SPINAL CORD INJURY (SCI) SWITCHES HOW GABA AFFECTS NOCICEPTIVE PLASTICITY

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2015

Major Subject: Neuroscience

ABSTRACT

Research has shown that spinal cord injury (SCI) can induce neural hyperexcitability within the spinal cord that facilitates nociceptive reflexes. Nociceptive inputs have been shown to sensitize spinal nociceptive systems, inducing a learning deficit and enhanced mechanical reactivity (EMR) in spinally transected rats. Nociceptive sensitization has been linked to abnormal GABA-mediated inhibition of nociceptive neurons within the spinal cord. However, underlying changes remain poorly understood. This dissertation were designed to test the effect of blocking GABA transmission on nociceptive sensitization after spinal cord injury.

Experiment 1 focused on the effect of bicuculline on shock-induced EMR in transected rats, finding blocking effect of bicuculline. Experiments 2 and 4 investigated whether bicuculline blocks inflammation-induced EMR. I found bicuculline pretreatment prevented both LPS and capsaicin-induced EMR. Further, capsaicin-induced EMR was reversed by bicuculline treatment (Experiment 5). Experiment 6 found that other GABA receptor antagonists also blocked the capsaicin-induced EMR.

Of clinical importance, bicuculline blocked indices of capsaicin-induced central sensitization at the mRNA level (Experiment 7) and protein level (Experiment 8). These results suggest that bicuculline blocks central sensitization in spinally transected rats and that GABA has an excitatory effect.

To explore whether a spinal transection alters GABA function, similar experimental manipulations were conducted in intact rats. Experiment 9 found that bicuculline treatment *per se* induced EMR and failed to block the capsaicin-induced EMR. Experiments 10 and 11 found that bicuculline did not block central sensitization at the cellular level. These results suggest that GABA inhibits nociceptive processing in intact rats, but promotes it after spinal injury.

Experiment 12 explored that spinal transection induced a downregulation of the membrane-bound KCC2, and thereby changed intracellular chloride homeostasis. To test whether drug manipulation targeting chloride co-transporters switch the role of GABA in nociceptive sensitization, channel blockers targeting KCC2 and NKCC1 were tested. Experiment 13 showed that blocking KCC2 in intact rats causes bicuculline to attenuate capsaicin-induced EMR. Conversely, Experiment 14 showed that blocking NKCC1 in transected rats switches how bicuculline affects capsaicin-induced EMR. Taken together, my results suggest that spinal cord injury switches the effect of GABA in nociceptive sensitization by altering the intracellular chloride homeostasis.

DEDICATION

For my family, and my friends

ACKNOWLEDGEMENTS

I would like to thank Dr. James W. Grau for his patient guidance and support throughout the years. This work would not be done without his valuable perspective, encouragement and willingness to exchange ideas. I would like to thank my committee members, Drs. Michelle Hook, Rajesh Miranda and Mary Magher, for their helpful feedback and enthusiasm. I would like to thank Drs. Kevin Hoy and Kuan-Hsien Lee for the examples they have set for me, both personally and professionally. I would also like to express my gratitude to Misty Strain, Joel Turtle, and Dr. Sandra Garraway who have never hesitated to help me; thank you for your friendship and encouragement. As always, I must thank my parents, my family, and my friends for their countless efforts for me.

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CHAPTER I

INTRODUCTION

Under normal condition, pain (nociceptive stimulation) subserves a protective function that promotes learning to avoid dangerous situations and prevent additional tissue damage. Nociceptive input begins with a specialized high-threshold nociceptor of the sensory system (the nociceptive system) and extends from the periphery (A-delta and C-fiber) through the spinal cord to the brain, where the sensation is perceived. When tissue damage occurs, injury-induced factors (inflammatory agents) lead to a hyperexcitable status of the peripheral nervous system (peripheral sensitization) and shift the system from protecting to promoting wound healing (Ji, Kohno, Moore, & Woolf, 2003). This inflammatory pain often produces allodynia (pain in response to a nonnociceptive stimulus) and hyperalgesia (increased pain sensitivity). Nociceptive pain, including protective acute pain and inflammation pain usually fade once the painful stimulus is removed and/or the wound has healed. However, sometimes pain extends beyond the expected period of healing and persists after the stimulus has been removed. Pain of this sort is viewed as neuropathic pain, which is associated with injury to the peripheral nerve and/or the central nervous system (spinal cord injury [SCI]), or sometimes disease (Woolf, 2004). The resulting malfunction of the nervous system has been related to changes within neurons (Ji et al., 2003; Woolf, 2004) and between neuro-glial interaction (Ji & Suter, 2007), that can enhance peripheral and central nociceptive processes (peripheral and central sensitization, respectively), and thereby enhance pain

by inducing allodynia and/or hyperalgesia. My dissertation examines the mechanisms that sensitize nociceptive systems within the spinal cord (central sensitization).

Evidence suggests that central sensitization is due in part to a change in γ-Aminobutyric acid (GABA) function (Gwak & Hulsebosch, 2011). Normally, GABA is actively modulated by the brain through descending projections (serotonin), and subsequently inhibits nociceptive circuits (Ciranna, 2006). Evidence suggests that the loss of the descending control from the brain after SCI promotes general hyperexcitability in the spinal cord (central sensitization), resulting in the facilitation of nociceptive reflexes (Curatolo et al., 2006; Millan, 2002). Here, I explore the possibility that this facilitatory effect reflects an alteration in GABA transmission, which causes GABA to have an excitatory effect that promotes nociceptive sensitization after SCI.

To explore how GABA contribute to the hyperexcited nervous system and whether GABA can switch from inhibitory to excitatory, I first review the mechanisms that sensitize nociceptive systems. Second, I review the role of GABA within hyperexcited nervous system. Third, I explain how GABA can have an excitatory effect.

Central Sensitization, Peripheral Sensitization and SCI

Central sensitization, a hyperactive state of nociceptive neurons within dorsal horn of the spinal cord, has been suggested to underlie neuropathic pain. It represents a condition where input in one set of nociceptor sensory fibers amplifies the subsequent responses to other non-stimulated non-nociceptor or nociceptor fibers, known as

heterosynaptic potentiation. In this phenomenon, nociceptor inputs can trigger a prolonged increase in the excitability and synaptic efficacy of neurons in central nociceptive pathways (Woolf & Salter, 2000). Pain hypersensitivity, such as tactile allodynia, secondary hyperalgesia, and enhanced temporal summation of action potential discharges are observed with central sensitization. The increased synaptic efficacy in somatosensory pathway can be attributed to several mechanisms: increased pre-synaptic excitatory transmitter release, increased response to the transmitter in the post-synaptic portion, increased membrane excitability, or the reduction of inhibition (Latremoliere & Woolf, 2009).

During tissue injury or inflammation, inflammatory mediators induce a peripheral sensitization and then ultimately increase the excitability of CNS neurons, generating central sensitization (Ji et al., 2003; Woolf, 2004). Peripheral sensitization only increases the pain sensitivity in an area that is restricted to the site of inflammation or injury, whereas central sensitization can heighten the sensitivity to peripheral region of the injury site (secondary hyperalgesia) and to low-threshold mechanosensory input (secondary mechanical allodynia). This activity-dependent form of central sensitization involves the activation of multiple intracellular signaling pathways including ion-gated NMDAR (NMDA receptor) and AMPAR (AMPA receptor), G-protein-coupled metabotropic receptors, substance-P receptor neurokinin-1 (NK1), mGluR, and tyrosine kinase receptors (trkB and Eph) in dorsal horn neurons (Ji et al., 2003). Central sensitization comprises two temporal phases, each with specific mechanisms. The early phosphorylation-dependent and transcription-independent phase has been linked to rapid

changes in glutamate receptor and ion channel properties. The later, longer-lasting, transcription-dependent phase results from the new proteins synthesis that yields a longer-lasting form of central sensitization observed in several pathological conditions (Ji et al., 2003; Woolf, 2004).

SCI can produce high concentrations of extracellular glutamate at both neuronal-neuronal and neuronal-glial cell appositions. Because neurons and glial cells express similar receptors and ion channels, glial activation may trigger similar intracellular cascades as those observed in neurons. Briefly, after SCI, high concentration of glutamate at neuronal-glial clefts activate the glutamate receptors on astrocyte and microglia, both ionotropic and metabotropic. This activation leads to the subsequent membrane depolarization that triggers a large influx of Na^+ and Ca^{2+} ions (both neuron and glia). Subsequently, the elevated Ca^{2+} concentrations in astrocytes and microglia initiate the activation of mitogen-activated protein kinase (MAPK, p38-MAPK and ERK) and phospholipase (PLA2) that result in the modulation of target protein expression or phosphorylation of membrane receptor and ion channels through activation of transcription factors, such as NF-κB or pCREB (Crown et al., 2006; Gwak & Hulsebosch, 2011; Ji et al., 2003). Finally, the activated glial cells release glutamate, ATP, proinflammatory cytokines, prostaglandins (PGs), and reactive oxygen (ROS) into the extracellular space. These pain mediating substances released by activated glia contribute to intracellular downstream biochemical pathways and provide an intracellular feed forward mechanism for continued phosphorylation/activation of receptors and ion channels. This mechanism ensures the induction and maintenance of

the central neuronal hyperexcitability. Glia activation also plays a crucial role in developing and maintaining the sensitization of PNS and CNS following SCI.

Inhibitory Tone and SCI

Research within the pain literature has revealed that the GABAergic system plays a crucial role in regulating the development of central sensitization and the spinally mediated changes induced by peripheral inflammation (Sivilotti & Woolf, 1994; Sluka, Willis, & Westlund, 1993, 1994). Under pathological conditions, such as after SCI, the inhibitory tone of GABA is modulated. Under normal condition, GABAergic system modulates inhibitory tone within the CNS. Treatment of $GABA_A$ receptor antagonist blocks the inhibitory effect of GABA, sensitizes the CNS, and thereby induces allodynia. Contrary to this common view, I hypothesize that the effect of GABA can switch from inhibitory to excitatory after SCI, and leads to the hyperexcitable status of CNS. Under these conditions, bicuculline could have an anti-allodynic effect.

Past studies have shown that GABA can have an excitatory effect under certain conditions. Ben-Ari et al. (1989) first showed that GABA is excitatory in the immature brain. GABA-releasing synapses are formed before glutamatergic contacts in a wide range of species and structures (GABAergic synapses are first formed as soon as neurons have an apical dendrite). It becomes inhibitory by the delayed expression of a chloride exporter, leading to a negative shift in the reversal potential for chloride ions in mature brains (Ben-Ari, 2002). The polarity of GABAergic signaling actions depends in part on

the intracellular concentration of chloride ($[Cl^-]_i$). When Cl^- concentration within the cell rises, engaging the $GABA_A$ receptor can have a depolarizing/excitatory effect. This is observed both early in development and in some neurological disorders (Ge et al., 2006; Gulledge & Stuart, 2003; Marty & Llano, 2005). These changes have been linked to alteration in the chloride-extruding- and-uptaking system (NKCC1 and KCC2) which regulates intracellular Cl^- concentration (Cramer et al., 2008; Hasbargen et al., 2010).

Other work has implicated central sensitization and GABA in the regulation of spinal plasticity (Grau et al., 2006). Prior work from our lab has shown that intermittent nociceptive stimulation can induce an over-excitation (hyperexcitable) of the spinal cord, and thereby produce a learning deficit and an enhanced mechanical reactivity (EMR; Baumbauer et al., 2008, 2012; Ferguson et al., 2003, 2006). Peripheral inflammation induced by capsaicin was also shown to impair learning (Hook et al., 2008). The shock-induced learning deficit has been linked to an alteration in the GABAergic system. Supporting this, treatment with bicuculline, a $GABA_A$ receptor antagonist, blocks the learning impairment (Ferguson et al., 2003). Because treatments that impair spinal learning also induce EMR, this finding implies that bicuculline would also block shock-induced EMR in spinally transected rats. This runs counter to the general view that bicuculline treatment should induce EMR by blocking GABAergic inhibition. I suggest bicuculline will have this paradoxical anti-allodynic effect in transected animals because spinal injury causes a rise in intracellular Cl^- that cause GABA to have an excitatory effect.

Underlying Mechanism of Excitatory GABA

In the nervous system, the strength and polarity of GABA-mediated neurotransmission is determined by the intracellular chloride concentration ($[Cl^-]_i$), because $GABA_A$ receptor is an ionotropic channel and selectively conducts Cl^- through its pore. Cl^- concentration is determined, in part, by the activities of the SLC12 cation–chloride cotransporters (CCCs). These transporters include the Na-K-2Cl cotransporter NKCC1, which mediates chloride influx, and various K-Cl cotransporters, such as KCC2 and KCC3, that extrude chloride. A precise balance between NKCC1 and KCC2 activity is necessary for inhibitory GABAergic signaling in the adult CNS, and for excitatory GABAergic signaling in the developing CNS and the adult neurogenesis. Altered chloride homeostasis, resulting from mutation or dysfunction of NKCC1 and/or KCC2, promotes neuronal hypoexcitability. In immature neonatal neurons, the intracellular $[Cl^-]_i$ is about 20–40 mM higher due to robust activity of the chloride-importing Na-K-2Cl cotransporter NKCC1. The binding of GABA to ligand-gated $GABA_A$ receptor triggers Cl^- efflux and depolarizing excitation. In adults, NKCC1 expression decreases and the expression of the genetically regulated chloride-extruding K-Cl cotransporter KCC2 increases which lowers the $[Cl^-]_i$. The activation of $GABA_A$ receptors triggers Cl^- influx and inhibitory hyperpolarization in this circumstance. Thus, chloride homeostasis is determined by the balance of NKCC1 and KCC2. Targeting these proteins could have clinical value in treating neurological

disorders such as spasticity and neuropathic pain (Fukuda, 2005; Jolivalt, Lee, Ramos, & Calcutt, 2008).

Several lines of research have shown that SCI upregulates NKCC1 whereas KCC2 is downregulated in the spinal cord, which correlates with allodynia and hyperalgesia (Cramer et al., 2008; Hasbargen et al., 2010). The upregulation of NKCC1 and downregulation of KCC2 produce high intracellular Cl^- concentration that facilitates efflux of Cl^- when $GABA_A$ receptor is activated, which generates depolarization (excitatory) rather than hyperpolarization (inhibitory). This process may underlie the switch of GABA from inhibitory to excitatory after SCI, and the resultant pain enhancement.

Specific Aims

When considering GABA effect and the role of $GABA_A$ receptor in nociceptive plasticity within the spinal cord after SCI, several questions arises: 1) Does $GABA_A$ receptor bicuculline block the shock-induced EMR after SCI, 2) Does $GABA_A$ receptor still have inhibitory effect on inflammation induced pain after SCI, 3) Does the effect of GABA switch from inhibitory to excitatory after SCI, and 4) If it does switch, what is the underlying mechanism?

The focus of this dissertation is on the role of GABAergic system in maladaptive plasticity of nociceptive system (central sensitization) and its underlying mechanism. My hypothesis is that GABA switches its effect from inhibitory to

excitatory after SCI; and that this biological switch results from the down regulation of KCC2 after SCI, which causes the high intracellular Cl^- concentration. To test my hypothesis, I examined the effect of spinal injury (by transection) and treatment with a GABA antagonist on behavioral and cellular indices of central sensitization. First, I tested whether the $GABA_A$ receptor antagonist, bicuculline, blocks the EMR induced by shock or inflammation after SCI (Chapter III). Second, I tested the effect of bicuculline on cellular indices of central sensitization (Chapter IV). Third, I tested whether bicuculline has different effect in the absence of SCI (Chapter V). Fourth, I explored the cellular mechanisms that underlie these effects (Chapter VI).

CHAPTER II

GENERAL METHOD

Subjects

Subjects were male Sprague-Dawley rats obtained from Harlan (Houston, TX). Rats were 70-90 days old and weighted 350-400g at the time of spinal cord transection. Food and water were available *ad libitum*. Subjects were housed in pairs and maintained on a 12 hour light-dark cycle. All experiments were carried out in accordance with NIH standards for the care and use of laboratory animals (NIH publications No. 80-23) and were approved by the University Laboratory Animal Care Committee at Texas A&M University. Every effort was made to minimize suffering and limit the number of animals used.

Surgery

Subjects were anesthetized with isoflurane gas, induced at 5%, and maintained at 2-3%. Each subject's head was rendered immobile in a stereotaxic apparatus with a small (5 X 4 X 2.5 cm) gauze pillow under the subject's chest to provide support for respiration. An anterior to posterior incision over the second thoracic vertebrae (T2) was made and the tissue just rostral to T2 was cleared using rongeurs, and the cord was exposed and cauterized. The remaining gap in the cord was filled with Gelfoam

(Pharmacia Corp., Kalamazoo, MI) and the wound was closed with Michel clips (Fisher Scientific, Waltham, MA). Following closure of the wound, intraperitoneal injections (3 mL) of 0.9% saline solution were administered post-operatively to prevent dehydration. Following surgery, rats was placed in a temperature-controlled environment (25.5 °C) and monitored until awake. All rats were checked every six to eight hours during the 18-24 hr post-surgical period. During this time, hydration was maintained with supplemental injections of saline, and the rats' bladders and colons were expressed as necessary.

Variable Intermittent Leg Shock

Subjects were treated with electrical stimulation 24 hours after surgery. Variable intermittent leg shock was applied while spinalized rats were loosely restrained in Plexiglas tubes as previously described (Ferguson et al., 2000; Grau et al., 2006). Leg shock was delivered using a BRS/LVE (Laurel, MD) constant current (60 Hz, AC) shock generator (Model SG-903). Electrical stimulation was applied by attaching one lead from the shock generator to a 2.5 cm stainless steel pin that was inserted 0.4 cm into the tibialis anterior muscles. The other lead was inserted through the skin over the tibia, 1.5 cm from the tarsals. Rats treated with intermittent nociceptive stimulation received 900, 80-ms leg shocks on a variable time schedule with a mean inter-stimulus interval (ISI) of 2 s (range 0.2 -3.8 s). Unshocked subjects were placed in the restraining tubes for an

equal amount of time as the shocked subjects, had the electrodes attached, but did not receive the electrical stimuli.

Drug Administration

Bicuculline (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% saline (1 μL). LPS (Sigma-Aldrich, St. Louis, MO) was dissolved in 10 μL of 0.9% saline. Phaclofen (Santa-Cruz Biotech, Dallas, TX) was dissolved in 1 μL of 0.9% saline. Gabazine (Sigma-Aldrich, St. Louis, MO) was dissolved in 10 μL saline. Twenty μg of DIOA (Santa-Cruz Biotech, Dallas, TX) was dissolved in 2 μL vehicle (DMSO [1%] and saline [99%]). One mM of bumetanide (Santa-Cruz Biotech, Dallas, TX) was dissolved in 10 μL vehicle (Tween20 [2%] and saline [98%]). All the drugs (Table 1) mentioned above were intrathecally injected into the subject and followed by a 20 μL saline (0.9%) flush over a period of 2 min.

Drugs	**Target**	**IC_{50}**	**Half-life**	**Loading dose**
Bicuculline	$GABA_A$ receptor antagonist	3 μM	45min	816 μM
Gabazine	$GABA_A$ receptor antagonist	349 nM	—	2.715 μM 271.5 μM
Phaclofen	$GABA_B$ receptor antagonist	118 μM	3.4 hr	4 mM 40 mM
DIOA	KCC2 blocker	50 μM	3.6 hr	50 mM
Bumetanide	NKCC1 antagonist	0.2 μM	0.8hr 6hr (intravenous in neonate)	1 mM

*Table 1***.** Pharmacological properties of drugs. IC_{50} represents the half maximal inhibitory concentration. Loading dose represents the dosage used in each experiment.

Capsaicin Injection

Three percent capsaicin (Sigma-Aldrich, St. Louis, MO) was dissolved in 50 μL of vehicle (Tween 20 [7%] and saline [93%]) and was injected subcutaneously into the dorsal surface of the hindpaw.

Mechanical Reactivity Testing

To determine if stimulation or drug administration produced a change in tactile reactivity, thresholds were assessed using von Frey filaments (Stoelting, Wood Dale, IL). Sensitivity was determined by stimulating the mid-plantar surface of each hindpaw in an ascending order until a flexion response is elicited. Stimuli were presented twice to each paw in an ABBA counterbalanced fashion (A = left, B = right), with testing on the same leg separated by a 2 min interval. Filament thickness/force is related to behavior using the transformation provided by the manufacturer: Intensity = log10 (10,000g). This transformation yields a scale that is approximately linear and amenable to parametric analyses. Data were converted to change from baseline scores for purposes of analysis.

