surgery and sham surgery were done on two groups of C57/B6 10 weeks old mice (n=6). Shake behavior was recorded at pre-SNL, 1, 3, 5, 7 and 14 days after surgery using Von Frey # 3.0 filament. Paw shake frequency reached 80% 1 week after SNL. Sham mice did not show any or showed very little shaking behavior. Data were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test. Data were presented as mean ± SEM (*, different from pre-drug within a treatment; #, different between treatments, $P<0.05$).

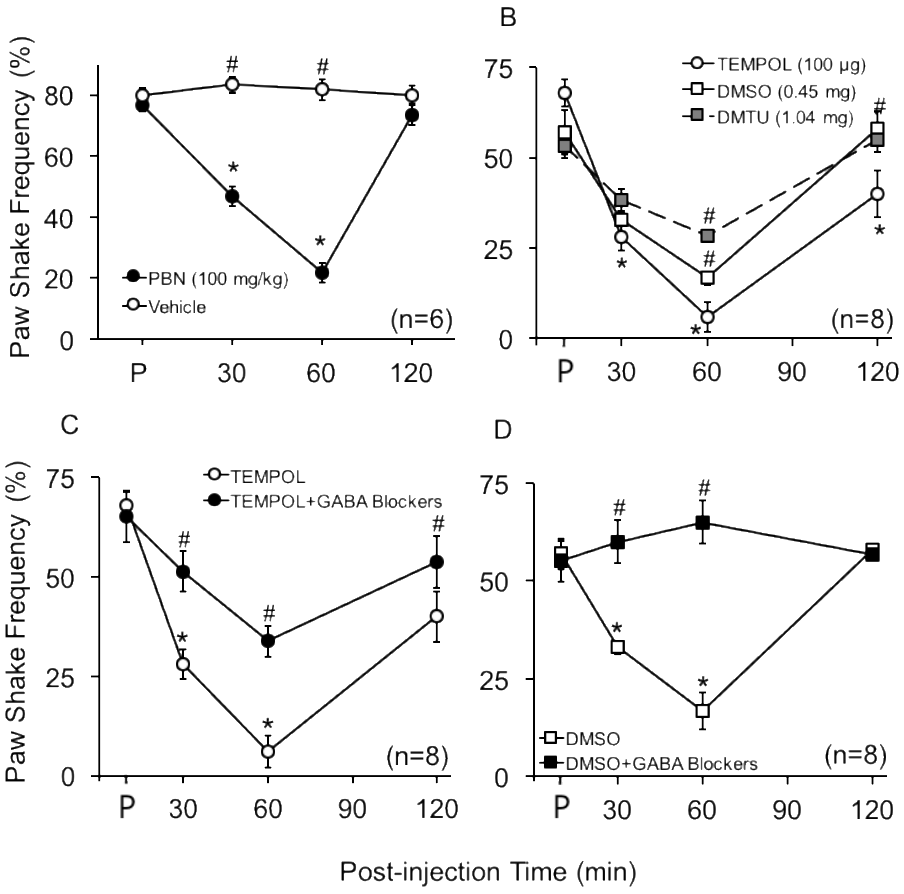


FIG 4.4: Specific ROS subtype scavengers differentially affect pain behaviors of neuropathic mice. Behavioral responses of C57BL/6 mice to VFF (# 3.00 = 0.1 g force) stimulations at pre- (P) and post-systemic (A) and intrathecal (5µL) (B,C,D) drug injections in neuropathic mice 7 days after SNL. (A) PBN, a non-specific ROS scavenger, (100 mg/kg) reduced shake frequency of neuropathic mice by 60% peaking at 1 hour in comparison to vehicle (saline 0.9 %). (B) Superoxide radical scavenger (TEMPOL; 100 µg, n=8) induced greater anti-hyperalgesia than hydroxyl radical scavengers (DMSO; 0.45 mg and DMTU; 1.04 mg, n=8) in neuropathic mice. (C) Combination of $GABA_A$ (Bicuculline, 1 µg) and $GABA_B$ (CGP46381, 0.5 µg) receptor antagonists partially blocked TEMPOL's anti-hyperalgesic effect in neuropathic mice (n=8). (D) The GABA receptor antagonists completely blocked DMSO's anti-hyperalgesic effect in neuropathic mice. Data were analyzed using two-way ANOVA followed by Bonferroni's post-hoc test. Data were presented as mean ± SEM (*, different from pre-drug within a treatment; #, different between treatments, $P<0.05$).

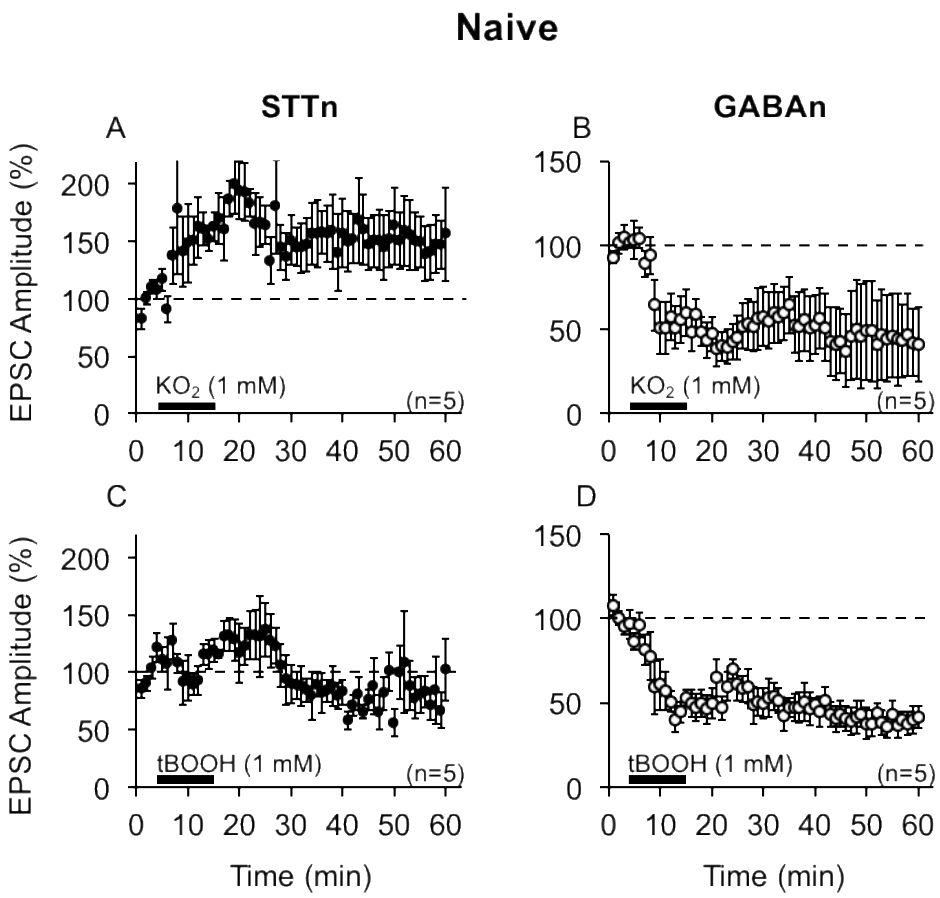


FIG 4.5: Superoxide radicals induce LTP in STTn and LTD in GABAn, but hydroxyl radicals are specific for GABAn-LTD in naïve mice. (A and B) Induction of STTn-LTP (n=5) and GABAn-LTD (n=5) by a superoxide radical donor (KO_2, 1 mM). (C and D) Induction of GABAn-LTD (n=5) but not STTn-LTP (n=5) by a hydroxyl radical donor (tBOOH, 1 mM). ROS donors were superfused into the recording chamber for 10 min (thick black bar) after obtaining whole cell configuration and a 3 min of baseline EPSC measurements. A 20% change on EPSC amplitude was enough to represent significance in terms of synaptic plasticity establishment in both LTP and LTD.

