

CHANGES IN GLUTAMATE CYCLE METABOLISM  
IN RESPONSE TO PERIPHERAL NERVE INJURY

By

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CHANGES IN GLUTAMATE CYCLE METABOLISM  
IN RESPONSE TO PERIPHERAL NERVE INJURY

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Title of Study: CHANGES IN GLUTAMATE CYCLE METABOLISM IN RESPONSE TO PERIPHERAL NERVE INJURY

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Abstract:

Scope and Method of Study: Neuropathic pain is a common complication of peripheral nerve injuries. In pain studies, it has been revealed that glutamate neurotransmission has a critical role in the noxious stimulus sensation. The glutamate cycle have several well established markers: vesicular glutamate transporters 1 & 2 and glutaminase. However, systemic studies for vesicular glutamate transporters 1 & 2 and glutaminase changes after peripheral nerve injuries have not been done. Thus, in this thesis project we studied the changes in vesicular glutamate transporters 1 & 2 and glutaminase systemically through immunohistochemistry at six different time points that represent the acute (day 1 & 2), subacute (day 7 & 14), and chronic (day 28 & 70) phases in peripheral nerve regeneration. Our peripheral nerve injury models, nerve crush and nerve transection, imitate two types of peripheral nerve injury in human, axonotmesis and neurotemsis, respectively.

Findings and Conclusions: The study of subacute phase of peripheral nerve regeneration in sciatic nerve crush model showed an elevation of dorsal root ganglia neurons VGLUT 2 immunoreactivity only at day 7 post injury. On the other hand, the study of VGLUT 1 & 2 and glutaminase dorsal root ganglia neurons immunoreactivity in the acute, subacute, and chronic phase of regeneration showed a pattern of elevation and reduction in the glutamate metabolism cycle. A minor increase in the three markers immunoreactivity at day 1 followed by marked decrease at day 2. In subacute phase VGLUT 1 & 2 and glutaminase immunoreactivity levels return to control levels at day 7, however, at day 14 they showed an increase above control levels. Finally at the chronic phase of regeneration, the three markers show a decrease in immunoreactivity levels initially (day 28) then return to control levels at day 70 post injury. In conclusion, our immunohistochemistry studies for vesicular glutamate transporters 1 & 2 and glutaminase gave insight into part of the story of neuronal response to injury. By understanding the molecular and physiological changes after peripheral nerve injuries, potential pharmacological or other treatments could be designed to improve the functional outcome of those injuries.

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## CHAPTER I

### INTRODUCTION

Peripheral nerve injury in the lower extremity is very common and serious nerve injuries affect 2-3 % of trauma patients [1]. Usually the injury affects either the sciatic or peroneal nerve [2]. The sciatic nerve originates from the sacral plexus (L4, L5, S1, S2, and S3) and it contains sensory, motor and autonomic axons. It bifurcates above the knee to the tibial and common peroneal nerve. These nerves innervate some of the muscles controlling knee and ankle joint flexion and extension as well as carrying the sensory information from lateral side of the calf and the fifth toe [3]. The causes of sciatic nerve injury vary from femoral fractures, laceration, contusions, and compression to gunshot wounds and total hip replacement surgery.

Peripheral nerve injuries in human are classified into three types depending on the severity of the injury [4]. The injuries are nerve compression with or without demyelination (neurapraxia), axon transection with both perineurium and epineurium remaining intact (axonotmesis), and nerve transection with disruption of the continuity of epineurium (neurotmesis). The recovery outcome of each type varies drastically. Usually, neurapraxia and axonotmesis have very good recovery outcomes with complete or near complete function. In neurotmesis, however, the recovery outcome is poor in most cases. Functional level of recovery is probable only in a few cases after nerve repair and other

correction surgeries [2]. The injured nerve usually takes a long time to regenerate. Typically, axon regeneration progresses at the speed of 1 mm/day in humans and 3 mm/day in animals [5]. Because of this slow regrowth, several complications may include muscle contraction and neuropathic pain [1].

Although peripheral nerves are capable of regenerating their axons after injury, patients typically have a poor functional recovery outcome. Patients with severe peripheral nerve injury usually have devastating permanent impairments. Thus, the need for better and faster treatment of nerve injuries inspires researchers to investigate peripheral nerve injuries and their complications. Several models of peripheral nerve injury have been developed that imitate the injuries to peripheral nerves seen in human. Injury of the sciatic nerve in rat is an optimal model to study the complexity of nerve injury and its recovery process [3]. Most of our understanding of regeneration of the peripheral nerve comes from models using injury to the sciatic nerve [1]. The nerve crush model imitates axonotmesis while the nerve transection model imitates neurotmesis. If nerve repair follows in the nerve transection model, it can lead to the development of neuropathic pain that manifests as an abnormal response to thermal and tactile stimuli. This is very similar to human nerve injury where neuropathic pain is a common complication. On the other hand, autotomy (self-mutilation), skin laceration, and limb contraction are common complication of the nerve transection model in rat [1].