RNA Extraction and RT-PCR

Subjects were deeply anesthetized with pentobarbital (50mg/kg) and a 1 centimeter of spinal cord around the lumbar enlargement (L3-L5) was rapidly removed

within 3 minute. The spinal cord was further subdivided into dorsal and ventral portions for determining the spatial (dorsal-ventral) changes in the expression of genes/proteins of interest. The spinal cord specimens were processed for extracting both total RNA (RNeasy Mini Kit; Qiagen, Valencia, CA) and total protein (see the protein extraction and western blot session). Total RNA (100 ng) was converted into cDNA by TaqMan EZ RT-PCR Core reagents (Applied Biosystems, Carlsbad, CA) and the mRNA levels of all targets were measured by TaqMan quantitative real-time (RT)-PCR using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA.). β-actin was used as the control gene. The sequences of probes, forward and reverse primers for β-actin, c-fos, and c-jun, were obtained from Applied Biosystems, Carlsbad, CA. The mRNA expression for each gene of interest was normalized to β-actin expression, and was presented as a fold change increase or decrease in experimental groups relative to the sham controls.

Protein Extraction and Western Blot

After RNA extraction, total protein was extracted from the organic layer, using the QIAzol^{TM} lysis reagent protocol for isolation of genomic DNA and/or proteins from fatty tissue (Qiagen, Valencia, CA). After determining the protein concentration by Bradford Assay (BioRad, Hercules, CA), protein samples were diluted in Laemmli sample buffer and were stored at -80^{o} C at known concentrations (usually 2-5μg/μl). Western blotting was used for the protein quantification of ERK1/2 and pERK1/2. Equal

amounts (30μg) of total protein were subjected to SDS-PAGE with 12% Tris-HEPES precast gels (Pierce, Rockford, IL) for ERK1/2 and pERK1/2 (~ 42/44 kDa). After transfering onto PVDF membranes (Millipore, Bedford, MA) by Bio-Rad Semi-dry transfer apparatus, the blots for non-phosphorylated proteins were blocked for one hour in 5% blotting grade milk (BioRad, Hercules, CA) in Tris-Buffered Saline Tween-20 (TBST). Blots for phosphorylated protein (pERK1/2) were blocked in 5% BSA in TBST. After blocking, the PVDF membranes were incubated overnight at 4^{o} C in one of the following primary antibodies generated in rabbit: ERK1/2 (1:2000; #06-182 - Millipore, Temecula, CA), pERK1/2 (1:500; #07-467 - Millipore, Temecula, CA), or β- actin (1:2500; #Ab8227 - Abcam, Cambridge, MA) which served as the control. All primary antibodies were diluted in blocking solution. The following day, PVDF membranes were washed in TBST (3 x 5 min) at room temperature and incubated in HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5,000; #31460 or 31430, respectively; Pierce, Rockford, IL) for 1 hour at room temperature. After another 3 x 5 min series of washes, the blots were incubated with ECL (Pierce, Rockford, IL) and were imaged with Fluorchem HD2 (ProteinSimple, Santa Clara, CA). The protein expression for each gene of interest was normalized to β-actin expression and presented as a fold change relative to the sham controls. Other targets of interest: GAD65/67 (1:500; #Ab1511 - Millipore, Temecula, CA), CAMKII (1:1000; #05-532 - Upstate, Lake Placid, NY), BDNF (1:500; R-066-500 - Novus Biological, Littleton, CO), TrkB (1:1000; #07-225 - Millipore, Temecula, CA), TNF-alpha (1:500; #ARC3012 - Invitrogen, Camarillo, CA), and KCC2 (1:500; #07-432 - Millipore, Temecula, CA) were assessed in the same fashion.

Fractionation

For Experiment 11, the spinal cord specimens were homogenized with dounce homogenizer (Kontes) followed by 5 passes through a 22 gauge needle in ice-cold buffer, pH 7.5, containing 10 mm Tris, 300 mm sucrose, and a complete mini protease inhibitor mixture (Roche). Crude homogenates were centrifuged at 5000 RCF for 5 min at 4°C. Supernatant was further fractionated at 13,000 RCF for 30 min. After centrifugation, supernatant was collected as cytoplasmic fraction and pellet was resuspended in PBS (50 μl) containing protease inhibitor as membrane rich fraction. All samples were sonicated and stored at −80°C for later processing. N-cadherin was used to confirm plasma membrane enrichment.

Statistics

All data were analyzed using an analysis of variance (ANOVA) or an analysis of covariance (ANCOVA). For behavioral measures, the individual variability was controlled by: (1) Analyzing the test data using an ANCOVA, entering the baseline score as a covariate; and (2) Computing a change from baseline score and analyzing the data using ANOVA. Both sets of analyses yielded similar patterns of statistical significance. An alpha value of .05 or below was considered statistically significant. Differences between group means were assessed using Duncan's New Multiple Range *post hoc* tests when necessary.

CHAPTER III

$GABA_AR$ ANTAGONIST'S EFFECT ON EMR IN SPINALLY TRANSECTED RATS

Prior studies have shown that variable intermittent shock (VIS; 900 shocks spaced with ISI: 0.2-3.8 s) produces an EMR and a learning deficit in instrumental learning (Ferguson et al., 2000; Ferguson et al., 2001). Bicuculline, a $GABA_A$ receptor antagonist, has been shown to block the induction and expression of the shock-induced learning impairment in spinally transected rats (Ferguson et al., 2003). However, the effect of bicuculline on EMR has not been tested. Here I examined whether bicuculline attenuates VIS-induced EMR in spinally transected rats. Mechanical reactivity was accessed using von Frey stimuli applied to the planar surface of each hind paw. Other treatments (e.g. lipopolysaccharide and capsaicin) that induce an EMR and learning impairment are tested in subsequent experiments. This set of experiments will elucidate whether GABA plays a role in the emergence of EMR, which is associated with allodynia and sensitization of nociceptive systems (central sensitization) within the dorsal horn.

Experiment 1

Experiment 1 examined whether bicuculline blocks the induction of VIS-induced EMR in spinally transected rats.

Procedure

The design used in experiments 1-3 is depicted in Figure 1. Spinally-transected (at second thoracic vertebra [T2]) and cannulized rat subjects (n=8 per group) were microinjected with either saline or 0.3% bicuculline (Ferguson et al., 2003) through the intrathecal catheter. Fifteen minutes after drug delivery, subjects received either VIS or nothing (unshock). This yielded a 2 (bicuculline vs. vehicle) X 2 (variable shock vs. unshocked) factorial design. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to leg shock, and again 0, 1, 2, 3 hr following shock treatment. A change from baseline score was also calculated to assess the impact of experimental manipulations.

<table>
<tr><td rowspan="4">Complete transection (T2)</td><td rowspan="4">24 hr</td><td rowspan="4">von Frey baseline</td><td>Vehicle</td><td rowspan="4">von Frey</td><td>Vehicle</td><td rowspan="4">von Frey 0, 1, 2, 3 hr</td></tr>
<tr><td>Vehicle</td><td>Treatment</td></tr>
<tr><td>Drug</td><td>Vehicle</td></tr>
<tr><td>Drug</td><td>Treatment</td></tr>
</table>

Figure 1. Experimental design for Experiment 1-3.

Results

Prior to drug treatment, mean baseline tactile reactive scores ranged from 6.27 ± 0.06 to 6.33 ± 0.06 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.0$, $p > .05$.

The effect of bicuculline on shock-induced EMR is depicted in Figure 2. Before shock treatment (Post Drug) bicuculline did not have a significant effect, $F(3, 28) < 1.0$, $p > .05$. As in previous studies (Ferguson et al., 2000; Ferguson et al., 2001),

shock (Post Shock) induced EMR in vehicle treated rats (Veh-Veh). Pretreatment with bicuculline blocked the development of EMR (Bic-Shk).

I first analyzed the raw data (Figure 2A). To control for variation in baseline reactivity, baseline scores were entered as a covariate using an analysis of covariance (ANCOVA). Both the main effect of bicuculline treatment and its interaction with shock were statistically significant, both $Fs > 5.48$, $p < .05$. The ANCOVA also revealed a significant time x shock interaction, F (3, 81) = 2.78, $p < .05$. No other terms were statistically significant, all $Fs < 2.78$, $p > .05$. *Post hoc* comparison confirmed that the vehicle treated group that received shock (Veh-Shk) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

Variation in baseline reactivity can also be addressed by computing a change from baseline score (Figure 2B). An analysis of variance (ANOVA) performed on the post shock scores again yielded a significant main effect of bicuculline treatment and bicuculline by shock interaction, both $Fs > 5.71$, $p < .05$. The ANOVA also revealed a significant time x shock interaction, F (3, 81) = 2.8, $p < .05$. No other terms were statistically significant, all $Fs < 2.02$, $p > .05$. *Post hoc* comparison confirmed that the vehicle treated group that received shock (Veh-Shk) differed from the other groups, $p < .05$. No other group comparisons were significant, $p > .05$.

An advantage of the change from baseline score is that our index of group reactivity (the standard error of the mean [SE]) is computed after we adjust for individual variability. Consequently, it is easier to judge relative group differences. For this reason, and because an ANCOVA performed on the raw scores, and an ANOVA

conducted on the change from baseline values, yielded the same pattern of significance in all subsequent experiments, only the latter is reported.

Vehicle Bicuculline	X	Unshock Shock

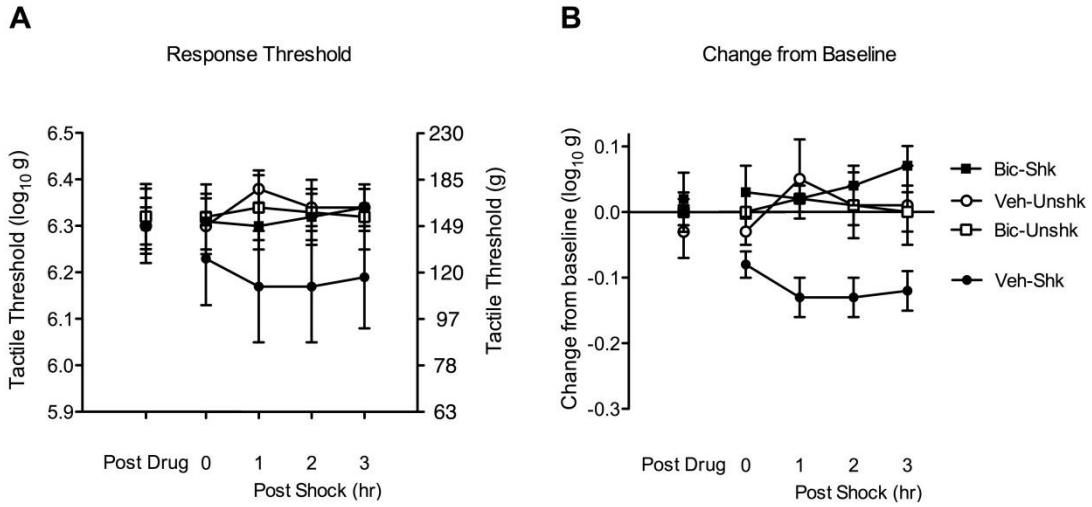

Figure 2. Bicuculline blocked shock-induced EMR. (A) Subjects that received bicuculline (Bic) or its vehicle (Veh) are depicted as squares and circles, respectively. Groups given VIS (Shk) or nothing (Unshk) are shown in black and white, respectively. The left y-axis depicts linearized tactile scores based on a transformation (log 10 [10,000g]) of the force required to bend the thinnest filament that produced paw withdraw after bicuculline treatment (Post Drug), and were then reassessed at 0, 1, 2, 3 hr after VIS treatment (Post Shock). The right y-axis depicts the gram force equivalents. (B) The change from baseline scores. The error bars depict ± SEM.

Discussion

Replicating previous results, VIS induced a lasting EMR. As predicted, this effect was blocked by pretreatment with the $GABA_A$ antagonist bicuculline.

Experiment 2

Experiment 1 showed that bicuculline pre-treatment blocks VIS-induced EMR. Experiments 2 and 3 examine the generality of this effect. The endotoxin, lipopolysaccharide (LPS), elicits a strong immune response in animals and induces both EMR (allodynia and hyperalgesia) and a spinally-mediated learning impairment (Reeve, Patel, Fox, Walker, & Urban, 2000; Vichaya et al., 2009; Young, Baumbauer, Elliot, & Joynes, 2007). LPS-induced EMR has been shown to correlate with the increase of peripheral and central proinflammatory cytokines such as TNF, IL-1β, and IL-6 that modulate pain response following injury (Kanaan et al., 2000; Watkins, Maier, & Goehler, 1995). The present experiment examines whether GABA plays a role in LPS-induced EMR.

Procedure

The design of experiment 2 is analogous to that used in Experiment 1 (Figure 1). Spinally-transected and cannulized rat subjects (n=8 per group) were microinjected with either vehicle or 0.3% bicuculline through an intrathecal (i.t.) catheter. Fifteen minutes after drug delivery, subjects in each group received either 100 μg LPS or vehicle (i.t.). This yielded a 2 (bicuculline vs. vehicle) X 2 (LPS vs. vehicle) factorial

design. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to LPS treatment, and again 0, 1, 2, 3 hr after LPS injection.

Results

Prior to treatment, mean baseline scores ranged from 6.26 ± 0.05 to 6.36 ± 0.62 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.0$, $p > .05$.

The effect of bicuculline on LPS-induced EMR is depicted in Figure 3. Before LPS treatment, bicuculline administration (Post Drug) had no effect on mechanical reactivity, $F(3, 20) < 1.0$, $p > .05$. LPS treatment (Post LPS) induced an EMR that lasted 3 hr (Veh-LPS). This effect was blocked by pretreatment with bicuculline (Bic-LPS). An ANOVA performed on the change from baseline scores revealed a significant main effect of LPS, $F(1, 20) = 5.71$, $p < .05$, and bicuculline treatment, $F(1, 20) = 9.28$, $p < .05$. Also, the LPS x bicuculline interaction was significant, $F(1, 20) = 6.16$, $p < .05$. No other terms were statistically significant, all $Fs < 2.49$, $p > .05$. *Post hoc* comparison confirmed that the group that received LPS alone (Veh-LPS) differed from the other groups, $p < .05$. No other group comparisons was significant, $p > .05$.

Vehicle Bicuculline	X	Vehicle LPS

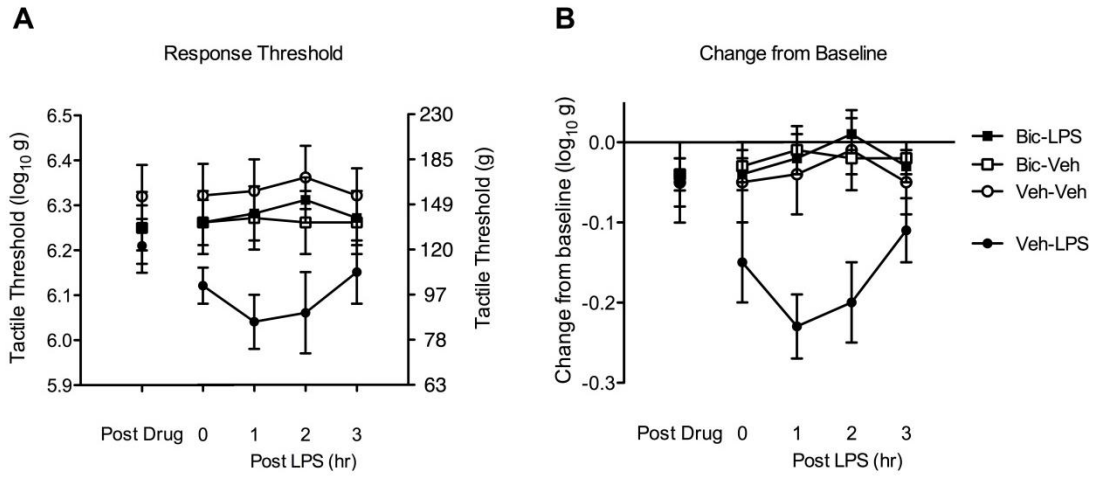

Figure 3. Bicuculline blocked LPS-induced EMR. (A) Subjects that received bicuculline (Bic) or its vehicle (Veh) are depicted as squares and circles, respectively. Groups with LPS or vehicle are shown in black and white, respectively. The left y-axis depicts linearized tactile scores based on a transformation (log 10 [10,000g]) of the force required to bend the thinnest filament that produced a paw withdraw. Tactile reactivity was reassessed after bicuculline treatment (Post Drug), and again at 0, 1, 2, 3 hr after LPS (Post LPS). The right y-axis depicts the gram force equivalents. (B) The change from baseline scores. The error bars depict ± SEM.

Discussion

As expected, LPS induced a lasting EMR. Pretreatment with bicuculline blocked the LPS-induced EMR. These data extend the generality of my finding and suggest that GABAergic system plays a critical role in inflammation-induced mechanical hypersensitivity.

Experiment 3

Experiment 2 showed that bicuculline blocks the induction of LPS-induced EMR. However, manipulations that affect the induction of EMR do not always prove capable of reversing the EMR after it has been induced. Whether drug treatment affects EMR after it is induced is especially important to clinical applications. The present experiment examined whether GABA plays a role in the maintenance of LPS-induced EMR.

Procedure

The design of experiment 3 is depicted in Figure 4. Spinally transected and cannulized subjects (n=8 per condition) were randomly assigned to one of three treatments: vehicle before LPS (Veh-LPS), bicuculline before LPS (Bic-LPS), and bicuculline given one hour after LPS (LPS-Bic). In the first two groups, bicuculline or its vehicle were given (i.t.) prior to LPS treatment. Fifteen minutes after drug delivery, subjects in each group received intrathecal LPS. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to LPS injection, and again at 0, 1, 2, 3 hr

following LPS treatment. In the third group (LPS-Bic), bicuculline (i.t.) was administered immediately after the tactile test 1 hour after LPS treatment. As reported above, the pattern of statistical significance was the same independent of whether the raw scores or a change from baseline scores were analyzed. For this reason, I focus on the change from baseline scores in this experiment.

<table>
<tr><td rowspan="3">Complete transection (T2)</td><td rowspan="3">24 hr</td><td rowspan="3">von Frey baseline</td><td>Vehicle</td><td rowspan="3">von Frey</td><td rowspan="3">LPS</td><td rowspan="3">von Frey 0, 1, hr</td><td>None</td><td rowspan="3">von Frey 2, 3 hr</td></tr>
<tr><td>Bicuculline</td><td>None</td></tr>
<tr><td>None</td><td>Bicuculline</td></tr>
</table>

Figure 4. Experimental design for Experiment 3.

Results

Prior to treatment, mean baseline scores ranged from 6.20 ± 0.04 to 6.3 ± 0.08 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.0$, $p > .05$.

The effect of bicuculline on the maintenance of LPS-induced EMR is depicted in Figure 5. Before LPS treatment, bicuculline administration (Post Drug) had no effect on mechanical reactivity, $F(2, 21) < 1.0$, $p > .05$. In the first hour (0-1 hr) after LPS treatment, LPS (Post LPS) induced a significant EMR (Veh-LPS and LPS-Bic). This effect was blocked by bicuculline pretreatment (Bic-LPS), replicating the results of Experiment 2. An ANOVA showed that the groups were significantly different, $F(2, 21) = 10.32$, $p < .05$. Also, the main effect of time, and its interaction with treatment were statistically significant, all $Fs > 3.69$, $p < .05$. *Post hoc* comparisons of the 0-1 hr means

confirmed that the group received bicuculline pretreatment (Bic-LPS) differed from the other groups, $p < .05$. No other group comparisons were significant, $p > .05$.