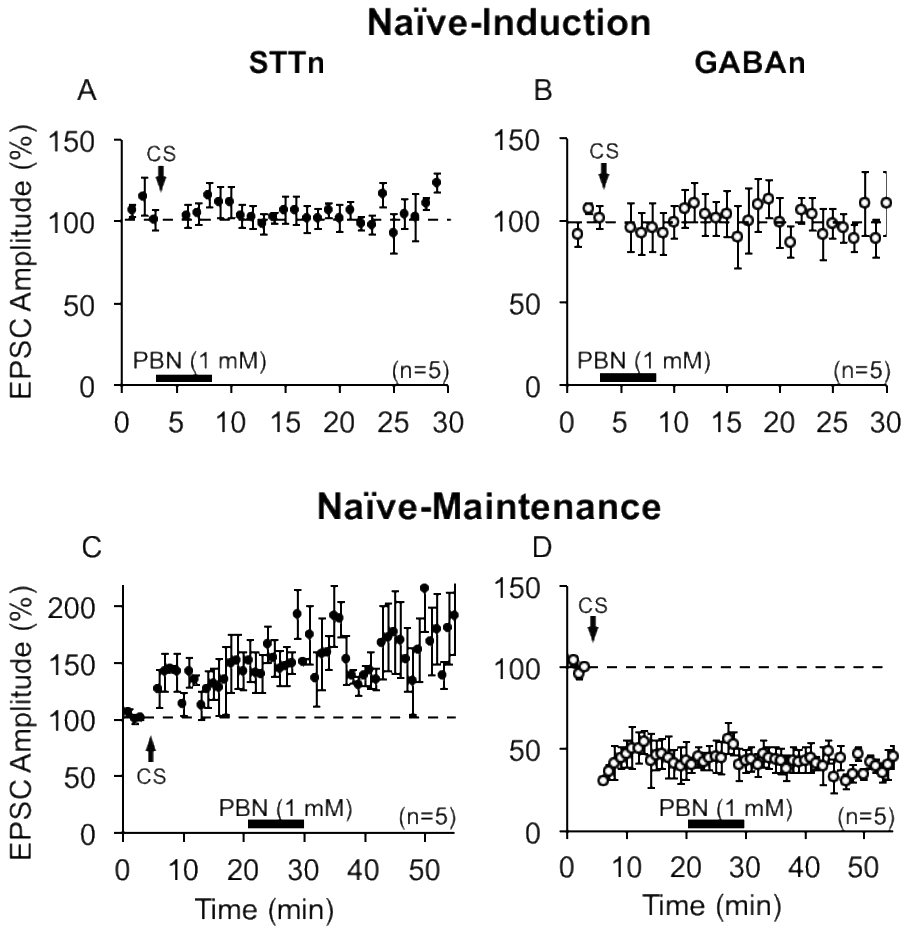


FIG 4.6: Non-specific ROS scavenger PBN blocks the CS-induced induction but not the maintenance of STTn-LTP and GABAn-LTD. (A and B) Induction of STTn-LTP (n=5) and GABAn-LTD (n=5) was blocked by PBN (1 mM) perfusion into the recording chamber right after CS application. (C and D) PBN failed to affect STTn-LTP and GABAn-LTD maintenance phase when applied to the recording chamber 16 min after CS application. The timing of PBN perfusion is represented by the black bar. A 20% change on EPSC amplitude was enough to represent significance in terms of synaptic plasticity establishment in both LTP and LTD.

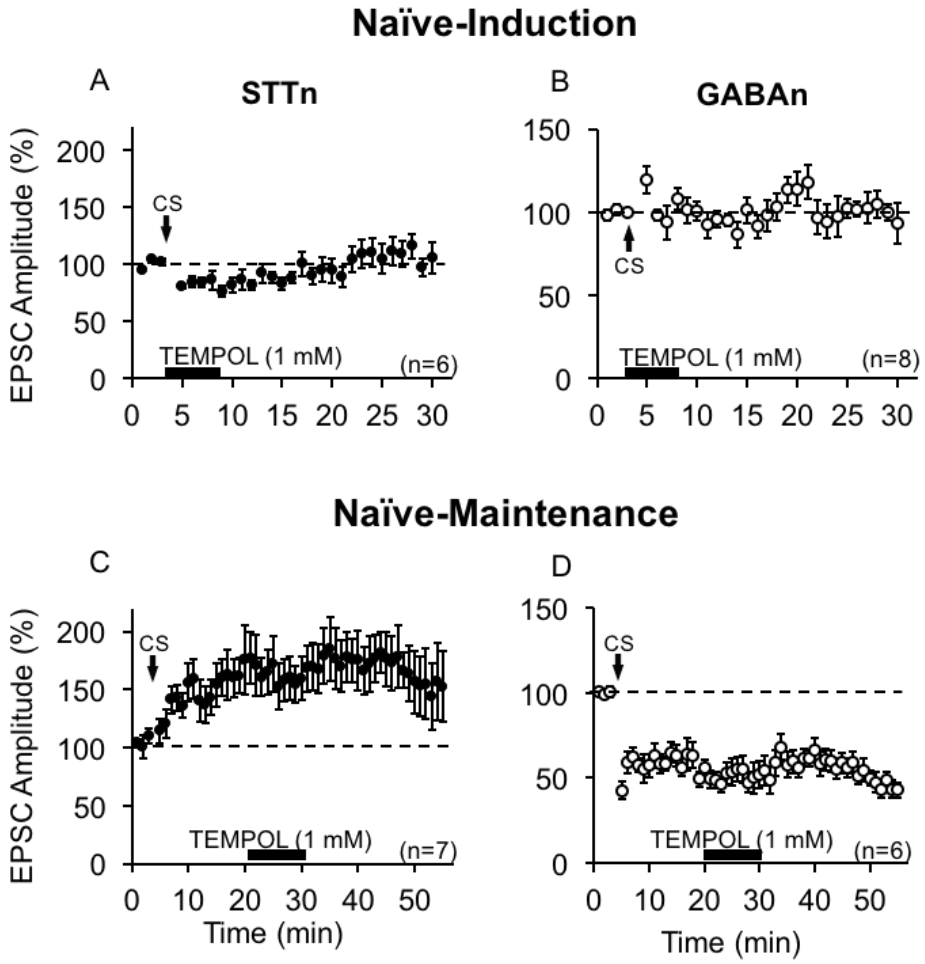


FIG 4.7: Superoxide radical scavenger blocks CS-induced induction but not maintenance of STTn-LTP and GABAn-LTD. A superoxide radical scavenger TEMPOL (1 mM), applied for 5 min during CS, blocked the CS-induced STTn-LTP (A, n=8) and GABAn-LTD (B, n=8). The same dose of TEMPOL (1 mM), applied for 10 min during the CS-induced maintenance phase (16 min after CS), failed to reverse STTn-LTP (C, n=7) or GABAn-LTD (D, n=8). A 20% change on EPSC amplitude was enough to represent significance in terms of synaptic plasticity establishment in both LTP and LTD.