The study of peripheral nerve injury in rat models has laid the groundwork of the molecular and physiological events that occur during this process. Nerve injury where the axonal continuity is disrupted triggers cell body responses that include chromatolysis, e.g., morphological changes including swelling, movement of the nucleus and nucleolus

to an eccentric position, and dissolution of the Nissl bodies. These morphological changes along with altered gene expression are required for axon regeneration. Gene alterations include the upregulation of growth associated genes such as actin, tubulin, and growth associated protein 43 (Gap43) and the downregulation of the neurofilament gene. At the injury site, the transected nerve (distal nerve stump) undergoes Wallerian degeneration, a process where severed axons exhibit an anterograde and retrograde degeneration [5]. After surgical repair of the transected nerve, however, outgrowing axons cross the surgical repair site in a staggered fashion over a prolonged period of 4 weeks in a rat [6].

In this study two models of peripheral nerve injury were used, sciatic nerve crush and sciatic nerve transection, which imitate the two types of peripheral nerve injury in humans, axonotmesis and neurotmesis, respectively. The primary goal of treatment in patients with sciatic nerve injury is the restoration of protective sensation in the sole [2]. In addition, a common consequence of nerve injury is neuropathic pain and providing pain relief is another goal following nerve injury. Thus, we investigated the molecular changes in the metabolism cycle of glutamate in cells of the dorsal root ganglia (DRG). Glutamate is an essential neurotransmitter regulating both normal and painful stimuli and it is probably used in the regenerating peripheral axon.

Glutamate from cells of the DRG is released as a neurotransmitter in both central and peripheral nerve terminals. The release of glutamate after specific types of stimulation in the periphery indicates that DRG neurons are participating in the peripheral metabolic cycle of glutamine [7]. In the glutamine cycle, glutamine is converted to glutamate to be used as neurotransmitter [8] through a multistep cycle (Figure 1.1). After



release of glutamate from DRG neurons nearby glial cells quickly remove excess glutamate from the intracellular space via a glutamate–aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1). In peripheral nerve, glutamate in Satellite or Schwann cells is converted to glutamine via glutamine synthetase (GS). Alternatively, glutamate is transported to the mitochondria to be a part of the TCA cycle. Converted glutamine is transported back to the neurons via sodium-coupled neutral amino acid transporters (SNAT) and converted back to glutamate by Glutaminase within the mitochondria of DRG neurons. Neurons transport glutamate from the mitochondria to the cytoplasm to be packaged into vesicles via vesicular glutamate transporter 1 or 2 [9].