During the second and third hour, bicuculline treatment after LPS (LPS-Bic) failed to reverse the LPS-induced EMR. An ANOVA revealed a significant effect of treatment, F (2, 21) = 5.46, $p < .05$. No other term was significant, all $Fs < 1.64$, $p > .05$. *Post hoc* comparisons of the 2-3 hr means confirmed that the group that received bicuculline pretreatment alone (Veh-Cap) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

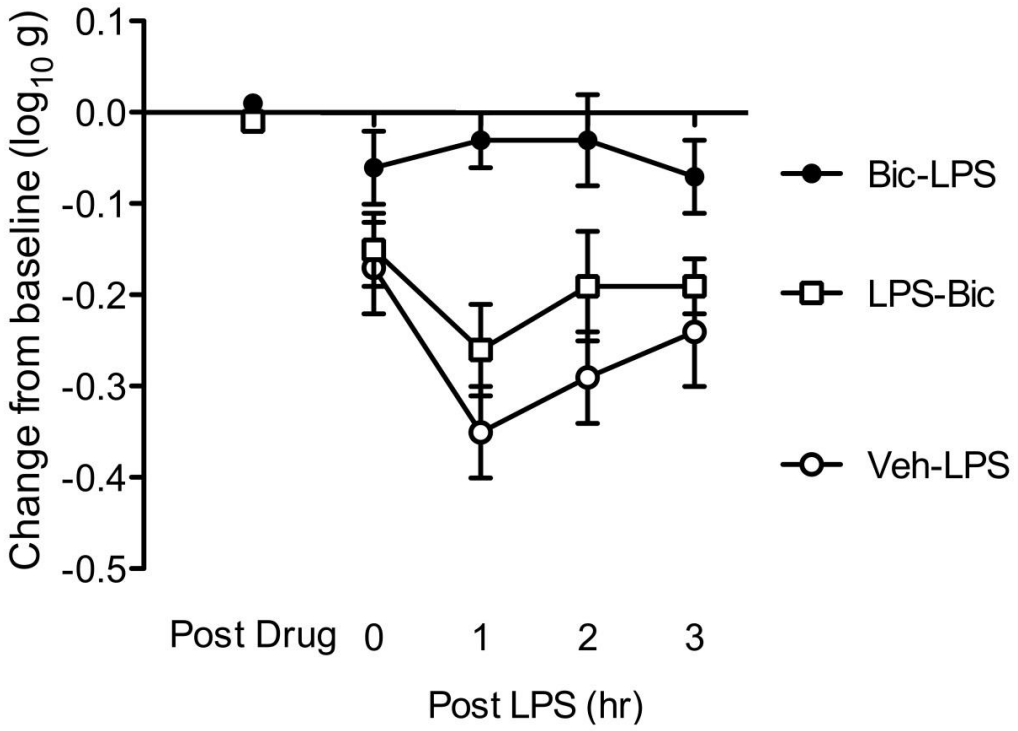

*Figure 5***.** Bicuculline blocked but failed to reverse LPS-induced EMR. Subjects that received vehicle and LPS (Veh-LPS) are depicted as open circles, subjects that received bicuculline before LPS (Bic-LPS) are depicted as black circles, and subjects that received bicuculline 1hr after LPS are depicted as open squares (LPS-Bic). The y-axis depicts the change from baseline after drug treatment (Post Drug), and 0, 1, 2, 3 hr after LPS treatment (Post LPS). The error bars depict ± SEM.

Discussion

Bicuculline pretreatment blocked the LPS-induced EMR, replicating the result from Experiment 2. However, bicuculline treatment 1 hr after LPS failed to reverse the LPS-induced EMR. These data imply that the $GABA_A$ receptor plays a critical role in the induction but not maintenance of LPS-induced EMR.

Experiment 4

Experiments 1 and 2 showed that the $GABA_A$ receptor antagonist bicuculline blocks the induction of shock and LPS induced EMR. These results suggest that spinal $GABA_A$ receptor transmission is a crucial component of the signaling cascade for enhanced mechanical reactivity. Elsewhere, Grau and his colleagues (Grau et al., 2006; Grau et al., 2014; Vichaya et al., 2009; Young et al., 2007) have suggested that VIS and LPS impair learning, and induce EMR, because these treatments diffusely sensitize spinal nociceptive systems (central sensitization). A more direct test of this hypothesis was provided by examining the impact of peripheral treatment with the irritant capsaicin. Capsaicin, a molecule that selectively binds to the TRPV1 receptor, has been widely used as a pain model (Caterina et al., 1997). It has also been shown to induce EMR and a learning deficit in spinalized rats (Hook et al., 2008). The activation of the TRPV1 receptor by capsaicin induces peripheral sensitization and ultimately increases the excitability of CNS neurons (central sensitization, Ji et al., 2003; Woolf, 2011). Here I examined whether bicuculline blocks capsaicin-induced EMR in spinally transected rats.

Procedure

The design of experiment 3 was analogous to that used in Experiments 1 and 2 (Figure 1). Spinally-transected and cannulized rat subjects (n=6 per group) were microinjected with either vehicle or 0.3% bicuculline through the intrathecal catheter (i.t.). Fifteen minutes after drug delivery, subjects in each group received either intradermal 3% capsaicin or its vehicle in the left or right hind paw (counter-balanced across subjects). This yielded a 2 (bicuculline vs. saline) X 2 (capsaicin vs. vehicle) factorial design. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to capsaicin injection, and again 0, 1, 2, 3 hr after capsaicin treatment.

Results

Prior to treatment, mean baseline scores ranged from 6.10 ± 0.05 to 6.29 ± 0.1 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 2.65$, $p > .05$.

The effect of bicuculline on capsaicin-induced EMR is depicted in Figure 6. Before capsaicin treatment, bicuculline administration (Post Drug) had no effect on mechanical reactivity, $F(3, 20) = 1.1$, $p > .05$. As expected, capsaicin induced a weaker EMR on the untreated leg (Figure 6C and 6B; -0.13 ± 0.03) relative to the treated leg (Figure 6A and 6B; -0.5 ± 0.03), $F(1, 20) = 8.37$, $p < .05$. Nevertheless, the overall pattern of results was similar on both legs. Capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Cap) and this effect was blocked by pretreatment with bicuculline (Bic-Cap).

Because similar results were obtained across legs (Figure 6E and 6F) in this and subsequent experiments, we collapsed the data across tested leg. An ANOVA performed on the mean change from baseline scores showed that the main effect of capsaicin and bicuculline, as well as their interaction, were significant, all *Fs* > 47.55, *p* < .05. The time x capsaicin interaction and the three-way interaction of time x capsaicin x bicuculline were also statistically significant, all *Fs* > 2.9, *p* < .05. No other terms were statistically significant, all *Fs* < 4.18, *p* > .05. *Post hoc* comparison confirmed the group that received capsaicin alone (Veh-Cap) differed from the other groups, *p* < .05. No other group comparison was significant, *p* > .05.

Vehicle Bicuculline	X	Vehicle Capsaicin

Treated leg

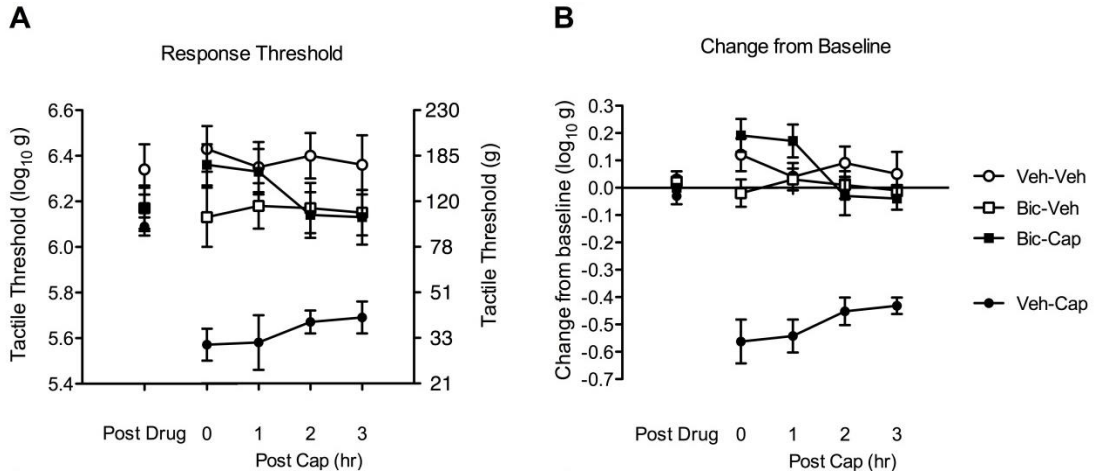

Figure 6. Bicuculline blocked capsaicin induced EMR. Subjects that received bicuculline (Bic) or its vehicle (Veh) are depicted as squares and circles, respectively. Groups treated with capsaicin (Cap) or its vehicle (Veh) are shown in black and white, respectively. (A) Raw data of capsaicin-treated leg. The left y-axis depicts linearized tactile scores based on a transformation (log 10 [10,000g]) of the force required to bend the thinnest filament that produced a paw withdraw. Mechanical reactivity was tested after bicuculline treatment (Post Drug), and again at 0, 1, 2, 3 hr after capsaicin (Post Cap). The right y-axis depicts the gram force equivalents. (B) The change from baseline scores of capsaicin-treated leg. (C) Raw data of untreated leg. (D) The change from baseline scores of untreated leg. (E) Raw data collapsed across test leg. (F) The change from baseline scores averaged over test leg. The error bars depict ± SEM.

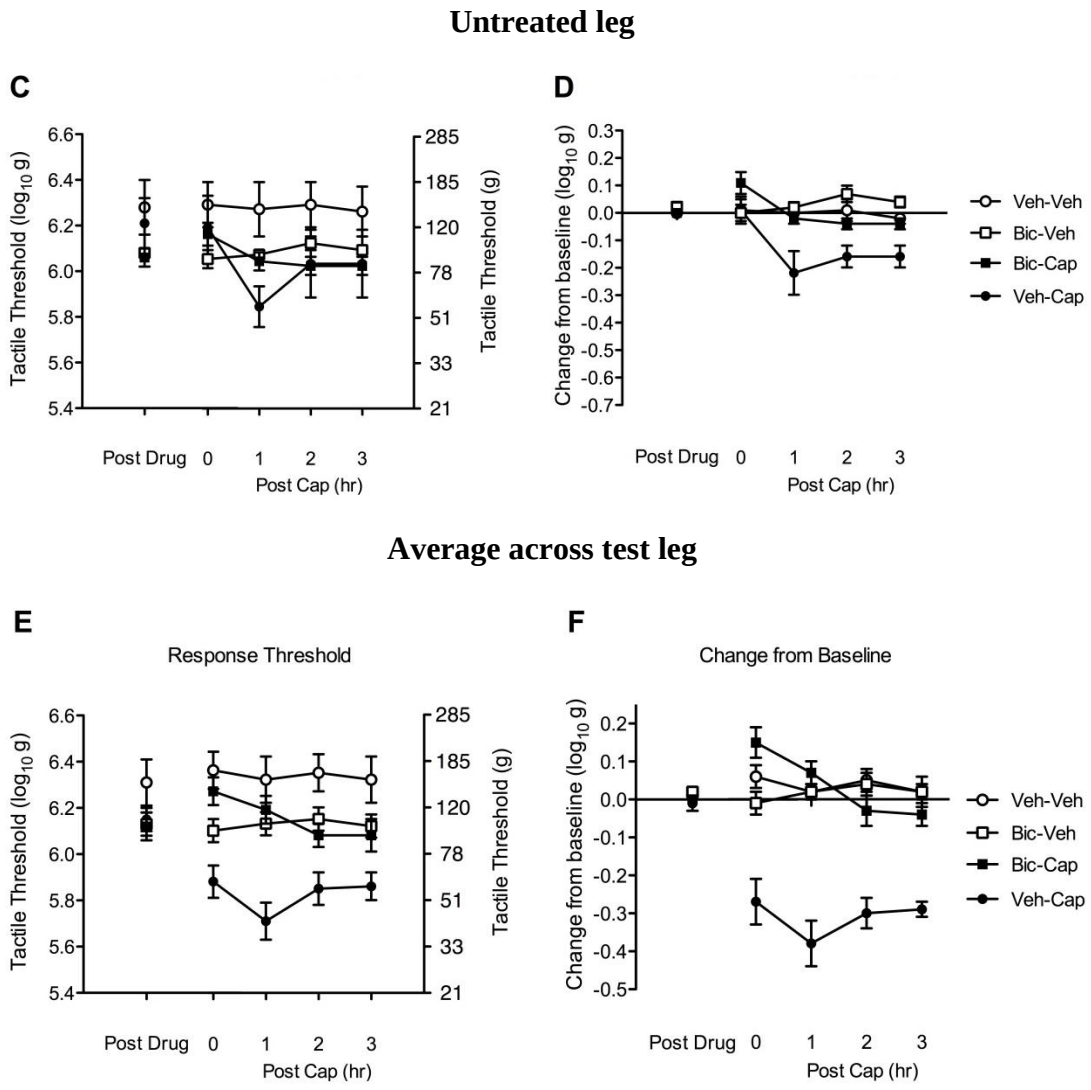

Figure 6. Continued.

Discussion

Capsaicin induced a lasting EMR, replicating previous results (Hook et al., 2008). Pretreatment with bicuculline blocked the capsaicin-induced EMR implying that GABAergic neurons play a role in inflammation-induced EMR.

Experiment 5

Experiment 4 showed that bicuculline blocks the induction of capsaicin-induced EMR. However, manipulations that affect the induction of central sensitization do not always prove capable of reversing the EMR after it has been induced (Grau et al., 2014; Sluka et al., 1994). Whether drug treatment affects central sensitization after it is induced is especially important to clinical applications. The present experiment examined whether GABA plays a role in the maintenance of capsaicin-induced EMR.

Procedure

The design of experiment 5 is depicted in Figure 7. Spinally transected and cannulized subjects (n=6 per condition) were randomly assigned to one of three treatments: vehicle before capsaicin (Veh-Cap), bicuculline before capsaicin (Bic-Cap), and bicuculline given one hour after capsaicin (Cap-Bic). In the first two groups, bicuculline or its vehicle were given (i.t.) prior to capsaicin treatment. Fifteen minutes after drug delivery, subjects in each group received intradermal capsaicin in the left or right hind paw (counter-balanced across subjects). Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to capsaicin injection, and again at 0, 1,

2, 3 hr following capsaicin treatment. In the third group (Cap-Bic), bicuculline (i.t.) was administered immediately after the tactile test 1 hour after capsaicin treatment. As reported above, the pattern of statistical significance was the same independent of whether the raw scores or a change from baseline scores were analyzed. Likewise, while a weaker effect was observed on the untreated leg, the overall pattern was the same. For these reasons, I focus on the change from baseline scores, collapsed across the treated and untreated legs, in this and subsequent experiments.

<table>
<tr><td rowspan="3">Complete transection (T2)</td><td rowspan="3">24 hr</td><td rowspan="3">von Frey baseline</td><td>Vehicle</td><td rowspan="3">von Frey</td><td rowspan="3">Capsaicin</td><td rowspan="3">von Frey 0, 1, hr</td><td>None</td><td rowspan="3">von Frey 2, 3 hr</td></tr>
<tr><td>Bicuculline</td><td>None</td></tr>
<tr><td>None</td><td>Bicuculline</td></tr>
</table>

Figure 7. Experimental design for Experiment 5.

Results

Prior to treatment, mean baseline scores ranged from 6.12 ± 0.07 to 6.19 ± 0.12 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.0$, $p > .05$.

The effect of bicuculline on the maintenance of capsaicin-induced EMR is depicted in Figure 8. Before capsaicin treatment, bicuculline administration (Post Drug) had no effect on mechanical reactivity, $F(2, 15) < 1.0$, $p > .05$. In the first hour (0-1 hr) after capsaicin treatment, capsaicin (Post Cap) induced a significant EMR (Veh-Cap and Cap-Bic). This effect was blocked by bicuculline pretreatment (Bic-Cap), replicating the results of Experiment 4. An ANOVA showed that the groups were significantly different,

F (2, 15) = 9.93, $p < .05$. Also, the main effect of time, and its interaction with treatment were statistically significant, all $Fs > 8.35$, $p < .05$. No other term was significant, all $Fs < 3.35$, $p > .05$. *Post hoc* comparisons of the 0-1 hr means confirmed that the group received bicuculline pretreatment (Bic-Cap) differed from the other groups, $p < .05$. No other group comparisons were significant, $p > .05$.

During the second and third hour, bicuculline treatment after capsaicin (Cap-Bic) reversed the capsaicin-induced EMR. An ANOVA revealed a significant effect of treatment, F (2, 15) = 12.67, $p < .05$. No other term was significant, all $Fs < 2.04$, $p > .05$. *Post hoc* comparisons of the 2-3 hr means confirmed that the group that received capsaicin alone (Veh-Cap) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

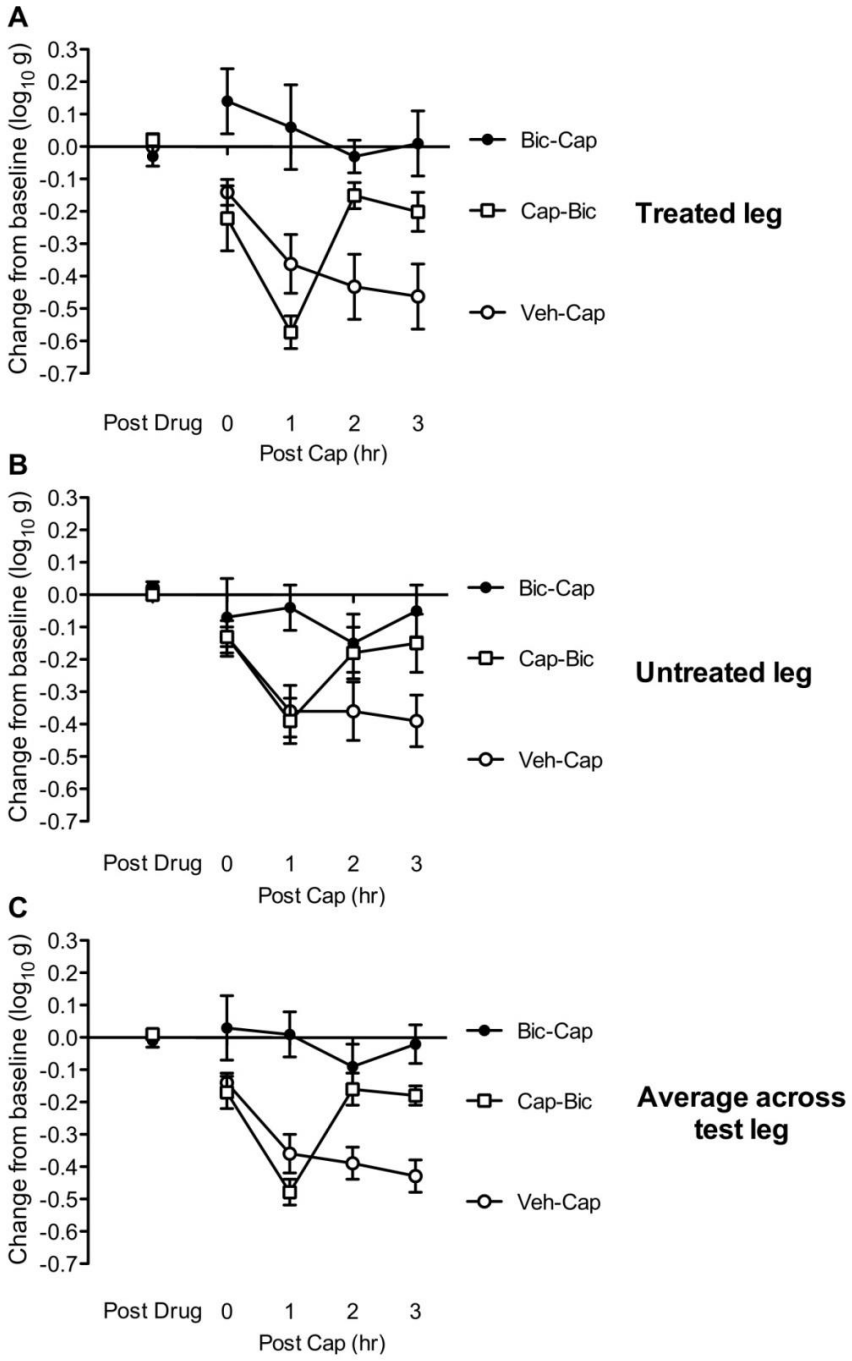

*Figure 8***.** Bicuculline blocked and reversed capsaicin-induced EMR. Subjects that received vehicle and capsaicin (Veh-Cap) are depicted as open circles, subjects that received bicuculline before capsaicin (Bic-Cap) are depicted as black circles, and subjects that received bicuculline 1hr after capsaicin are depicted as open squares (Cap-Bic). (A) The change from baseline scores of capsaicin-treated leg. The y-axis depicts the change from baseline after drug treatment (Post Drug), and 0, 1, 2, 3 hr after capsaicin treatment (Post Cap). (B) The change from baseline scores of untreated leg. (C) The change from baseline scores collapsed across test leg. The error bars depict ± SEM.

Discussion

Capsaicin induced a lasting EMR, replicating the result from Experiment 4. Bicuculline treatment blocked and reversed the capsaicin-induced EMR. These data imply that the $GABA_A$ receptor plays a critical role in both the induction and maintenance of capsaicin-induced EMR.

Experiment 6

Experiments 4 and 5 showed that the $GABA_A$ receptor antagonist bicucullilne can block and reverse capsaicin-induced EMR in transected rats. However, bicuculline also affects Ca^{2+}-activated potassium channels (Khawaled, Bruening-Wright, Adelman, & Maylie, 1999). Given this, I sought further evidence that the $GABA_A$ receptor plays a critical role. This was accomplished by assessing the impact of another $GABA_A$ receptor antagonist (gabazine) on capsaicin-induced EMR in transected rats. This experiment also explores whether a $GABA_B$ receptor antagonist (phaclofen) affects capsaicin-induced EMR (Malan, Mata, & Porreca, 2002).