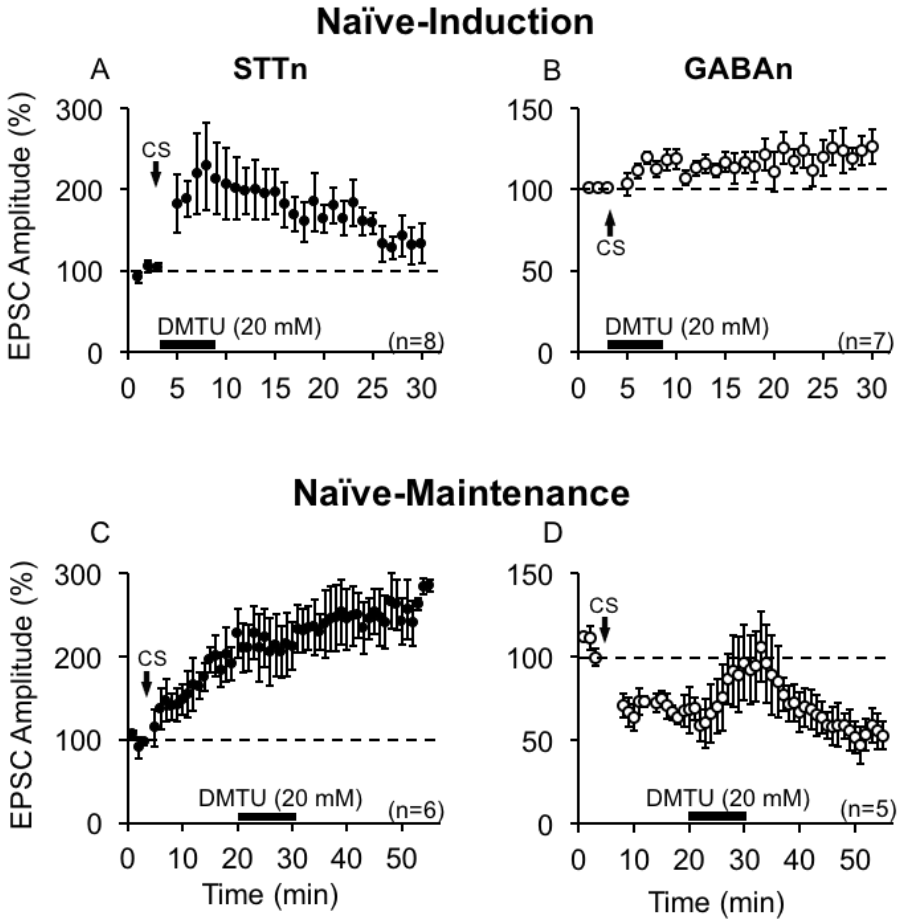


FIG 4.8: Hydroxyl radical scavenger blocks both the CS-induced induction and maintenance of GABAn-LTD but does not interfere with STTn-LTP. A hydroxyl radical scavenger DMTU (20 mM), applied for 5 min during CS, blocked the CS-induced GABAn-LTD (B, n=7) but not STTn-LTP (A, n=8). The same dose of DMTU (20 mM), applied for 10 min during the maintenance phase (16 min after CS), reversed GABAn-LTD (D, n=5) but not STTn-LTP (C, n=6). A 20% change on EPSC amplitude was enough to represent significance in terms of synaptic plasticity establishment in both LTP and LTD.

4.6 DISCUSSION:

The results of the present study suggest that two different biologically active ROS subtypes, superoxide and hydroxyl radicals, are contributing to cell-type specific synaptic plasticity. The results further indicate that superoxide radicals are responsible for the induction of both STTn-LTP and GABAn-LTD, whereas hydroxyl radicals are only involved in GABAn-LTD induction. The maintenance phase of CS-induced GABAn-LTD is also hydroxyl radicals-dependent, but that of CS-induced STTn-LTP is not dependent on either superoxide or hydroxyl radicals.

Our behavioral data show that a superoxide radical scavenger, TEMPOL, induces greater analgesic effect than a hydroxyl radical scavenger, DMTU or DMSO, in neuropathic mice. Similarly, a superoxide radical donor, KO_2, induces higher levels of hyperalgesia than a hydroxyl radical donor, tBOOH, in naïve mice. In addition, when spinal cord GABA function is blocked with GABA-R antagonists, DMSO loses its analgesic effect, while TEMPOL stays partially effective. Collectively, these data suggest that hydroxyl radicals downregulate only the GABA-mediated pain modulatory system, whereas superoxide radicals' effects extend to more than the GABA modulatory system. A study done by Yowtak et al. (2011) supports these results by showing that tBOOH (hydroxyl radical donor) suppresses GABAergic inhibitory transmission without affecting either glycinergic or excitatory transmission in substantia gelatinosa (SG) neurons. This suggests the significance of hydroxyl radicals in inhibitory transmission more so than in excitatory transmission. No other studies have investigated the distinct involvement of different ROS subtypes in the excitatory-inhibitory balance in the pain circuitry of the dorsal horn at the behavioral level.

At the synaptic plasticity level, superoxide and hydrogen peroxide are currently the most studied reactive oxygen species because of their high abundancy and stability in comparison to other ROS types like hydroxyl radicals (Takahashi et al., 2007). Where superoxide induced and maintained LTP, hydrogen peroxide, which is potentially a hydroxyl radical precursor (Lu et al., 2010), was shown to attenuate LTP and even facilitate LTD by exploiting LTD signaling machinery—such as calcineurin— in the brain (Beckhauser et al., 2016). Along similar lines, hydrogen peroxide was also shown to increase both amplitude and frequency of miniature inhibitory postsynaptic currents of GABAergic interneurons in the substantia gelatinosa (SG) of the spinal cord. This last study suggested that hydrogen peroxide acts both post- and pre-synaptically to regulate IP3 receptor-regulated calcium pools, leading to prolonged GABAergic inhibitory depression (Takahashi et al., 2007). A hydroxyl radical donor tBOOH produced similar effects and was, above all, selectively effective in depressing SG inhibitory and not excitatory transmission (Yowtak et al 2011). Together, these findings support the notion suggested in our study stating that hydroxyl radicals are important for GABAergic inhibitory more than for excitatory function of dorsal horn circuitry.

Knowing that the maintenance phase of STTn-LTP and GABAn-LTD could be more relevant to the chronic nature of neuropathic pain, we tested the effects of ROS scavengers on the maintenance of STTn-LTP and GABAn-LTD. The doses of TEMPOL and DMTU, which blocked the induction phase, were tested on the established LTP and LTD. A significant reversal of already established LTD in GABAn was shown during DMTU application. DMTU, however, failed to reverse STTn-LTP, reaffirming the exclusive influence of hydroxyl radicals to GABAn and supporting DMTU-induced partial

anti-hyperalgesia. The puzzling result however was that TEMPOL did not significantly reverse either the established LTP in STTn or the established LTD in GABAn, but still induced significant anti-hyperalgesia. When higher doses of TEMPOL, 5 and 10 mM, were tested, cell viability was compromised and any firm conclusion could not be drawn. At this point, further experiments were warranted to explain the mismatch between the electrophysiological and behavioral data of TEMPOL. Aim 3 presents a detailed explanation of those experiments and the obtained results by which TEMPOL's effect of SNL-induced changes in synaptic efficacy shown, and in a cell-type specific manner.

The results of this aim suggest that superoxide and hydroxyl radicals determine the polarity of synaptic plasticity in a cell type-specific manner; in that superoxide radicals are involved in both STTn-LTP and GABAn-LTD, whereas hydroxyl radicals are only involved in GABAn-LTD. Moreover, full anti-hyperalgesic effect can only be achieved by reducing STTn-mediated pain transmission as well as restoring GABA inhibition. This aim highlights the important roles of specific subtypes of ROS in synaptic plasticity in the spinal cord and points toward new treatment approaches and tools for neuropathic pain.

CHAPTER 5

CELL TYPE SPECIFIC SYNAPTIC PLASTICITY IS ESTABLISHED IN NEUROPATHIC MICE

5.1 ABSTRACT:

Following nerve injury, aberrant afferent inputs lead to neuropathic pain. Long-term synaptic plasticity in the spinal dorsal horn underlie a state of increased excitatory but decreased inhibitory tones, leading to central sensitization and pain. However, long-term synaptic plasticity is seldom assessed in neuropathic animals. Rather, it is often induced in naïve intact spinal cords via a conditioning stimulus mimicking neuropathic conditions. Therefore, this study investigated whether long-term changes in the synaptic strength on two types of pain-relaying neurons were actually established in the spinal nerve ligation model (SNL). We hypothesized that long-term potentiation (LTP) would already be established in spinothalamic tract neurons (STTn), whereas long-term depression (LTD) would be established in GABAergic interneurons (GABAn) in the dorsal horn of SNL mice, leading to central sensitization. Long-term changes were assessed by comparing the following between naïve and SNL mice: 1) The induction of synaptic plasticity following conditioning stimulus. 2) Amplitudes of raw excitatory post synaptic currents (EPSCs) in STTn and GABAn. 3) The effect of ROS scavengers on EPSCs in STTn and GABAn. Results showed that, following conditioned stimulus (CS), LTP was induced in the STTn of the contralateral side of SNL but was occluded in the STTn of the ipsilateral side of SNL. LTD in GABAn of the ipsilateral side of SNL still developed after CS, but in a slower manner than LTD in GABAn of the contralateral side of SNL. Furthermore, ROS

scavengers alleviated SNL-induced changes in EPSC amplitudes. These results suggest that, in SNL mice, STTn are sensitized with increased excitatory synaptic efficacy, whereas GABAn are desensitized with decreased excitatory synaptic efficacy, leading to central sensitization and neuropathic pain.

