EARLY PREGABALIN TREATMENT SUPPRESSES AUTONOMIC DYSREFLEXIA FOLLOWING SPINAL CORD INJURY IN RATS

by

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ABSTRACT

Following spinal cord injury (SCI), up to 70% of patients develop a condition known as autonomic dysreflexia (AD). This study investigates the use of Pregabalin as a preemptive treatment to mitigate the development of AD following SCI in an animal model.

Saline-treated and dPGB rats (first Pregabalin treatment 7 days post-SCI) demonstrated typical signs of AD, with mean arterial pressure (MAP) increases of 23.5% and 27.4% respectively, following colon distension. In contrast, iPGB animals (first Pregabalin treatment 1 hour post-SCI) had MAP increases of 14.6%; significantly lower than saline-treated animals. Additionally, iPGB animals had significantly lower urine volumes than saline-treated animals on days 9 and 10 post-SCI, indicating a more rapid return of spontaneous bladder voiding. It was concluded that only treatment with Pregabalin immediately following SCI can alleviate large increases in blood pressure that accompany AD episodes. Immunostaining for substance P revealed a significantly higher density in both the dorsal horn and central autonomic area in iPGB animals when compared to saline-treated and uninjured animals, indicating a possible mechanism of sympathetic inhibition following iPGB treatment.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
β	Beta
δ	Delta
5-HT	Serotonin
ACh	Acetylcholine
AD	Autonomic Dysreflexia
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
BBB	Basso, Beattie, and Breshnahan 21-point rating scale
BP	Blood Pressure
Ca^{2+}	Calcium
CAA	Central Autonomic Area
CCK	Cholecystokinin
CD	Colon Distension
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
CTB	Cholera Toxin B
CV	Cardiovascular
CVLM	Caudal Ventrolateral Medulla
DH	Dorsal Horn
dPGB	Delayed Pregabalin Treatment
DSD	Detrusor Sphincter Dyssynergia

ENS	Enteric Nervous System
g	Gram
GABA	*gamma*-Aminobutyric Acid
GAP	Growth Associated Protein
GBP	Gabapentin
GPCR	G Protein-Coupled Receptor
HR	Heart Rate
Hz	Hertz
IB4	Isolectin B4
IML	Interomediolateral Cell Column
IP	Intraperitoneal
iPGB	Immediate Pregabalin Treatment
IR	Immunoreactivity
IU	International Unit
kg	Kilogram
Kv3.1	Potassium Voltage-gated Channel 3.1
L	Liter
LI	Lamina I of the Spinal Cord
LII	Lamina II of the Spinal Cord
LIII	Lamina III of the Spinal Cord
LIV	Lamina IV of the Spinal Cord
LV	Lamina V of the Spinal Cord
LX	Lamina X of the Spinal Cord

L2	Second Lumbar Segment of the Spinal Cord
L3	Third Lumbar Segment of the Spinal Cord
L4	Fourth Lumbar Segment of the Spinal Cord
L5	Fifth Lumbar Segment of the Spinal Cord
L6	Sixth Lumbar Segment of the Spinal Cord
M1	Muscarinic Receptor Type 1
M2	Muscarinic Receptor Type 2
M3	Muscarinic Receptor Type 3
MAP	Mean Arterial Pressure
mg	Milligram
ml	Milliliter
NA	Nucleus Ambiguus
NE	Norepinephrine
NGF	Nerve Growth Factor
NK1R	Neurokinin 1 Receptor
NMDA	*N*-Methyl-D-Aspartic Acid
NOS	Nitric Oxide Synthase
NTS	Solitary Tract Nucleus
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PBS-G	Phosphate Buffered Saline (with Glucose)
PFA	Paraformaldehyde
PGB	Pregabalin
PMC	Pontine Micturition Center

PNS	Peripheral Nervous System
RVLM	Rostral Ventrolateral Medulla
S1	First Sacral Spinal Cord Segment
S2	Second Sacral Spinal Cord Segment
S3	Third Sacral Spinal Cord Segment
S4	Fourth Sacral Spinal Cord Segment
SAP	Saporin
SBP	Systolic Blood Pressure
SCI	Spinal Cord Injury
SE	Standard Error
SNS	Sympathetic Nervous System
SP	Substance P
SPN	Sympathetic Preganglionic Neuron
T1	First Thoracic Spinal Cord Segment
T2	Second Thoracic Spinal Cord Segment
T3	Third Thoracic Spinal Cord Segment
T4	Fourth Thoracic Spinal Cord Segment
T5	Fifth Thoracic Spinal Cord Segment
T6	Sixth Thoracic Spinal Cord Segment
T7	Seventh Thoracic Spinal Cord Segment
T8	Eigth Thoracic Spinal Cord Segment
T9	Ninth Thoracic Spinal Cord Segment
T10	Tenth Thoracic Spinal Cord Segment

T11	Eleventh Thoracic Spinal Cord Segment
T12	Twelfth Thoracic Spinal Cord Segment
T13	Thirteenth Thoracic Spinal Cord Segment
TPBS	Tris Phosphate Buffered Saline
TPBS-X	Tris Phosphate Buffered Saline (with Triton-X)
TSP	Thrombospondin
UTI	Urinary Tract Infection
WDR	Wide Dynamic Range

CHAPTER 1: INTRODUCTION

1.1 – Spinal Cord Injury

Currently, there are approximately 86 000 individuals in Canada living with spinal cord injuries; a number expected to increase to 121 000 by the year 2030. Furthermore, there are an additional 4 300 new spinal cord injury (SCI) cases each year in Canada. The most common causes of these injuries are vehicle collisions (55%), sports or medical conditions (27%), and falls (18%). Additionally, these injuries most frequently occur in males (80%) between the ages of 16-34. While this may seem like a considerable age range, it is important to note that these injuries most commonly occur in younger individuals with a significant proportion of their lives ahead of them (Urban Futures Institute, 2010). As a result, the lifetime financial care requirements of afflicted individuals can vary from $1.6 million in paraplegic patients up to $3.0 million in quadriplegic patients. As such, the economic impact of traumatic spinal cord injury in Canada alone is estimated at $3.6 billion per year, taking into account health care, specialized equipment and modifications, and long-term care. Furthermore, patients with SCI are re-hospitalized and require contact with a physician almost three times more often than the general population, and can also expect a lifespan 15-30 years shorter than those in the general population as well (Rick Hansen Institute, 2013; Urban Futures Institute, 2010).

Egyptian accounts of spinal cord injuries have been found dating back to 1700 B.C., describing SCI as “an ailment not to be treated”. It wasn’t until the seventh century A.D. that basic spinal surgery began to help alleviate paralysis through the removal of

bone fragments. During the twentieth century, both a standardized method of treating spinal cord injuries and the anti-inflammatory drug methylprednisolone were introduced, both of which have helped to improve the prognosis of those with spinal cord injuries (NINDS, 2012).

The primary factor contributing to SCI is the mechanical damage, caused by either injury or disease, which disrupts communication between the brain and the spinal cord below the site of injury (Carlson and Gorden, 2002). Following this, secondary acute events occur that further contribute to the development of SCI. Reduction in blood flow at the site of injury results in ischemia, preventing the normal delivery of oxygen and nutrients to neurons in this region causing them to die. In addition, for reasons that are not fully understood, injured neurons release a copious amount of the neurotransmitter glutamate. This massive release of glutamate results in excitotoxicity, which is lethal to nearby neurons. Furthermore, the injury causes a sudden influx of immune cells, triggering an inflammatory response. Additional secondary events include hemorrhage, edema, free radical formation and apoptosis. Cumulatively, these secondary events act to significantly increase the area of damage to the spinal cord as further axon degeneration and demyelination occur. Glial cells enclose the injury site, sealing off necrotic tissue and forming a scar. This, in turn, creates a barrier for any remaining axons that might be able to regenerate and reconnect through the lesion. Finally, aberrant axonal sprouting and synaptic plasticity occurring rostral and caudal to the site of injury ultimately result in abnormal control of neuronal circuits (NINDS, 2012; Wang et al., 2012; Schwab et al., 2006; Thuret et al., 2006; Kwon et al., 2004; Hausmann, 2003; Nashmi and Fehlings, 2001; Popovich et al., 1997; Schwab and Bartholdi, 1996). As a result of the abnormal

neuronal circuit control, SCI patients also commonly experience symptoms of hyperalgesia and autonomic dysfunction (Fehlings and Tator, 1995).

While most current SCI research prioritizes the restoration of motor function within the SCI population as a primary goal, patients with these injuries often have differing priorities. Anderson et al. (2004) reported that 40% of quadriplegics and 38% of paraplegics listed the restoration of bladder and bowel function and the elimination of autonomic dysreflexic symptoms as their top two priorities, ahead of the restoration of motor function. In light of the priorities of this population, more focus should be placed on autonomic research within SCI research communities. Currently, therapeutic SCI research can be divided into several categories: neuroprotective, anti-inflammatory, rehabilitative, or neuroregenerative, and future clinical treatment of SCI will likely utilize a combination of these therapies (Schwab et al., 2006). Regardless of the category, therapeutic research generally aims to reduce secondary spinal cord damage and to restore neuronal communication at the site of injury (Kwon et al., 2004; McDonald and Sadowsky, 2002).

The present study investigates the development of autonomic dysreflexia, a condition identified by an exaggerated sympathetic response to noxious stimuli following an SCI at T6 or above. Symptoms of this condition most commonly present as episodic hypertension (Khastgir et al., 2007; Thuret et al., 2006). Additional symptoms include profuse sweating, hot flashes, severe headache, and in extreme cases, hemorrhagic stroke. The reduction of autonomic dysfunction-induced episodic hypertension, changes in associated spinal neural circuitry, and the development of this potentially fatal condition are investigated.

1.2 – Autonomic Nervous System

The autonomic nervous system (ANS) is a key component of the peripheral nervous system (PNS), acting to maintain bodily homeostasis and regulate visceral functions such as digestion, and cardiovascular function. Visceral functions are regulated in the ANS through interactions with the endocrine system and peripheral autonomic reflex circuits (Shields, 1993). ANS nuclei in the brain receive information from visceral sensory receptors. This information allows these centers to form the appropriate ANS efferent motor response and transmit the response to the relevant smooth muscles, cardiac muscles, or glands (Mallory, 2003; Shields, 1993).

The ANS is conventionally divided into three distinct systems: the sympathetic nervous system (SNS), the PNS, and the enteric nervous system (ENS) (Mallory, 2003; Shields, 1993). This study, however, will be restricted to the SNS and PNS. The efferent cell bodies of the SNS and PNS are located in the spinal cord and brainstem (Shields, 1993). In these systems, myelinated axons of these preganglionic neurons leave the central nervous system (CNS) via cranial nerves or ventral roots. Preanglionic neurons synapse, in specialized peripheral autonomic (sympathetic and parasympathetic) ganglia, with postganglionic neurons, which give rise to unmyelinated axons that directly innervate smooth muscles, cardiac muscles, and exocrine glands. Generally, both target organs and viscera of the ANS have both sympathetic and parasympathetic innervation. Often these two systems generate antagonistic effects, allowing them to work together to help maintain homeostasis and regulate visceral functions (Shields, 1993).

1.2.1 – The Parasympathetic Nervous System

Often considered the "rest and digest" system, the cell bodies of preganglionic neurons in the PNS are located in the brainstem and the intermediolateral (IML) cell column of the sacral spinal cord (S2-S4) (Mallory, 2003; Way, 1999; Shields, 1993). The cranial preganglionic axons leave the CNS via cranial nerves III, VII, IX, and X, while the sacral portion of the PNS leaves through the ventral roots of S2-S4, travelling in the pelvic splanchnic nerves. The preganglionic axons of the parasympathetic nervous system are long and myelinated, terminating in peripheral ganglia located very near or directly on the effector organ. Comparatively short, unmyelinated axons of postganglionic neurons leave these ganglia and synapse on the effector organ (Mallory, 2003; Way, 1999; Shields, 1993).

1.2.2 – The Sympathetic Nervous System

Opposite to the PNS, the SNS is often referred to as the "fight or flight" system and is responsible for such functions as inhibiting digestion and increasing heart rate (Mallory, 2003; Shields, 1993). The preganglionic component of the SNS is located entirely within the spinal cord (Mallory, 2003; Shields, 1993). Sympathetic preganglionic neurons (SPNs) are located in the interomediolateral (IML) cell column of the thoracic and upper lumbar spinal cord, with their axons exiting the CNS through the ventral roots from segments T1-L2. As such, the SNS outflow is commonly referred to as the thoracolumbar outflow. The preganglionic fibers that leave the ventral roots of the spinal cord are short, myelinated fibers referred to as the white rami communicante and enter the sympathetic trunk, a paired bundle of nerve fibers running from the skull to coccyx,

lateral to the vertebral column (Mallory, 2003; Shields, 1993). Subsequent to leaving the ventral roots, these fibers may: directly synapse with one or more neurons in the sympathetic trunk; ascend or descend the sympathetic trunk before synapsing with ganglionic neurons; directly stimulate the release of epinephrine or norepinephrine from the adrenal medulla; or pass through the sympathetic trunk without synapsing, instead synapsing within a collateral ganglion outside of the trunk. The collateral ganglia, also referred to as the prevertebral ganglia, that are innervated by this last group of fibers (referred to as the splanchnic nerves) include the celiac ganglion, superior mesenteric ganglion, and the inferior mesenteric ganglion (Mallory, 2003; Shields, 1993).

1.2.3 – Neurotransmitters of the Autonomic Nervous System

All physiological effects of the ANS are mediated by three neurotransmitters: acetylcholine (ACh), epinephrine, and norepinephrine (NE). The neurotransmitter utilized by all preganglionic fibers (sympathetic and parasympathetic), as well as postganglionic parasympathetic fibers is ACh. The postganglionic sympathetic neurotransmitter, with the exception of cholinergic fibers innervating sweat glands, is NE (McCory, 2007; Shields, 1993). ACh binds to two types of cholinergic receptors: muscarinic and nicotinic receptors. Muscarinic receptors, found on the cell membranes of effector tissues, are bound by ACh released by all parasympathetic postganglionic neurons and sympathetic postganglionic neurons innervating sweat glands. Muscarinic receptors are commonly divided into two types based on their function: M1 or M2, although M3, M4, and M5 subtypes also exist within the ANS (McCory, 2007; Shields, 1993).

Found on the cell bodies of sympathetic and parasympathetic postganglionic neurons, nicotinic receptors bind ACh. Similar to muscarinic receptors, nicotinic receptors can also be subdivided into three groups: those related to muscle, brain, and autonomic ganglia (McCory, 2007; Shields, 1993).

There are two distinct divisions of adrenergic receptors for epinephrine and NE; alpha (α) and beta (β). As a general rule, α receptors exert excitatory influences while β receptors exert inhibitory influences. As with most rules, however, there are notable exceptions. Both α and β receptors can be divided into two subtypes: α_1 and α_2; β_1 and β_2. Generally, both α_1 and β_1 receptors are excitatory, while α_2 and β_2 receptors are inhibitory. There are, however, also exceptions to this rule. The two subtypes of both α and β receptors can be differentiated by their mechanisms of action.

1.2.4 – Higher ANS Centers

Supraspinal neurons responsible for the control of ANS systems are located in the nucleus of the tractus solitarius (NTS), nucleus ambiguus (NA), dorsal motor nucleus of vagus, dorsal raphe nucleus, medullary reticular formation nuclei, locus ceruleus, hypothalamus, limbic system, and the primary sensory and motor cortex (Mallory, 2003; Shields, 1993; Loewy, 1990). The hypothalamus receives a wide array of sensory information. After receiving this information, the hypothalamus interacts with the pituitary and the endocrine system, and also sends descending pathways to the midbrain and subsequently the lateral pons and medullary reticular formation. These pathways interact with interneurons in the spinal cord that influence the activity of the IML cells (Mallory 2003; Shields, 1993; Loewy, 1990).