Procedure

The design of experiment 6 is depicted in Figure 9. Spinally-transected and cannulized rat subjects (n=6 per group) were microinjected with either saline or one of three doses of gabazine (Figure 9A; 0.0 [vehicle], 0.001, 0.01 μg) or phaclofen (Figure 9B; 0.0 [vehicle], 1, 10 μg) through intrathecal catheter (i.t.). Fifteen minutes after drug delivery, subjects in each group received intradermal capsaicin to the left or right hind

paw (counter-balanced across subjects). Tactile reactivity was assessed on each paw prior to drug delivery (baseline), prior to capsaicin injection, and again 0, 1, 2, 3 hr following capsaicin treatment.

A

Complete transection (T2)	24 hrs	von Frey baseline	Vehicle	von Frey	Capsaicin	von Frey 0, 1, 2, 3 hr
			Gabazine 0.001 μ g			
			Gabazine 0.01 μ g			

B

Complete transection (T2)	24 hrs	von Frey baseline	Vehicle	von Frey	Capsaicin	von Frey 0, 1, 2, 3 hr
			Phaclofen 1 μ g			
			Phaclofen 10 μ g			

Figure 9. Experimental design for Experiment 6.

Results

Prior to gabazine or phaclofen treatment, mean baseline scores ranged from 5.99 ± 0.04 to 6.28 ± 0.14 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.7$, $p > .05$.

The effect of gabazine on capsaicin-induced EMR is depicted in Figure 10A. Before capsaicin treatment, drug administration (Post Drug) had no effect on mechanical reactivity, $F(2, 15) < 1.0$, $p > .05$. Capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Cap). This effect was blocked by pretreatment with gabazine (GBZ 0.01, GBZ 0.001). An ANOVA showed that the main effect of drug and time, as well as their interaction, were statistically significant, all $Fs > 29.5$, $p < .05$. No other term was

statistically significant, all *Fs* < 1.0, *p* > .05. *Post hoc* comparison confirmed that the group that received capsaicin alone (Vehicle) differed from the other groups, *p* < .05.

The effect of phaclofen on capsaicin-induced EMR is depicted in Figure 10B. Before capsaicin treatment, drug administration (Post Drug) had no effect on mechanical reactivity, *F* (2, 15) = 1.84, *p* > .05. Capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Cap). This effect was blocked by pretreatment with both dosages of phaclofen (Phaclofen 1, Phaclofen 10). An ANOVA showed that the main effect of drug and time, as well as their interaction, were statistically significant, all *Fs* > 11.64, *p* < .05. No other term was significant, all *Fs* < 1.77, *p* > .05. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Vehicle) differed from the other groups, *p* < .05.

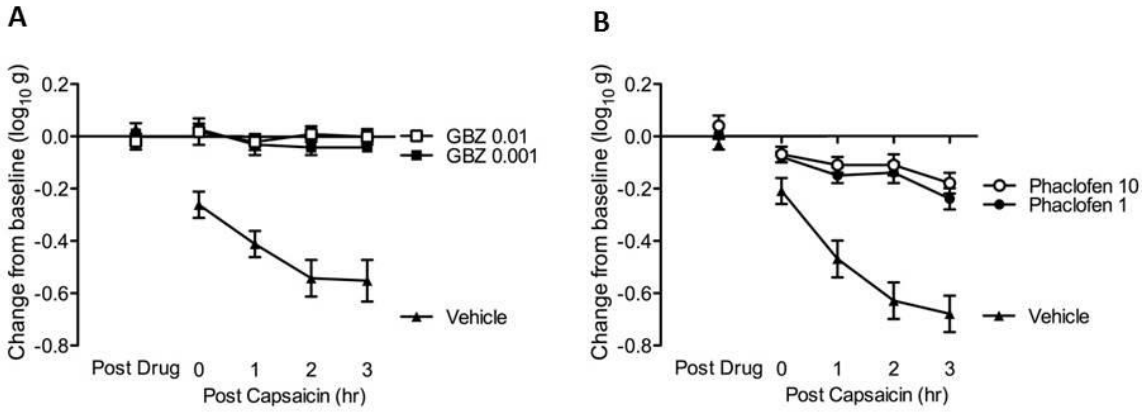

Figure 10. Gabazine and phaclofen blocked capsaicin-induced EMR. Subjects that received gabazine (GBZ) are shown in squares, subjects that received phaclofen are shown in circles, and subjects that received vehicle are shown in triangles. (A) Effect of gabazine on capsaicin induced EMR. The y-axis depicts the change from baseline after drug treatment (Post Drug), and 0, 1, 2, 3 hr after capsaicin treatment (Post Cap). (B) Effect of phaclofen on capsaicin induced EMR. The error bars depict ± SEM.

Discussion

Capsaicin induced a lasting EMR, replicating Experiment 4. Both gabazine and phaclofen treatment blocked the capsaicin-induced EMR. These data provide further evidence that GABAergic transmission plays a critical role in inflammation-induced EMR. While both drugs attenuated capsaicin-induced EMR, only gabazine appeared to fully block this effect, and a significant difference between these two groups was shown. Presumably, this difference in effectiveness is due to difference in how $GABA_A$ versus $GABA_B$ receptors affect cellular function, and/or where the receptors are localized. I will discuss these issues in more detail in the General Discussion.

CHAPTER IV

$GABA_AR$ ANTAGONIST'S EFFECT ON CELLULAR INDICES OF CENTRAL SENSITIZATION

The previous experiments showed that blocking the $GABA_A$ receptor eliminates shock and inflammation induced EMR after SCI (Chapter III). These results suggest that GABAergic mechanisms play a crucial role in EMR induction and expression after spinal transection. The results also imply that these GABAergic systems are involved in the sensitization of nociceptive processes (central sensitization) within the dorsal horn. Behaviorally, central sensitization is evident from pain hypersensitivity, tactile allodynia, and secondary punctate or pressure hyperalgesia. At a cellular level, central sensitization is associated with increased expression of the immediate early gene *c-fos* and phosphorylation of the protein ERK. Here I examine whether bicuculline treatment impacts these cellular indices of central sensitization.

Experiment 7

The induction of central sensitization is correlated with the activation of the immediate early proto-oncogene *c-fos* within the dorsal horn (Gao & Ji, 2009). In this experiment, I tested whether bicuculline treatment attenuates capsaicin-induced *c-fos* expression using qRT-PCR. Another early transcription factor gene, *c-jun*, which forms AP-1 early response transcription factor in combination with *c-fo*s, was also assessed.

Procedure

The design of Experiment 7 is similar to that used in Experiment 4 (see Figure 1). The one change is that von Frey mechanical testing was only conducted for 2 hr after capsaicin treatment. Immediately after the last behavior test, subjects (n=6 per group) were sacrificed. A 1 centimeter of spinal cord around the lumbar enlargement (L3-L5) region was rapidly removed. The spinal cord samples were hemi-dissected into dorsal and ventral halves, and were then subjected to RNA extraction for qRT-PCR.

Results

Prior to treatment, mean baseline scores ranged from 6.16 ± 0.02 to 6.24 ± 0.06 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 2.69$, $p > .05$.

The behavioral data are depicted in Figure 11. Before capsaicin treatment, bicuculline administration (Post Drug) had no effect on mechanical reactivity, F (3, 20) = 2.5, $p > .05$. Replicating the previous result (Experiment 4), capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Cap). This effect was blocked by pretreatment with bicuculline (Bic-Cap). An ANOVA showed that the main effect of capsaicin and bicuculline, as well as their interaction, were significant, all $Fs > 17.71$, $p < .05$. Also, the main effect of time, and its interaction with bicuculline and capsaicin, as well as the time x capsaicin x bicuculline three-way interaction were statistically significant, all $Fs > 3.38$, $p < .05$. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Veh-Cap) differed from the other groups, $p < .05$. In addition, the group that

received bicuculline before capsaicin (Bic-Cap) differed from the other three groups, $p < .05$. No other group comparison was significant, $p > .05$.

Vehicle Bicuculline	X	Vehicle Capsaicin

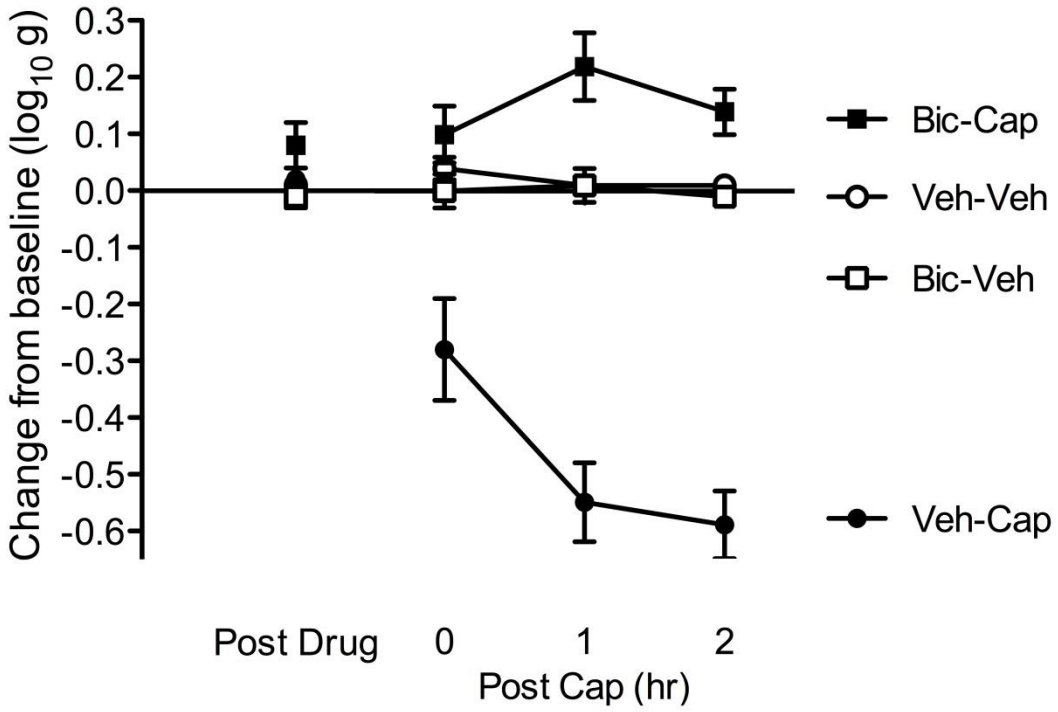

Figure 11. Bicuculline blocked capsaicin-induced EMR. Subjects that received bicuculline (Bic) or vehicle (Veh) are depicted as squares and circles, respectively. Groups treated with capsaicin (Cap) or its vehicle (Veh) are shown in black and white, respectively. The y-axis depicts the change from baseline after bicuculline treatment (Post Drug), and 0, 1, 2 hr after capsaicin treatment (Post Cap). The error bars depict ± SEM.

The PCR results are depicted in Figure 12. The mRNA expression for each gene of interest was normalized to β-actin expression level, and is presented as a fold change relative to the sham controls. In the dorsal region (Figure 12A), capsaicin induced an increase in *c-fos* mRNA expression level. Bicuculline pretreatment reduced the capsaicin induced elevation of *c-fos* level. An ANOVA revealed a significant main effect of capsaicin treatment, F (1, 20) = 23.88, $p < .05$. No other terms were statistically significant, all $Fs < 3.42$, $p > .05$. *Post hoc* comparisons confirmed that the group that received bicuculline before capsaicin (Bic-Cap) differed from the other groups, $p < .05$. Also, the group that received capsaicin alone (Veh-Cap) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

In the ventral region (Figure 12B), capsaicin induced an increase in *c-fos* mRNA expression level that was not affected by bicuculline pretreatment. An ANOVA showed that the main effect of capsaicin treatment was statistically significant, F (1, 20) = 21.22, $p < .05$. No other term was significant, all $Fs < 1.0$, $p > .05$. *Post hoc* comparisons showed that the groups that received capsaicin alone (Veh-Cap) and bicuculline before capsaicin (Bic-Cap) were significantly different from the groups that received bicuculline alone (Bic-Veh) and the control group (Veh-Veh), $p < .05$. No other group comparison was significant, $p > .05$.

Capsaicin treatment also induced an increase in *c-jun* mRNA expression level within the dorsal horn (Figure 12A). However, bicuculline pretreatment did not reduce *c-jun* expression. An ANOVA revealed a significant main effect of capsaicin treatment, F (1, 20) = 13.64, $p < .05$. No other term was statistically significant, all $Fs < 4.17$, p

> .05. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Veh-Cap) differed from the control group (Veh-Veh) and bicuculline alone group (Bic-Veh). Also, the group that received bicuculline before capsaicin (Bic-Cap) differed from the bicuculline alone group (Bic-Veh). No other group comparison was significant, $p > .05$.

A similar pattern of *c-jun* mRNA expression level was observed within the ventral horn (Figure 12B). An ANOVA revealed a significant main effect of capsaicin treatment, $F(1, 20) = 22.47$, $p < .05$. No other term was statistically significant, all $Fs < 1.84$, $p > .05$. *Post hoc* comparisons showed that the groups that received capsaicin alone (Veh-Cap) and bicuculline before capsaicin (Bic-Cap) were significantly different from the groups that received bicuculline alone (Bic-Veh) and the control group (Veh-Veh), $p < .05$. No other group comparison was significant, $p > .05$.

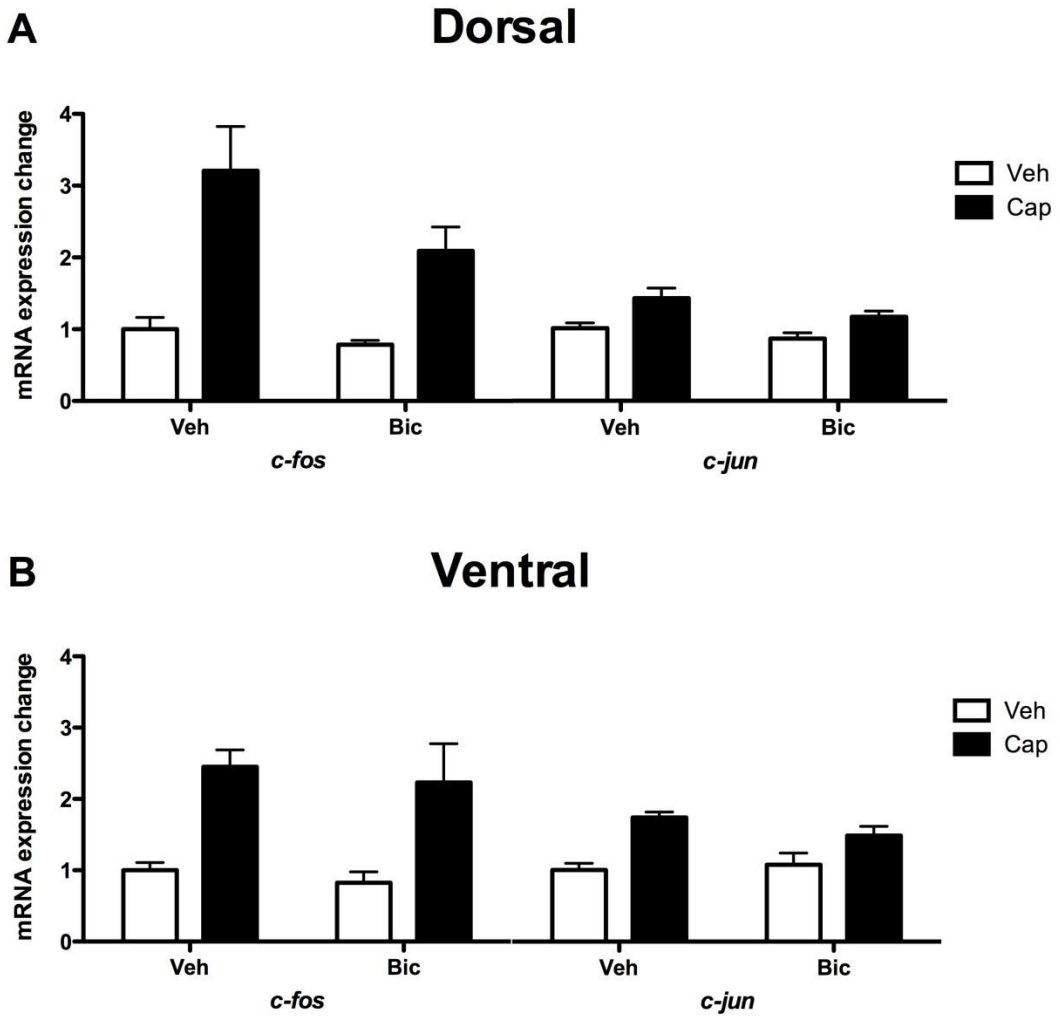

Figure 12. mRNA expression levels after bicuculline and capsaicin treatment. (A) mRNA expression level of *c-fos* and *c-jun* in the dorsal region of the spinal cord. Bicuculline (Bic) or saline (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in white and black bars, respectively. The y-axis depicts the fold change in mRNA expression for each group relative to the control (Veh-Veh) (B) mRNA expression level of *c-fos* and *c-jun* in the ventral region of the spinal cord. The error bars depict ± SEM.

Discussion

As found in Experiment 4, pretreatment with bicuculline blocked the capsaicin-induced EMR. Here, I showed that bicuculline also attenuates *c-fos* mRNA expression within the dorsal horn, a cellular index of central sensitization.

Experiment 8

Experiment 7 showed that bicuculline attenuates capsaicin-induced *c-fos* mRNA expression level within the dorsal horn. Here I examine another cellular marker of central sensitization, the phosphorylation of ERK protein (Gao & Ji, 2009).

Procedure

After RNA extraction in Experiment 7, total protein was extracted from the organic layer of the 48 samples (24 dorsal, 24 ventral), and was used for Western Blotting. The protein expression for each target was normalized to β-actin expression level, and was presented as a fold change relative to the sham controls. Subsequently, the protein expression of pERK1/2 was normalized to ERK1/2 expression yielding a pERK/ERK ratio. Other proteins of interest including CaMKII, BDNF, GAD65/67, TrkB92/145, TNF-α were also tested.

Results

ERK protein levels are depicted in Figure 13. In the dorsal region (Figure 13A), capsaicin induced an increase in ERK phosphorylation (pERK ratio) for both ERK1

(ERK42) and ERK2 (ERK44). Bicuculline pretreatment reduced the capsaicin induced elevation of ERK phosphorylation ratio in both ERK isoforms. For ERK1, an ANOVA revealed a significant main effect of capsaicin treatment, F (1, 20) = 6.98, $p < .05$. For ERK2, an ANOVA showed that the main effect of capsaicin and bicuculline, as well as their interaction, were statistically significant, all $Fs > 5.32$, $p < .05$. No other term was statistically significant, all $Fs < 3.59$, $p > .05$. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Veh-Cap) differed from the other groups for both ERK1 and ERK2, $p < .05$. No other group comparison was significant, $p > .05$.

In the ventral region (Figure 13B), capsaicin induced an increase in ERK phosphorylation (pERK ratio) for both ERK1 and ERK2. Bicuculline pretreatment reduced the capsaicin induced elevation of ERK phosphorylation ratio in both ERK isoforms. For both ERK1 and ERK2, an ANOVA showed that the main effect of bicuculline and capsaicin, as well as their interaction, were statistically significant, all $Fs > 8.13$, $p < .05$. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Veh-Cap) differed from the other groups for both ERK1 and ERK2, $p < .05$. No other group comparison was significant, $p > .05$.

The results for other protein targets are depicted in Figures 14, 15, and 16. In the dorsal region, capsaicin and bicuculline treatment did not affect GAD65 and GAD67 expression (Figure 14A), all $Fs < 1.64$, $p > .05$. In the ventral region (Figure 14B), bicuculline reduced GAD65 expression and capsaicin increased GAD67 expression. An ANOVA showed that the main effect of bicuculline treatment on GAD65, as well as the main effect of capsaicin treatment on GAD67 expression, was statistically significant,

both *Fs* > 5.62, *p* < .05. No other term was significant, all *Fs* < 2.55, *p* > .05. For GAD65, *post hoc* comparisons showed that the group that received capsaicin alone (Veh-Cap) differed from groups that received bicuculline (Bic-Veh, Bic-Cap), *p* < .05. For GAD67, the group that received capsaicin alone (Veh-Cap) differed from the groups that received capsaicin vehicle (Bic-Veh, Veh-Veh), *p* < .05. No other group comparison was significant, *p* > .05.