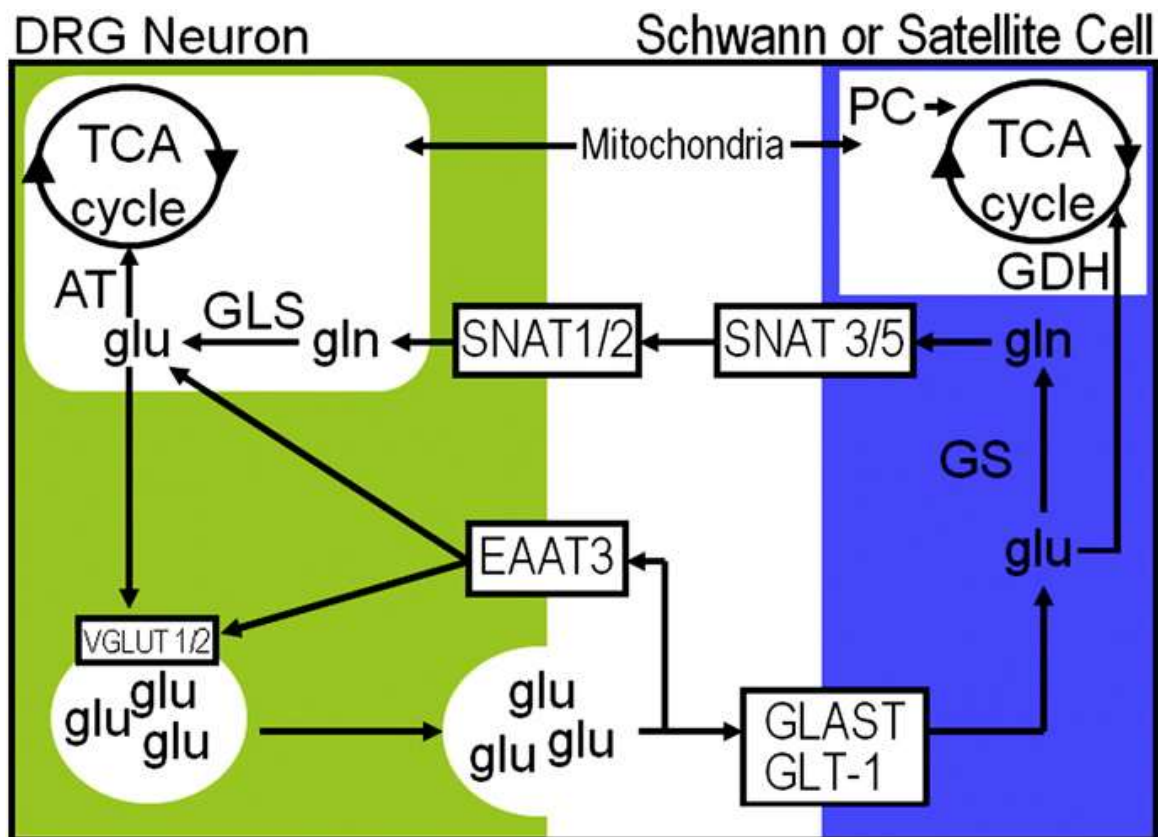


Figure 1.1: Glutamine cycle in the peripheral nervous system.

In this series of studies, I hypothesized that glutamate metabolism in dorsal root ganglia neurons would change following peripheral nerve injury in response to a transient

inflammatory state and as an accommodation for the needs of the regenerating nerve. I utilized two peripheral nerve injury models: sciatic nerve crush and sciatic nerve transection to study the changes in vesicular glutamate transporter 1 & 2 and glutaminase; known markers for glutamate metabolism cycle. In the sciatic nerve transection model, I sutured the nerve for alignment to prevent the regenerating nerve forming a neuroma. I utilized immunohistochemistry to evaluate the changes in vesicular glutamate transporter 1 & 2 and glutaminase at six time points: 1, 2, 7, 14, 28, 70 days. Each pair of two time points, e.g., 1, 2 or 7, 14 or 28, 70; represent acute, subacute and chronic phases in the regenerative process in rats.

## CHAPTER II

### VESICULAR GLUTAMATE TRANSPORTER 2 EXPRESSION IS ALTERED IN RAT DORSAL ROOT GANGLION NEURONS DURING THE REGENERATIVE PHASE FOLLOWING SCIATIC NERVE CRUSH

#### 1. Introduction

Glutamate is the most abundant excitatory neurotransmitter in the adult central nervous system (CNS) [10]. It has critical role in CNS function and many defects in glutamate signaling have been linked to several diseases [11]. Furthermore, several studies show a direct link between peripheral injuries or inflammation and changes in the activity of glutamate metabolism [9, 12]. The discovery of vesicular glutamate transporters 1, 2, and 3 had an enormous role in the advancement of glutamate related studies. Hence, these vesicular glutamate transporters enabled researchers to accurately identify glutamatergic neurons [11].

Dorsal root ganglion (DRG) neurons are glutamatergic using glutamate as the primary neurotransmitter at central terminals. Many DRG neurons also release glutamate from their peripheral terminals in response to noxious or injurious stimuli [9]. Previous studies indicate that glutamate metabolism is altered in DRG neurons after axonal injury. Following sciatic nerve transection in rats, glutamate levels double in the nerve at 15 days post-injury [13]. After dorsal root crush in cats, glutamate levels in the DRG

decrease from 2-4 days, but regain normal levels from 10-25 days [14]. Glutamate levels are diminished 4-25 days in dorsal roots following dorsal root crush [14]. Sciatic nerve transection in mice at 7 days decreases the overall number of DRG neurons for vesicular glutamate transporters 1 & 2 (VGLUT 1, 2) [12]. Despite an overall decrease in neuronal cells, VGLUT 2 increases in a subpopulation of small diameter neurons after 7 days of sciatic nerve transection [12]. However, the effect of peripheral nerve injury or regeneration on DRG glutamate metabolism has not been studied systematically.

In the present study, we evaluated vesicular glutamate transporter 2, a marker of glutamatergic function, in DRG cell bodies during the regenerative phase following sciatic nerve crush in the rat. Glutamate is produced from glutamine by glutaminase, the primary synthetic enzyme in the neuronal/glial glutamine cycle [9]. After synthesis, glutamate is packaged into synaptic vesicles by VGLUT2 [9]. In addition, to verify if our crush model indeed produces a regenerative nerve injury, we simultaneously evaluated growth associated protein (Gap43). Gap 43 is a known marker for axonal regeneration. I evaluated VGLUT2 and Gap 43 in DRG with immunohistochemistry at 7 and 14 days after crush of the sciatic nerve

## 2. Materials and Methods

### 2.1. Animals:

Adult Sprague Dawley rats (n=9; 250-350 g) were housed in a 12-hr-light: 12-hr-dark cycle and given free access to food and water. Procedures were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. All appropriate efforts were made to minimize the number of animals used in

this study. Rats were randomly assigned to specific experiments, irrespective of their gender. Gender differences were not investigated in this study.