1.3 – Autonomic Nervous System Regulation of Cardiovascular Function

1.3.1 – Intact Spinal Cord

Neuronal control of cardiovascular (CV) function is modulated by the responses of both the SNS and PNS to stimuli (Guyenet, 2006) (Figure 1.1). Systemic vascular chemoreceptors and baroreceptors relay sensory information to the brainstem through afferents associated with the vagal and glossopharyngeal nerves. Input to the spinal cord from somatic and visceral nociceptors, as well as skeletal muscle chemoreceptors and proprioceptors help to further impact cardiovascular control (Guyenet, 2006; Dampney, 1981). Other factors, including the renin-angiotensin system, can also affect cardiovascular function through their influence on effector organs. Although current research suggests that the brain may play a significant role in the regulation of blood pressure, the mechanisms used by the brain to elicit the changes in blood pressure are poorly understood (Guyenet, 2006).

Autonomic cardiovascular nuclei are found in the medulla oblongata of the brainstem. In addition to interpreting and processing afferent information, the systemic changes influencing blood pressure from these nuclei (i.e. vasoconstriction/dilation) are components of a response signaled by higher centers, including the midbrain, limbic forebrain, cortex, and hypothalamus (Guyenet, 2006). These nuclei also influence vagal output from the brainstem and SNS output from the spinal cord.

Cardiovascular control is integrated mainly in the solitary tract nucleus (NTS) of the medulla. Afferents receiving input from arterial baroreceptors, and many other types of cardiovascular afferents, synapse in the NTS. In addition, afferent information from the spinal cord is also integrated in the NTS, after arriving via second order projection neurons. As well as integration, the NTS also relays afferent information to other

brainstem nuclei responsible for cardiovascular control, including the rostral ventrolateral medulla (RVLM), caudal ventrolateral medulla (CVLM), and midline raphe nuclei (Dampney, 1981).

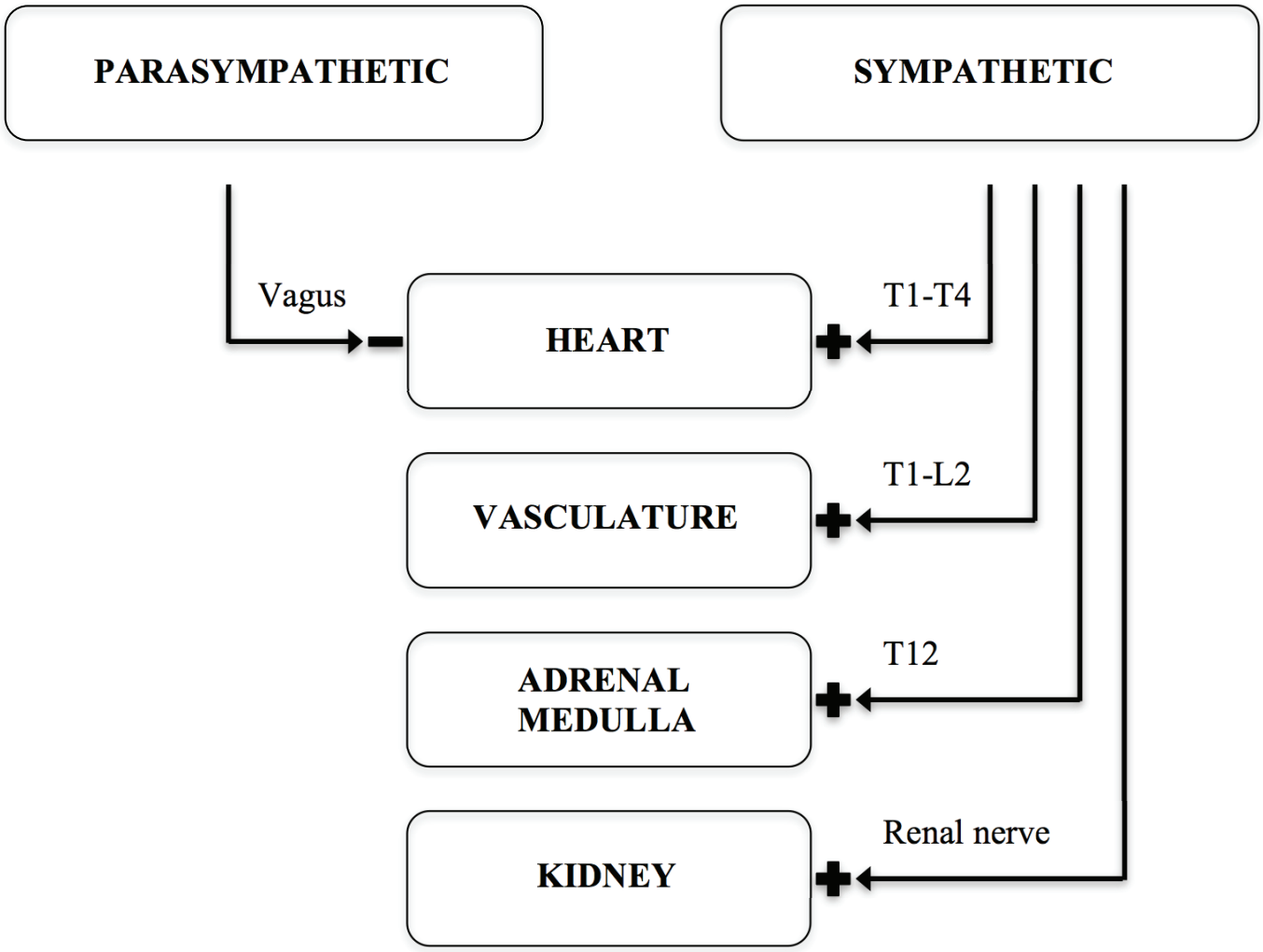


Figure 1.1 – Autonomic cardiovascular control. The PNS and SNS act through different systems, respectively providing inhibitory and excitatory inputs, to maintain cardiovascular homeostasis. In addition, inputs to the kidney and adrenal medulla help to indirectly maintain cardiovascular homeostasis. Sympathetic input to the kidney, for example, utilizes the renin-angiotensin system to increase blood pressure. Adapted from Cormier (2010).

1.3.1.1 – The Rostral Ventrolateral Medulla

The RVLM is an excitatory pressor area responsible for sympathetic vasomotor activity. Many RVLM neurons synapse with sympathetic preganglionic neurons (SPNs) in the spinal cord or with spinal interneurons. RVLM neurons exhibit tonic and reflexive control over these groups of neurons (Koganezawa et al., 2008; Guyenet, 2006; Dampney, 1981). All RVLM neurons produce glutamate, but roughly 70% of these neurons also produce adrenaline. These neurons are referred to as C1 neurons, while those that also produce serotonin are referred to as B3 neurons. Interestingly, both adrenergic and serotonergic terminals are found in the IML, a region containing the cell bodies of SPNs (Llewellyn-Smith et al., 2006; Mallory, 2003). Elimination of the excitatory response from the RVLM has been achieved through the injection of GABA agonist muscimol, demonstrating its importance in sympathetic vasomotor activity (Kogenzawa, 2008). C1 neurons from the RVLM also innervate the hypothalamus. These neurons are thought to influence blood pressure through water balance and activation of the hypothalamic-pituitary axis (Guyenet, 2006).

1.3.1.2 – The Caudal Ventrolateral Medulla

Functionally opposite to the RVLM, the CVLM is a depressor area responsible for the reduction of blood pressure and heart rate. This is achieved through the inhibition of SPNs in the spinal cord and the stimulation of vagal preganglionic neurons in the NA. Inhibition of SPNs in the spinal cord is achieved through the GABAergic inhibition of RVLM C1 neurons (Guyenet, 2006).

1.3.1.3 – Sympathetic Preganglionic Neurons

The majority of spinal SPNs are located in the IML, with smaller populations located in the central autonomic area (CAA; dorsal to central canal) (Llewellyn-Smith et al., 2006; Dampney, 1981). SPNs primarily express receptors for ACh, although they also express other receptors including those for substance P, glutamate, GABA, and 5-HT (Llewellyn-Smith et al., 2006; Llewellyn-Smith, 2002; Grossman et al., 2000; McNair et al., 1998; Llewellyn-Smith et al., 1997; Deuchars et al., 1995; Coote, 1990). Through the action of these receptors, SPNs receive a number of excitatory and inhibitory inputs that help contribute to resting vasomotor tone and heart rate (Llewellyn-Smith et al., 1997; Dampney, 1981). Supraspinal inputs come from areas such as the RVLM, caudal raphe nuclei, and hypothalamus. Spinal neurons involved in the modulation of SPN activity are excitatory interneurons originating in the dorsal horn and a group of inhibitory interneurons originating in the central autonomic area (Llewellyn-Smith et al., 2006; Deuchars et al., 2005). Both of these groups of interneurons receive information from sensory afferent fibers.

1.3.1.4 – The Baroreflex

The baroreflex is an important homeostatic mechanism in the body for the maintenance of short-term blood pressure control. This reflex provides a negative feedback loop wherein elevated blood pressure inhibits sympathetic cardiovascular activity while stimulating parasympathetic activity (Guyenet, 2006; Dampney, 1981). Afferent information is relayed by baroreceptors in the carotid and aortic sinuses to the NTS. Upon integration of these inputs, the NTS acts to stimulate vagal neurons in the NA

and inhibit sympathetic output from the RVLM via activation of the CVLM (Kogenazawa, 2008). This increase in vagal parasympathetic activity in conjunction with the inhibition in sympathetic activity acts to rapidly decrease blood pressure. Similarly, sympathetic activation with corresponding parasympathetic inhibition allows this reflex to work in reverse as well.

1.3.2 – Following Spinal Cord Injury

The balance between the PNS in the brainstem and the SNS in the spinal cord becomes disrupted following spinal cord injury. Spinal cord sympathetic reflexes remain intact, but the descending parasympathetic signals from the cardiovascular nuclei become blocked at the site of SCI (Krassioukov and Claydon, 2006, Krassioukov, 2006; Blackmer, 2003) (Figure 1.2). In the intact spinal cord, excitatory glutamatergic input from sensory afferents is regulated by these descending inputs, including B3 (serotonergic) and C1 (adrenergic) neurons from the RVLM, attenuating the excitatory response. Following spinal cord injury, however, these descending regulatory signals are blocked, allowing for an uninhibited sympathetic or parasympathetic excitatory response. This lack of excitatory regulation can lead to the development of abnormal cardiovascular, reproductive, thermoregulatory, motor, reproductive, and urinary spinal reflexes (Mathias, 2006; Krassioukov and Weaver, 1996).

Cardiovascular dysfunction is the leading cause of death among the SCI population. The degree of cardiovascular dysfunction is related to both the severity of injury (degree of disruption of descending bulbospinal neurons) and the level of injury (Krassioukov and Fehlings, 1999). The greatest degree of dysfunction appears to be

associated with injuries at the T6 level or above (Mathias, 2006). This is due in large part to the disruption of innervation in the splanchnic vasculature, which is important in blood pressure homeostasis due to its large area (Mathias, 2006; Karlsson, 1999).

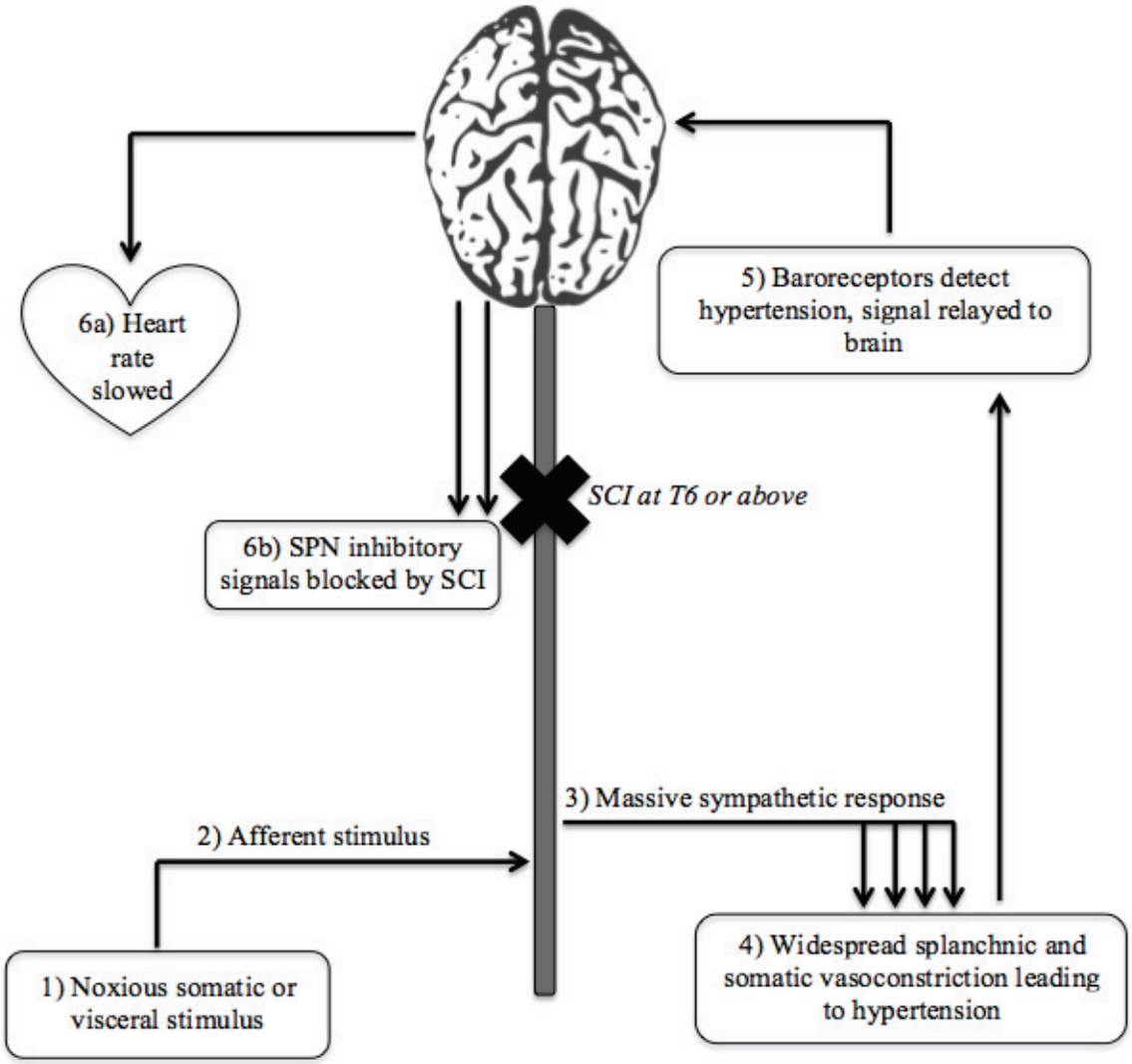


Figure 1.2. Autonomic cardiovascular control following spinal cord injury. Noxious stimulus leads to widespread sympathetic vasoconstriction, but compensatory inhibitory signals from the brain are blocked at the injury site.

1.4 – Autonomic Dysreflexia

1.4.1 – Symptoms and Triggers

Reports estimate that 19-70% of patients with an SCI at T6 or above suffer from a condition known as autonomic dysreflexia (AD) (Blackmer, 2003). Despite the injury, some spinal reflex mechanisms remain intact below the site of injury. AD is characterized by an exaggerated sympathetic reflex resulting in hypertension. This occurs in conjunction with a baroreceptor-mediated increase in parasympathetic activity, resulting in bradycardia (Blackmer, 2003; Karlsson, 1999). AD episodes can be triggered by a multitude of sources, including, but not limited to, gastric dilation, uterine contraction during pregnancy, menstruation, burns, ingrown toenails, muscle spasms, and ejaculation (Mathias, 2006). Most commonly, however, AD episodes are triggered by bladder distension (75-85%) or bowel distension (13-19%) (Blackmer, 2003). In humans, these triggers lead to a sympathetic response capable of increasing systolic and diastolic blood pressures to 250 and 200 mmHg, respectively, more than double normal resting blood pressure (Blackmer, 2003; Karlsson, 1999). Interestingly, stimuli from the bronchio-pulmonary system have not been shown to induce AD (Karlsson, 1999).