For TrkB92 and TrkB145 within the dorsal region (Figure 15A), capsaicin treatment reduced expression in vehicle treated subjects, but enhanced expression in bicuculline treated rats, yielding a significant interaction, both *Fs* > 4.47, *p* < .05. No other term was statistically significant, all *Fs* < 3.8, *p* > .05. *Post hoc* comparison showed that the group that received bicuculline alone (Bic-Veh) differed from the sham group (Veh-Veh) for TrkB145, *p* < .05. No other group comparison was significant, *p* > .05. In the ventral region (Figure 15B), capsaicin induced an increase in TrkB92 expression and bicuculline reduced TrkB145 expression. An ANOVA showed that the main effect of capsaicin treatment on TrkB92 expression, and the main effect of bicuculline treatment on TrkB145 expression, were statistically significant, both *Fs* > 12.47, *p* < .05. No other term was significant, all *Fs* < 1.84, *p* > .05. For TrkB92, *post hoc* comparisons showed that the group that received capsaicin alone (Veh-Cap) differed from the groups that received capsaicin vehicle (Veh-Veh, Bic-Veh), *p* < .05. For TrkB145, the groups that received bicuculline (Bic-Veh, Bic-Cap) differed from the groups that did not receive bicuculline (Veh-Veh, Veh-Cap), *p* < .05. No other group comparison was significant, *p* > .05.

For CAMKII in the dorsal region (Figure 16A), neither capsaicin nor bicuculline treatment had a significant effect, all $Fs < 2.77$, $p > .05$. In the ventral region (Figure 16D), capsaicin induced a significant increase of CAMKII expression. An ANOVA showed that the main effect of capsaicin was statistically significant, F (1, 20) = 14.56, $p < .05$. No other term was significant, all $Fs < 1.58$, $p > .05$. *Post hoc* comparisons showed that the group that received capsaicin alone (Veh-Cap) differed from the groups that did not receive capsaicin (Veh-Veh, Bic-Veh), $p < .05$. No other group comparison was significant, $p > .05$.

For BDNF in the dorsal region (Figure 16B), neither capsaicin nor bicuculline treatment had a significant effect, all $Fs < 2.25$, $p > .05$. In the ventral region (Figure 16E), capsaicin induced an increase in BDNF expression. An ANOVA showed that the main effect of capsaicin was statistically significant, F (1, 20) = 7.01, $p < .05$. No other term was significant, all $Fs < 1.29$, $p > .05$. *Post hoc* comparisons showed that the group that received bicuculline alone (Bic-Veh) differed from the groups that received capsaicin (Veh-Cap, Bic-Cap), $p < .05$. No other group comparison was significant, $p > .05$.

For TNFα in the dorsal region (Figure 16C), neither capsaicin nor bicuculline had a significant effect, all $Fs < 3.85$, $p > .05$. In the ventral part (Figure 16F), capsaicin induced an increase in TNFα expression. An ANOVA showed that the main effect of capsaicin was statistically significant, F (1, 20) = 46.04, $p < .05$. No other term was significant, all $Fs < 2.4$, $p > .05$. *Post hoc* comparisons showed that the groups that

received capsaicin (Veh-Cap, Bic-Cap) differed from the groups that received its vehicle (Veh-Veh, Bic-Veh) $p < .05$. No other group comparison was significant, $p > .05$.

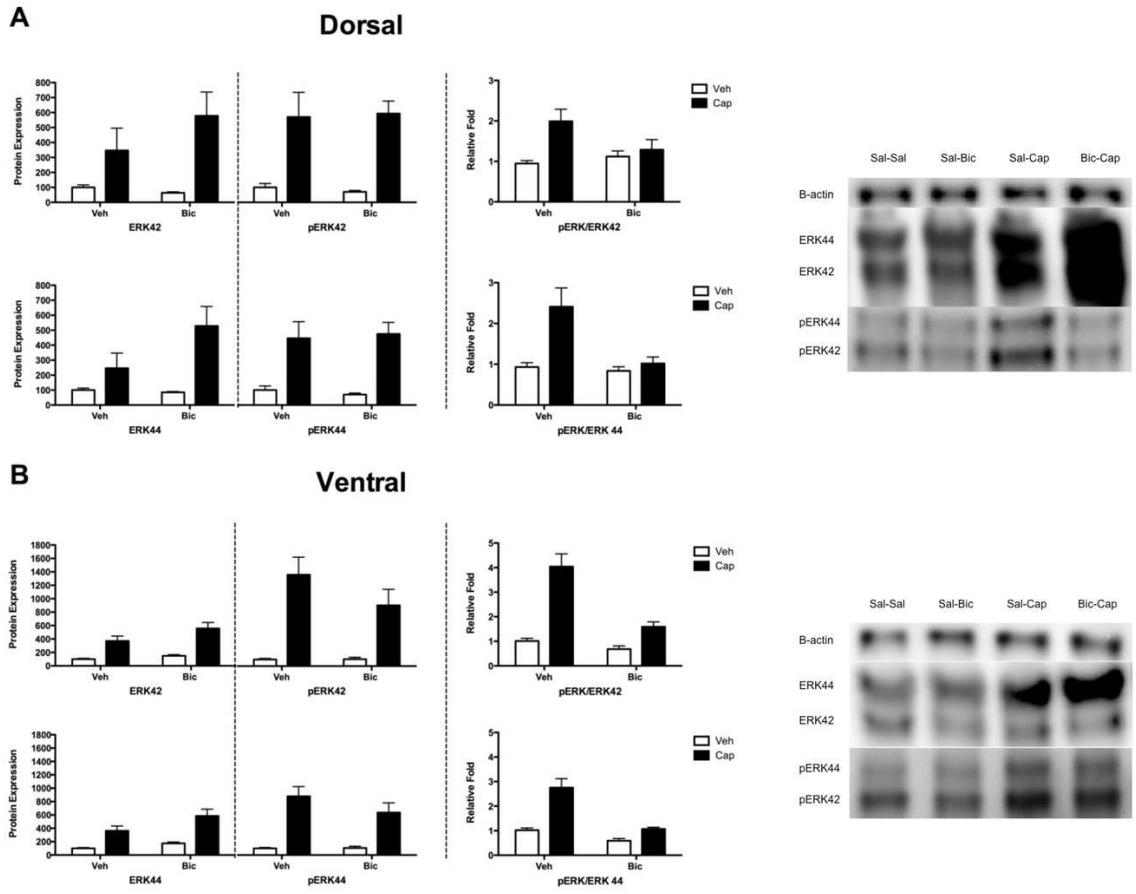

Figure 13. Protein expression of ERK, pERK and the pERK ratio after bicuculline and capsaicin treatment. (A) Expression in the dorsal region of the spinal cord. Bicuculline (Bic) and vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in black and white bars, respectively. ERK42 is shown in the upper part, and ERK44 is shown in the lower part of the panel. The y-axis depicts the fold change of protein expression for each group relative to the control (Veh-Veh). (B) Protein expression in the ventral region of the spinal cord. The error bars depict ± SEM.

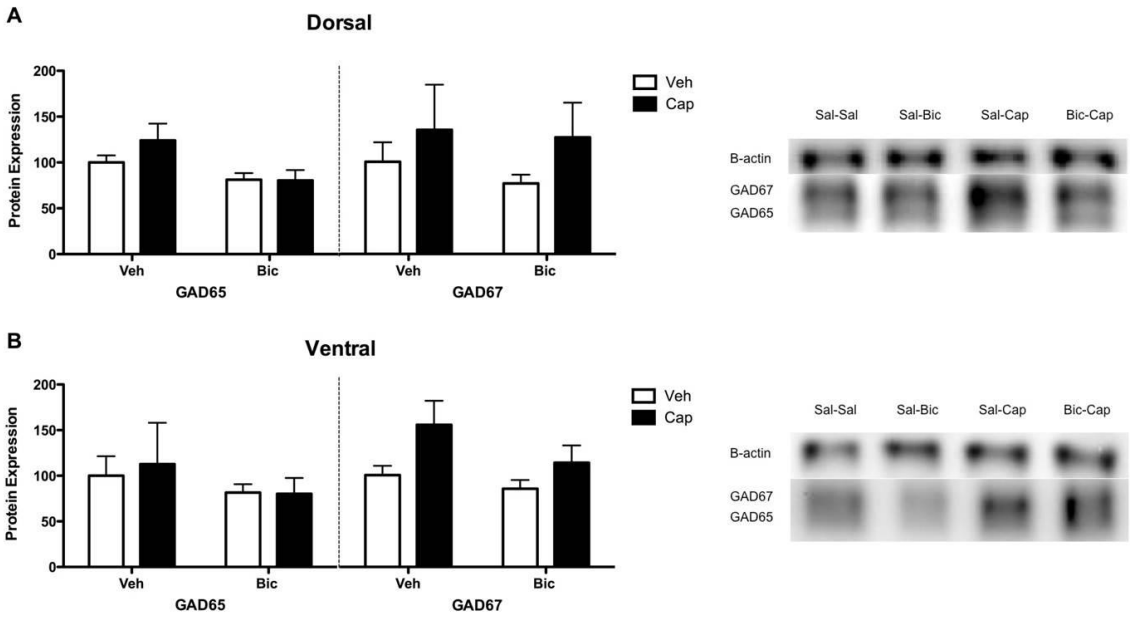

Figure 14. Protein expression of GAD65 and GAD67 after bicuculline and capsaicin treatment. (A) Expression in the dorsal region of the spinal cord. Bicuculline (Bic) or vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in black and white bars, respectively. GAD65 is shown in the left part, and GAD67 is shown in the right part of the figure. The y-axis depicts the fold change of protein expression for each group relative to the control (Veh-Veh). (B) Protein expression level in the ventral region of the spinal cord. The error bars depict ± SEM.

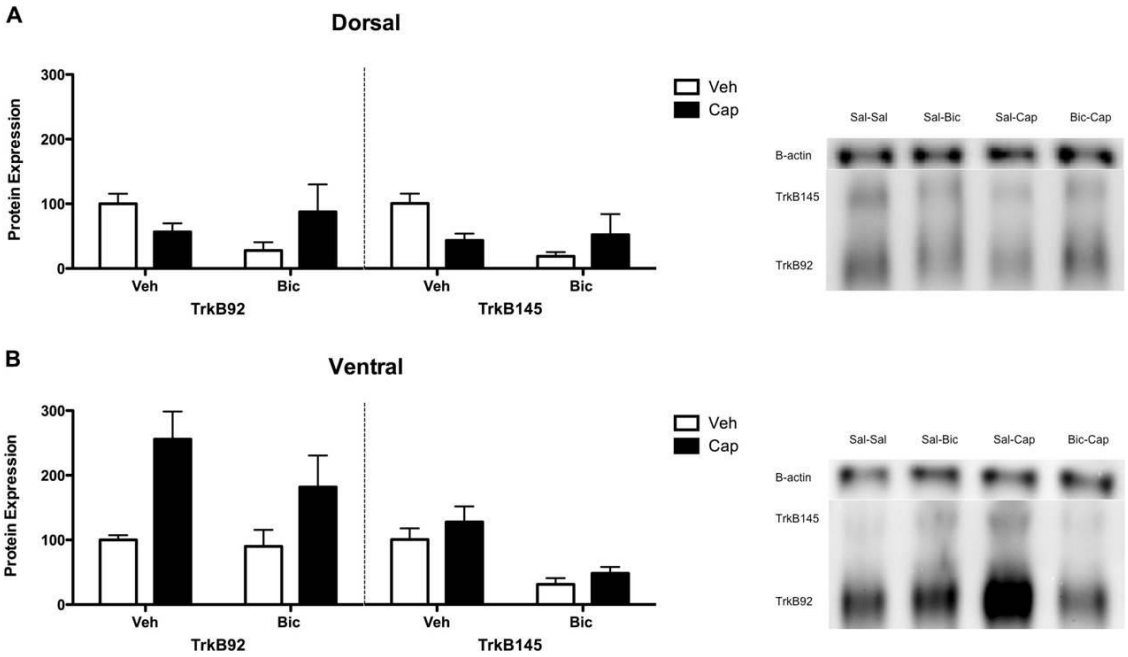

Figure 15. Protein expression of Trk92 and Trk145 after bicuculline and capsaicin treatment. (A) Expression in the dorsal region of the spinal cord. Bicuculline (Bic) or vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in black and white bars, respectively. Trk92 is shown in the left part, and Trk145 is shown in the right part of the figure. The y-axis depicts the fold change of protein expression for each group relative to the control (Veh-Veh). (B) Protein expression level in the ventral region of the spinal cord. The error bars depict ± SEM.

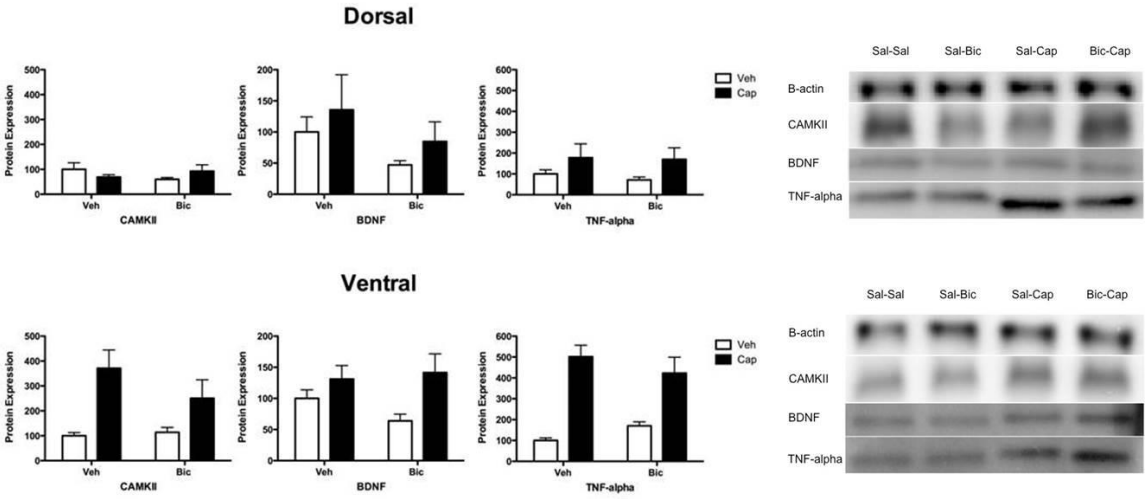

Figure 16. Protein expression of CAMKII, BDNF, and TNFα after bicuculline and capsaicin treatment in the dorsal (A-C) and ventral (D-F) tissue. (A and D) Protein expression level of CAMKII. Bicuculline (Bic) or vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in black and white bars, respectively. The y-axis depicts the fold change of protein expression for each group relative to the control (Veh-Veh). (B and E) Protein expression level of BDNF. (C and F) Protein expression level of TNFα. The error bars depict ± SEM.

Discussion

Capsaicin treatment increased expression of ERK, pERK, and pERK ratio in both dorsal and ventral region of the spinal cord. Bicuculline pretreatment was shown to attenuate the capsaicin-induced pERK ratio. These data provide further cellular evidence that bicuculline attenuates central sensitization in transected rats. For proteins involved in GABA synthesis, GAD65 and GAD67, bicuculline treatment reduced GAD65 expression and capsaicin treatment increased GAD67 expression in the ventral region of the spinal cord. The unique distributions and expression patterns of GAD67 and GAD65 suggest divergent functional roles of them. GAD67 predominates early in development and after neuronal injury and may subserve an intracellular GABA pool, whereas GAD65 is usually expressed later in development and is subject to regulation by cofactor binding and neuronal activity (Pinal & Tobin, 1998). The reduction of GAD65 in bicuculline treated rats presumably implies a decrease of GABAergic signaling, because bicuculline has already blocked central sensitization. The increase of GAD67 expression in capsaicin treated group could reflect the contributions of synaptogenesis and protection to capsaicin treatment.

Bicuculline treatment was shown to reduce BDNF expression in both dorsal and ventral region. For the BDNF receptor protein, TrkB, capsaicin treatment showed a tendency to reduced expression of TrkB proteins in vehicle treated subjects, but enhanced expression in bicuculline treated subjects. Thus, bicuculline has a reducing effect on BDNF signaling, whereas capsaicin has the opposite effect — induces BDNF signaling. A possible explanation for the opposing effect of bicuculline and capsaicin on

BDNF signaling is that BDNF protects capsaicin-induced central sensitization. From this perspective, BDNF signaling is reduced in bicuculline treated subjects because central sensitization has already been blocked by bicuculline. In addition, capsaicin treatment enhanced CAMKII and TNF-alpha expression in the ventral region of the spinal cord.

CHAPTER V

$GABA_AR$ ANTAGONIST'S EFFECT ON EMR IN SHAM-OPERATED INTACT RATS

In Chapter III and IV, the results showed that the $GABA_A$ receptor plays a role in inflammation induced EMR and central sensitization. These data stand in contrast to work examining the effect of bicuculline treatment in intact rats, which has generally found that the drug induces EMR (Sorkin, Puig, & Jones, 1998; Zhang, Hefferan, & Loomis, 2001). These observations suggest that the same experimental manipulation may have opposite effects on capsaicin-induced EMR in intact and transected subjects. Here, I explore this possibility by testing the effect of bicuculline treatment on capsaicin-induced EMR in intact (sham-operated) rats.

Experiment 9

I first accessed whether the $GABA_AR$ antagonist bicuculline enhances, rather than blocks capsaicin-induced EMR in intact rats. To facilitate comparison across experiments, all details (including surgery) were the same as Experiment 4, except the spinal cord was not transected.

Procedure

The design of experiment 9 is similar to that used in Experiment 7 (see Figure 1). The one change is that subjects received a sham surgery rather than transection. Immediately after the last behavior test, subjects (n=8 per group) were sacrificed.

Results

Prior to treatment, mean baseline scores ranged from 5.97 ± 0.04 to 6.22 ± 0.08 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 2.4$, $p > .05$.

The behavioral data are depicted in Figure 17. Before capsaicin treatment, bicuculline administration (Post Drug) had a significant effect on mechanical reactivity, $F\ (1, 28) = 50.31$, $p > .05$. Capsaicin and bicuculline treatment (Post Cap) both induced a lasting EMR (Bic-Veh, Veh-Cap). In addition, bicuculline treatment failed to block the capsaicin-induced EMR (Bic-Cap). An ANOVA showed that the main effect of bicuculline was significant, $F\ (1, 28) = 34.6$, $p > .05$. Also, the main effect of time, and its interaction with bicuculline and capsaicin were statistically significant, all $Fs > 5.13$, $p < .05$. *Post hoc* comparisons confirmed that the group that received capsaicin alone (Veh-Cap) differed from the other groups, $p < .05$. In addition, the vehicle treated group (Veh-Veh) differed from groups that received bicuculline (Bic-Veh, Bic-Cap), $p < .05$. No other group comparison was significant, $p > .05$.

Vehicle Bicuculline	X	Vehicle Capsaicin

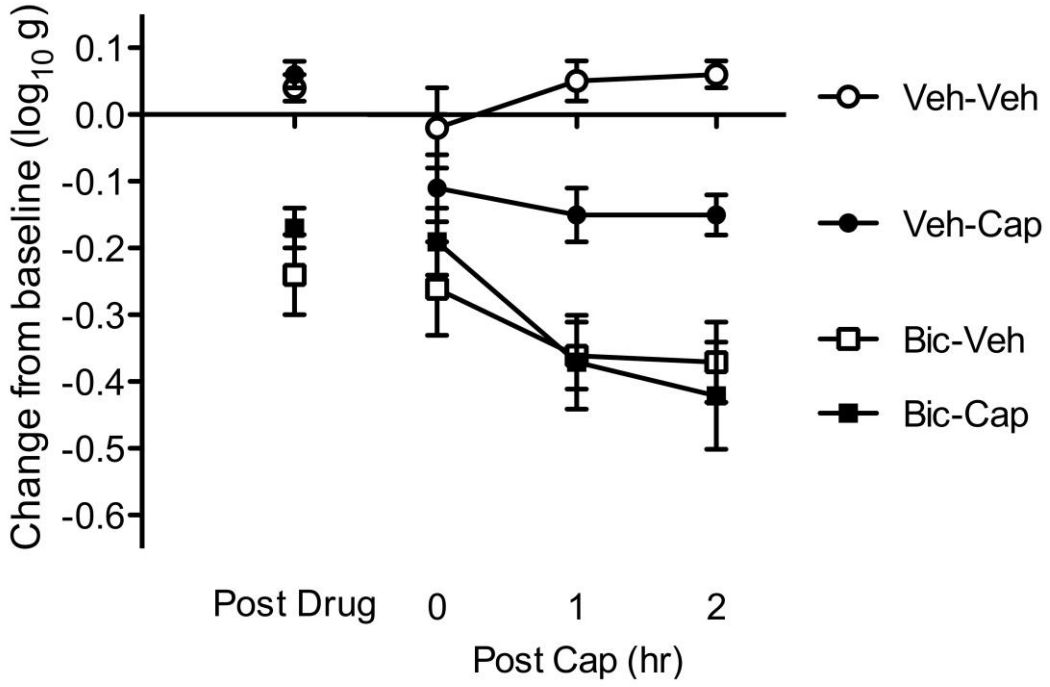

*Figure 17***.** Bicuculline failed to block capsaicin-induced EMR in intact rats. Subjects that received bicuculline (Bic) and vehicle (Veh) are depicted as squares and circles, respectively. Groups treated with capsaicin (Cap) or its vehicle (Veh) are shown in black and white, respectively. The y-axis depicts the change from baseline after bicuculline treatment (Post Drug), and 0, 1, 2 hr after capsaicin treatment (Post Cap). The error bars depict ± SEM.