5.2 INTRODUCTION:

Lomo and Bliss, in 1966, were the first to discover the phenomenon of long term potentiation (LTP). LTP was discovered in the hippocampus, and was described as a prolonged (minutes to hours) augmentation in synaptic strength in response to a brief and intense stimulation (100 Hz) (Lomo and Bliss, 1966). It was not until 25 years later that the first report of synaptic plasticity in the spinal cord emerged. As mentioned in aim 2 of this study, Randic and her colleagues were able to induce LTP as well as long term depression (LTD) in the cells of the superficial laminae of the spinal cord using conditioned dorsal root stimulation. As it is known that the superficial laminae of the spinal cord are imperative in sensory and nociceptive transmission (Todd, 2010), Randic and her colleagues, therefore, speculated that physiological long term changes like LTP and LTD might underlie pain transmission in the spinal cord as a result of nerve injury (Randic et al., 1993). Since then, conditioned stimulation-induced (CS-induced) synaptic plasticity in the spinal cord became the most commonly used cellular model that symbolizes nerve injury-induced synaptic plasticity to study neuropathic pain cellular mechanisms.

In more details, CS-induced synaptic plasticity is a model that utilizes artificial electrical stimulation paradigms to mimic aberrant nociceptive transmission induced by physiological nerve injury (Randic et al., 1993). In this model, neurons from naïve mice

are used to obtain a baseline measurement of excitatory post-synaptic potentials (EPSCs). Afterwards, a conditioned stimulus is delivered to the neuron to induce synaptic plasticity. However, no studies, so far, have shown the establishment of LTP or LTD in neuropathic mice. Knowing that changes in the frequency and amplitude of miniature post synaptic currents in the superficial dorsal horn have been reported in neuropathic mice (Yowtak et al., 2013), miniature currents do not reflect the status of a single synapse like an evoked EPSC.

In this study, we present a piece of evidence pointing towards the establishment of STTn-LTP and GABAn-LTD in neuropathic mice at least up to 7 days after SNL. The data show that baseline EPSC levels in cells taken from the ipsilateral side of SNL are elevated in comparison to those of cells taken from the contralateral side of SNL. In addition, LTP was occluded in STTn of ipsilateral side of SNL following CS. LTD developed in GABAn of ipsilateral side of SNL following CS. Moreover, TEMPOL alleviated SNL induced changes in EPSC levels in both STTn and GABAn. DMTU only affected GABAn SNL-induced depression of EPSCs. Results indicate that cell-type specific synaptic plasticity is established in SNL model of neuropathic pain. In addition, results support the crucial role of ROS in long term pain-related synaptic strength changes as well as the differential role of superoxide and hydroxyl radicals in STTn-LTP and GABAn-LTD in neuropathic mice.

5.3 METHODS:

Please refer to materials and methods section (Chapter 2).

5.4 RESULTS:

5.4.1 EPCS amplitudes are elevated in STTn of SNL-mice in comparison to their naïve mice counterparts, and STTn-LTP is occluded in SNL-mice.

Neuropathic pain-related synaptic plasticity is rarely investigated in neuropathic pain animal models. Rather, it is often investigated in naïve mice and artificially induced via a conditioning stimulus (CS). As reported in aim 1, CS-induced synaptic plasticity in STTn and GABAn of the dorsal horn is cell-type specific (Kim et al., 2015). In this study, we ought to seek evidence for STTn-LTP establishment in spinal nerve-ligated mice (SNL mice) to better correlate our experimental conditions to pathophysiological conditions. **Fig. 5.1** explores long-term potentiation in STTn after SNL. After obtaining a whole cell patch condition, we collected and compared raw baseline EPSC values at four states (lowercase letters correspond to regions where representative EPSC traces were collected, as shown in the figures): (a) Baseline (pre-CS) in naïve mice, (b) LTP state (post-CS) in naïve mice, (c) Baseline in the ipsilateral side of SNL mice, and (d) Baseline in the contralateral side of SNL mice. **Fig. 5.1A** shows that the averages of raw EPCS amplitudes taken from states (b) and (c) are significantly higher than the averages of those taken from (a) and (d). This indicates that baseline EPSC levels in STTn in the ipsilateral side of SNL mice are already elevated (in comparison to baseline EPSCs in naïve STTn or SNL-contralateral STTn) and fall in the same range of LTP state EPSCs in STTn from naïve mice. **Fig. 5.1B** shows CS-induced LTP in STTn from naïve mice. STTn-LTP induction experiment here was repeated to confirm previous results and directly compare naïve CS-induced and SNL CS-induced STTn-LTP in the same figure.

When the same CS was applied to STTn in the ipsilateral and contralateral side of SNL, LTP was occluded in the former but normally developed in the latter (**Fig. 5.1C**). This suggests that STTn in the ipsilateral side of SNL are already in the LTP state and thus cannot be further potentiated by CS.

5.4.2 EPCS amplitudes are depressed in STTn of SNL-mice in comparison to their naïve mice counterparts, however, GABAn-LTD still develops in SNL-mice

The same experiments done in figure 5.1 on STTn were repeated on GFP-labeled GABAn (**Fig. 5.2A**). Similarly, we compared the averages of raw baseline (pre-CS) EPSC magnitudes recorded from GABAn in (a) naïve mice, (c) in the ipsilateral side of SNL mice and (d) in the contralateral side of SNL mice with the magnitude of (b) LTD state EPSCs from GABAn in naïve mice. Baseline EPSCs in the ipsilateral side of SNL mice were comparable with LTD state EPSCs in naïve mice, being smaller than baseline EPSCs in naïve or the contralateral side of SNL mice (**Fig. 5.2A**). Following CS, GABAn-LTD developed in naïve mice (**Fig. 5.2B**) as well as in both the ipsilateral and contralateral sides of SNL mice (**Fig. 5.2C**).

5.4.3 TEMPOL alleviate SNL-induced changes in EPSC amplitude in STTn and GABAn

The lack of effect of TEMPOL on the maintenance phase of CS-induced STTn-LTP and GABAn-LTD (reported in aim 2) does not correlate with the significant analgesic effect of TEMPOL observed behaviorally. It becomes worthy to mention that TEMPOL's analgesic effect was observed in 'SNL mice', whereas CS-induced STTn-LTP was done in 'naïve mice', therefore not entirely reflecting the pathophysiological environment of a

chronic pain model. In addition, EPSCs in STTn were shown to be potentiated and those in GABAn depressed at rest in the ipsilateral side of SNL mice (**Figs. 5.1 and 5.2**). Therefore, we examined the effect of TEMPOL (1 mM) on evoked EPSCs recorded from STTn and GABAn in the ipsilateral side of SNL mice. Baseline (pre-drug in this case) was obtained for 10 min, then TEMPOL was superfused for 5 min. TEMPOL was able to significantly decrease STTn EPSC amplitudes (n=5) (**Fig. 5.3A**) and increase GABAn EPSC amplitudes (n=5) (**Fig. 5.3B**), indicating that TEMPOL is effective in alleviating the chronic maintenance phase of STTn-LTP and GABAn-LTD in a neuropathic pain condition. These results agree with TEMPOL's analgesic effect observed 7 days after SNL.