Bradycardia is often associated with episodes of AD (Mathias 2006; Blackmer, 2003; Karlsson, 1999). This is due to vagal stimulation from the NA (Guyenet, 2006). This is not always the case, however, as tachycardia is commonly seen in AD episodes as well. This difference may be explained by the disruption of the SNS connection to the heart in some patients, although more investigation is needed (Karlsson, 1999). Other symptoms of the condition include facial flushing, headache, chest tightness, pupillary dilation, anxiety, nausea, and nasal congestion. If the AD episode is allowed to

perpetuate, more severe symptoms can result, including intracranial hemorrhage, seizure, stroke, and death (Mathias, 2006; Blackmer, 2003; Karlsson, 1999).

1.4.2 – Mechanisms of Autonomic Dysreflexia

Autonomic dysreflexia has been observed 2 weeks following injury but does not fully develop until approximately 3-4 weeks following SCI (Marsh and Weaver, 2004; Krassioukov and Weaver, 1995). During SCI, supraspinal inputs are destroyed causing morphological changes in SPNs. SPN dendrites become withdrawn and the cell body of the SPN shrinks (Llewellyn-Smith and Weaver, 2001). These morphological changes are restored to their original state within a month following SCI, although this may result in the formation of aberrant synapses contributing to abnormal blood pressure regulation (Krassioukov and Weaver, 1996). Following injury, disruption of supraspinal inputs leads to sensory afferents being the only source of input to SPNs. Sensory afferents synapse on $Kv3.1^{+}$ interneurons which, in turn, synapse on the SPNs (Schramm, 2006; Deuchars et al., 2001). These interneurons express substance P and other neurotransmitters to interact with SPNs. Previously, growth-associated protein (GAP)-43 immunostaining has been utilized to detect axonal sprouting and regeneration. Following SCI, it has been demonstrated that GAP-43 immunoreactivity (IR) increases in sympathetic spinal interneurons and sensory afferents (Weaver et al., 1997). Furthermore, GAP-43 IR has been detected at several post-SCI timepoints, indicating that reorganization and regeneration of spinal circuits is an ongoing process (Weaver et al., 1997). A clear correlation has been demonstrated between the amount of afferent sprouting and the severity of AD (Cameron et al., 2006). Some exceptions to this correlation have been

found, however. Gris et al. (2005) found that increases in $CGRP^+$ afferent arbor were not related to symptoms of autonomic dysreflexia. Also, Marsh et al. (2004) found that the expansion of substance P^+ afferent arbors was not related to the development of autonomic dysreflexia. Indeed, following SCI there is an increased expression in nerve growth factor (NGF) in the spinal cord, leading to the sprouting of calcitonin gene-related peptide $(CGRP)^+$ afferents. CGRP IR has been associated with Aδ and C nociceptive afferents (Rabchevsky, 2006; Wong et al., 2000). An increased arbor of $CGRP^+$ afferents has been observed in the spinal cord following injury, and is believed to be due to the loss of descending spinal inputs (Wong et al., 2000; Krenz and Weaver, 1998). Furthermore, the time-course of afferent sprouting coincides with the development of autonomic dysreflexia (Wong et al., 2000). Isolectin-B4 $(IB4)^+$ sensory afferents have also been found to express GAP-43 following SCI, further demonstrating their sprouting following SCI (Zinck and Downie, 2008; Wong et al., 2000). Therefore, it is possible that the aberrant sprouting of these afferents leads to the inappropriate activation of spinal interneurons and SPNs, in turn leading to the development and maintenance of AD.

1.4.2.1 – Substance P

Substance P (SP) is a tachykinin neuropeptide found in the brain and spinal cord. Found in both Aδ and C primary afferent fibers, it is believed to play a role in nociception and cardiovascular control (Braz et al., 2005; Seki et al., 2005; Llewellyn-Smith et al., 1997; Lembeck and Holzer, 1979). Substance P interacts with the neurokinin 1 receptor (NK1R), a G-protein coupled receptor (GPCR) in the tachykinin receptor subfamily found in the dorsal horn of the spinal cord and also in the rough endoplasmic reticulum

and golgi apparatus of SPNs (Seki et al., 2005; Llewellyn-Smith et al., 1997; Gerard et al., 1991). Substance P has been found to be released in the spinal cord following noxious stimulation and selectively excite spinal nociceptive neurons (Allen et al., 1997). Substance P causes increases in BP, HR, and plasma catecholamines when administered intrathecally, responses thought to be evoked through SPN excitation (Llewellyn-Smith et al., 1997). Additionally, substance P^+ nerve fibers have been found in autonomic nuclei in the spinal cord and substance P-containing neurons have been found to synapse on SPNs (Llewellyn-Smith et al., 1997).

Interestingly, there have been conflicting reports regarding substance P appearance following SCI. Marsh (2004), following clip compression SCI in rat, determined that there was no change in dorsal horn substance P. In contrast, Klimachewski (2001) determined there was an increase in substance P IR in all sympathetic areas when compared to SPNs. These studies, however, have also been inconsistent with regard to which spinal cord level and lamina was assessed. Given this, and its known role in nociception, substance P may play a role in the development and maintenance of AD.

1.4.3 – Treatment of Autonomic Dysreflexia

Acute treatment of AD is essential in order to avoid the progression of symptoms from mild to life threatening. Upon detection of AD symptoms, the patient should be sat upright, in order to help trigger an orthostatic drop in blood pressure (Blackmer, 2003; Karlsson, 1999). Meanwhile, a Foley catheter may be inserted in patients utilizing intermittent catheterization, or for those using a permanent catheter, the catheter should

be checked for blockages. These steps will help void the bladder, the most common precipitant of AD (Blackmer, 2003). If neither Foley catheterization nor manual bowel disimpaction is effective in reducing the patient's blood pressure, antihypertensive medications can be administered. Commonly, captopril or nifedipine are used in the treatment of AD (Blackmer, 2003; Karlsson, 1999).

1.4.4 – Spinal Cord Injury and Autonomic Dysreflexia Models

Clip compression SCI models of AD have been well established in rats (Cormier et al., 2010; Marsh and Weaver, 2004; Weaver et al., 2001). The severity of cardiovascular dysfunction and axonal degeneration is proportional to the severity of injury (Cormier et al., 2010; Fehlings and Tator, 1995). AD can be induced in these animals through both noxious somatic and visceral stimulation, such as skin pinch or colon distension, respectively. Following induction of AD, accompanying symptoms can be assessed by changes in cardiovascular function (Marsh and Weaver, 2004; Krassioukov et al., 2002). Colon distension in animal models is used to replicate a human impacted colon. It has been shown to evoke both avoidance behavior and vigorous and reproducible cardiovascular reflexes in rats (Ness and Gebhart, 1988). Colon distension has been shown to stimulate afferents transmitting to both the T13-L2 and L6-S1 spinal segments (Ness, 1988). Additionally, compression SCI models in rats have been shown to be a reliable model for human SCI (Cormier et al., 2010; Marsh and Weaver, 2004).

1.4.4.1 – Visceral Nociception

Visceral afferents can be classified as muscle, tension-sensitive, tension/mucosal, serosal, or mesenteric according to the location of their respective receptor endings (Sengupta, 2009). The majority of these afferents are mechanoreceptive, while a smaller population is chemoreceptive. Mechanoreceptive muscle afferents in the thoracolumbar and sacral pathways respond to distension and stretch in hollow viscera and as such are commonly referred to as 'distension-sensitive receptors' or 'tension receptors' (Sengupta, 2009). The majority of these fibers (70-85%) have a low threshold for distension and are capable of transmitting both noxious and non-noxious stimuli. A smaller population of these fibers, however, has a high threshold for distension, indicating that they may be implicated in visceral nociception (Sengupta, 2009).

Primary sense cells, including endochromaffin cells and enteroendocrine cells (which release serotonin (5-HT) and cholecystokinin (CCK), respectively), release mediators upon visceral stimulation. These mediators generate action potentials in the visceral sensory afferents; unmyelinated C and lightly myelinated Aδ fibers. These fibers carry both somatic and visceral sensory information, often referring visceral pain to somatic sites as the two groups of afferents converge in the dorsal horn of the spinal cord (Grundy, 2004). These visceral afferent fibers synapse in laminae I, II, V and X (including the CAA) of the spinal cord. Laminae I-II and lamina V have increased c-Fos expression, a marker for neuronal activity, in response to colon distension (Sengupta, 2009). In lamina I, nociceptive and wide dynamic range (WDR) neurons from Aδ and C fibers travel up the contralateral ventrolateral funiculus to the brain. Lamina V contains WDR that transmits noxious stimuli to the brain. Projections traveling up the contralateral ventrolateral funiculus travel in the spinthalamic, spinoreticular, or spinomesencephalic

pathways. The spinothalamic pathway projects directly to the thalamus, but also projects collateral axons that synapse in the brainstem. Some of these collateral axons synapse in the RVLM and raphe nuclei; regions that have been implicated in cardiovascular regulation and nociception (Dampney, 1981).

1.5 – Gabapentin and Pregabalin

The thrombospondin (TSP) family of proteins are secreted by astrocytes and are involved in the formation of synapses. The application of TSPs (TSP-1 and -2) has been shown to be sufficient to induce excitatory synaptogenesis in cultured retinal glial cells (Christopherson et al., 2005). It has also been shown that TSPs are upregulated following spinal cord injury (Zeng et al., 2013). TSPs bind to the α2δ-1 subunit of voltage-gated Ca^{2+} channels, which are expressed extensively in pre-synaptic terminals of sensory afferents in the dorsal horn of the spinal cord (Bauer et al., 2009). Overexpression of α2δ-1 has been shown to increase excitatory synaptogenesis. These results suggest an important role of thrombospondin and its interaction with α2δ-1 in the aberrant synaptogenesis following SCI.

In addition to TSPs, the α2δ-1 receptor is also the receptor for gabapentinoid drugs Gabapentin and Pregabalin. Originally, it was believed that treatment with gabapentinoids inhibited the influx of Ca^{2+} after binding α2δ-1. Recently, however, it has instead been shown that treatment with Gabapentin or Pregabalin limits the trafficking of α2δ-1 to the plasma membrane (Bauer et al., 2009; Hendrich et al., 2008). Additionally, Gabapentin has been demonstrated to non-competitively antagonize TSP binding to α2δ-1, and in turn, inhibit excitatory synapse formation *in vitro* and *in vivo* (Eroglu et al., 2009). Furthermore, injection of Gabapentin into the neonatal mouse brain has been

shown to markedly reduce the number of excitatory synapses (Eroglu et al., 2009). The administration of both Gabapentin and Pregabalin has been shown to attenuate symptoms of neuropathic pain following spinal cord injury in humans (Siddall et al., 2006; Levendoglu et al., 2004). Interestingly, the development of neuropathic pain is thought to be mechanistically similar to the development of autonomic dysreflexia. The combined sprouting of sensory afferents and lack of descending inhibition, as seen following spinal cord injury, are believed to contribute to the development and maintenance of this condition (Woolf and Mannion, 1999).

Pregabalin was developed as an alkylated analogue of GABA (Dworkin, 2005). Pregabalin is the successor to Gabapentin, owning a greater oral bioavailability, linear dose-dependent plasma concentration, and increased half-life (~6 hours). Additionally, it was found to be more potent than Gabapentin in models of neuropathic pain (Dworkin and Kirkpatrick, 2005). Pregabalin is rapidly absorbed by the body and is able to reach peak plasma concentrations within an hour. Once absorbed, Pregabalin undergoes slight metabolism, with 98% undergoing renal excretion as non-metabolized Pregabalin (EMA, 2013).

1.6 – Current Study

1.6.1 – Rationale

The primary cause of autonomic dysreflexia is the loss of supraspinal pathways and bulbospinal inhibitory mechanisms below the site of injury. Following this, synaptic plasticity and the NGF-induced enlargement of sensory afferent fiber arbor provides increased input to interneurons and enhances the spinal reflex. The increased number of

synaptic contacts and expanded SPN dendritic arbor may lead to the development and maintenance of autonomic dysreflexia (Llewellyn-Smith et al., 2006).

As mentioned previously, the interaction of thrombospondin with α2δ-1 acts to promote excitatory synaptogenesis. Pregabalin administration, however, has been shown to limit the trafficking of α2δ-1 to the plasma membrane, limiting its expression at synaptic terminals (Bauer et al., 2009). Through this action, and non-competitively antagonizing thrombospondin at α2δ-1, it is possible that Pregabalin may be an inhibitor of synaptogenesis following SCI. Furthermore, Pregabalin treatment has been shown to decrease the release of glutamate in the spinal cord, as well as that of substance P and CGRP (Kumar et al., 2010; Fehrenbacher et al., 2003). Perioperative administration of Gabapentin and Pregabalin has been shown to reduce the incidence of chronic postsurgical pain (Clarke et al., 2012). Similarly, data from the Marsh lab has demonstrated that acute Pregabalin treatment following SCI significantly attenuated tactile allodynia in mice (Meisner et al., 2011). If this treatment is delayed one week, however, tactile allodynia develops post-SCI as it does in untreated groups. This may indicate that there is a 'therapeutic window' for Pregabalin treatment following SCI. In addition, autonomic dysreflexia has been shown to develop within 2 weeks following SCI, demonstrating a delay following injury that suggests a requirement of neuroplasticity (Marsh and Weaver, 2004; Krassioukov and Weaver, 1995). Given the evidence, it is possible that acute Pregabalin treatment immediately following SCI may help to mitigate the development and maintenance of autonomic dysreflexia by suppressing excitatory synaptogenesis, reducing the release of excitatory neurotransmitters, and reducing the aberrant sprouting of sensory afferents.

1.6.2 – Purpose

The objective of this animal study is to validate the clinical use of Pregabalin as a pre-emptive treatment to prevent changes of spinal circuits and neuronal excitability that are thought to lead to the development of autonomic dysreflexia following SCI.

1.6.3 – Hypotheses

1) The immediate administration of Pregabalin following SCI will attenuate changes in spinal circuits that are believed to lead to the development of autonomic dysreflexia following SCI**.**

2) Effects of Pregabalin treatment on suppression of SCI-induced alteration of spinal circuits will manifest as diminished symptoms of autonomic dysreflexia and improved bladder function in rats following SCI.

3) Delayed (7-days post-SCI) Pregabalin treatment will have little to no effect upon changes in spinal circuits, as the treatment will be outside of the 'therapeutic window' for Pregabalin treatment. Neurological symptoms seen following SCI will still be present.

4) Sensory afferent fiber sprouting and the subsequent increase in the density of type C sensory afferent fibers in the dorsal horn, central autonomic area and the interomediolateral cell column caudal to the injury will be suppressed by immediate Pregabalin treatment, compared to saline-treated and delayed Pregabalin treated SCI animals.

CHAPTER 2: MATERIALS AND METHODS

2.1 – Animals

Female Wistar rats (mean weight = 218.43 g; Charles River) were used in all experiments for this study. Female rats were utilized in order to maintain consistency with previous studies conducted in the Marsh lab. Experimental animals were housed in pairs at the Dalhousie University Carleton Animal Care Facility. Animals were kept on a 12h/12h light/dark cycle with food and water available *ad libitum*. All animal experimental procedures were approved by the University Committee on Laboratory Animals in accordance with the Canadian Council on Animal Care guidelines.

2.2 – Experimental Groups

Animals were randomly assigned to 4 groups: uninjured, saline-treated SCI, delayed Pregabalin-treated (dPGB) SCI, and immediate Pregabalin-treated (iPGB) SCI. The dPGB group received their first dose of PGB one week following SCI, after which standard dosing procedures were followed (Section 2.4, below). The iPGB group received their first dose of PGB one hour following SCI, after which standard dosing procedures were followed. The saline-treated group did not receive any doses of PGB, but instead received an injection of saline. The experimental groups are summarized in Table 2.1, below:

Table 2.1 - Experimental rat groups. Rats were divided into different experimental groups based on the timing of initial Pregabalin administration.