## 2.2. Surgery:

All surgeries were performed on animals anesthetized by 2%-3% isoflurane inhalation. Under deep anesthesia, the rat right sciatic nerve was exposed at mid-thigh level. With fine hemostats (2.5mm), the nerves were strongly compressed for 30 s twice. Muscles and skin were sutured in layers. The animals were allowed to recover from the anesthesia in a warmed cage and then were housed individually until the day of perfusion. Sham-operated rats were used as control.

## 2.3. Tissue Preparation:

Rats were anesthetized with tribromoethanol [Avertin; 2.5% (w/v)] and xylazine and perfused through the ascending aorta with 75 ml of calcium-free Tyrode's solution (pH 7.3), followed by 300 ml of fixative 0.2% paraformaldehyde (w/v), 0.8% (w/v) picric acid in 0.1M phosphate buffer, pH 7.3 [15,16]. Right L4 DRG was removed and placed for 4hrs in the same fixative used for perfusion at 4C. DRGs were transferred to 20% sucrose in 0.1M phosphate buffer, pH 7.3 for 24–48 hrs at 4C.

## 2.4. Immunofluorescence:

Immunohistochemical localization of VGLUT 2 and Gap43 were performed for two different experimental time points: 7 and 14 days post right sciatic nerve crush (SNC), and sham rats. The tissue was frozen, sectioned at 12  $\mu$ m with a Leica CM 1850 Leica (Microsystem Inc. Bannockburn, IL). Sections were thaw mounted to gelatin-

coated SuperFrost slides (Fisher Scientific; Pittsburg, PA) and dried for 1 hr at 37C. Every fifth section was used to reduce the possibility of evaluating a neuron twice. Sections were washed three times for 10 min in phosphate buffered saline (PBS) and incubated in 0.5% bovine serum albumin (BSA), and 0.5% polyvinylpyrrolidone (PVP) in PBS with 0.3% Triton X-100 (PBS-T) and primary antiserum for 4 days at 4C. For VGLUT 2 detection, mouse antiserum against VGLUT 2 was used at 1:4000 dilution and for Gap43 detection, rabbit antiserum (AbDSerotec) was used at 1:4000 dilution. The tissue was rinsed three times in PBS and incubated in secondary antisera: Biotinylated Goat anti-Mouse IgG 1:1500 and FITC-Avidin 1:500 in PBS-T (VGLUT2 detection) and in Cy3 Donkey anti-Rabbit IgG in PBS-T 1:1500 (Gap43 detection) for 1 hr at room temperature. Sections were rinsed two times in PBS following secondary antibody incubation, and then incubated in DAPI for nucleus staining for 15 min. All sections were rinsed three more times in PBS and coverslipped with ProLong Gold mounting medium (Molecular Probes).

## 2.5. Image analysis:

Images were acquired using a 20X objective on an Olympus BX51 epifluorescence microscope (Olympus; Center Valley, PA) equipped with a SPOT RT740 quantitative camera (Diagnostic Instruments; SterlingHeights, MI). Three random fields of view were imaged for each DRG section. Images were saved as uncompressed TIFF files. The exposure time for all images was the same for all tissue sections from all animals. The exposure time was determined empirically so that weakly stained neurons could be distinguished for tracing, but that intensely stained neurons were not oversaturated [16, 17]. This approach allowed images to be evaluated along the linear

aspect of immunofluorescence intensity [17]. Captured images were  $1600 \times 1200$  pixels with 2.69 pixels per micrometer. All neuron profiles with clearly visible nuclei that were not touching the edge of the image were traced with a Cintiq 21UX interactive pen display (Wacom; Kita Saitama-Gun, Saitama, Japan), using the freehand selection tool in Image J software (NIH). Nuclei were excluded from the regions of interest, making each region of interest correspond to the cytoplasmic profile of a single DRG neuron [16,17] Images were acquired at a bit depth of 8, so pixel intensities ranged from 0 (darkest) to 255 (lightest) on a grayscale. The mean gray value and area in  $\mu\text{m}^2$  for each cytoplasmic profile were measured and copied to an Excel spreadsheet. The values for area ( $\mu\text{m}^2$ ) were binned into small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ) sized categories to indicate three broadly defined neuronal subpopulations that corresponded to C, A $\delta$ , and A $\alpha/\beta$  fibers, respectively [18, 19].