Discussion

Bicuculline induced a lasting EMR, replicating previous results (Sorkin et al., 1998; Zhang et al., 2001), and failed to block the capsaicin-induced EMR. These data imply that spinal injury alters GABAergic function and transforms how bicuculline affects capsaicin-induced EMR.

Experiment 10

Experiment 9 showed that bicuculline does not block capsaicin-induced EMR in intact subjects. In this experiment, I tested whether bicuculline treatment affects *c-fos* expression in uninjured rats using qRT-PCR. Again, *c-jun* was also assessed.

Procedure

Two hour after capsaicin treatment, subjects from Experiment 9 were sacrificed. A 1 centimeter of spinal cord around the lumbar enlargement (L3-L5) region was rapidly removed. These spinal cord samples were hemi-dissected into dorsal and ventral halves, and were then subjected to RNA extraction for qRT-PCR.

Results

The PCR results are depicted in Figure 18. The mRNA expression for each gene of interest was normalized to β-actin expression level, and is presented as a fold change relative to the sham controls. In the dorsal region (Figure 18A), capsaicin induced an increase in *c-fos* mRNA expression level. Bicuculline pretreatment failed to

reduce the capsaicin induced elevation of *c-fos* level. An ANOVA revealed a significant main effect of capsaicin treatment, F (1, 20) = 6.33, $p < .05$. No other term was statistically significant, all Fs < 1.0, $p > .05$. *Post hoc* comparisons confirmed that the group that received bicuculline before capsaicin (Bic-Cap) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$. In the ventral region (Figure 18B), neither capsaicin nor bicuculline treatment had a statistically significant effect on *c-fos* expression, all Fs < 1.17, $p > .05$.

Neither capsaicin nor bicuculline treatment had a statistically significant effect on *c-jun* mRNA expression within the dorsal or ventral region (Figure 18A, 18B), all Fs < 1.0, $p > .05$.

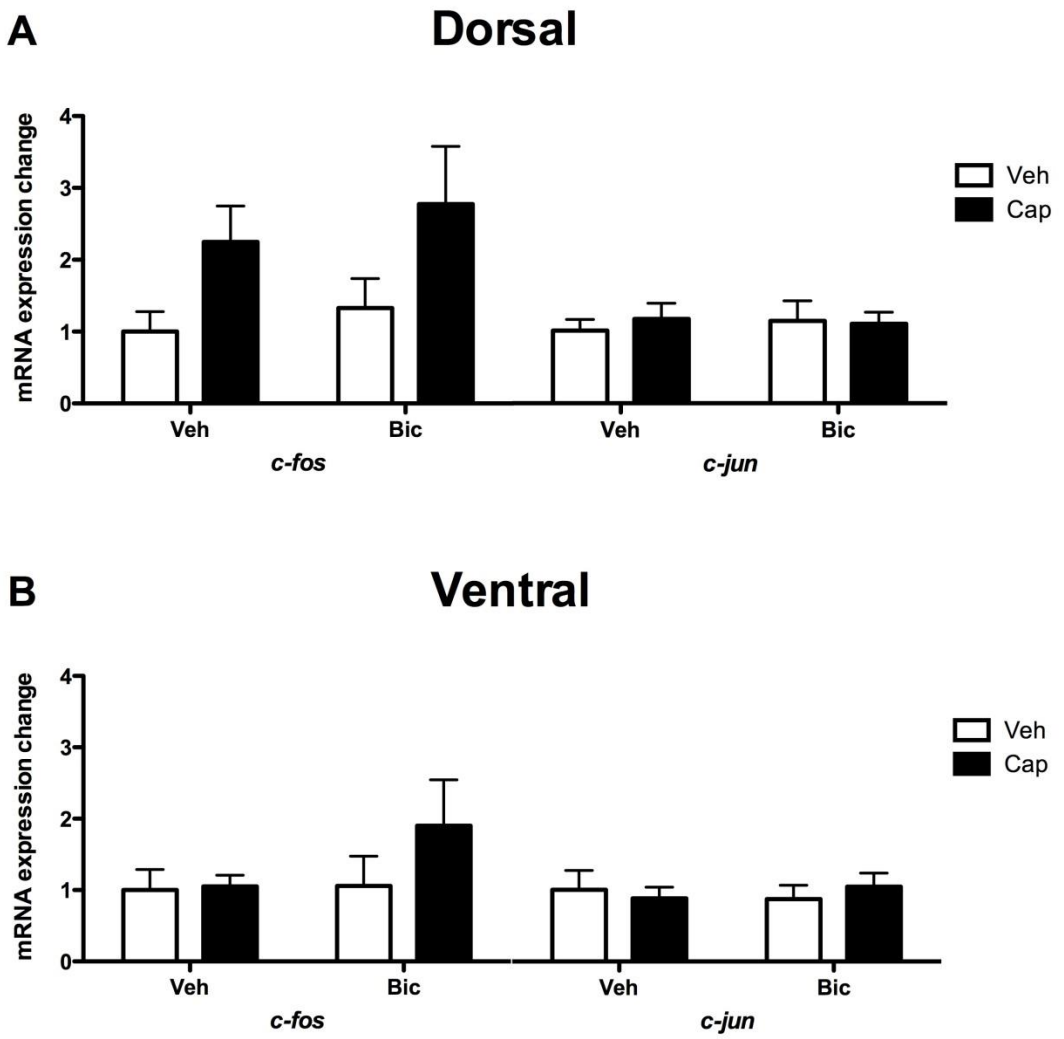

Figure 18. c-fos and *c-jun* mRNA expression after bicuculline and capsaicin treatment in intact rats. (A) mRNA expression in the dorsal region of the spinal cord. Bicuculline (Bic) or vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in white and black bars, respectively. The y-axis depicts the fold change in mRNA expression relative to the control (Veh-Veh) (B) mRNA expression in the ventral region of the spinal cord. The error bars depict ± SEM.

Discussion

In contrast to Experiment 7, which showed bicuculline attenuate capsaicin-induced *c-fos* expression in transected rats, drug treatment had no effect in intact rats.

Experiment 11

Experiment 10 showed that bicuculline does not attenuate capsaicin-induced *c-fos* mRNA expression level within the dorsal horn in intact rats. Here I examine another cellular marker of central sensitization, phosphorylate on ERK (pERK) protein.

Procedure

After RNA extraction in Experiment 10, total protein was extracted from the organic layer of the 48 samples (24 dorsal, 24 ventral), and was used for Western Blotting. The protein expression for each target was normalized to β-actin expression level, and was presented as a fold change in experimental groups relative to the sham controls. Subsequently, the protein expression of pERK1/2 was normalized to ERK1/2 expression yielding a pERK/ERK ratio.

Results

ERK protein levels are depicted in Figure 19. In the dorsal region (Figure 19A), bicuculline and capsaicin treatment both induced an elevation in ERK1 (ERK44) and ERK2 (ERK42), all $Fs > 4.55$, $p < .05$. *Post hoc* comparisons confirmed that the vehicle-treated (Veh-Veh) group differed from the other groups in ERK1, $p < .05$. For ERK2,

the vehicle-treated group (Veh-Veh) differed from the groups that received bicuculline (Bic-Veh, Bic-Cap), $p < .05$. No other group comparison was significant, $p > .05$. For pERK, capsaicin induced an elevation in both pERK1 and pERK2, both $Fs > 9.04$, $p < .05$. *Post hoc* comparisons confirmed that the groups that received bicuculline before capsaicin (Bic-Cap) differed from the groups that received capsaicin vehicle (Veh-Veh, Bic-Veh) in pERK1. In addition, the vehicle treated group (Veh-Veh) differed from the groups that received capsaicin (Veh-Cap, Bic-Cap) in pERK2, $p < .05$. No other group comparison was significant, $p > .05$. For pERK ratio, neither capsaicin nor bicuculline had statistically significant effect on ERK phosphorylation for ERK1 or ERK2, all $Fs < 2.98$, $p > .05$.

In the ventral region (Figure 19B), neither bicuculline nor capsaicin had significant effect on ERK and pERK expression for both isoforms, all $Fs < 2.65$, $p > .05$. For pERK ratio, capsaicin induced an increase in ERK phosphorylation in ERK1. Bicuculline pretreatment failed to reduce the capsaicin induced elevation of ERK phosphorylation ratio. An ANOVA showed that the main effect of capsaicin was statistically significant, $F(1, 20) = 6.48$, $p < .05$. No other term was statistically significant, all $Fs < 3.38$, $p > .05$. *Post hoc* comparisons showed that the group that received bicuculline before capsaicin treatment (Bic-Cap) differed from the group that received bicuculline alone (Bic-Veh) in ERK1, $p < .05$. No other group comparison was significant, $p > .05$.

In addition, the overall expression of ERK and pERK protein in dorsal region of the spinal cord in intact and transected rats was examined. A comparison of the results

(Figure 13 versus Figure 19) suggests that spinal transection increased the expression of ERK and pERK protein. An ANOVA showed that the main effect of transection was statistically significant for both ERK and pERK, all $Fs > 4.74$, $p < .05$.

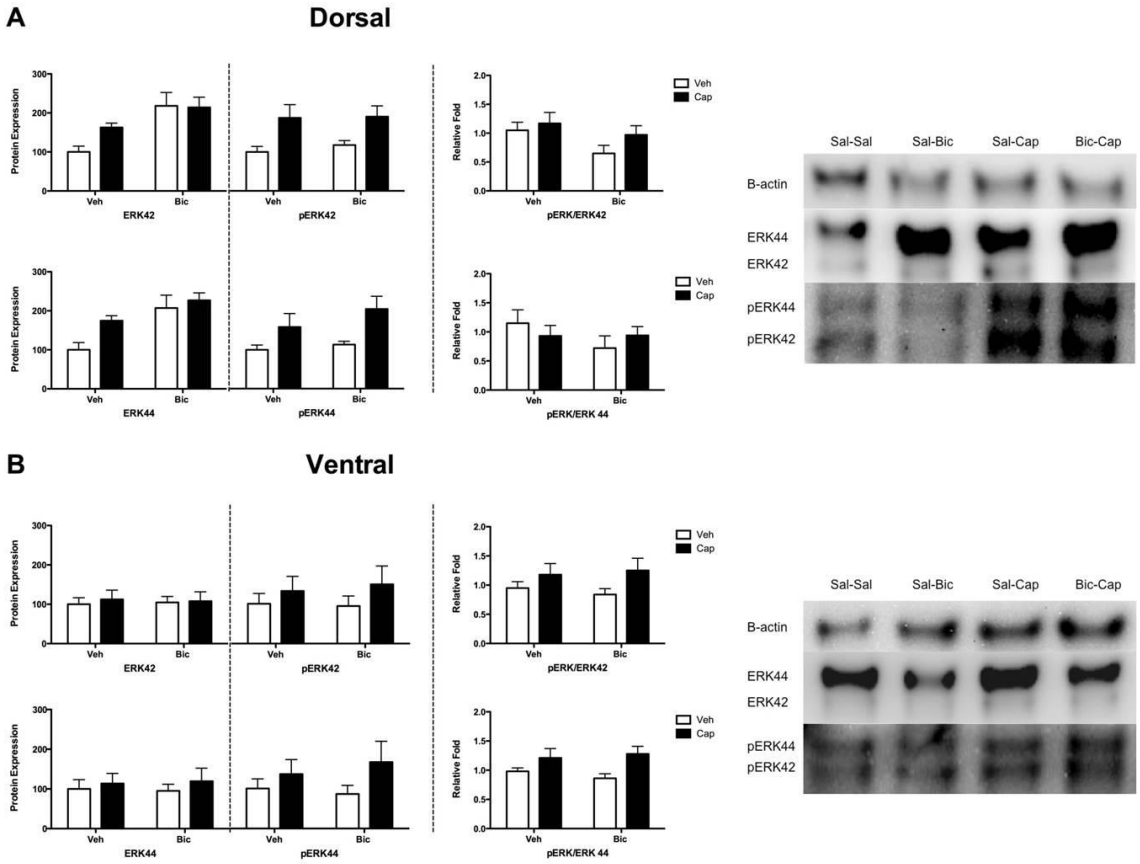

Figure 19. Protein expression of ERK, pERK, and the pERK ratio after bicuculline and capsaicin treatment in intact rats. (A) Expression in the dorsal region of the spinal cord. Bicuculline (Bic) or vehicle (Veh) treatment are shown on the x-axis. Subjects that received capsaicin (Cap) or its vehicle (Veh) are shown in black and white bars, respectively. ERK42 is shown in the upper part, and ERK44 is shown in the lower part of the figure. The y-axis depicts the fold change of protein expression for each group relative to the control (Veh-Veh). (B) Protein expression in the ventral region of the spinal cord. The error bars depict ± SEM.

Discussion

I found that capsaicin induced ERK and pERK expression in the dorsal horn and that the effect was not blocked by bicuculline. These data provide further cellular evidence that bicuculline pretreatment fails to block central sensitization in intact subjects. Comparing these results to those obtained in transected rats (Experiment 8), it appears that the impact of bicuculline on capsaicin-induced central sensitization depends on the integrity of spinal circuits — that a spinal transection switches GABAergic function. Further, the protein expression level changes of transected rats are much larger than those of intact rats, suggesting that descending system normally inhibits the development of central sensitization.

CHAPTER VI

SPINAL TRANSECTION SWITCHES GABA FROM INHIBITORY TO EXCITATORY

Results from Chaper IV and V showed that blocking the $GABA_A$ receptor yields opposite effects in spinally transected and intact rats. This alternation in GABAergic function may be due to an injury-induced change in cation-chloride cotransporters. The chloride gradient in the neural membrane is critically important to $GABA_A$ receptor mediated inhibitory function because the $GABA_A$ receptor is anion-permeable (Kaila, 1994). Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and K^+-Cl^- cotransporter 2 (KCC2) are the two chloride transporters that regulate the intracellular chloride concentration ($[Cl^-]_{intra}$). NKCC1 transports the Cl^- into the cell and KCC2 transports the Cl^- out of cell into the extracellular space. Research has shown that SCI upregulates NKCC1 whereas KCC2 is downregulated in the spinal cord, which correlates with allodynia and hyperalgesia (Cramer et al., 2008; Hasbargen et al., 2010). The upregulation of NKCC1 and downregulation of KCC2 produce high $[Cl^-]_{intra}$ that facilitates efflux of Cl^- when the $GABA_A$ receptor is activated, which leads to depolarization (excitatory) rather than hyperpolarization (inhibitory). This $GABA_AR$-mediated membrane depolarizations can cause activation of cation channels, such as voltage-dependent Na^+ and Ca^{2+} channels that generate excitation of neurons, and lead to the enhanced nociceptive transmission, rather than inhibition after SCI. Here I examine whether spinal injury affects KCC2 membrane-bound levels and whether a change in

channel function can explain why bicuculline treatment has opposite effects in injured and intact rats.

Experiment 12

Previous work suggests that spinal transection transforms the action of GABA, causing it to have an excitatory effect. This biological switch of GABA from inhibitory to excitatory may be due to the down-regulation of membrane-bound KCC2 and loss of descending control from the brain (Ben-Ari, 2002; Bos et al., 2013; Boulenguez et al., 2010). To investigate whether the opposing effect of $GABA_A$ receptor blockage in the transected and intact rats is due to a change in KCC2 protein expression, I evaluated KCC2 protein expression levels using Western Blotting.

Procedure

The design of experiment 12 is depicted in Figure 20. Twelve rats were randomly assigned to receive a spinal transection at T2 or sham-operation. Baseline behavioral reactivity was tested using von Frey stimuli 24 hr later. Subjects were then sacrificed and a one-centimeter section of the spinal cord containing the lumbar enlargement (L5-L6) region was rapidly removed. These spinal cord samples were hemi-dissected into dorsal and ventral halves, and then went through homogenization, protein extraction, and fractionation (membrane-bound and cytoplasmic fraction) for Western Blotting. KCC2 protein expression was normalized to β-actin expression level, and was presented as a fold change in the transected group relative to the sham group.

Subsequently, the protein expression of membrane-bound fraction was normalized to the cytoplasmic fraction yielding a membrane-bound/cytoplasmic ratio.

Complete transection (T2)	24 hr	von Frey Baseline	Tissue collection
Sham operated			

Figure 20. Experimental design for Experiment 12.

Results

The behavioral data are depicted in Figure 21. Intact rats were more responsive than spinally transected subjects. An ANOVA revealed a significant main effect of transection, F (1, 10) = 18.34, $p < .05$.

KCC2 protein expression levels are depicted for the membrane-bound and cytoplasmic fractions (Figure 22A and B). Transection induced a reduction in membrane-bound KCC2 and an increase in cytoplasmic KCC2. An ANOVA showed that the main effect of fraction, and its interaction with transection, were statistically significant, both $Fs > 10.75$, $p < .05$. *Post hoc* comparisons showed that the cytoplasmic fraction of transected rats differed from intact subjects, $p < .05$. No other group comparisons were significant, $p > .05$.

For the membrane-bound/cytoplasmic ratio (Figure 22C), transection induced a 46% reduction in KCC2 protein expression. An ANOVA revealed a revealed a significant main effect of transection, F (1, 10) = 9.31, $p < .05$.

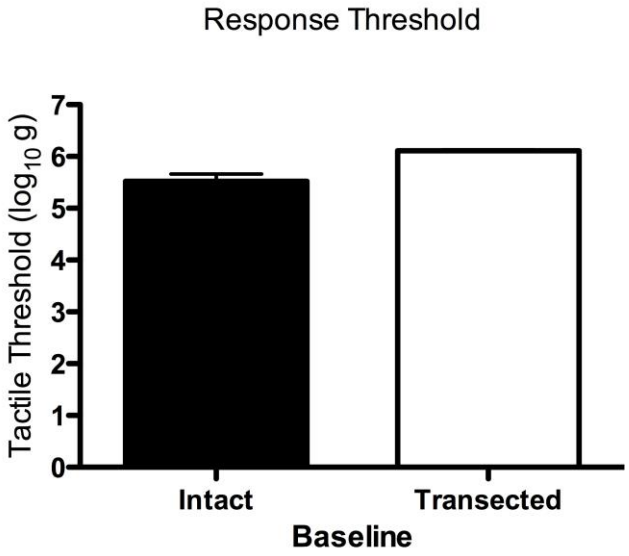

Figure 21. Baseline tactile reactivity for intact and transected rats. The y-axis depicts the linearized tactile scores of baseline. The error bars depict ± SEM.