Experimental Group	Initial PGB Administration
Uninjured	None
SCI + Saline	None
SCI + dPGB	One week following SCI
SCI + iPGB	One hour following SCI

2.3 – Spinal Cord Injury

2.3.1 – Pre-operative Preparation

Animals were anesthetized with isofluorane (Pharmaceutical Partners of Canada). Induction was achieved at 4-5% isofluorane and anesthesia was maintained at 2.5% with an oxygen flow of 1L/min (Porter Instrument Co.). Isofluorane was reduced to 1.5-2% if the animals' respiration became laboured while anesthetized. The surgical level of anesthesia was determined by the disappearance of hind limb (toe pinch) and blink reflexes. Upon reaching the desired level of anesthesia, the dorsal surface of the animals were shaved between the lower cervical and upper thoracic region and cleaned with iodine solution (Betadine; West Chemical Products of Canada Ltd.). Following this, animals were given subcutaneous injections of Baytril (5 mg/kg; Bayer Inc.), saline (5 ml, 0.9% NaCl; Hospira, Inc.), Ketoprofen (5 mg/kg; Merial), and atropine (0.05 mg/kg; Abbott) and ocular lubricant (Refresh Lacri-lube; Allergan, Inc.) was applied to the eyes.

During the procedure, body temperature was maintained using a heating pad. All surgical instruments, including towels, gauze, and cotton-tipped applicators, were autoclaved before use. Surgical tools were further sterilized prior to surgeries through a 15-minute immersion in Cavacide (Metrex Research Corp.) and subsequent rinsing in

75% ethanol.

2.3.2 – Cholera Toxin B Injection

After adequate anesthetic depth was reached, a dorsal cut in the skin was made just above the kidney, posterior to the last rib and lateral to the midline. Rat-toothed forceps were used to pull the fat surrounding the adrenal gland out of the incision. Care was taken to avoid grabbing the adrenal gland with the forceps. Once the adrenal gland was exposed, 10 µl of 1% CTB solution (List Biological Laboratories, Inc.) was injected into the adrenal gland using a Hamilton Syringe (Hamilton Company). The injection site was sealed using tissue adhesive (Vetbond; 3M). The fat surrounding the adrenal gland was returned through the incision and the muscle and skin were then sutured (muscle and fat pad: 4.0 braided Vicryl suture, Ethicon, Inc.; skin: 5.0 Prolene suture, Ethicon, Inc.).

2.3.3 – Spinal Cord Injury

While anesthetized, surgery to induce SCI in the animals began with a midline dorsal incision. Following the incision, fat and muscle tissue was spread apart, revealing the large T2 dorsal process, which was used as a reference landmark to identify the T4 vertebrae. A laminectomy was performed at this site, revealing the T4 spinal segment. SCI was achieved using clip-compression with a modified aneurysm clip, calibrated to apply 35g of force (Toronto Western Research Institute, University Health Network, Toronto). Once the clip encompassed the spinal segment extradurally, the clip was released. Upon release, the clip applied a force of 35 g to the spinal cord for a period of

60 s, after which it was removed (Figure 2.1). Injury completeness was confirmed through visual inspection.

Following the injury, the muscle, fat pad, and skin were sutured (muscle and fat pad: 4.0 braided Vicryl suture, Ethicon, Inc.; skin: 5.0 Prolene suture, Ethicon, Inc.) and the animals were placed in an empty cage, lined with paper towel, on a heating pad to allow them to recover from the anesthetic. Within 30 minutes, the animals were able to move about their cages having recovered from the anesthetic.

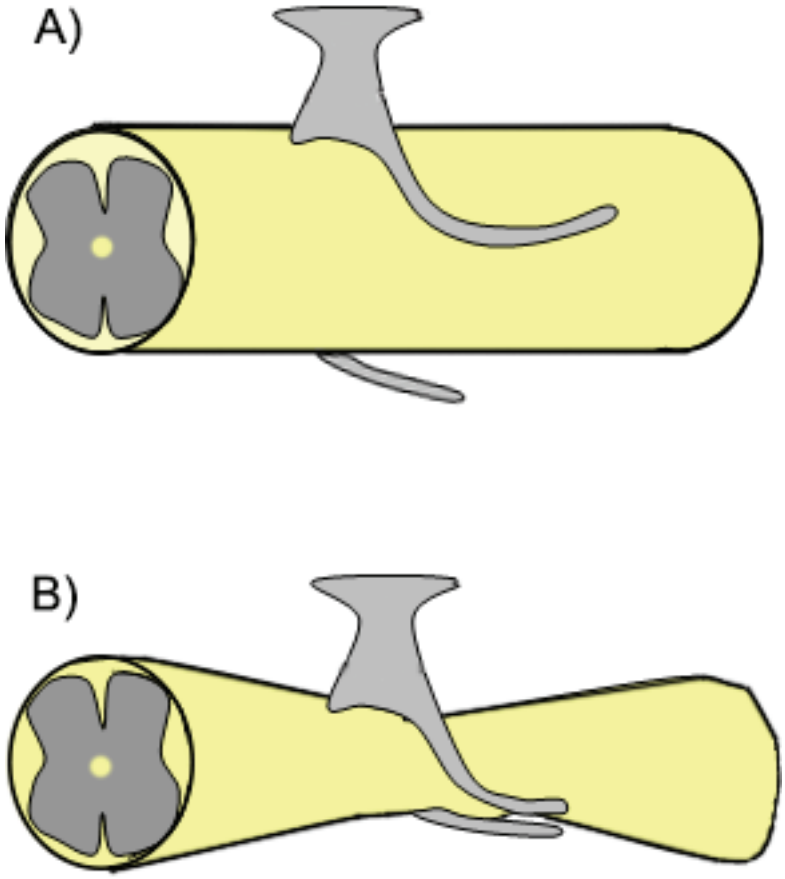


Figure 2.1. Clip compression injury. Modified aneurysm clip, modified to apply 35 g of force, is placed extradurally around the spinal cord (A). Upon release, force is applied for 60 s (B) before the clip is removed.

2.3.4 – Post-operative Care

Animals were removed from the empty cage and placed in a shallow, clean cage after approximately 30 minutes with access to food and water. A plastic grid placed at the bottom of these cages, covered in bedding, allowed the animals to easily maneuver around the cage using their forelimbs. Animals remained on heating pads for 12 h following surgery.

Daily subcutaneous injections of Baytril (5 mg/kg; Bayer, Inc.), saline (5 ml; 0.9% NaCl; Hospira, Inc.), and Ketoprofen (5 mg/kg; Merial) were administered for 72 h following surgery. Due to detrusor sphincter dyssynergia associated with SCI, post-operative care included manually expressing the animals' bladders 3 times a day for the duration of the study (18 d); morning, midday, and evening. Urinary tract infections (UTIs) were indicated by the appearance of cloudy or bloody urine. UTI-afflicted animals were given subcutaneous Baytril (5 mg/kg; Bayer, Inc.) to help combat the infection. In addition, due to the increased incidence of perforated ulcerated stomachs found in SCI rats in previous studies, the water supply of SCI animals was treated with Baytril (1 ml; Bayer, Inc.) for approximately 2 weeks following surgery. To help speed recovery and encourage eating, animals were provided with mash, corn, cereal, and fruit in addition to standard food pellets. Hay, wood blocks, and empty food trays were provided to the animals to help create a more stimulating environment. During post-operative care, animals were monitored for weight loss, behavioral changes, and symptoms of stress.

2.4 – Pregabalin Administration

Pregabalin (Pfizer, Inc.) was prepared in saline (0.9% NaCl; Hospira, Inc.) at a concentration of 10 mg/ml and administered to animals subcutaneously at a dose of 10 mg/kg. PGB was first administered based on experimental group as shown in **Table 2.2**. Following initial dose, PGB was administered twice daily, approximately every 12 hours.

Table 2.2 - Experimental groups and PGB dosing.

Experimental Group	Number of Animals	Initiation of PGB Treatment	Subsequent PGB Administration
Uninjured	3	N/A	N/A
SCI + Saline	9	N/A	N/A
SCI + dPGB	5	1 week post-SCI	Twice daily (every 12h) for 18d
SCI + iPGB	10	1 hour post-SCI	Twice daily (every 12h) for 18d

2.5 – Cannulation of the Carotid Artery

2.5.1 – Fabrication of Carotid Cannulas

The day preceding the cannulation procedure, carotid cannulas were constructed using 7 cm of Micro-Renathane tubing (MRE-025, .025" O.D. x .012" I.D., Braintree Scientific, Inc.) and 10 cm of tygon tubing (.02" I.D. x .06" O.D., Cole Parmer). One end of the tygon tubing was immersed for 5 minutes in 1-2-dichloroethane, allowing for relaxation of the tubing. MRE tubing was inserted approximately 1 cm into the relaxed tygon tubing and allowed to dry. The tygon tubing shrunk back to its original size, forming a tight seal around the MRE tubing, once dry. Additionally, super glue (SuperGlue; Loctite) was applied to the insertion point to further solidify the joint. Once constructed, the cannulas were left to dry overnight for use the following day.

2.5.2 – Pre-operative Preparation

Animal anesthesia induction and surgical instrument sterilization were performed as described above in *2.3.1*. During the procedure, the animal's body temperature was maintained using a heating pad. Prior to surgery and subsequent to anesthetic induction, the ventral surface of the animal was exposed, and the animal's neck was shaved and washed with iodine solution (Betadine; West Chemical Products of Canada Ltd.). To allow improved access to the carotid artery, an empty 10 ml syringe was placed under the animal's neck for the duration of the procedure.

2.5.3 – Carotid Artery Cannulation

Following anesthetic induction, a midline ventral incision located rostral to the sternum was made. The underlying muscle and tissue were spread to reveal the left common carotid artery. Once isolated, a ligature (6.0 braided silk nonabsorbable suture; Ethicon, Inc.) was utilized to tie off the artery. A small incision was then made in the artery using small surgical scissors. The cannula was filled with heparizined dextrose (500 IU/ml) (Heparin Sodium Injection; Pharmaceutical Partners of Canada, Inc.) prior to insertion into the carotid artery. The distal end of the cannula was then clamped using Kelly forceps and the proximal end of the cannula was fed into the small incision in the artery until it entered the descending aorta. A ligature was then tied around the artery to secure the cannula. The cannula was briefly unclamped to ensure that the cannula was working properly. A custom-made steel plug was then inserted into the distal end of the cannula. The cannula was then fed under the subcutaneous tissue to a cutaneous incision in the ventral surface, through which it was fed. The cannula was then secured using both sutures (4.0 braided Vicryl suture; Ethicon, Inc.) and tissue adhesive (Vetbond; 3M).

Following the procedure, the animals were placed in an empty cage on a heating pad for 30 minutes to allow them to recover from the anesthetic.

2.6 – Bladder Function Assessment

During the course of the study, the urine volume of each animal was assessed 3 times daily (as described by Ramsey et al., 2010). Bladders were manually expressed into a collection dish and the animal was washed clean with soap and warm water. Urine was then measured using a 5 ml serological pipette (Corning, Inc).

2.7 – Hind Limb Locomotor Function Assessment

At 7 and 21 days following SCI, each animal underwent an assessment of locomotor function in an open field test. This was done using the Basso, Beattie, and Breshnahan (BBB) 21-point rating scale (Basso et al., 1995). Articulation of the ankle, knee, and hip was observed and scored by a trained observer. Animals with normal locomotor function received a full score of 21; animals showing slight movement of the three joints received a score of 3, and animals lacking locomotion in the three joints received a score of 0. Animals with no movement of some joints and movement (full or slight) in others received varying scores.

2.8 – Cardiovascular Function Assessment

Cardiovascular function assessment was done 23 days following SCI. Pregabalin administration was ceased 2 days prior to cardiovascular function assessment to ensure any circulating PGB did not interfere with the recordings. A quiet, closed room was used

to record cardiovascular measurements during the day. This was done to mitigate any possible disturbances to the animals, that could affect the cardiovascular recordings. Animals remained in their cages throughout the recording. The cannula was clamped and the steel plug was removed before being connected to a pressure transducer outside of the cage measuring pulsatile pressure (Utah Medical Products). The pressure transducer was connected to a computer running Power Lab software (ADInstruments Ltd.), which allowed transmission and subsequent derivation and recording of the animals' arterial pressure, mean arterial pressure (MAP), and heart rate from the obtained pulsatile pressure. Treats were given to the animals during testing to help mitigate stress.

The animal's MAP and heart rate were measured both during rest and during noxious visceral stimulation. Noxious visceral stimulation was achieved through colon distension. Colon distension was initiated through the insertion of a lubricated balloon tip of a foley catheter (Bardex All-Silicone foley catheter; C.R. Bard, Inc.) approximately 5 cm into the rat's colon via the anus. Once inserted into the colon, the catheter was secured for the duration of the recordings by taping it to the base of the tail. The balloon was inflated with 1.5 ml of air using a 3 ml syringe (Becton Dickinson) over a period of 10 s to help mitigate any possible damage to the colon. The balloon remained inflated for a period of 60 s to initiate an autonomic dysreflexia response.

Baseline measurements were obtained for roughly 10 minutes following the onset of recordings, allowing the MAP to stabilize. After this, 3 colon distension episodes were initiated each with 20 minutes of rest between them to allow the animal to recover.

2.9 – Mean Arterial Pressure, Heart Rate, and Spectral Analysis

Power Lab Software (ADInstruments Ltd.) was used to derive the MAP, heart rate, and spectral data for each animal. For MAP and HR data, baseline measurements were obtained for 60 s prior to each colon distension. Measurements were also obtained for each colon distension episode for both the 60 s autonomic dysreflexia period and for a 5 s period during which the MAP was the highest; the "peak" MAP. Differences between the baseline and colon distension episode measurements were also obtained. For spectral information, data from two full colon distension events from each animal were processed using Power Lab Software (ADInstruments Ltd.).

2.10 – Transcardial Perfusion and Collection of Spinal Cord Tissue

Animals were anesthetized through an IP injection of ketamine (Broniche Animal Health Canada) and xylazine (Bayer, Inc.). Foot pinch and blink responses were used to determine anesthetic depth. Once these reflexes were absent, large scissors were used to make an incision at the xiphoid process. The ribcage was then cut on each side to allow the sternum to be moved, revealing the heart. A peristaltic pump (Masterflex console drive; Cole Parmer Instrument Company) was used to pump phosphate-buffered saline with glucose (PBS-G) solution through tygon tubing at a rate of 1 ml/s. A flattened needle (20G, 1 ½ inch; Becton Dickinson) was attached to the end of the tygon tubing, inserted at the apex of the heart into the left ventricle, and clamped into place prior to starting the flow of PBS-G. An incision was made in the right atrium using small surgical scissors and the peristaltic pump was subsequently turned on, initiating the transcardial perfusion with PBS-G. Once the flow leaving the right atrium appeared colourless (50-100 ml of PBS-G), the perfusate was switched to 4% paraformaldehyde (PFA) in PBS. Fixation

occurred following perfusion with approximately 400 ml of PFA. The peristaltic pump was stopped during this perfusate change to avoid the accumulation of air in the tygon tubing. Successful perfusion was determined first through the muscle spasms of the animals and subsequent body rigidity. Following perfusion, the spinal cord was removed from the spinal column using scissors, rongeurs, and foreceps. The spinal cord was segmented after referencing lumbar enlargements and the injury site at T4. Spinal segments were placed in scintillation vials containing 2% PFA/2.5% sucrose overnight at $4^{\circ C}$ before being transferred into vials containing 5% sucrose at $4^{\circ C}$. Segments were further equilibrated in 15% and 30% sucrose solutions at $4^{\circ C}$ after which they were frozen in 30% sucrose at $-80^{\circ C}$.

2.11 – Sectioning Spinal Cord Tissue

Spinal cord segments were cut after being encased and frozen in OCT (Tissue-Tek O.C.T. Compound, Sakura Finetek). T10-T12 segments were cut using a cryostat (Leica Biosystems) into two series of 30 µm transverse sections and thaw-mounted onto slides (Superfrost Plus, 25 x 75 x 1.0 mm; Fisherbrand). Sections were alternated between slides to ensure each series provided an accurate representation of the complete spinal segment.