## 2.6. Statistic:

Data from the analyses are reported as mean value with standard error of the mean. A one-way ANOVA test, followed by Tukey test was used to determine differences between nerve injury and control groups (Prism version 5.01, GraphPad Software Inc., San Diego, CA). In all analysis, P values less than 0.05 were considered significant.

## 3. Results

### 3.1. VGLUT2 IR:

At 7 days, VGLUT2 immunoreactivity (IR) in L4 DRG neurons post SNC qualitatively was increased compared to DRG neurons from sham operated rats (Figure

2.1 a, d, & g). Therefore, the VGLUT2 IR intensities of the total immunoreactive neurons and of the three different sizes of DRG cell bodies were analyzed (Figure 2.2). The overall VGLUT 2 IR intensity of L4 DRG cell bodies (Figure 2.2) from the SNC rats ( $155.3 \pm 1/\mu\text{m}^2$ ) was greater ( $P < 0.05$ ) than sham operated rats ( $113.3 \pm 20/\mu\text{m}^2$ ). This increase in VGLUT2 intensity was diminished by day 14 post injury ( $126.2 \pm 2.3/\mu\text{m}^2$ ). For both 7 and 14 days post SNC there was no significant difference in any of the size subgroups in VGLUT2 IR in comparison with sham operated DRG neurons (Figure 2.2).

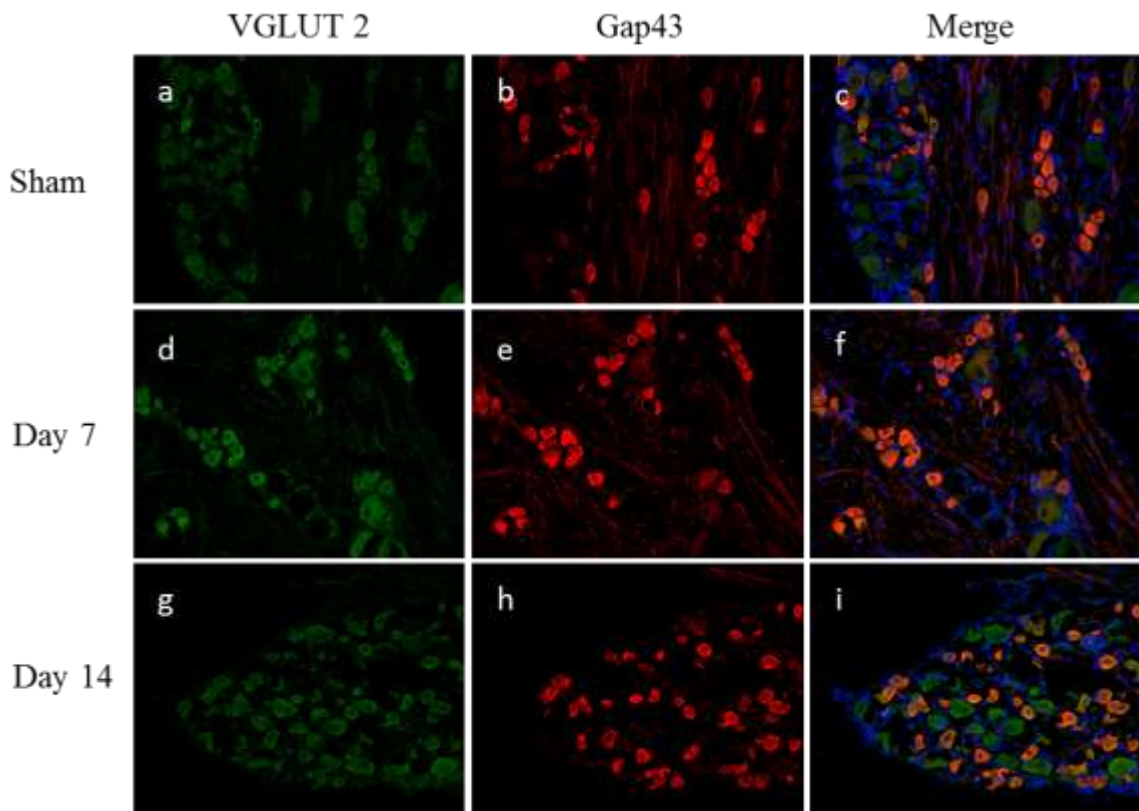


Figure 2.1: Vesicular glutamate transporter 2 (green), Gap43 (red), and merge of both immunoreactivities (IR) in rat L4 dorsal root ganglia (DRG) following 7 (subfigures d,e, f) and 14 (subfigures g,h, i) days post right sciatic nerve crush (SNC). DRG sections were processed simultaneously with a mouse VGLUT 2 and rabbit Gap43 antiserum and photographed under identical conditions. In sham operated sections, VGLUT 2 IR (a) was light to moderate in all neuronal cell sizes. Gap43 IR (b) was light to moderate in small and medium neuronal cell sizes. Elevated VGLUT 2 IR (d) in all neuronal cell sizes occurred in the DRG 7 days post SNC. Elevated Gap43 IR (e & h) in small and medium neuronal cell sizes occurred in the DRG 7 and 14 days post SNC.