5.4.4 DMTU alleviates SNL-induced changes in EPSC amplitude only in GABAn and not in STTn

DMTU (20 mM) was applied on STTn and GABAn in the ipsilateral side of SNL mice. DMTU significantly increased evoked EPSC amplitudes in GABAn (**Fig. 5.4A**) but failed to significantly alter evoked EPSC amplitudes in STTn (**Fig. 5.4B**) from the neuropathic mice. Results thus suggest that hydroxyl radicals are crucial for GABAn-LTD but not for STTn-LTP induction and maintenance confirming the differential role of specific ROS subtypes in cell-type specific synaptic plasticity. We also attempted to use DMSO as a hydroxyl radical scavenger in this experiment due to its significant analgesic effect; however, giga-seal integrity was often compromised and no reliable recording could be obtained.

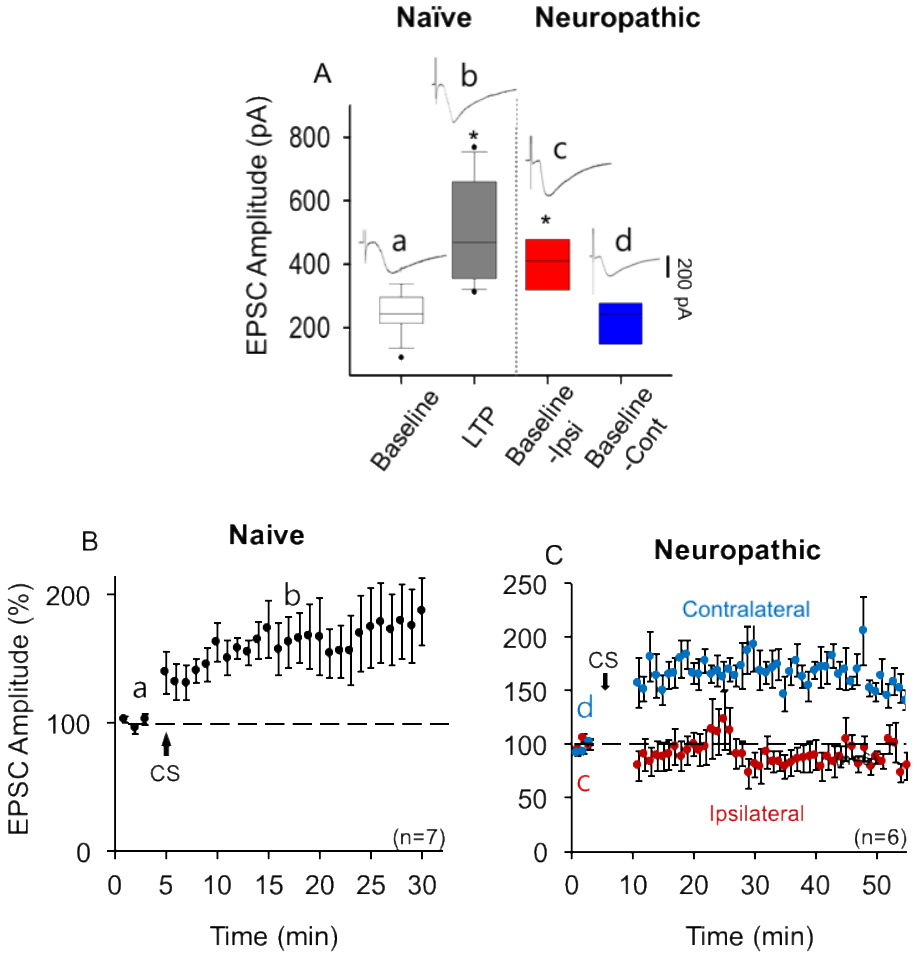


FIG 5.1 LTP is already established in STTn in the ipsilateral side of SNL. (A) Box-plot showing medians, ranges between 25th and 75th percentiles (boxes), and whiskers representing minimum and maximum values of raw EPSC amplitudes (pA) of (a) baseline (pre-CS) and (b) LTP state (15 min after CS) in naïve mice, compared to baselines in the (c) ipsilateral (Baseline-Ipsi) and (d) contralateral (Baseline-Cont) sides of SNL mice. Baseline-Ipsi was significantly higher in amplitude than the baseline in naïve STTn and lies in the range of LTP observed in naïve STTn, whereas Baseline-Cont was similar to the baseline in naïve STTn under our stimulation parameters. Individual EPSC traces, designated by lowercase letters show their corresponding areas of collection. (B) LTP developed in naïve STTn after CS (2 Hz for 40 seconds, holding potential of +30 mV). (C) EPSCs were not potentiated in STTn in the ipsilateral side of SNL after CS application (red), whereas they developed normally in the contralateral side (blue). Stimulation event interval was set at 20 seconds resulting in three EPSC values per minute. Individual EPSC values were normalized to the mean of baseline (before CS). Then each of the three normalized EPSC values (per minute) were averaged and plotted vs. time. (A) Data were analyzed using One-way ANOVA followed by Bonferroni post-hoc test (*, different from (a); $P < 0.05$).