2.12 – Immunohistochemistry

The thaw-mounted T10-T12 sections were first washed in TPBS-X (0.1 M tris phosphate buffered saline with 0.2% Triton-X (Sigma-Aldrich Co.); 3 x 10 minutes). Following the wash series, slides were then placed in sodium citrate solution (0.6 M) for 30 minutes at 60°C. Slides were incubated for 1.5 hours in blocking serum (5% horse

serum in TPBS-X) and then subsequently incubated for 24 hours with primary antibodies diluted in blocking serum (Rabbit anti-NOS @ 1:500 (Immunostar, Inc.); Guinea pig anti-Substance P @ 1:1000 (Cedarlane); Goat anti-CTB @ 1:5000 (List Biological Laboratories, Inc.)). Following primary antibody incubation, slides were washed 3 x 10 minutes in 1X TBS (no Triton-X) and then incubated for 2 hours in darkness with 1X TBS containing diluted biotinylated secondary antibody (Biotin anti-Rabbit @ 1:5000 (Molecular Probes)). Excess solution was removed from the slides followed by incubation for 1.5 hours in 1X TBS containing diluted fluorochrome secondary antibodies (Donkey anti-Guinea pig Cy2 @ 1:300 (Jackson ImmunoResearch Inc.) and Donkey anti-Goat 633 @ 1:300 (Invitrogen)) as well as Streptavidin conjugated Alexafluor 555 @ 1:300 (Invitrogen) in darkness. Slides were washed 3 x 10 minutes in TPBS in darkness, mounted using PermaFluor Aqueous Mounting Medium (Thermo Fisher Scientific Inc.), and coverslipped (20 x 40 mm; VWR).

2.13 – Microscopy

Fluorescent images were taken of the dorsal horn, IML, and CAA from T10-T12 transverse sections using an Axiovert 200M microscope (Carl Zeiss AG) with attached Hamamatsu Orca R2 digital camera (Hamamatsu Photonics K.K.) at 20x magnification.

2.14 – Image Analysis

All images were converted to grayscale using Adobe Photoshop CS6 (Adobe Systems Inc.). Quantification of substance P was performed on the inverted image using ImageJ software (NIH). The region of interest was identified for each image (CAA, LI-II,

LIII-IV, IML; Figure 2.2) and a threshold value was set to quantify each region. Cell counts were then calculated per 1000 μm^2 to normalize counts between spinal cord regions.

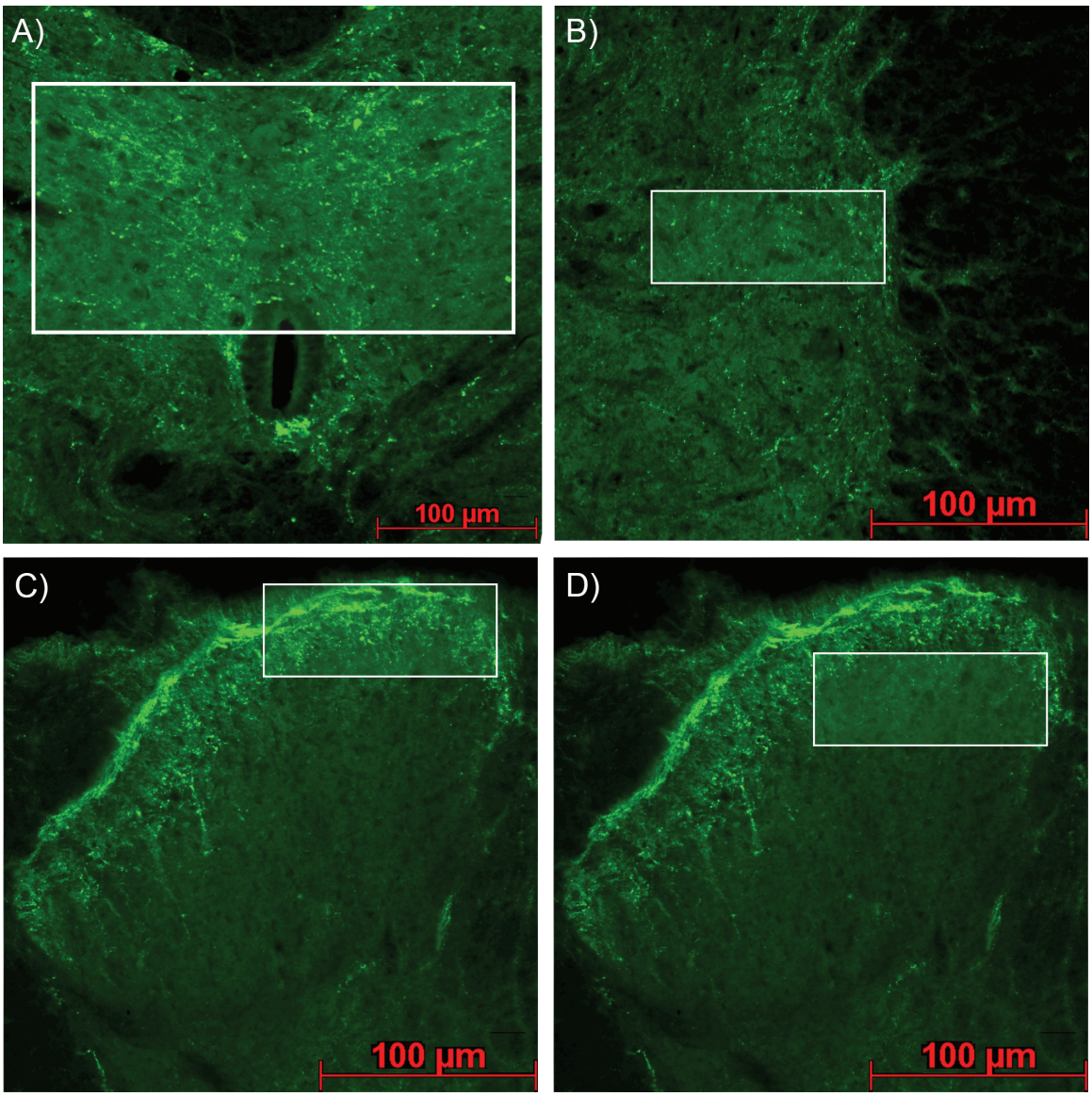


Figure 2.2 – Identification of the region of interest in the spinal cord. The region of interest was identified using a 339 µm x 174.1 µm rectangle (central autonomic area, (A)). Only cells within the rectangle were quantified with ImageJ. A similar approach was used in the other spinal cord regions (intermediolateral cell column, (B); Laminae I-II (C), Laminae III-IV (D)), although the rectangle dimensions were reduced to accommodate smaller spinal cord regions (215.8 µm x 87.3 µm).

2.15 – Statistical Analysis

Statistical analysis was performed using Minitab 15 software (Minitab Inc.). A one-way analysis of variance (ANOVA) was used to determine significant differences between experimental groups. Additional post-hoc analysis was performed using a Tukey test. A repeated measures ANOVA was also used to test for significance when analyzing values obtained on each day of the study.

CHAPTER 3: RESULTS

3.1 – Assessment of Hind Limb Locomotor Function

Hind limb locomotor function for all groups was assessed during open-field locomotion using the BBB 21-point scoring system (Basso et al., 1995) at 7 and 21-day time points following SCI. During all testing, the uninjured control group scored normal locomotor function while at 7 days post SCI (Figure 3.1a), the BBB scores of the SCI rats showed 'slight' or no movement at the hip, knee, and ankle joints. At 21 days post SCI (Figure 3.1b), the SCI animals displayed 'slight' movement at the hip, knee, and ankle joints – a minor improvement of the 7-day post-injury score. No significant differences were found between experimental groups at either the 7-day or 21-day post-injury time points. The BBB results of the SCI animals are indicative of a successful spinal cord injury.

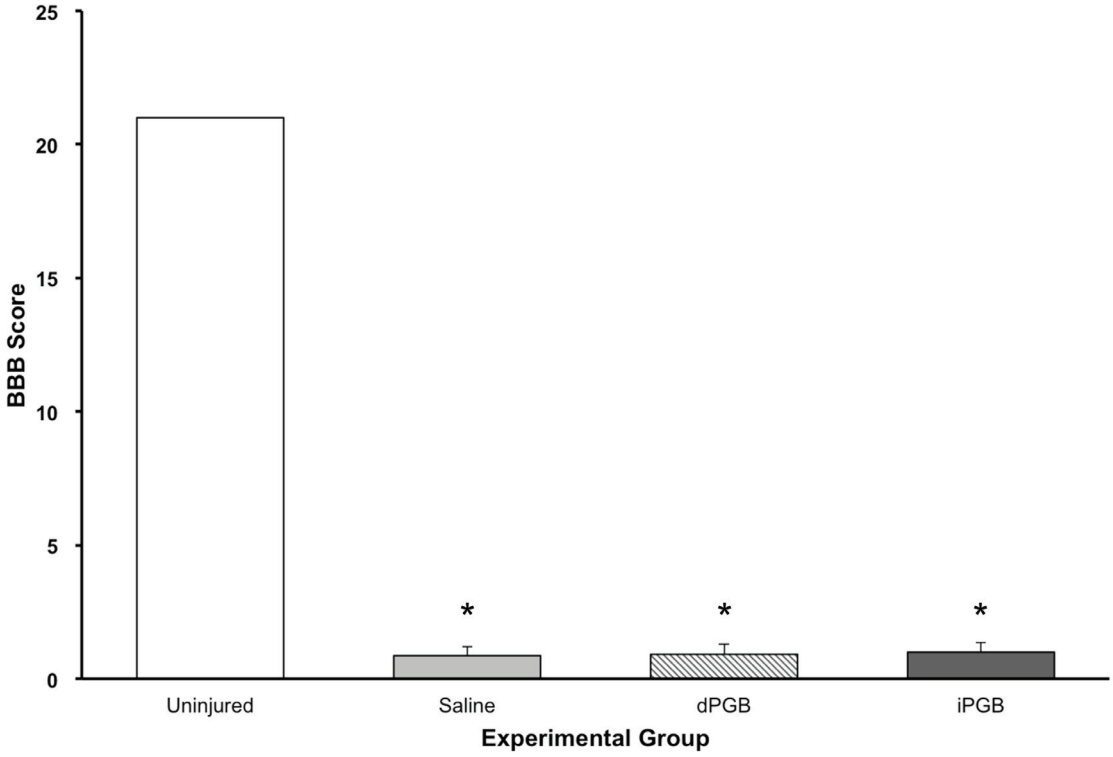


Figure 3.1. Mean BBB scores for hind limb locomotor function. The groups were assessed 7 days (above) and 21 days (not shown) post-injury. * denotes significance from the uninjured group ($p < 0.05$). Error bars represent the standard error.

3.2 – Assessment of Cardiovascular Function

3.2.1 – Assessment of Heart Rate

Data reflecting the changes in heart rate throughout the course of the cardiovascular physiology experiments were obtained for each animal (Table 3.1). No significant differences were found in resting heart rate or heart rate during colon distension stimulation between the experimental groups.

Table 3.1. Experimental heart rate data. Heart rate measurements were recorded 3 weeks post-SCI. Values were obtained for HR during three conditions: a) at rest; b) the 60s colon distension event; and c) the peak 5s of the colon distension event. Additionally, colon distension data was also analyzed as a percentage change from resting HR values. No significant differences were found between experimental groups in each condition.

	Experimental Group (ΔHR ± SE)		
	Saline-treated	**dPGB-treated**	**iPGB-treated**
Resting HR	452.2 ± 34.7	549.5 ± 7.4	503.4 ± 14.8
Average change in HR during CD	-39.4 ± 10.9	-44.00 ± 14.5	-31.2 ± 17.6
Minimum change in HR during CD	-58.0 ± 13.0	-63.1 ± 10.0	-41.0 ± 16.0
Percent change in average HR during CD	10.0 ± 2.2	10.5 ± 2.8	16.1 ± 5.1
Percent change in minimum HR during CD	17.0 ± 3.7	13.8 ± 2.7	16.9 ± 4.7

3.2.2 – Resting Cardiovascular Function

Resting MAP (Figure 3.2) was obtained for each animal to establish a baseline prior to colon distension. No significant differences ($p > 0.05$) were found among the uninjured (123.2 ± 2.2 mmHg), saline-treated (113.3 ± 2.8 mmHg), dPGB-treated (110.4 ± 5.4 mmHg), or iPGB-treated (124.1 ± 5.3 mmHg) experimental groups for resting MAP[1]. Though not statistically significant, SCI tended to decrease resting MAP in saline- and dPGB-treated animals.

[1] Resting MAP values for uninjured animals were collected previously in the Marsh lab (Cormier et al., 2010).

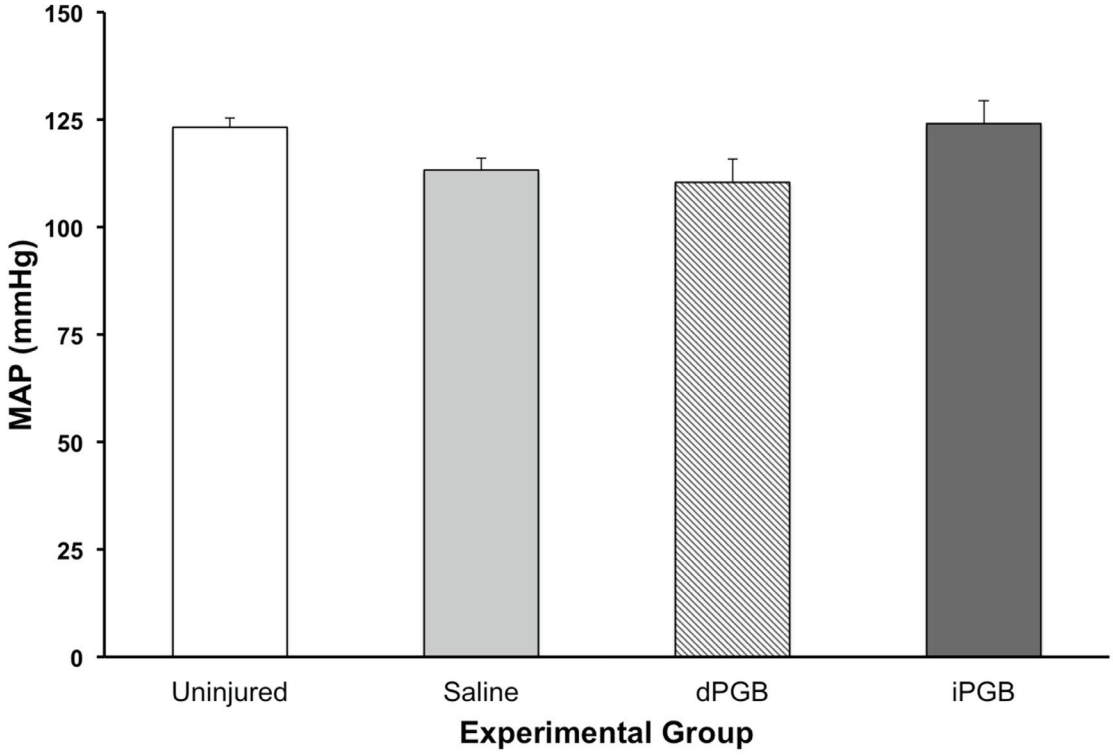


Figure 3.2. Mean resting cardiovascular function. Resting mean arterial pressure (MAP) measurements were recorded 3 weeks post-SCI. MAP was calculated for the 60-second period prior to each colon distension event and then averaged. Resting MAP values for uninjured animals were collected previously in the Marsh lab (Cormier et al., 2010). No significant differences were found between the uninjured, saline, delayed PGB-treated, and immediate PGB-treated groups. Error bars represent the standard error.