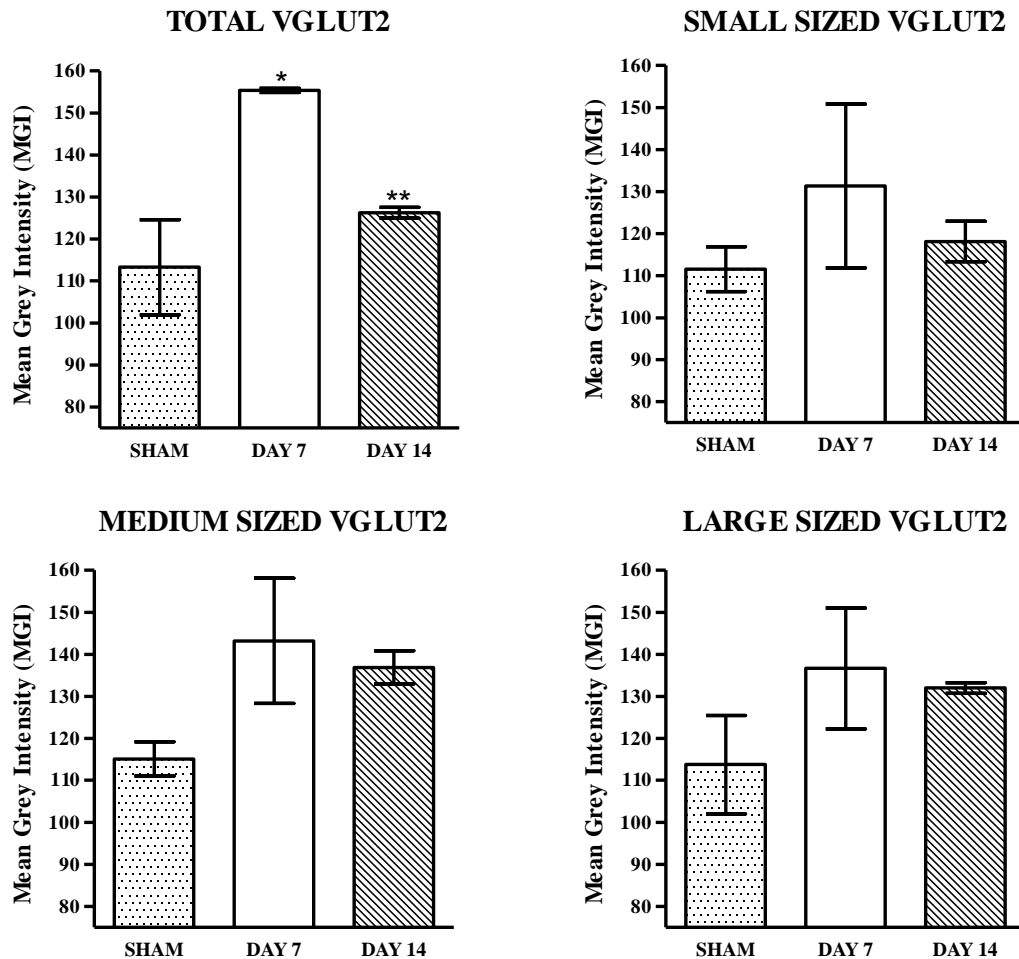


Figure 2.2: Image analysis of vesicular glutamate transporter 2 (VGLUT 2) immunoreactivity (IR) in L4 DRG neurons after 7 and 14 days of sciatic nerve crush (SNC). Data are presented as intensity divided by the area of the cell (mean grey intensity, MGI). DRG neurons were categorized into three area size groups: small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ). Overall VGLUT 2 IR increased at 7 days compared to sham. Although diminished at day 14, VGLUT 2 was still elevated compared to sham. This overall increase was similar to what was observed in small, medium, and large subgroups.\* 7 days compared to sham, \*\* 7 days compared to 14 days. (\*, \*\*  $P < 0.05$ ). One-way ANOVA followed by Tukey test were used.