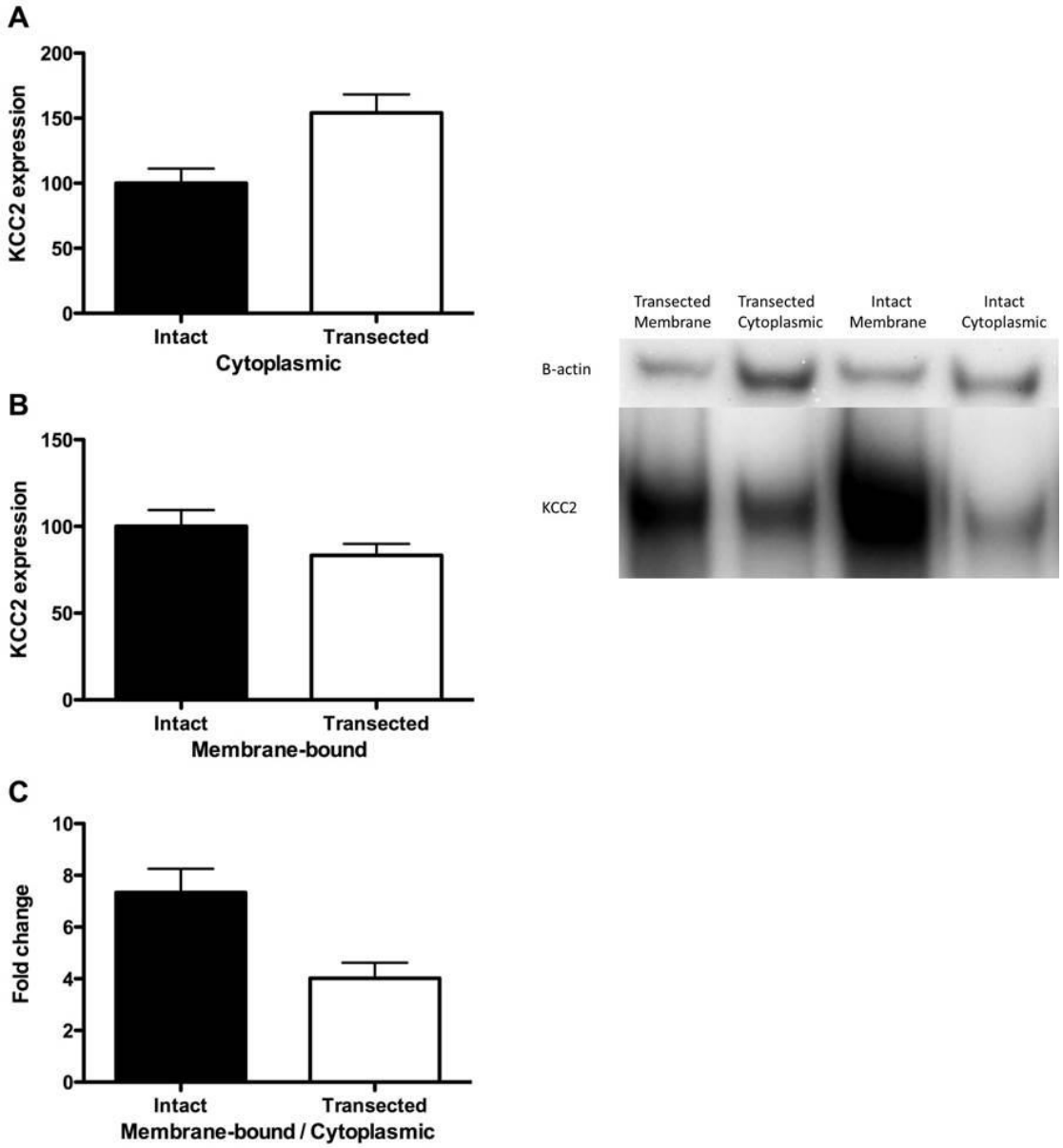

Figure 22. Transection induced a decrease of membrane-bound KCC2 expression. (A) KCC2 in the cytoplasmic fraction. (B) KCC2 in the membrane-bound fraction. (C) The fold change of membrane-bound/cytoplasmic ratio. The error bars depict ± SEM.

Discussion

I found that a spinal transection enhanced tactile reactivity relative to intact rats. This may be due to the lack of descending inhibition from the brain. I also found that transection increased the cytoplasmic KCC2 and decreased the membrane-bound form. This suggests that a reduction in the membrane-bound KCC2 transporter, through internalization, underlies the alternation in GABA function observed after transection.

Experiment 13

The results of Experiment 12 imply that a transection-induced reduction in membrane-bound KCC2 underlies a biological switch that alters how $GABA_A$ receptor blockade affects capsaicin-induced EMR (Experiment 4 and 7). KCC2 plays a critical role in controlling $[Cl^-]_{intra}$, which determines whether GABA is inhibitory or excitatory. Given this, pharmacologically blocking the KCC2 channel in intact rats should emulate the effect of spinally-transection and switch how bicuculline treatment affects capsaicin-induced EMR. Here, I test whether the KCC2 blocker DIOA induces an injury-like state wherein bicuculline has an anti-allodynic effect.

Procedure

The design of experiment 13 is depicted in Figure 23. Sham operated (with intact spinal cord) and cannulized rats (n=8 per group) were microinjected with either vehicle or 20 ug DIOA (i.t.). Fifteen minutes after drug delivery, subjects in each group received either the vehicle (saline) or bicuculline (i.t.). Fifteen minutes after drug

delivery, subjects in each group received an intradermal injection of capsaicin on one hindlimb (balanced across subjects). This yielded a 2 (DIOA vs. vehicle) X 2 (bicuculline vs. vehicle) factorial design. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), post drug treatment, and again 0, 1, 2, 3 hr following capsaicin treatment. A change from baseline score was calculated to assess the impact of the experimental manipulations.

<table>
<tr><td rowspan="4">Sham operated</td><td rowspan="4">24 hr</td><td rowspan="4">von Frey baseline</td><td rowspan="2">Vehicle</td><td rowspan="4">von Frey</td><td>Vehicle</td><td rowspan="4">von Frey</td><td rowspan="4">Capsaicin</td><td rowspan="4">von Frey 0, 1, 2, 3 hr</td></tr>
<tr><td>Bicuculline</td></tr>
<tr><td rowspan="2">DIOA</td><td>Vehicle</td></tr>
<tr><td>Bicuculline</td></tr>
</table>

Figure 23. Experimental design for Experiment 13.

Results

Prior to drug manipulations, mean baseline scores ranged from 5.63 ± 0.08 to 5.76 ± 0.05 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 1.08$, $p > .05$.

The effect of bicuculline on capsaicin-induced EMR after DIOA treatment is depicted in Figure 24. Before bicuculline treatment, DIOA administration (Post Drug) increased mechanical reactivity. An ANOVA revealed a significant effect of DIOA treatment, $F(3, 28) = 7.79$, $p < .05$. Before capsaicin treatment, bicuculline *per se* (Veh-Bic) induced EMR, which replicates the result of Experiment 9. An ANOVA showed that the main effect of DIOA and bicuculline, as well as their interaction, were statistically significant, all $Fs > 6.16$, $p < .05$. *Post hoc* comparisons confirmed that the

group that received bicuculline alone (Veh-Bic) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

Capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Veh). This effect was blocked by pretreatment with DIOA and bicuculline (DIOA-Bic). Replicating Experiment 9, bicuculline treatment failed to block capsaicin-induced EMR without DIOA treatment (Veh-Bic). An ANOVA showed that the main effect of DIOA and bicuculline, as well as their interaction, were statistically significant, all $Fs > 23.9$, $p < .05$. Also, the main effect of time, and its interaction with bicuculline, were statistically significant, both $Fs > 6.9$, $p < .05$. No other term was significant, all $Fs < 2.53$, $p > .05$. *Post hoc* comparisons confirmed that the group that received DIOA and bicuculline (DIOA-Bic) differed from the other groups, $p < .05$. In addition, the group that received DIOA alone (DIOA-Veh) differed from the group that received bicuculline alone (Veh-Bic). No other group comparison was significant, $p > .05$.

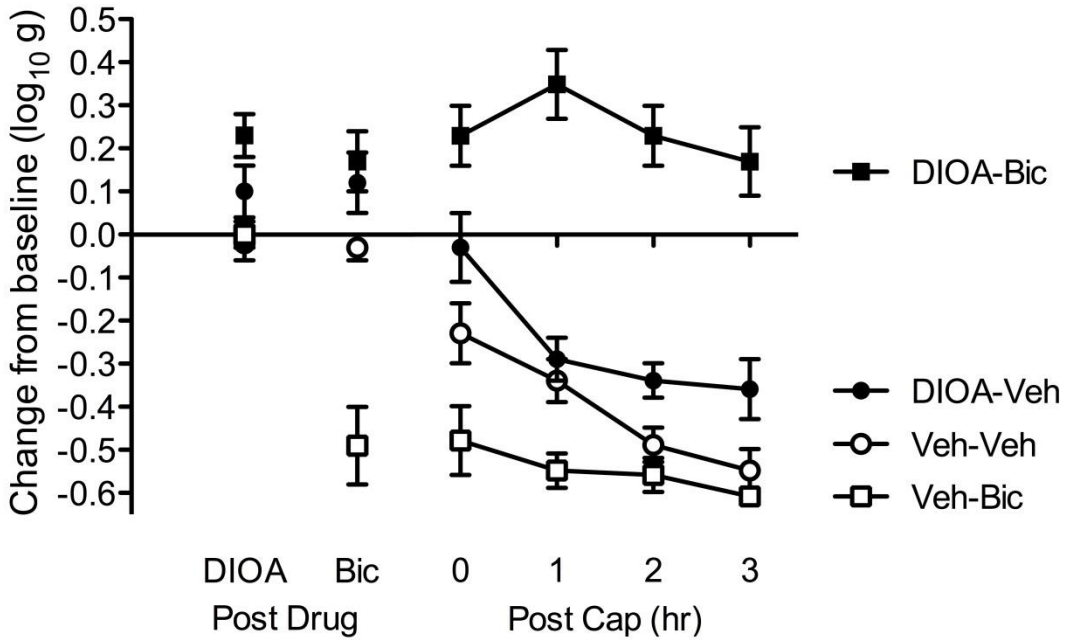

*Figure 24***.** Bicuculline blocked capsaicin-induced EMR in intact rats after DIOA treatment. Subjects that received DIOA and its vehicle (Veh) are shown in black and white, respectively. Groups given bicuculline (Bic) or its vehicle (Veh) are depicted as squares and circles, respectively. The y-axis depicts the change from baseline after DIOA and bicuculline treatment (Post Drug), and 0, 1, 2, 3 hr after capsaicin treatment (Post Cap). The error bars depict ± SEM.

Discussion

DIOA reduced mechanical reactivity and blocked the allodynia inducing effect of bicuculline. Also, bicuculline blocked the capsaicin-induced EMR in DIOA treated intact rats. Comparing the results to those obtained in Experiments 4 and 9, it appears that DIOA treatment induced a state that emulated the effect of spinal transection, eliminating the EMR induced by bicuculline and flipping the effect of bicuculline on capsaicin-induced EMR. This change of GABA effect is presumably due to an increase in $[Cl^-]_{intra}$.

Experiment 14

The effect of GABA (inhibitory or excitatory) depends on intracellular chloride concentrations. This is regulated by both KCC2 and NKCC1. Prior work has shown that the effect of decreased KCC2 on intracellular chloride gradient can be countered by blocking the inward flow of chloride using a NKCC1 antagonist (Cramer et al., 2008; Hasbargen et al., 2010). This suggests that administration of a NKCC1 antagonist (bumetanide) could reinstate inhibitory GABAergic tone in transected rats. Given this, I examined whether bumetanide treatment could switch the effect of bicuculline, causing it to enhance capsaicin-induced EMR in spinally-transected rats.

Procedure

The design of Experiment 14 is depicted in Figure 25. Spinally-transected and cannulized rats (n=8 per group) were microinjected with either vehicle or 1mM

bumetanide (BUM; i.t.). Fifteen minutes after drug delivery, subjects in each group received either the vehicle (saline) or bicuculline (i.t.). Fifteen minutes after drug delivery, subjects in each group received an intradermal injection of capsaicin on one hindlimb (balanced across subjects). This yields a 2 (bumetanide vs. vehicle) X 2 (bicuculline vs. vehicle) factorial design. Tactile reactivity was assessed on each paw prior to drug delivery (baseline), post drug treatment, and again 0, 1, 2, 3 hr following capsaicin treatment. A change from baseline score was calculated to assess the impact of the experimental manipulations.

<table>
<tr><td rowspan="4">Complete transection (T2)</td><td rowspan="4">24 hr</td><td rowspan="4">von Frey baseline</td><td rowspan="2">Vehicle</td><td rowspan="4">von Frey</td><td>Vehicle</td><td rowspan="4">von Frey</td><td rowspan="4">Capsaicin</td><td rowspan="4">von Frey 0, 1, 2, 3 hr</td></tr>
<tr><td>Bicuculline</td></tr>
<tr><td rowspan="2">Bumetanide</td><td>Vehicle</td></tr>
<tr><td>Bicuculline</td></tr>
</table>

Figure 25. Experimental design for Experiment 14.

Results

Prior to drug treatment, mean baseline scores ranged from 6.0 ± 0.03 to 6.16 ± 0.08 (mean ± standard error of the mean [SE]) across groups. These differences were not statistically significant, all $Fs < 2.22$, $p > .05$.

The effect of bicuculline on capsaicin-induced EMR after BUM treatment is depicted in Figure 26. Before bicuculline treatment, BUM administration (Post Drug) had no effect on mechanical reactivity, $F(3, 20) = 1.22$, $p > .05$. Before capsaicin treatment, bicuculline induced EMR in BUM treated subjects (BUM-Bic). An ANOVA showed that the interaction of BUM and bicuculline was statistically significant, $F(1, 20) = 4.68$, $p < .05$. No other term was significant, all $Fs < 3.58$, $p > .05$. *Post hoc*

comparisons confirmed that the group that received BUM and bicuculline (BUM-Bic) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

Capsaicin treatment (Post Cap) induced a lasting EMR (Veh-Veh, BUM-Veh). This effect was blocked by pretreatment with bicuculline alone (Veh-Bic). Bicuculline treatment failed to block capsaicin-induced EMR in BUM treated subjects (BUM-Bic). An ANOVA showed that the main effect of BUM and bicuculline, as well as their interaction, were statistically significant, all $Fs > 5.2$, $p < .05$. Also, the main effect of time and its interaction with BUM and bicuculline, as well as the time x BUM x bicuculline three way interaction, were significant, all $Fs > 2.97$, $p < .05$. *Post hoc* comparisons confirmed that the group that received bicuculline alone (Veh-Bic) differed from the other groups, $p < .05$. No other group comparison was significant, $p > .05$.

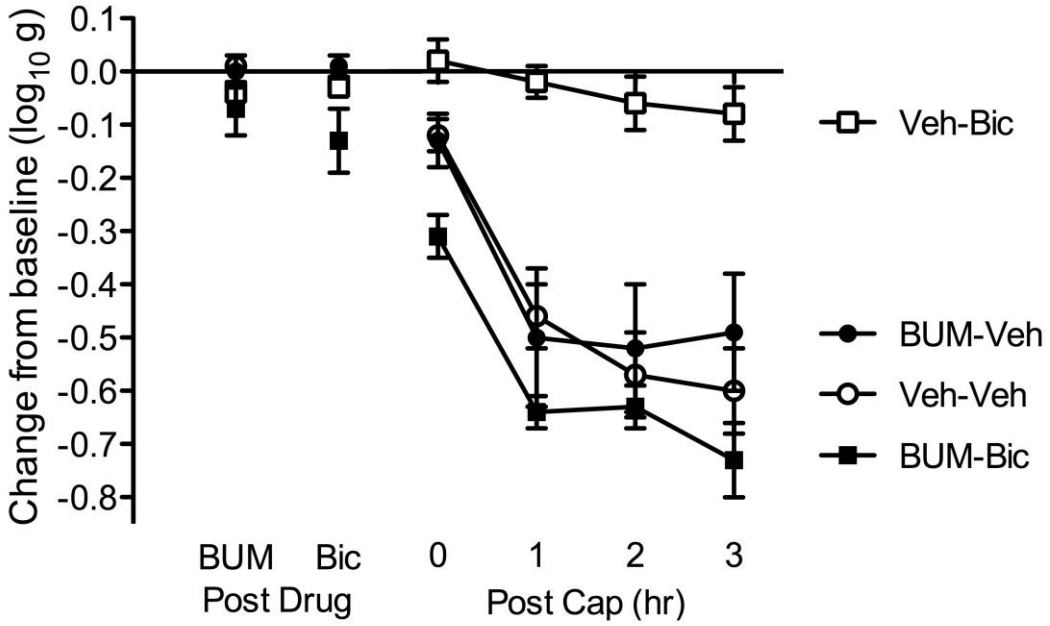

*Figure 26***.** Bicuculline failed to block capsaicin-induced EMR in transected rats after BUM treatment. Subjects that received BUM or its vehicle (Veh) are shown in black and white, respectively. Groups given bicuculline (Bic) or its vehicle (Veh) administration are depicted as squares and circles, respectively. The y-axis depicts the change from baseline after BUM and bicuculline treatment (Post Drug), and 0, 1, 2, 3 hr after capsaicin treatment (Post Cap). The error bars depict ± SEM.

Discussion

After bumetanide treatment, the allodynia inducing effect of bicuculline was reinstated. As in Experiment 4, bicuculline alone blocked capsaicin-induced EMR. Pretreatment with bumetanide eliminated this effect. Thus, blocking NKCC1 channel with bumetanide in transected rats emulated the pattern of results observed in intact rats (Experiment 9), producing a state wherein blocking the $GABA_A$ receptor induces EMR and fails to attenuate capsaicin-induced EMR. Together with Experiment 13, these data suggest that spinal injury modifies GABAergic function by inducing a change in $[Cl^-]_{intra}$.

CHAPTER VII

GENERAL DISCUSSION AND SUMMARY

Prior studies have shown that pathological conditions can induce a lasting increase in nociceptive excitation within the CNS (central sensitization; Ji et al., 2003; Woolf, 2004). A variety of events, including VIS, LPS, and peripheral inflammation, have been shown to induce EMR, which is linked to a NMDAR-mediated sensitization of spinal neurons (Baumbauer et al., 2008; Baumbauer et al., 2012; Hook et al., 2008; Reeve et al., 2000). In addition to the NMDAR-mediated signaling, malfunctioning of GABAergic system has been suggested to contribute to central sensitization (Gwak & Hulsebosch, 2011). Prior works from out laboratory has shown that treatments (VIS and LPS) that induce central sensitization impair spinal learning (Ferguson, Crown, & Grau, 2006; Ferguson et al., 2003; Young et al., 2007). It has also been observed that the inhibitory effect VIS has on learning is blocked by bicuculline, a $GABA_A$ receptor antagonist (Ferguson et al., 2003). This leads to the prediction that GABA may be excitatory after SCI, and bicuculline treatment may also block the VIS-induced EMR. Indirect support for this comes from studies showing that GABA can have an excitatory effect in other conditions (Ben-Ari, 2002; Ge et al., 2006; Gulledge & Stuart, 2003; Marty & Llano, 2005). These findings suggest that GABA effect can switch from inhibitory to excitatory after SCI, and thereby contribute to the emergence of central sensitization.

The present dissertation examines a number of questions raised by prior studies: first, would bicuculline block VIS-induced and inflammation-induced EMR in transected subjects (LPS and capsaicin); second, does blocking GABA function have opposite effects in intact rat versus spinally-transected subject; third, does SCI alter the role of GABA by modifying membrane concentration of the proteins that regulate intracellular Cl^- concentration?

I first established whether bicuculline administration blocks the shock (VIS) induced EMR (Experiment 1). I found that VIS induced a lasting EMR, which replicates the previous findings (Ferguson et al., 2000; 2001). This effect was blocked by pretreatment with the $GABA_A$ antagonist bicuculline.

To explore the generality of this effect, I tested whether bicuculline treatment would block LPS-induced EMR (Experiment 2). LPS has been shown to induce both EMR and a spinally-mediated learning impairment (Reeve et al., 2000; Vichaya et al., 2009; Young et al., 2007). I found that bicuculline pretreatment blocks LPS induced EMR. For the maintenance of LPS induced EMR, I found that bicuculline failed to reverse it (Experiment 3).

Experiments 1, 2, and 3 showed that spinal $GABA_A$ receptor transmission is a crucial component of signaling cascade for enhanced mechanical reactivity and suggest a role in central sensitization. The next two experiments (Experiment 4 and 5) provide a more direct test of this hypothesis by examining the impact of peripheral treatment with the irritant capsaicin. Capsaicin has been shown to induce central sensitization, as well as the concomitant EMR and learning deficit (Hook et al., 2008; Woolf, 2011). I found

that bicuculline pretreatment blocked the induction (Experiment 4) and maintenance (Experiment 5) of capsaicin-induced EMR. Further, the nociceptive agent capsaicin applied to one hind leg induced EMR on both the ipsilateral and contralateral legs, implying an increase in the excitability and synaptic efficacy of neurons in central nociceptive pathways. This effect suggests a heterosynaptic potentiation and implicates central sensitization (Woolf & Salter, 2000). Hence, the data suggest that bicuculline treatment is capable of blocking and reversing the capsaicin induced central sensitization.

Experiment 6 provided a more detailed analysis of the role of GABA in capsaicin-induced EMR. At issue is whether bicuculline blocked capsaicin-induced EMR through $GABA_A$ receptor rather than Ca_{2+} activated potassium channels (Khawaled et al., 1999). To address this issue, subjects were given another $GABA_A$ receptor antagonist, gabazine. As expected, gabazine pretreatment blocked capsaicin-induced EMR (Experiment 6A). I also assessed the effect of the $GABA_B$ receptor antagonist, phaclofen (Experiment 6B). Surprisingly, phaclofen pretreatment blocked the capsaicin-induced EMR, though it appeared to be less effective than gabazine.