3.2.3 – Cardiovascular Function During Noxious Visceral Stimulation

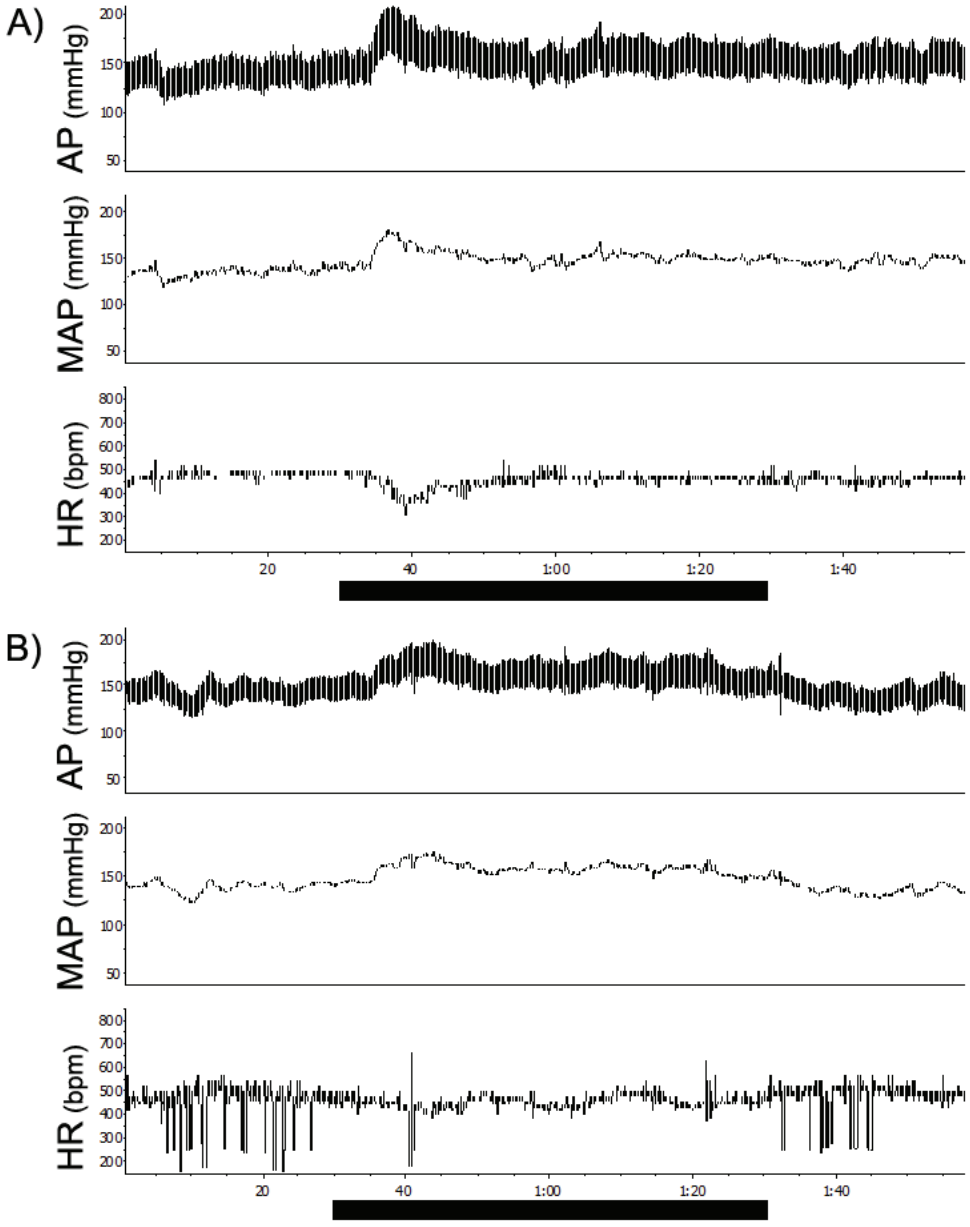
Representative cardiovascular recordings during noxious visceral stimulation can be found in Figure 3.3. Saline-treated (31.4 ± 2.6 mmHg) and dPGB-treated (33.1 ± 7.9 mmHg) animals had a significantly greater increase in average MAP ($p = 0.008$) during the course of the 60-second colon distension compared to uninjured animals (11.0 ± 1.6 mmHg). MAP in iPGB-treated animals during this time period (21.2 ± 2.6 mmHg) was significantly lower ($p = 0.016$) than both the saline- and dPGB-treated groups (Figure 3.4A). Saline-treated (41.5 ± 2.6 mmHg) and dPGB-treated (42.9 ± 8.2 mmHg) animals also had a significantly greater peak MAP ($p = 0.005$) during the course of the 60-second colon distension in comparison to uninjured animals (19.8 ± 2.6 mmHg). Peak MAP in iPGB-treated animals during this time period (34.0 ± 1.9 mmHg) was also significantly higher ($p = 0.005$) than uninjured animals. Though higher than uninjured animals, the peak MAP in iPGB-treated animals during the 60-second colon distension was significantly ($p = 0.005$) lower than saline- and dPGB-treated animals (Figure 3.4B). Saline- and dPGB-treated animals display MAP changes typical of autonomic dysreflexia following colon distension, while iPGB-treated animals display lower MAP changes in response to the same stimuli.

Due to the differences in baseline resting MAP between groups, the MAP during colon distension of the three experimental groups was further analyzed as a percentage change. Saline-treated animals had a significantly higher ($p = 0.005$) average change in MAP from baseline (23.5 ± 2.5%) during the 60-second colon distension when compared to uninjured animals (11.0 ± 0.9%) (Figure 3.5A). During this time, saline-treated

animals also had a significantly higher (p = 0.01) peak change in MAP from baseline (28.4 ± 2.1%) when compared to uninjured animals (19.8 ± 1.7 %) (Figure 3.5B). Average change in MAP from baseline in iPGB-treated animals (14.6 ± 1.8%) was significantly lower than both saline- and dPGB-treated animals (p = 0.02) (Figure 3.5A). In addition, iPGB-treated animals also had a significantly lower (p = 0.01) peak change in MAP from baseline (21.5 ± 1.2%) during this time when compared to saline- and dPGB-treated animals (Figure 3.5B)[2].

[2] MAP values for uninjured animals during colon distension were collected previously in the Marsh lab (Cormier et al., 2010).

Figure 3.3. Examples of arterial pressure (AP), MAP, and HR recordings during colon distension. Experimental groups, saline-treated (A) and iPGB-treated (B), were assessed 3 weeks post-SCI. Horizontal black lines indicate the duration of the colon distension.


A)
AP (mmHg)
200
150
100
50
MAP (mmHg)
200
150
100
50
HR (bpm)
800
700
600
500
400
300
200
20
40
1:00
1:20
1:40
B)
AP (mmHg)
200
150
100
50
MAP (mmHg)
200
150
100
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HR (bpm)
800
700
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200
20
40
1:00
1:20
1:40

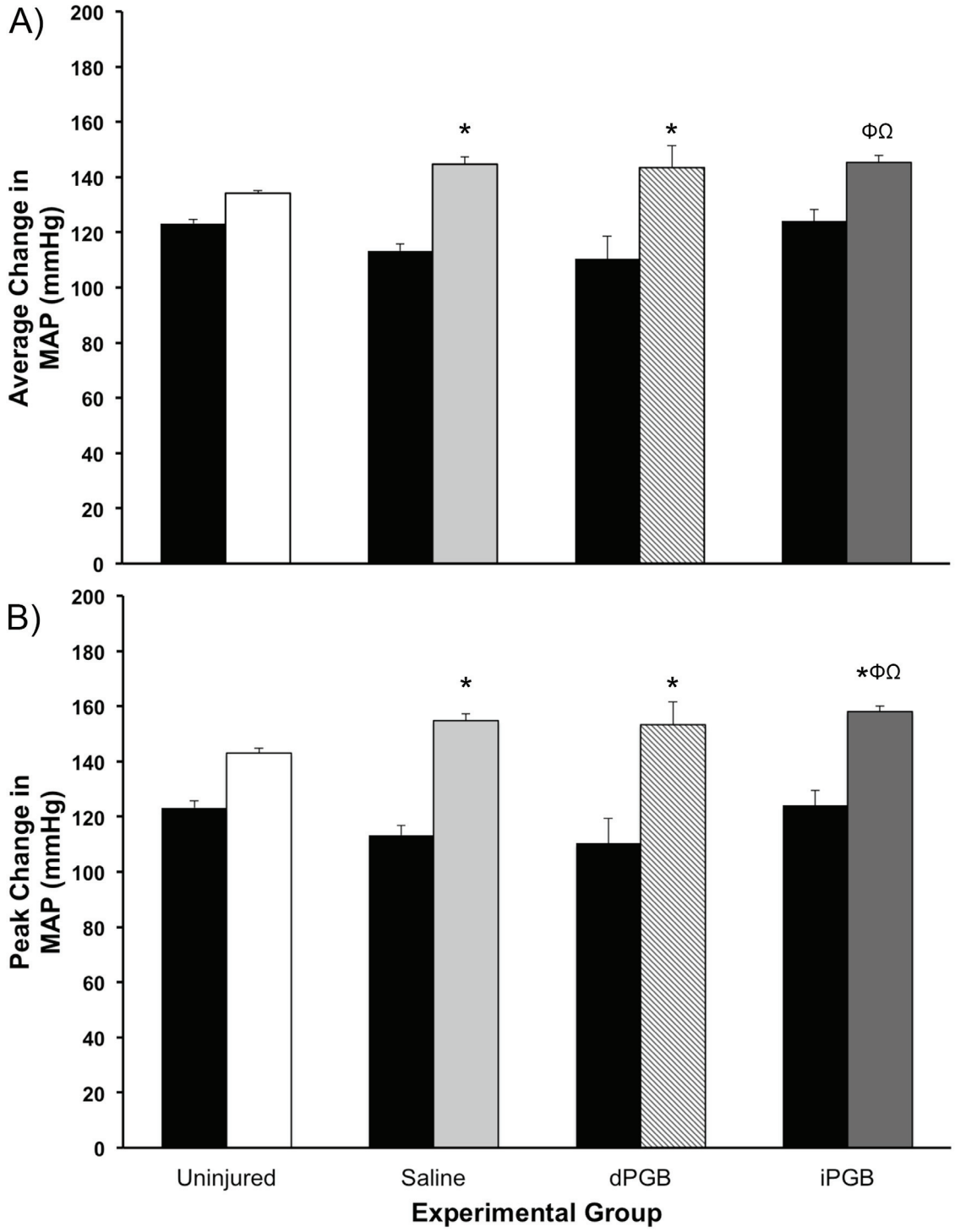


Figure 3.4. MAP changes during CD event. Mean values were obtained for the change in mean arterial pressure (MAP) during the 60s colon distension (CD) (A) and during the peak 5s of the CD (B), 3 weeks post-SCI. Corresponding resting MAP values are shown in black. * denotes significance from uninjured group, Φ denotes significance from saline-treated group, Ω denotes significance from dPGB-treated group. Error bars represent the standard error.

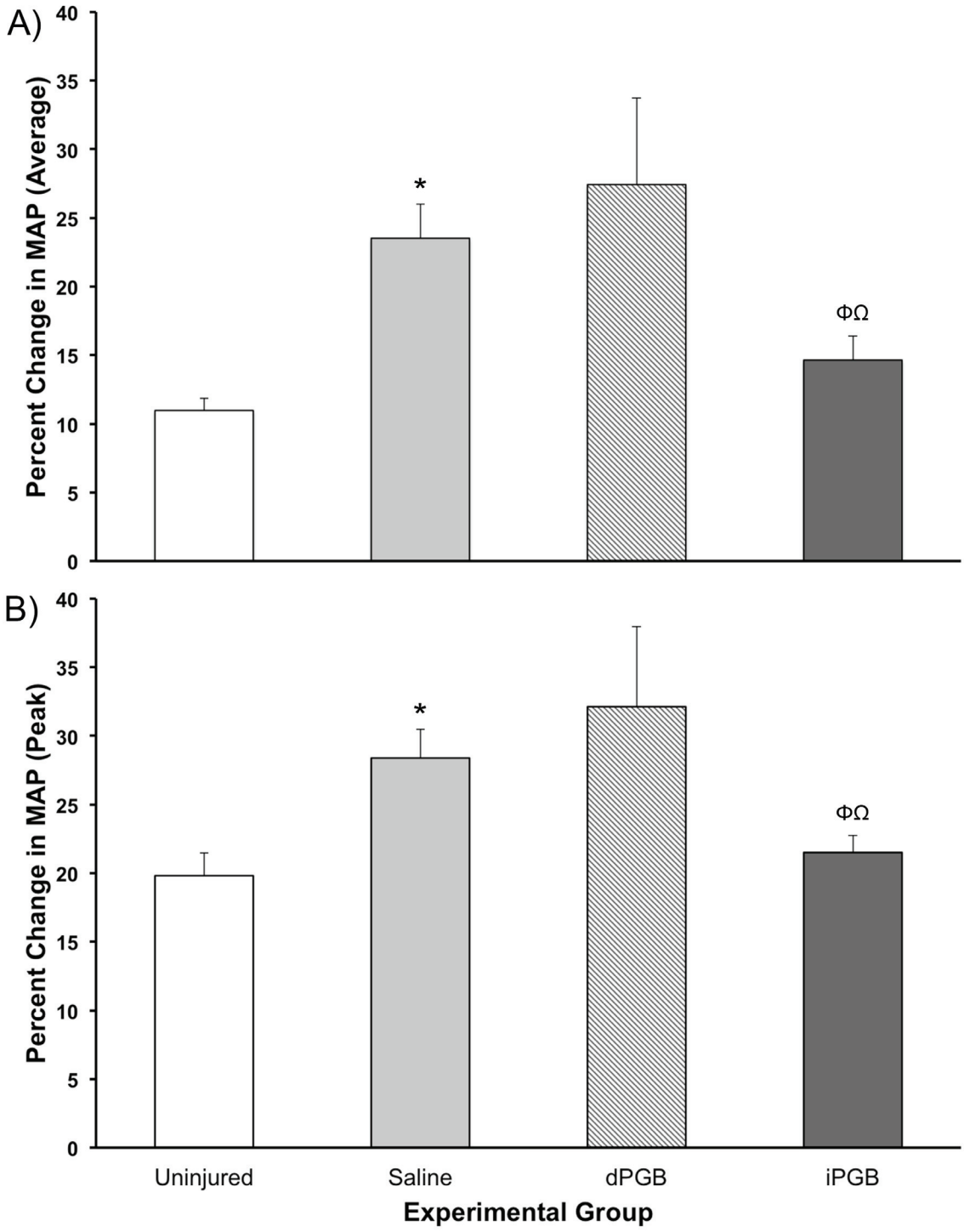
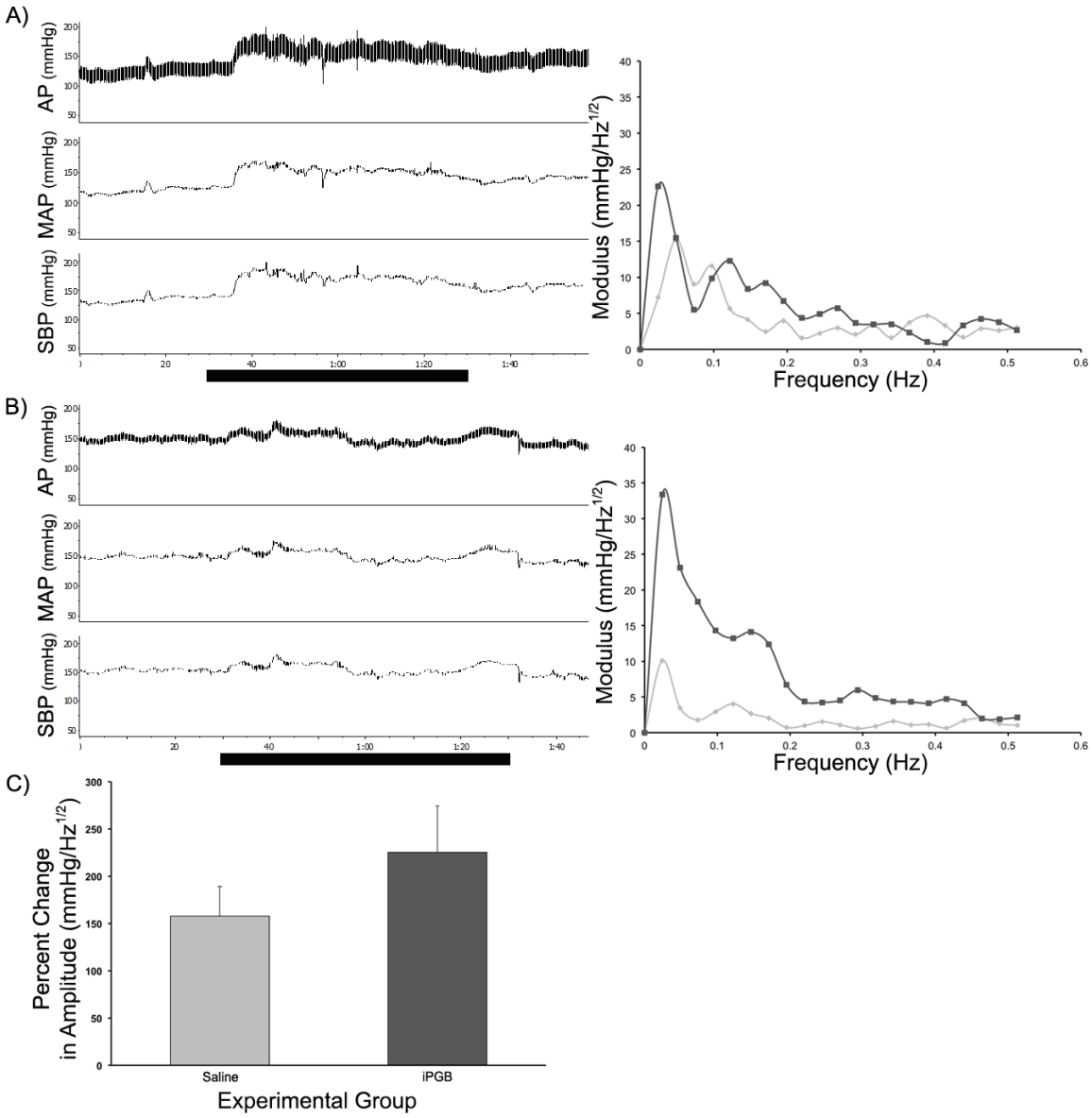