### 3.2. Gap43 IR:

Gap43 IR was elevated mainly in L4 small and medium sized DRG neurons post SNC at day 7 and 14 (Figure 2.1b, e, & h) [6, 7]. At day 7, the overall Gap43 IR intensity of L4 DRG cell bodies (Figure 2.3-A) from the SNC rats ( $63.64 \pm 1/\mu\text{m}^2$ ) was greater

( $P < 0.05$ ) than sham operated rats ( $39.66 \pm 1.4/\mu\text{m}^2$ ). At day 14, the overall Gap43 IR intensity of L4 DRG cell bodies from the SNC rats ( $54.67 \pm 2/\mu\text{m}^2$ ) was greater ( $p < 0.05$ ) than sham operated rats ( $39.66 \pm 1.4/\mu\text{m}^2$ ) but less than 7 days post SNC ( $63.64 \pm 1/\mu\text{m}^2$ ). This pattern of intensity increasing at day 7 post SNC then decreasing at day 14 post SNC was consistent in all DRG neuronal subgroups.

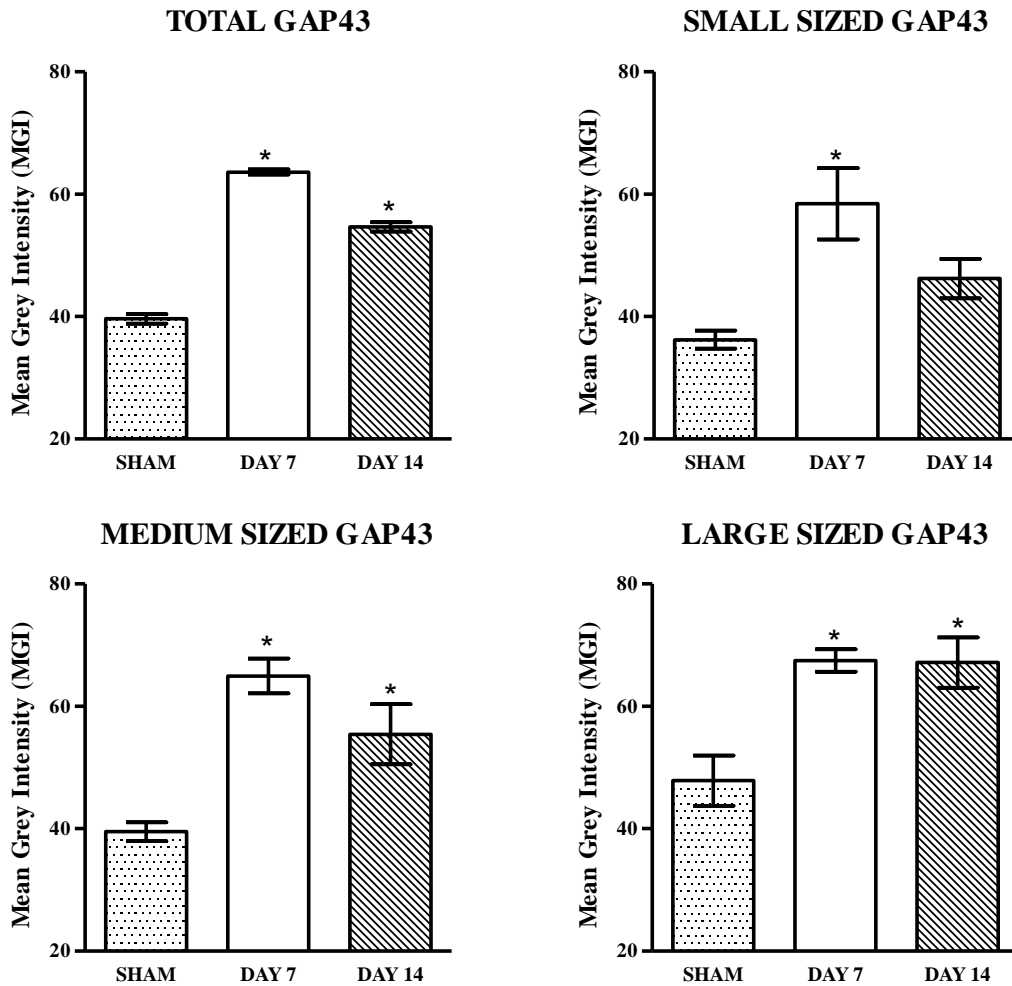


Figure 2.3: Image analysis of Gap43 immunoreactivity (IR) in L4 DRG neurons after 7 and 14 days of sciatic nerve crush (SNC). Data are presented as intensity divided by the area of the cell. DRG neurons were categorized into three area size groups: small ( $< 400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ). Overall GAP 43 immunoreactivity increased at 7 and 14 days compared to sham. This overall increase was similar to what was observed in small, medium, and large subgroups. (\*  $P < 0.05$ ). One-way ANOVA followed by Tukey test were used.