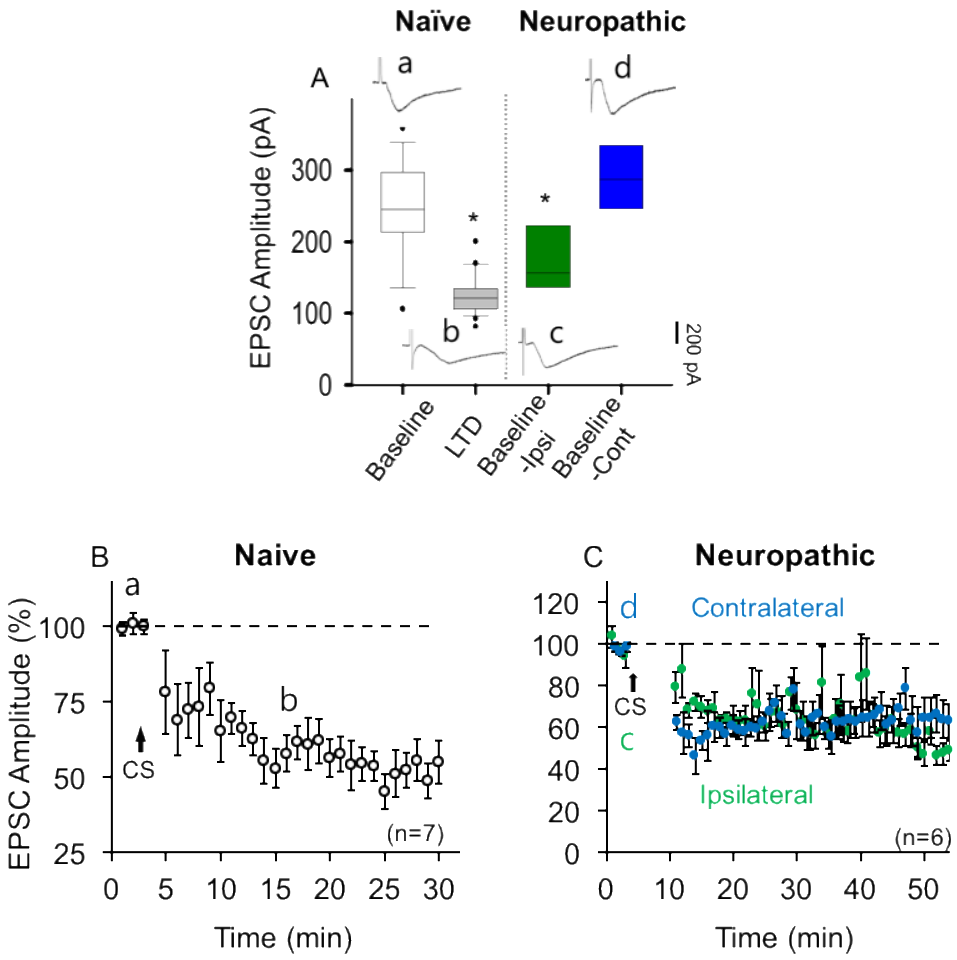


FIG 5.2 LTD is already established in GABAn in the ipsilateral side of SNL. (A) Box-plot showing medians and ranges between 25th and 75th percentiles (boxes), and whiskers representing minimum and maximum values of raw EPSC amplitudes (pA) of (a) baseline (pre-CS) and (b) LTD state (15 min after CS) in naïve mice, compared to baselines in the (c) ipsilateral (Baseline-Ipsi) and (d) contralateral (Baseline-Cont) sides of SNL mice. Baseline-Ipsi was lower in amplitude than the baseline in naïve GABAn and lies in the range of LTD observed in naïve GABAn, whereas Basline-Cont was similar to the baseline in naïve GABAn under our stimulation parameters. Individual EPSC traces, designated by lowercase letters, show their corresponding areas of collection. (B) LTD developed in GABAn in naïve mice and (C) both in the ipsilateral and contralateral sides of SNL mice after CS (2 Hz for 40 seconds, holding potential at +30 mV). Stimulation event interval was set at 20 seconds resulting in three EPSC values per minute. Individual EPSC values were normalized to the mean of baseline (before CS) EPSC values. Then each of the three normalized EPSC values (per minute) were averaged and plotted vs. time. (A) Data were analyzed using One-way ANOVA followed by Bonferroni post-hoc test (*, different from (a); $P< 0.05$).

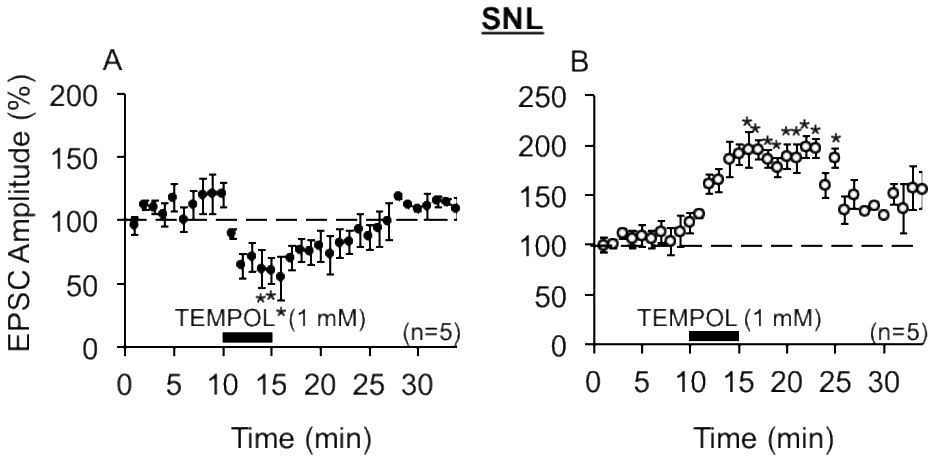


FIG 5.3 TEMPOL reduces SNL-induced elevation in EPSC amplitude of STTn and GABAn in the ipsilateral side of SNL. (A)1 mM of TEMPOL, applied 10 min after obtaining EPSC baseline 7 days post-SNL, successfully decreased evoked EPSC amplitude in STTn in ipsilateral side of SNL (n=5) and increased evoked EPSC amplitude in GABAn in ipsilateral side of SNL (n=5). No CS was applied in A and B. Data in A and B were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test (*, different from pre-drug within a treatment; $P<0.05$).

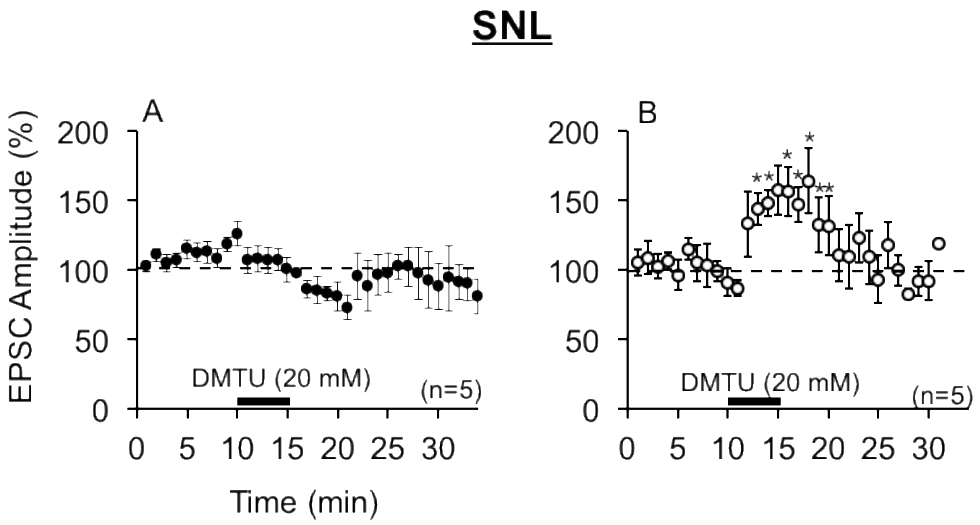


FIG 5.4 DMTU reduces SNL-induced reduction in EPSC amplitude only in GABAn and fails to affect those of STTn in SNL mice. Consistent selectivity to GABAn was observed when DMTU (20 mM) was applied 10 min after obtaining baseline EPSCs in SNL mice. DMTU significantly increased EPSC amplitude in GABAn in the ipsilateral side of SNL (B, n=5), whereas it failed to affect EPSC amplitude in STTn in the ipsilateral side of SNL (A, n=5). No CS was applied in A and B. Data in A and B were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test (*, different from pre-drug within a treatment; $P<0.05$).

5.5 DISCUSSION:

As reported in aim 1, cell-type specific synaptic plasticity develops in the laminae I and II of the dorsal horn in response to CS. LTP develops in STTn and LTD develops in GABA-interneurons under both low frequency and high frequency conditioned stimulus. However, the question remains whether this cell-type specific synaptic plasticity is expressed in neuropathic mice after peripheral nerve injury. In this aim, SNL model was used to induce peripheral nerve injury and synaptic plasticity was investigated in spinal nerve-ligated mice. We found that STTn from the ipsilateral side of SNL were unable to develop LTP, unlike their counterparts from the contralateral side of SNL. GABAn from the ipsilateral side of SNL however, still developed LTD, however in a slower manner than their counterparts of contralateral side of SNL. Upon these results, we speculate that STTn and GABAn affected by SNL are already excited and depressed, respectively in comparison to their counterparts from the contralateral side of SNL, or from naïve mice (as reported in aim 1 of the study). Comparing baseline evoked EPSC magnitudes from the same groups pf neurons confirmed our speculation.

Going back to aim 2 where TEMPOL was shown ineffective in significantly reversing CS-induced STTn-LTP and GABAn-LTD; a disconnection between the analgesic effect of TEMPOL and its role in the maintenance phase of pain-related synaptic plasticity. After showing that STTn and GABAn excitability is altered by SNL, we tested the effect of TEMPOL and DMTU on evoked-EPSCs STTn and GABAn in the ipsilateral side of SNL. Interestingly, TEMPOL, superoxide radical scavenger, significantly reduced STTn and elevated GABAn evoked EPSC amplitudes indicating an important role of ROS in pain-related synaptic plasticity. More interestingly, DMTU, a hydroxyl radical

scavenger only alleviated SNL-induced changes in GABAn evoked-EPSCs, leaving STTn evoked-EPCSs unaffected. This suggests the differential role of superoxide and hydroxyl radicals in SNL-induced cell type specific synaptic plasticity.