Figure 3.5. Cardiovascular changes during colon distension as a percent change from resting values. Values were obtained for mean arterial pressure (MAP) during the 60s colon distension (CD) (A) and the peak 5s of the CD (B), 3 weeks post-SCI, and analyzed as a percentage change from the groups' resting MAP values. * denotes significance from uninjured group, Φ denotes significance from saline-treated group, Ω denotes significance from dPGB-treated group. Error bars represent the standard error.

3.3 – Spectral Analysis of Cardiovascular Function

The same cardiovascular data were additionally subject to spectral analysis. Data used for spectral analysis were generated from the raw systolic blood pressure (SBP) obtained during the cardiovascular measurements. Measurements comprised the 200 Hz digitization of cardiovascular data, followed by continuous computation and recording of arterial pressure (AP) and mean arterial pressure (MAP). The SBP was derived from the MAP. The spectrum computation of the SBP was completed using a discrete fast Fourier transform algorithm within Power Lab Software (ADInstruments Ltd.). Figure 3.6 displays representative time-course and corresponding SBP spectra for both saline-treated (A) and iPGB-treated (B) groups. Resting moduli (light grey) express the amplitude at rest during the 60s preceding the colon distension event. Colon distension event moduli (dark grey) express the amplitude during the 60 s colon distension (Figure 3.6A and Figure 3.6B, right). Figure 3.6C displays the absolute percentage change in amplitude from corresponding resting amplitude values. No statistical differences were found between the experimental groups.

Figure 3.6. Representative samples of arterial pressure (AP), mean arterial pressure (MAP), and systolic blood pressure (SBP) recordings and corresponding SBP spectra. Saline-treated (A) and iPGB-treated (B) groups were assessed 3 weeks post-SCI. Horizontal black lines indicate the duration of the colon distension. Corresponding spectra (A and B, right) are shown at rest (light grey) and during colon distension (dark grey). Change in amplitude from rest (C) was recorded as a percentage between the groups.


A)
AP (mmHg)
MAP (mmHg)
SBP (mmHg)
Modulus (mmHg/Hz^1/2)
Frequency (Hz)
B)
C)
Percent Change in Amplitude (mmHg/Hz^1/2)
Saline
iPGB
Experimental Group

3.4 – Assessment of Bladder Function

Throughout the 18-day study, the urine volume of each rat was measured and recorded three times daily. Point analysis using a 2-sample t-test on Day 10 and 11 post-SCI revealed a significant difference in urine volumes on these days between groups ($p < 0.05$) (Figure 3.7).

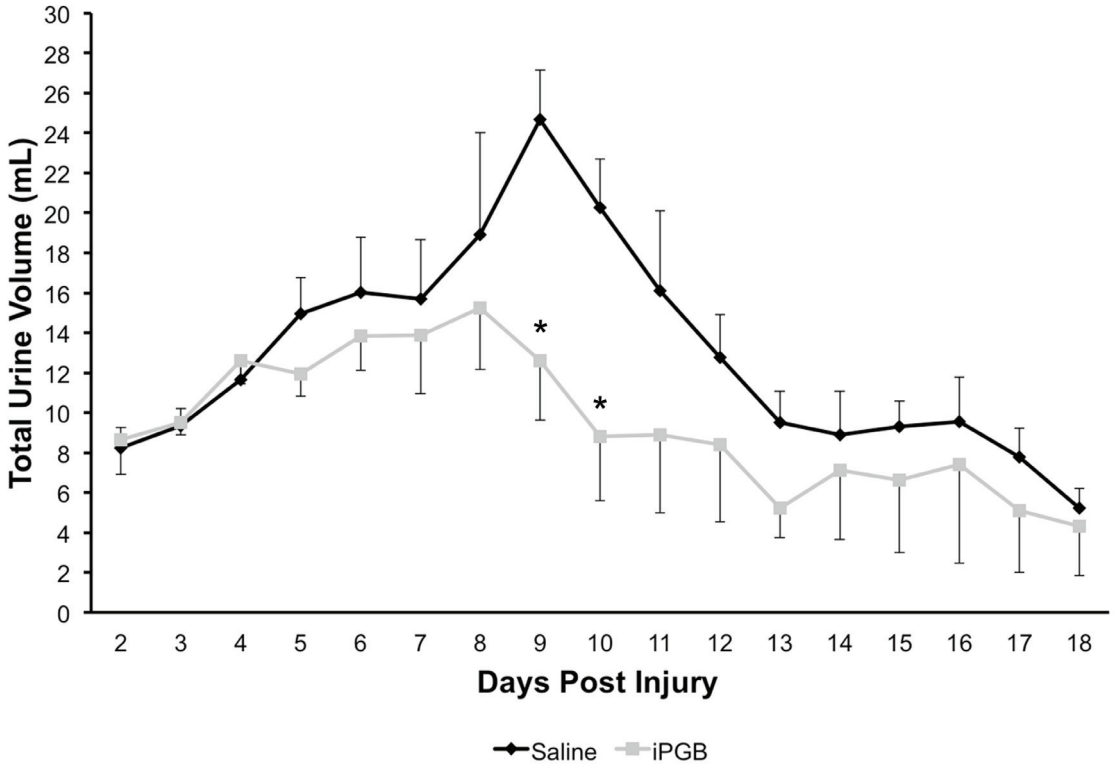


Figure 3.7. Daily urine volume. Daily urine volumes were obtained for the two groups. * denotes significance from the saline-treated group. Error bars represent the standard error. Outlier values from one rat (saline-treated) were removed; values lay outside of 2 standard deviations from the mean.

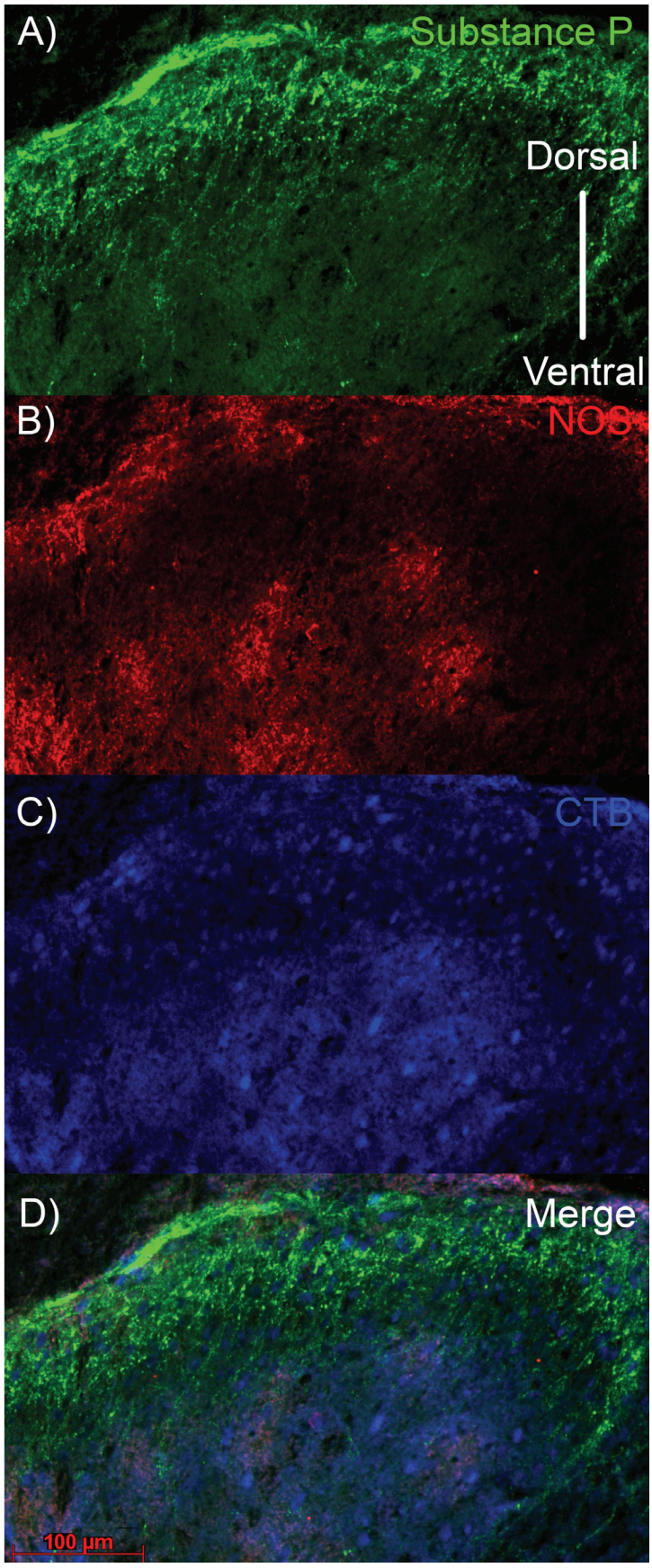
3.5 – Substance P Immunoreactivity in the Spinal Cord

The substance P immunoreactivity was determined from transverse spinal cord sections (T10-12) in the central autonomic area (CAA), laminae I-II, laminae III-IV, and the intermediolateral (IML) cell column (Table 3.2). Compared to the uninjured control group, the integrated optical density of substance P immunoreactive sensory afferent fibers increased in the saline-treated and iPGB-treated groups in all regions but the IML (Figure 3.8).

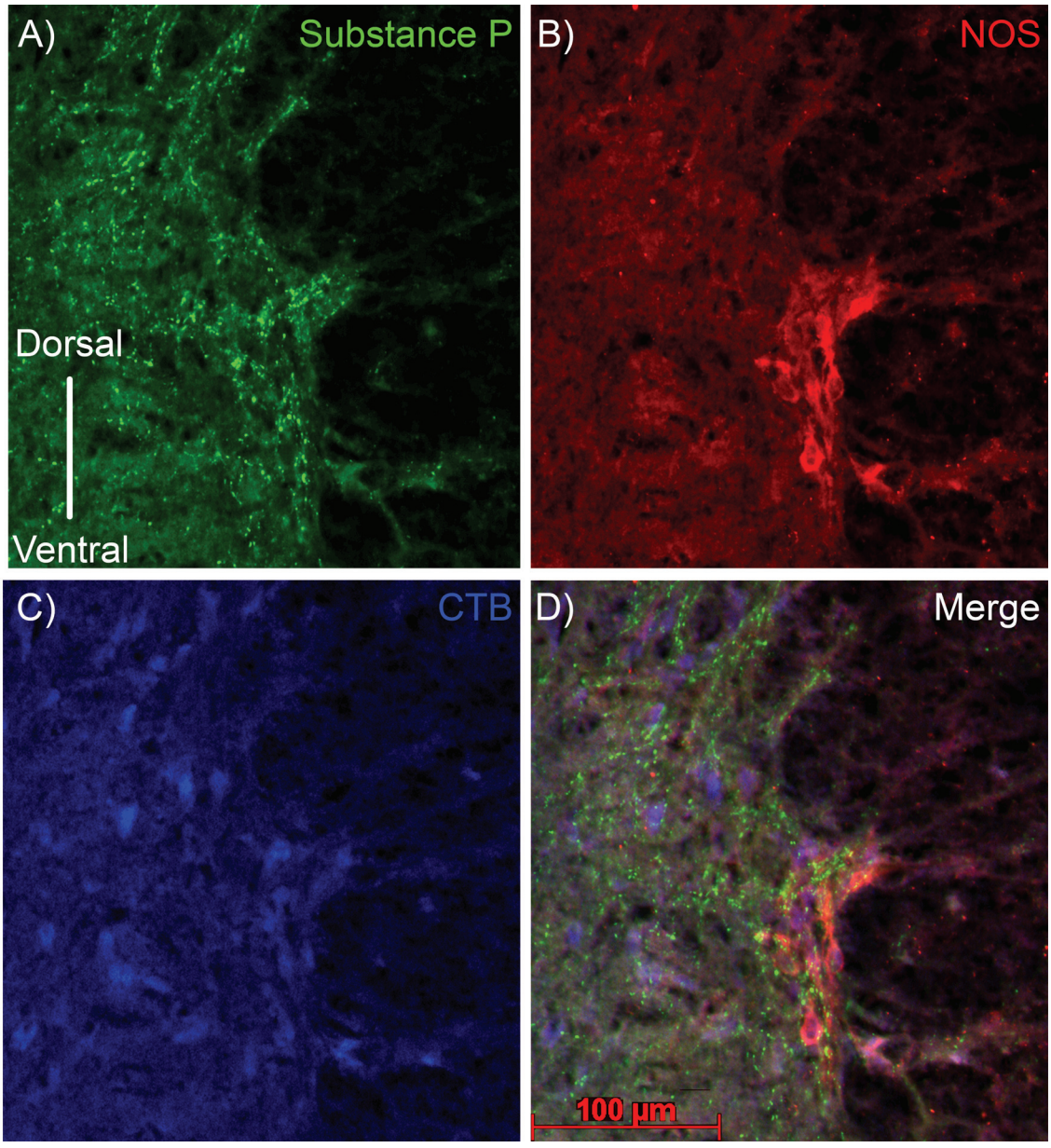
Table 3.2. Substance P immunoreactive sensory afferent fibers in transverse T10-T12 spinal cord sections. Integrated optical density of substance P-immunoreactive cells was calculated per 1000 μm^2 in the central autonomic area (CAA) and laminae (L) I-IV in uninjured control animals, saline-treated animals, and iPGB-treated animals. * denotes significance from uninjured group, Φ denotes significance from saline-treated group, $p < 0.05$.