Experiments 4-6 provide behavioral evidence that the GABAergic system is involved in the sensitization of nociceptive processes (central sensitization). Next, I examined the impact of bicuculline treatment on cellular indices (mRNA and protein) of nociceptive sensitization. PCR and western blotting (Experiment 7 and 8 respectively) showed that capsaicin induced *c-fos* and pERK expression in the dorsal horn and that these effects were reduced by bicuculline pretreatment. Although capsaicin induced *c-fos* expression within the ventral horn was not affected by bicuculline pretreatment, pERK

protein expression showed a pattern similar to that observed in the dorsal horn. There was no significant change in GAD65 and 67 in dorsal horn which suggests that the effect of bicuculline depends on GABA receptors rather than GABA synthesis. Bicuculline treatment did reduce BDNF expression in both dorsal and ventral region. For the BDNF receptor protein, TrkB, capsaicin treatment reduced expression in vehicle treated subjects, but enhanced expression in bicuculline treated subjects. There was a trend towards higher BDNF expression in capsaicin treated subjects, which agrees with prior work showing that capsaicin induces a dose-dependent release of BDNF (Lever et al., 2001). This may suggest that capsaicin induces BDNF signaling, whereas bicuculline blocks it. A possible explanation is that BDNF has a protective effect on capsaicin-induced central sensitization. This BDNF-dependent mechanism may not be engaged in bicuculline treated subjects, because central sensitization has already been blocked.

Results from Experiments 4 to 8 imply that pretreatment with the $GABA_A$ receptor antagonist bicuculline blocks capsaicin-induced central sensitization in spinally transected rats, implying that GABA is excitatory. This stands in contrast to previous work examining the effect of bicuculline treatment in intact rats, which has generally found that the drug induces EMR (Sorkin et al., 1998; Zhang et al., 2001). Given this discrepancy, Experiments 9 to 11 explored the impact of my experimental manipulations in intact (sham operated) subjects. Pretreatment of bicuculline *per se* induced EMR (allodynia) in intact rats and failed to block the capsaicin induced EMR. Likewise, bicuculline pretreatment failed to block cellular indices of central sensitization in the dorsal horn. Comparing these results to those observed in transected rats (Experiment 7

& 8), bicuculline pretreatment showed the opposite effect on capsaicin-induced central sensitization in intact subjects. This implies that spinal injury switches how GABA affects nociceptive systems. Further, cellular indices of central sensitization are higher in transected rats. The loss of descending inhibition from the brain in transected rats is the probable cause of this difference.

In the process of examining how spinal injury affects GABAergic system, Experiment 12 revealed that spinal transection *per se* increases mechanical reactivity threshold. This may indicate that, in intact rats, the withdrawal response is partially mediated (facilitated) by brain-dependent systems. Alternatively, the PNS may down-regulate nociceptive signaling after spinal injury to compensate for the centrally-mediated hyperexcitability that emerges after the loss of descending inhibition. Also, as previously reported (Boulenguez et al., 2010), spinal transection reduced membrane-bound KCC2 expression. This downregulation of KCC2 is thought to depend on a PKC-dependent pathway (Lee, Deeb, Walker, Davies, & Moss, 2011). These data suggest that spinal transection induces downregulation of membrane-bound KCC2, which results in the high $[Cl^-]_{intra}$, and GABA-mediated depolarization.

To further confirm that $[Cl^-]_{intra}$ underlies the alteration in GABA function, Experiments 13 and 14 tested whether drug manipulations that target the $[Cl^-]_{intra}$ controlling cotransporters, NKCC1 and KCC2, affect how $GABA_A$ receptor blockage impact nociceptive sensitization. Results showed that blocking KCC2 in intact rats is capable of emulating the blocking effect of bicuculline on capsaicin-induced EMR in

transected rats. Conversely, blocking NKCC1 in transected rats emulates the EMR-inducing effect of bicuculline in intact rats.

Taken together with previous findings (Boulenguez et al., 2010; Cramer et al., 2008; Hasbargen et al., 2010), this dissertation revealed that GABA shows excitatory effect after SCI, relative to the inhibitory effect in intact subjects. This biological switch in GABAergic mechanism results from the expressional change of KCC2 cotransporter, which controls the intracellular chloride concentration, through a PKC dependent pathway (Lee et al., 2011).

Role of GABA in SCI

GABA is the main inhibitory neurotransmitter in the nervous system, and was further divided into two modes of neuronal inhibition- phasic and tonic (Farrant & Nusser, 2005). It has been suggested that neurons are not the only type of cell that synthesizes GABA; glia cells have also been shown to synthesize GABA (Velez-Fort, Audinat, & Angulo, 2012). Interaction between neurons and glia synergistically control inhibitory system functioning. After spinal cord injury, hyperexcitable neurons and glial activation disrupts the balance of chloride ions, glutamate and GABA distribution in the spinal dorsal horn and results in chronic neuropathic pain (Gwak & Hulsebosch, 2011). In short, SCI activate both ionotropic and metabotropic glutamate receptors on astrocyte and microglia. This activation leads to the subsequent membrane depolarization that triggers increased influx of Na^+ and Ca^{2+} ions into both neurons and glia. Subsequently,

the elevated Ca^{2+} concentrations in astrocytes and microglia initiate the activation of mitogen-activated protein kinase (MAPK, p38-MAPK and ERK) and phospholipase (PLA2) that result in the modulation of target protein expression or phosphorylation of membrane receptor and ion channels through activation of transcription factors. The activated glial cells release glutamate, ATP, proinflammatory cytokines, prostaglandins (PGs), reactive oxygen (ROS) and nitrogen species (NOS) into the extracellular space (Ji et al., 2003). These pain mediating substances that are released by activated glia contribute to intracellular downstream biochemical pathways and provide an intracellular feed forward mechanism for continued activation (phosphorylation) of receptors and ion channels. In concomitant with the excitatory GABA and glycine, this mechanism ensures the induction and maintenance of the central neuronal hyperexcitability.

Hypofunction or malfunction of GABAergic inhibition is one of the most important factors in the enhanced synaptic transmission and may underlie neuronal hyperexcitability in dorsal horn neurons following spinal cord injury. A hypothetical model is shown in Figure 27. Under normal conditions, phasic GABA release allows the rapid and precise modulation of pre- and post-synaptic signaling at the synaptic site, whereas tonic GABA increases the input conductance persistently through extrasynaptic receptors. The low $[Cl^-]_{intra}$ caused by NKCC1 and KCC2 endows the hyperpolarizing effect of GABA in both CNS and PNS. After SCI, especially during the acute phase of spinal transection, the loss of descending inhibition from the brain leads to a hyper-excited state within the spinal cord. The loss of descending projections (serotonin) from

the brain cause the down-regulation of KCC2 and the subsequent increase in $[Cl^-]_{intra}$ (Bos et al., 2013; Boulenguez et al., 2010). Synergy of SCI-induced hyperexcitable neurons and glial activation disrupts the normal physiological homeostasis, and underlies the depolarizing GABA in the CNS. Research has shown that neurons can tune themselves to maintain levels of excitation by modulating different ion channels (Grashow, Brookings, & Marder, 2010; Rush et al., 2006; Waxman, Cummins, Dib-Hajj, Fjell, & Black, 1999). In the hyperexcited CNS, firing properties of dorsal root ganglion (DRG) neurons (PNS) may decrease by altering sodium channel expression. By changing the excitability of primary afferent sensory neurons, this lowers the input conductance of the PNS and is able to protect the CNS from excitotoxicity. This could also explain the higher mechanical reactivity of the transected rats (see Figure 21, Experiment 12) and the DIOA treated intact rats (see Figure 23, Experiment 13).

Malfunctioning of GABA has been suggested to be the underlying mechanism of neuropathic pain (Gwak & Hulsebosch, 2011), such as inflammation induced neuropathic pain (Sluka et al., 1993, 1994) and spontaneous pain in diabetes (Jolivalt et al., 2008). Similar to my result, the blocking effect of $GABA_A$ receptor antagonist on neuropathic pain mentioned above has also been shown. This drug effect on nociceptive activation is attributed to KCC2 depletion within the CNS which then underlies the malfunction of GABA and the following central sensitization. Further, Reichl et al. (2012) showed that intrathecal but not peripheral administration of muscimol ($GABA_A$ receptor agonist) and baclofen ($GABA_B$ receptor agonist) reduced mechanical and thermal hyperalgesia after plantar incision in rats. It seems that the GABA functioning

within the CNS is the major factor that determines the homeostasis of nociceptive plasticity, and GABA can be either hyperpolarizaing or depolarizaing depending on the $[Cl^-]_{intra}$. From this view, CNS works as a shunt and PNS acts as a receiver and conductor.

GABA-dependent depolarization has also been linked to the dorsal root reflex (DDR) triggered by primary afferent depolarization (PAD). The GABAergic input is normally inhibitory and mediates presynaptic inhibition. It can, however, have an excitatory effect when DRR is triggered. Inflammatory stimuli activate NKCC1 within the DRG. This leads to an increase in intracellular Cl^- within the afferent neuron, which cause GABAergic input to have an excitatory effect (Delpire & Austin, 2010; Pitcher & Cervero, 2010). In the presence of inflammation, engaging the $GABA_A$ receptor on the primary afferent terminal causes an efflux of chloride ions, inducing depolarization in the primary afferent (Cervero & Laird, 1996; Rudomin & Schmidt, 1999; Willis, 1999). As a result, GABA release can increase nociceptive input and enhance pain reactivity.

PAD and DRR have been suggested to contribute to inflammation-induced hyperalgesia and the accompanying flare, swelling, and increased temperature. These symptoms are unilateral, limited to the affected region (Lin, Wu, & Willis, 1999; Sluka et al., 1993, 1994; Willis, 1999). Evidence that these changes have a functional role rises from studies demonstrating that pretreatment with bicuculline reduces inflammation-induced flare and hyperalgesia (Lin et al., 1999; Sluka et al., 1993, 1994). Because this could also reduce capsaicin-induced EMR, it is possible that the DRR and PAD contribute to the effects I reported. However, the contribution of this effect would be

limited to the treated dermatone; PAD and DRR could not explain the effect of bicuculline treatment on the EMR observed on the contralateral leg. Nor could it explain the effect of bicuculline on LPS-induced EMR. Finally, I examined whether i.t. treatment with bicuculline affects capsaicin-induced swelling and paw temperature. Drug treatment did not have a significant effect. These observations suggest that the GABAergic mechanism studied within my dissertation modulates nociceptive processes in a general fashion.

Yin-yang of GABAergic system

Like Yin-yang, everything is relative and nothing is absolute, GABA can be either excitatory or inhibitory depending on different circumstances. As discussed above, it is clear that GABA can have bidirectional effect, leading to inhibition in intact rats and excitation in spinally transected rats. The $GABA_A$ receptor, an ionotropic channel receptor, seems to play a major role in mediating this switch, through KCC2 expressional change and the following turnover of intracellular chloride concentration. However, $GABA_B$ receptor has also been shown to play a role. Experiment 6 showed that phaclofen, a $GABA_B$ receptor antagonist, blocked capsaicin-induced EMR, which implies that the $GABA_B$ receptor also contributes to the excitatory effect of GABA, though less effective. This difference of effectiveness is presumably linked to the characteristic difference between $GABA_A$ and $GABA_B$ receptor, which are ionotropic and metabotropic, respectively. Similar to my result, Hirono et al. (2001) have also

shown that postsynaptic $GABA_B$ receptor can contribute GABA-dependent excitation by interacting with mGluR1. This has been shown to enhance mGluR1 mediated excitatory transmission at cerebellar parallelfiber-Purkinje cell excitatory synapses. In addition, VIS has been shown to induce both a spinally-mediated learning deficit (Ferguson et al., 2003) and EMR (Ferguson et al., 2001; Experiment 1). Activation of mGluR1 was found to be both necessary and sufficient for this metaplastic inhibition of spinal learning (Ferguson et al., 2008). These findings suggest that the $GABA_B$ receptor contributes to the excitatory effect of GABA through its interaction with mGluR1. Taken together, my results suggest that both $GABA_A$ and $GABA_B$ receptor contribute to the excitatory effect of GABA after spinal injury.

Descending Input from the Brain

Descending control of spinal nociception originates from many brain regions and plays a critical role in determining the experience of both acute and chronic pain. The periaqueductal gray matter (PAG), the nucleus raphe magnus and adjacent structures of the rostral ventromedial medulla (RVM), send projections to the spinal dorsal horn. Prior research suggests that supraspinal systems can have a dual action, facilitating or inhibiting nocicpetive transmission (Dogrul, Ossipov, & Porreca, 2009; Suzuki & Dickenson, 2005; Suzuki, Rygh, & Dickenson, 2004). As in models of inflammation (Millan, 2002), descending inhibition generally predominates over descending facilitation in the primary pain circuits with input from the inflamed tissue; while in the

secondary pain circuit, descending facilitation predominates over descending inhibition with input from neighboring tissues. Also, the inhibitory descending control from the PAG-RVM system preferentially suppresses nociceptive inputs mediated by C-fibers, preserving sensory-discriminative information conveyed by more rapidly conducting A-fibers (Heinricher, Tavares, Leith, & Lumb, 2009; Suzuki & Dickenson, 2005; Suzuki et al., 2004; Vanegas & Schaible, 2004). Different 5-HT receptor subtypes have been suggested to underlie either facilitatory or inhibitory descending control. For example, the descending facilitatory pathway from the RVM act ultimately on spinal cord in acute and chronic pain states through 5HT3 receptor whereas the inhibitory pathway is through 5HT7 (Dogrul et al., 2009; Suzuki & Dickenson, 2005; Suzuki et al., 2004). Additionally, several receptors, including the 5HT1 (1A and 1B), 5HT2 (2A and 2C), 5HT3, and 5HT4 receptors are expressed in the spinal cord dorsal horn and can produce an inhibitory effect on spinal nociception transmission (Liu et al., 2010). As a whole, the descending control seems to have an inhibitory effect on spinal neurons under normal condition. Loss of descending inhibition after spinal cord transection can contribute to hyperexcitability in spinal circuits, resulting in the facilitation of nociceptive reflexes. Further, recent research has demonstrated that activation of 5-HT2A receptors upregulates KCC2 function and expression through a PKC-dependent mechanism, which decreases the intracellular chloride concentration of neuron in the spinal cord, thereby increasing the inhibitory tone (Bos et al., 2013; Lee et al., 2011; Medina et al., 2014). These results parallel my finding that membrane-bound KCC2 was decreased by internalization after spinal transection (loss of descending control). Taken together, it is

clear that the transection-induced switch of GABAergic signaling is due to the lack of descending control from the brain. This work also confirms that descending inhibition from the brain is a key regulator for the neuronal plasticity of pain sensation in the CNS.

Clinical Implications

The regulation of GABA function by $[Cl^-]_{intra}$ may be an important therapeutic strategy for treating pain or neurological disorders. For painful diabetic neuropathy, Jolivalt et al. (2008) has shown that KCC2 downregulation and increased GABA release contribute to spinally-mediated hyperalgesia in diabetes. The KCC2 downregulation and the consequent $[Cl^-]_{intra}$ elevation have also been related to neuropathic pain (Coull et al., 2003), spasticity following SCI (Boulenguez et al., 2010), and adult neurogenesis (Ge et al., 2006). These studies imply that impaired KCC2 function is the principal cause of the $[Cl^-]_{intra}$ increase that subsequently lead to the depolarizing effect of GABA. Although the primary cause and mechanisms of KCC2 downregulation are still not fully-understand, further research focusing on the signaling pathways upstream the expressional change of KCC2 or even NKCC1 should reveal strategies to treat pain or neurological disorder.

Summary

Maladaptive pain is associated with injury to a peripheral nerve or the central nervous system (neuropathic pain). Central sensitization, a hyperactive state of

nociceptive neurons within dorsal horn of the spinal cord, has been suggested as a possible causal mechanism of neuropathic pain, which is manifested as allodynia and hyperalgesia. This dissertation examined one of the mechanisms, reduction of inhibition, that contributes to the increased synaptic efficacy and the central sensitization of the somatosensory pathway. I showed that GABA can have excitatory effect after spinal transection, when the descending input from the brain has been disrupted. Other research has shown that GABA is also excitatory in neonatal CNS (Ben-Ari, Khalilov, Kahle, & Cherubini, 2012), and that this effect contributes to neurite growth in mature CNS (Ge et al., 2006), and allodynia and hyperalgesia in diabetic rats (Jolivalt et al., 2008). Research indicates that KCC2 expressional changes underlie the excitatory effect of GABA. Further, regulatory action of BDNF has also been linked to the expressional change of KCC2. In mature neurons, application of exogenous BDNF induces a down-regulation of the KCC2 function, whereas application of BDNF to immature hippocampal cultures significantly increases the expression of KCC2 (Medina et al., 2014). SCI-induced switch of GABAergic system is probably linked to the developmental switch of GABA from excitatory to inhibitory during the postnatal period. Under pathological condition in mature neurons, the down regulation of KCC2 may reflect a rejuvenation of the system which emulates neonatal circumstance for neurite regrowth and re-establishment of the damaged tissue. This may be a prerequisite for the repair and recovery of the system after SCI.

In summary, the descending input from the brain, probably through dorsal funiculus, is crucial for maintaining GABAergic inhibition. By regulating the balance of

the potential across cell membrane by KCC2 expressional change or interaction with other metabotropic receptor (mGluR1), GABA can have a dual effect and thereby enhance or diminish neuropathic pain after SCI. The switch in GABA function after SCI enables the CNS to mimic neonatal-like circumstance where GABA is excitatory. This can be beneficial for lesion repair and neurite regrowth after SCI. GABAergic system is important in controlling neuronal homeostasis and is involved in many neurological disorders. Understanding how GABAergic system is modified by SCI is important to deriving more effective methods to treat pain. As mentioned before, the primary mechanisms initiating KCC2 downregulation during pathologies are still not well understood. Further studies are needed to explore the underlying mechanisms, as well as creating new agents that target KCC2-related signaling pathways.

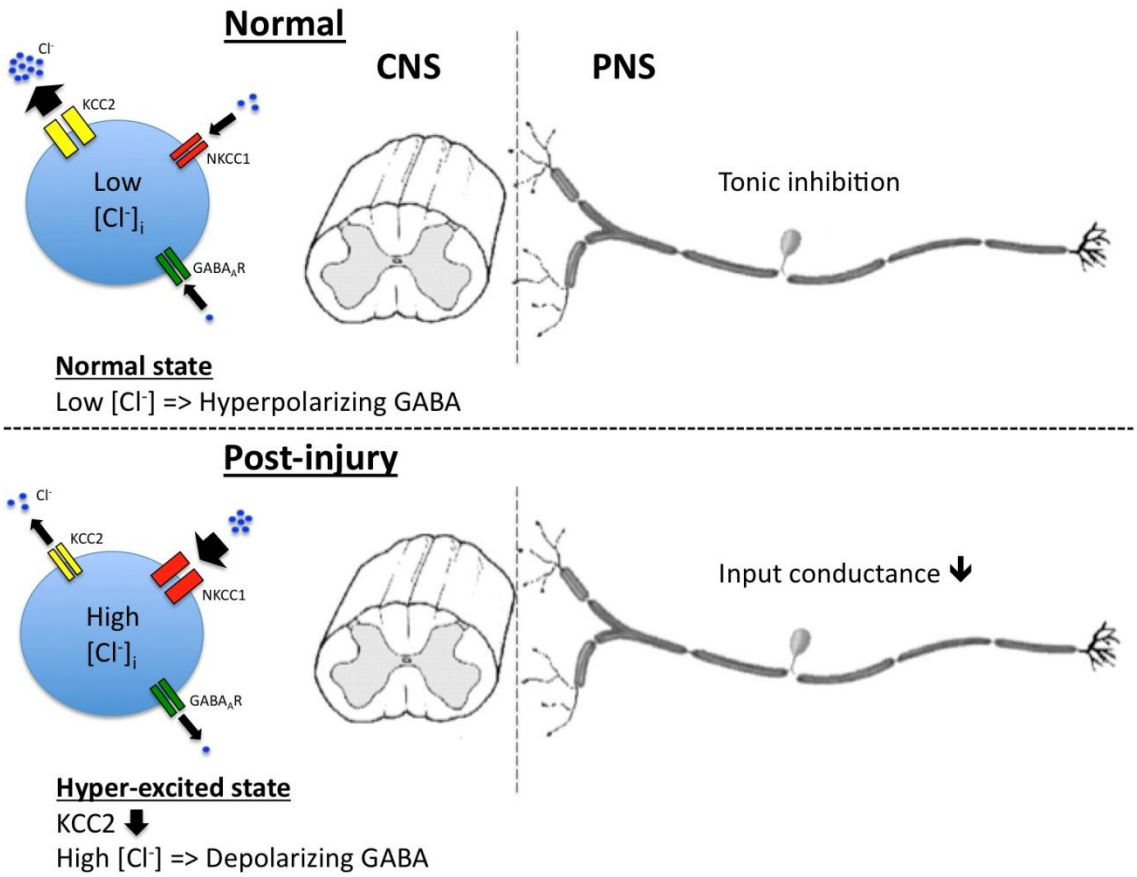

Figure 27. Hypothetical model of depolarizing GABA after SCI.

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