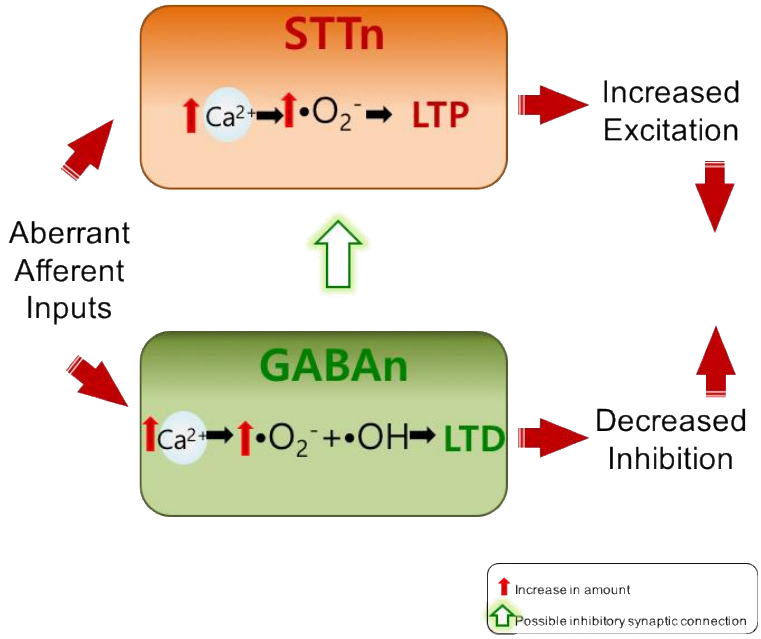
The interest in ROS molecules as specific and selective signaling molecules is growing, however, not much has been uncovered concerning the differential involvement of ROS subtypes in excitatory vs. inhibitory synaptic plasticity in the spinal cord. As mentioned before, superoxide and hydrogen peroxide are two of the most commonly implicated reactive oxygen species in pain signaling. Hydrogen peroxide in particular is very stable and travels around the cell, and therefore can act as a signaling molecule. In the spinal cord, hydrogen peroxide was shown to increase the frequency of miniature inhibitory post-synaptic currents in GABAn of the substantia gelatinosa (SG) suggesting that it mediates pain disinhibition (Takahashi et al., 2007). Nonetheless, hydrogen peroxide was shown to induce hyperalgesia only when injected in the intraplantar but not in the intrathecal space (Kim et al., 2015). This suggests that hydrogen peroxide might not directly mediate pain-related synaptic plasticity mechanisms in the central nervous system. Instead, hydrogen peroxide's role in synaptic plasticity might be mediated by its derivatives, hydroxyl radicals. Therefore, we speculate that hydroxyl radicals, that are continuously generated as a result of continuous hydrogen peroxide accumulation induced by nerve injury, contributes to synaptic plasticity. This speculation is supported by the findings that a hydroxyl radical donor tBOOH produces hyperalgesia when injected intrathecally, and is selectively effective in depressing SG inhibitory and not excitatory transmission (Yowtak et al., 2011). Together, with our previous results, these findings

support the notion suggested in our study stating that hydroxyl radicals are important for GABAergic inhibitory more than for excitatory function of dorsal horn circuitry.

6. CONCLUSION

This study sheds the light on the types of synaptic plasticity in the dorsal horn contributing to central sensitization and neuropathic pain behavior. It also demonstrated the importance of reactive oxygen species as very specific and selective signaling molecules in the initiation and the maintenance of this dorsal horn synaptic plasticity and central sensitization. Our findings are summarized here. We found that synaptic plasticity in the dorsal horn of the spinal cord is cell-type specific rather than stimulation frequency dependent. In other words, we found that long term potentiation develops in spinothalamic tract neurons whereas long term depression develops in GABAergic interneurons by both high and low frequency stimulations. In addition, we were able to show that this cell-type specific synaptic plasticity develops not only in response to conditioned stimulus in naïve mice, but is also established in neuropathic mice after spinal nerve ligation. Concerning the role of ROS in synaptic plasticity, we found that ROS mediate cell-type specific synaptic plasticity induction and maintenance in naïve and neuropathic mice respectively. However, the highlight of the study was finding a differential role of superoxide and hydroxyl radicals in cell-type specific synaptic plasticity. In other words, Superoxide radicals were shown to mediate both long term potentiation and long term depression in spinothalamic tract neurons and GABA interneurons, respectively. Nevertheless, hydroxyl radicals were found to be involved only in GABA interneurons' long term depression. This suggests different subtypes of ROS might be driving cell-type specific synaptic plasticity and the excitatory/ inhibitory imbalance that underlies central sensitization and neuropathic pain after nerve injury.

6.1 Conceptual representation of the possible role of specific ROS subtypes in cell type-specific synaptic plasticity



The illustration above shows a proposed summary box diagram of the role of specific ROS which is formulated from the obtained results and previous studies in our laboratory. Excitatory STTn and inhibitory GABAn receive excitatory inputs. Following nerve injury and aberrant afferent stimulation, calcium enters the cells through NMDA and AMPA receptor channels. This, in turn, leads to downstream production of secondary messengers including ROS. Our interpretation of the present results is that superoxide radicals are the main form of ROS accumulating in STTn and driving LTP. However, in GABAn, both superoxide radicals and hydroxyl radicals accumulate and lead to the induction and maintenance of LTD. LTP in STTn will increase STTn excitability. Moreover, LTD in GABAn will inhibit GABAn inhibition of STTn and further increase STTn excitability. Mechanisms of the differential increase in ROS subtypes in STTn vs. in GABAn are currently under investigation.

FUTURE DIRECTIONS

Future directions aim to tackle a few questions that remain unclear, and that will help compose a more complete picture of this study, when answered. Some of these questions revolve around the specific signaling mechanisms involved in inducing opposing directions of synaptic plasticity in STTn and GABAn. Other questions ask whether other ROS, other than superoxide and hydroxyl radicals, act differentially on STTn and GABAn. In addition, one of the big questions relates to the translatable (to clinical settings) potential of ROS scavengers in being used to make clinically safe pharmacological therapeutics. In the future, it would be suitable to study the possibility and the means available to take these ROS scavengers into a new level closer to clinical stages of research.

After showing that pain-related synaptic plasticity in the dorsal horn STTn and GABAn is cell-type specific, we ought to further investigate the signaling mechanisms underlying this specificity, and examine how different ROS types differentially influence the signaling machinery. Previously, we showed that the intracellular calcium increase is similar in STTn and GABAn after conditioned stimulus. This finding suggests that the opposite polarity of synaptic plasticity is probably induced by a factor downstream of calcium signaling. Calcium affects the functions of many proteins shown to be involved in synaptic plasticity like kinases and phosphatases (Latremoliere et al., 2009). Therefore, it would be beneficial to investigate an array of kinases and phosphatases such as PKC, PKA, CamKII, calcineurin, phosphatase 1A in STTn and GABAn of spinal nerve-ligated mice specifically using immunohistochemistry techniques. These proteins have been shown to be regulated by ROS and play a significant role in the overall cellular kinase/phosphatase balance. (Kamsler and Segal, 2003).

One of the end targets of kinases and phosphatases responsible for the maintenance phase of synaptic plasticity has been shown to be AMPA receptors. Therefore, it would be critical to investigate the regulation of AMPA receptors in STTn vs. GABAn in naïve vs. neuropathic mice using immunohistochemical techniques. In addition, it would also be interesting to examine whether different types of ROS differentially regulate AMPA receptors directly or through kinases and phosphatases only. This will help better understand membrane excitability differences between STTn and GABAn as well as better target future investigations towards the signaling mechanisms upstream of AMPARs.

The level of ROS specificity observed in the findings of this study is intriguing, and the success of the electrophysiological recordings, during ROS exposure, suggests that ROS levels were far from inducing neuronal toxicity. However, the observed changes in synaptic plasticity and pain behavior in response to ROS donors or scavengers suggest that endogenous ROS were not scavenged properly by the cellular scavenging machinery. Therefore, it becomes interesting to quantitatively assess ROS levels in STTn and GABAn after SNL. Technical difficulties including consistent STTn labeling and a lack of specific markers to differentiate superoxide from hydroxyl radicals halted the progress of this experimental aim. It would be beneficial in the future, to develop an assay that makes detecting the level of ROS in individual STTn and GABAn possible.