	Integrated optical density of substance P-immunoreactive (area/1000 μm^2 ± SE)			
	CAA	**LI-II**	**LIII-IV**	**IML**
Uninjured animals (n = 6, 6, 6. 5)	6.89 ± 0.42	11.90 ± 0.51	8.50 ± 0.66	13.14 ± 1.05
Saline-treated animals (n = 6, 7, 7, 6)	13.49 ± 0.73*	15.12 ± 0.61*	12.08 ± 0.50*	11.78 ± 0.84
iPGB-treated animals (n = 6, 5, 5, 6)	15.47 ± 0.51*Φ	16.82 ± 0.58*	14.19 ± 0.63*Φ	13.45 ± 0.75

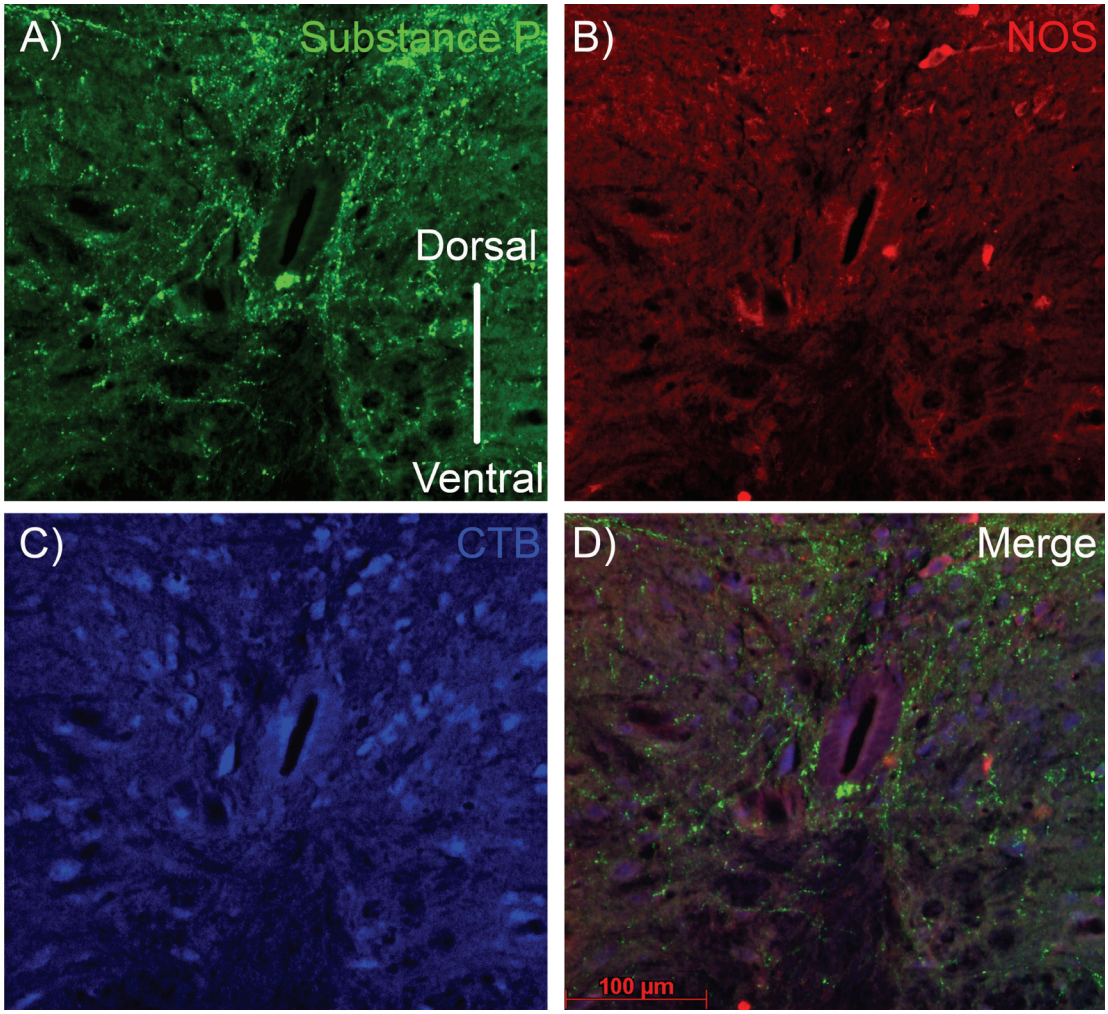
Figure 3.8. Representative substance P immunoreactivity in T10-T12 spinal cord segments. 20x images of the dorsal horn (DH) (i; iPGB-treated rat), intermediolateral cell column (IML) (ii; saline-treated rat), and central autonomic area (CAA) (iii; saline treated rat) displaying substance P (A), nitric oxide synthase (NOS) (B), and cholera toxin B (CTB) (C) IR. Spinal cords were processed and imaged 3 weeks post-SCI. Scale bar is shown in (D).


i)
A)
Substance P
Dorsal
Ventral
B)
NOS
C)
CTB
D)
Merge
100 µm

ii)



iii)



CHAPTER 4: DISCUSSION

4.1 – Effect of Pregabalin Treatment in Autonomic Dysreflexia

4.1.1 – Clinical Relevance

This study investigated the use of Pregabalin as a pre-emptive treatment in order to prevent the changes in spinal circuitry that lead to the development and maintenance of autonomic dysreflexia following spinal cord injury. As a pre-clinical animal experiment, if proven effective, it is possible that pre-emptive Pregabalin treatment could be translated to human SCI. In humans, Pregabalin treatment following SCI may act to greatly improve the quality of life of SCI- and autonomic dysreflexia-afflicted individuals. Furthermore, hypertensive episodes that accompany autonomic dysreflexia could be attenuated, allowing for more effective rehabilitation programs for these patients.

4.1.2 – Pregabalin Inhibitory Mechanisms

Following SCI, sprouting of afferent fibers due to NGF and CGRP regulation, thrombospondin-stimulated synaptogenesis, and the lack of descending inhibition can lead to the development and maintenance of autonomic dysreflexia. Given the differences between experimental groups in this study in terms of cardiovascular and bladder function, it appears that the immediate administration of Pregabalin following SCI may help to attenuate the neuroplastic changes commonly associated with the development of autonomic dysreflexia. It has been previously documented that the acute administration of Pregabalin and Gabapentin can reduce the trafficking of α2δ-1 to the plasma membrane (Bauer et al., 2009), reduce the release of CGRP (Fehrenbacher et al., 2003), and reduce the spinal release of glutamate (Kumar et al., 2010), thereby limiting the excitatory input to the spinal cord and subsequent reduction of exaggerated sympathetic responses to

noxious stimuli (Rabchevsky et al., 2011). In this study, however, administration of Pregabalin was ceased 48 hours prior to cardiovascular recordings. Therefore, any circulating systemic Pregabalin would be eliminated prior to recording and any changes observed would be the result of chronic changes in spinal circuitry. Following colon distension, the saline-treated group displayed percentage increases in MAP (23.5%) comparable to those previously reported (Marsh and Weaver, 2004; Cormier et al., 2010; Mayorov et al., 2001), and were significantly higher than the uninjured group (Figure 3.5A). The iPGB-treated group, however, had percentage increase in MAP (14.6 ± 1.8 mmHg) similar to the uninjured group (11.0 ± 0.9 mmHg) (Figure 3.5A). These results indicate that immediate Pregabalin treatment following SCI can significantly reduce the increase in MAP associated with autonomic dysreflexia when compared to untreated groups. In addition, administration of Pregabalin did not affect resting MAP between experimental groups (Figure 3.2). Resting MAP for the saline-treated (113 ± 2.8 mmHg) and dPGB-treated (110.4 ± 5.4 mmHg) groups tended toward a lower value than uninjured animals (123.2 ± 2.2 mmHg). This is consistent with previous reports that resting MAP decreases following SCI (Marsh and Weaver, 2004; Mayorov et al., 2001; Krassioukov and Weaver, 1995). Interestingly, the iPGB-treated group had a similar resting MAP (124.1 ± 5.3 mmHg) to the uninjured group. This suggests that Pregabalin treatment may help MAP return to baseline measurements following SCI.

Our results show that if Pregabalin treatment is delayed one week following SCI, the pre-emptive effect is lost and symptoms of autonomic dysreflexia develop as they do in saline-treated animals. From this, it can be concluded that chronic changes such as sensory afferent sprouting may have already irreversibly occurred and that spinal cord circuitry changes associated with autonomic dysreflexia develop within one week.

Therefore, there is a finite 'therapeutic window' for Pregabalin and other putative "pre-emptive" treatments. This coincides with previous data from the Marsh lab, indicating the same therapeutic window for Pregabalin administration in the treatment of tactile allodynia (Gehrig et al., 2012; Meisner et al., 2011). Additionally, the time-course associated with autonomic dysreflexia onset put forth by Marsh and Weaver (2004) and Krassioukov and Weaver (1995) coincides with this therapeutic window. Within this therapeutic window, several maladaptive changes occur including the release of growth factors (such as NGF), excess release of glutamate causing excitotoxicity, cell death due to ischemia, and free radical formation (Schwab et al., 2006).

In addition to the previously documented effects of Pregabalin administration following SCI, our results suggest that the immediate Pregabalin treatment following SCI may help to return spinal reflexes back to baseline through compensation for the lack of descending inhibition. Substance P is an excitatory neuropeptide released during noxious stimulation (Allen et al., 1997). Contrary to our hypothesis, our results show that the integrated optical density of substance P$^-$immunoreactive sensory afferent fibers is significantly higher in the DH (LIII-IV) and CAA in the iPGB-treated group than both the saline-treated group and uninjured group. The increase in substance P in the CAA amongst iPGB-treated animals is especially intriguing. Previously it has been shown that a large group of GABAergic interneurons in the CAA directly inhibit SPN activity in the IML (Deuchars et al., 2005). Stimulation of these interneurons through glutamate microinjections resulted in hyperpolarization of IML SPNs and subsequent inhibition. Additionally, no difference was found between the integrated optical density of substance P$^-$immunoreactive sensory afferent fibers in the IML of uninjured or iPGB-treated animals, indicating a similar level of neuropeptide innervation. Therefore, we believe that

the increase in substance P^+ immunoreactive sensory afferent fibers leads to increased excitation of GABAergic interneurons synapsing on SPNs in the IML and CAA. This increased inhibition of SPNs in the CAA and the IML may lead to decreased sympathetic outflow of spinal reflexes, resulting in attenuation of severe vasoconstriction and subsequent rise in BP following noxious stimuli. We propose that Pregabalin treatment may utilize increased substance P release to stimulate inhibitory interneurons in the CAA in order to compensate for the lack of descending inhibitory pathways from higher ANS centers. Interestingly, the density of substance P^+ immunoreactive sensory afferent fibers was also significantly higher in the saline-treated group than the uninjured group. We believe this, however, to be purely excitatory substance P release and is due to the changes that normally follow SCI, including increased sensory afferent arbor and increased synaptogenesis. This, in turn, would lead to increases in glutamate and substance P release following excitatory stimuli, resulting in exaggerated sympathetic outflow. This coincides with our cardiovascular results as saline-treated animals had a much higher cardiovascular response to colon distension than either uninjured or iPGB-treated animals.

This proposed mechanism also is consistent with our bladder function data. Control of micturition is moderated by the pontine micturition center (PMC), located in the brainstem, and its connections to the sacral spinal cord. At sufficient levels of bladder distension, the PMC becomes activated through input from bladder afferents (Aδ and C fibers) and subsequent ascending input (Cruz and Cruz, 2011). This results in the relaxation of the external urethral sphincter through inhibition of its somatic innervation, and contraction of the bladder's detrusor muscle through parasympathetic innervation (Cruz and Cruz, 2011). This coordinated control of relaxation and contraction allows for

successful voiding of the bladder. Following SCI, however, the descending and ascending pathways from the PMC to the spinal cord become blocked at the site of injury, leading to detrusor sphincter dyssynergia (DSD) (Castro-Diaz and Taracena Lafuente, 2006). In this case, both the detrusor and external urethral sphincter contract simultaneously. This is thought to occur due to the increased sprouting of sensory afferents following SCI causing contraction of the external urethral sphincter (Zinck and Downie, 2008). Furthermore, Zinck and Downie (2008) demonstrated that saporin conjugated to IB4 (IB4-SAP), a ribosomal inactivating protein neurotoxic to $IB4^+$ afferents, can help to improve voiding efficiency in SCI rats when compared to untreated SCI rats. In addition, they describe that lamina X contains a population of inhibitory interneurons responsible for mediating proper control of the external urethral sphincter (Zinck and Downie, 2008). This coincides with our data as we see an increase in substance P activity in this region in iPGB-treated rats, suggesting these same inhibitory interneurons are being stimulated. It appeared that iPGB-treated animals regained their bladder function (spontaneous voiding) more quickly than saline-treated animals, regaining most of their function by approximately 9-10 days post-SCI versus approximately 13 days post-SCI in saline-treated animals (Figure 3.7). These results indicate that immediate Pregabalin following SCI may help to prevent changes in these bladder afferents and therefore work to attenuate the development of DSD following SCI. Both the abolition of $IB4^+$ afferents and increased excitation of inhibitory CAA interneurons have been shown to help to reduce the severity of DSD and the return of spontaneous voiding (Zinck and Downie, 2008). Given this coincidence with our results, we believe our proposed mechanism of inhibitory interneuron compensation of descending spinal pathways can help explain how Pregabalin administration immediately following SCI attenuates symptoms associated

with autonomic dysreflexia. If these results are translatable to human SCI patients, the administration of Pregabalin could help to mitigate the hypotension commonly seen following SCI, episodic hypertension, and increase voiding efficiency, all greatly improving the quality of life of future SCI patients.

4.2 – Limitations

Pregabalin (10 mg/ml) was administered by subcutaneous injection every 12 h at a concentration of 10 mg/kg. This is similar to previous studies involving Pregabalin administration following SCI (Kumar et al., 2010; Wallin et al., 2002). Kumar et al. (2010) has shown that doses of 10 mg/kg are sufficient to suppress neuropathic pain. Other studies have shown, however, that tactile allodynia is suppressed by Pregabalin in a dose-dependent manner up to 100 mg/kg. Therefore, additional changes in spinal circuits and resulting cardiovascular and bladder function may occur at higher Pregabalin doses.

In this study, heart rate data was obtained through resolution of pulsatile pressure data from carotid cannulation. The many stages involved in this process made it difficult to accurately obtain HR data for each animal. Other systems, including the use of radiotelemetric devices implanted in the abdominal aorta, could provide more accurate HR data for each animal. In addition, these devices transmit a higher resolution signal of 500 Hz, where our data was collected at 200 Hz. This would increase the accuracy and detail of both obtained HR and MAP data, as well as spectral data. In addition, data would not have to be derived from pulsatile pressure, leading to further accuracy. Implantation of such devices would also allow each SCI animal to serve as its own control, as cardiovascular data could be obtained pre- and post-injury.

Many studies employ immunohistochemistry to quantify proteins of interest. Often, a threshold value is attributed to each image in order to automate cell counting. This, however, is not without its limitations. It is possible that software may detect false-positive afferents or group several afferents together based on the attributed threshold value. Although great care was taken when image thresholds were determined, these consequences are still possible. This, however, is not unique among one experimental group and it would be expected that it would happen with a similar frequency between groups.

Substance P IR in the IML was quantified in transverse sections. Due to the clustering of SPNs in the IML at ventral roots, however, it is possible that horizontal sections would have provided a more representative quantification of substance P in the IML as each individual cluster would have been visible along the length of the spinal cord. Transverse sections only provide a small number of sections where the IML proper is fully visible.

4.3 – Conclusions

1) Cardiovascular function and immunohistochemical results from this study have demonstrated that the acute administration of Pregabalin following SCI can attenuate increases in blood pressure associated with autonomic dysreflexia following SCI.

2) Results demonstrated that administration of Pregabalin outside of the 1-week 'therapeutic window' following SCI has little to no effect upon changes in blood pressure associated with autonomic dysreflexia following SCI.

3) Cardiovascular and bladder function results from this study demonstrate that the effects of immediate Pregabalin treatment following SCI manifest as diminished symptoms of autonomic dysreflexia and improved bladder function.

4) Sprouting and the subsequent increase in type C sensory afferent density in the dorsal horn and central autonomic area was not suppressed by immediate Pregabalin treatment. On the contrary, immediate Pregabalin treatment was associated with an increase in substance P immunoreactivity in these regions when compared to saline-treated and delayed-Pregabalin treated animals.

In conclusion, it is possible that the immediate administration of Pregabalin following spinal cord injury may be used as a pre-emptive treatment to prevent the development of autonomic dysreflexia following spinal cord injury.

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