In addition to superoxide and hydroxyl radicals, other ROS types might be involved in STTn-LTP and GABA-LTD. For instance, nitrogenous species like peroxynitrite (PN) was shown to be involved in inflammatory and peripheral sensitization after peripheral nerve injury. It was also shown to contribute to central sensitization and mechanical hypersensitivity in several neuropathic pain models like diabetic neuropathy and

chemotherapy induced neuropathic pain. Therefore, it is important to investigate the role of PN in STTn-LTP and GABAn-LTD (Salvemini et al., 2011).

Interestingly, several studies stress on Peroxynitrite-decomposing catalysts (PNDCs), which are specific PN scavengers, as potential therapeutics for neuropathic pain. PDNCs were shown eliminate PN but spare superoxide radicals, and thus avoid the many side effects associated with excessive ROS removal (Salvemini et al., 2011). Despite the abundant evidence supporting the positive effects of PNDCs in relieving some types of neuropathic pain, PNDCs have not been taken to clinical studies yet. In the future, it would be interesting to develop methods that specifically target hydroxyl radicals, the most toxic form of ROS. It has been shown that glutathione peroxidases prevent the accumulation of hydrogen peroxides which subsequently protects against hydroxyl radicals production. Organoselenium compounds such as ebselen have been shown to function as glutathione peroxidase mimetics and protect against neuronal damage in stroke models (Carbone et al., 2015). Therefore, it would be important to test the potency of organoselenium in neuropathic pain relief. Further experimentation is warranted to investigate the ability of organoselenium compounds to preferentially target hydrogen peroxides.

In conclusion, this study demonstrated that the accumulation of ROS promotes the development and maintenance of neuropathic pain in a peripheral nerve injury model. In addition, the effects of ROS are not arbitrary, but are rather discriminative in regulating neuronal excitability and synaptic plasticity. Mechanisms underlying the specificity of ROS are still under investigation. This study presents novel findings that help better understand the mechanisms of synaptic plasticity in neuropathic pain, the role of ROS in cell-type specific synaptic plasticity, and offer a new perspective in viewing ROS as

molecules that are specific in action. These findings help in directing neuropathic pain research and creating novel therapeutics.

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Bachelor of Science; Biology Major-Chemistry Minor
Graduated Magna cum laude (July 2009-May 2012)

RESEARCH ACTIVITIES:

Graduate Assistant, University of Texas Medical Branch, Chung Laboratory
Neuropathic Pain (February 2014-Present)
Graduate Assistant, University of Texas Medical Branch, Neugebauer Laboratory
Inflammatory Pain (February 2013- February 2014)
Graduate Assistant Rotation, University of Texas Medical Branch, Carney Laboratory,
Biochemical Research; Drug targeting (December 2012- February 2013)
Graduate Assistant Rotation, University of Texas medical Branch, Finnerty Laboratory
Burn Research (October 2012- December 2012)
Undergraduate Research, Bradley University, Peoria, IL (August 2010- May 2012)
Research Assistant at OSF Saint Francis Medical Center, Peoria, IL (May 2010- July 2010)

MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS:

Former president of Neuroscience Graduate Program-Journal Club
Society for Neuroscience SfN- Active member
American Pain Society-Active member
Global Brigades Organization
Phi-Kappa-Phi Honor Society

AWARDS AND HONORS:

Best oral presentation at Neuroscience and Cell Biology annual retreat (December 2016)

Jeane B Kempner Scholarship/Award (September 2016)

Best oral presentation at Neuroscience Graduate Program Symposium (July 2016)

Invited as a distinguished trainee to talk at Gulf Coast Consortium annual meeting (April 2016)

Dr. and Mrs. Seymour Fisher Academic Excellence Award (September 2015)

Bradley University Dean's List (May 2010-May 2012)

Bradley University Alumni Award (May 2011)

TEACHING RESPONSIBILITIES:

Training a visiting student from UT Dallas on patch clamp technique (August 2016)

Bench Mentor Program, training/ teaching a high school student research principals and laboratory basic techniques (June 2015- May 2016)

Lecturer at TAMUG, Lecture on cardiovascular toxicology, as part of toxicology course for undergraduates. (March 2015)

Teaching/ Training a prep-student on behavioral research techniques in Dr. Neugebauer's laboratory at University of Texas Medical Branch. (February 2013- February 2014)

COMMUNITY OUTREACH:

Poster judge at UTMB SURP program (June 2015)
United to serve, University of Texas Medical Branch Volunteers (July 2015)
Poster judge at Austin Middle School, Galveston. (2014)
Organizer at first UTMB Brain Fair for Ball High School, Galveston, Tx (2014)
Ronal McDonald House/Shriners Hospital Volunteer-English/Arabic translator (2015)
Society for Neuroscience Galveston Chapter (2013-2016)
Global Water Brigades, University of Texas Medical Branch Chapter (2013-2014)
United to Serve, University of Texas Medical Branch volunteer, The Children's Center & Jesse Tree at Galveston, Tx. (2013-2014)
Committee for Career Development (CCD), University of Texas Medical Branch. (2012-2013)
Honor Society of PHI KAPPA PHI (2011-2012)
Volunteer at Easter Seals organization, Peoria, IL (2010-2011)
Volunteer OSF Saint Francis Medical Center, Peoria IL/ Emergency Room (2011-2012)
Lebanese Red Cross - Paramedical and Emergency Department (2008-2011)
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PUBLISHED:

A. ARTICLES IN <u>PEER-REVIEWED</u> JOURNALS:

Kim HY, Jun J, Wang J, **Bittar A**, Lu Y, Chung K, Chung JM. (2015). The induction of LTP and LTD is cell-type specific in the spinal cord. Pain. 156(4):618-25.

Alvin M. Shih, Lincy Varghese, **Alice Bittar**, Jin Mo Chung, and Ok-Ho Shin Dysregulation of norepinephrine release in the absence of functional Synaptotagmin 7. J Cell Biochem. 117(6):1446-53.

B. ABSTRACTS:

Bittar A, Wang J, La JH, Bae C, Shim HS, Chung JM. Electrophysiological evidence of spinal cord central sensitization in neuropathic mice. (2016)

La JH, Wang J, **Bittar A**, Shim HS, Bae C, Chung JM. Capsaicin-induced secondary mechanical hyperalgesia and allodynia are mediated by different central sensitization mechanisms involving reactive oxygen species. (2016)

Bae C, Wang J, **Bittar A**, Shim HS, La JH, Tang SJ, Chung JM. Mitochondrial reactive oxygen species regulate excitatory and inhibitory synapses on dorsal horn neurons in neuropathic pain. (2016)

Bittar A, Jun J, Wang J, Chung K, Chung J.M. Differential effects of specific ROS scavengers on synaptic plasticity induction and mechanical hypersensitivity in neuropathic mice. (2015)

Jun J, **Bittar A,** Wang J, Chung K, Chung J.M. Different types of free radicals are involved in synaptic plasticity in the spinal cord. (2015)

Bittar A, Crofton L, Neugebauer V. Cognitive performance is negatively correlated with pain behavior in rats. (2013)

Marek CJ, Rahman SJ, **Bittar A**, Stabeneau E. Identify muscle progenitor cells in culture and assess distribution and functionality of the inner mitochondrial membrane proteins in Pyrene Exposed and Control Frogs. (2011)

PUBLICATIONS - SUBMITTED:

Bittar A, Jun J, La JH, Wang J, Leem J, Chung JM. Differential effect of ROS subtypes on synaptic plasticity and neuropathic pain behavior.

INVITED LECTURES AT SYMPOSIA AND CONFERENCES:

Differential effects of ROS subtypes on synaptic plasticity in the spinal cord. Gulf Coast Consortium, TRP Symposium, Houston Tx, April 2015.