### 3.3. Co-localization of VGLUT2 and Gap43:

VGLUT2 and Gap43 IR showed co-localization behavior primarily in L4 small and medium sized DRG neurons (Figure 2.1c, f, & i). Most of small to medium Gap43 IR neurons were double stained with VGLUT2. However, large DRG neurons showed no or little double staining for both VGLUT2 and Gap43.

## 4. Discussion

DRG neurons primarily use glutamate as their excitatory neurotransmitter [9]. In pathological conditions, DRG neurons constantly modify their neurotransmitter concentration and production [15]. One valid marker of glutamate cycle metabolism is vesicular glutamate transporter 2 (VGLUT2) [11]. For nerve injury experiments, the SNC model is an accepted model for axonotmesis to study nerve regeneration [20]. In this study, I evaluated the changes in immunoreactivity for VGLUT2 and Gap 43, a known marker for regeneration, in DRG neuronal cell bodies 7 and 14 days post sciatic nerve crush.

Our results showed Gap43 to be elevated at day 7 and 14 post SNC (Figure 2.3). This is consistent with previous studies in the regeneration field demonstrating increases in Gap43 IR at day 4. Gap43 IR continues to be elevated 9 weeks post injury with a peak at day 21 in DRG neuronal cell bodies [21, 22]. Thus, I can say my SNC model indeed produces a nerve injury that results in a regenerative phase.

VGLUT 2 is elevated in DRG neuronal cell bodies during the regenerative phase following SNC in the rat (Figure 2.2). Since VGLUT2 is important for vesicular release of glutamate, the elevation in VGLUT2 immunoreactivity may indicate increased

glutamatergic neurotransmission in the spinal cord to provide information to the CNS concerning the regenerative process. Alternatively, vesicular release of glutamate may be occurring at the regenerating peripheral process to regulate the local environment of the outgrowing axons.

Further studies are needed to establish VGLUT2 activity changes in DRG following different model of regeneration. In addition, to determine if changing VGLUT2 levels is an acute response or a prolonged activity change, longer time periods post injury need to be investigated.

## CHAPTER III

### VESICULAR GLUTAMATE TRANSPORTER 2 EXPRESSION IS ALTERED IN RAT DORSAL ROOT GANGLION NEURONS DURING THE REGENERATIVE PHASE FOLLOWING SCIATIC NERVE TRANSECTION

#### 1. Introduction

Pain is a defense mechanism produced by the central nervous system to alert the body of dangerous conditions either internally or externally. One of the many examples of a painful situation is peripheral nerve injury [23]. This painful stimulus is detected by dorsal root ganglia (DRG) neurons and transmitted to the spinal cord neurons via glutamatergic neurotransmission utilizing vesicular glutamate transporters in presynaptic nerve terminals [16]. Glutamate is the most abundant excitatory neurotransmitter in the adult central nervous system (CNS) [10]. Glutamate is transported into vesicles by the VGLUT's 1, 2, or 3 [24]. Discovery of these vesicular glutamate transporters provided a mechanism for the identification of glutamate signaling neurons [11]. In addition, by controlling the activity of VGLUT proteins, neurons can modify the efficiency of glutamatergic neurotransmission [24]. Previous studies indicate that glutamate metabolism is altered in DRG neurons after axonal injury. Following sciatic nerve transection in rats, glutamate levels double in the nerve at 15 days post-injury [13].

Sciatic nerve transection in mice at 7 days decreases the overall number of DRG neurons immunoreactive for vesicular glutamate transporters 1 & 2 [12]. Despite an overall decrease, VGLUT 2 increases in a subpopulation of small diameter neurons after 7 days of sciatic nerve transection in mice [12]. However, the effect of peripheral nerve injury or regeneration on DRG glutamate metabolism has not been studied systematically.

In the present study, I evaluated vesicular glutamate transporter 2 a marker of glutamatergic function in DRG neuronal cell bodies during the inflammatory and regenerative phases following sciatic nerve transection and resection in the rat. Transecting the nerve without any surgical repair results in that axons regenerate without any directionality and that lead to the formation of neuroma. By suturing the proximal and distal ends of the transected nerve, I allow the nerve to regenerate in the correct direction toward the distal end rather than forming a neuroma. VGLUT2 was evaluated in DRG neuronal cell bodies and peripheral axons with immunohistochemistry at day 1, 2, 7, 14, 28, and 70 after transection and resection of the sciatic nerve (SNT).

## 2. Materials and Methods

### 2.1. Animals:

Adult Sprague Dawley rats (n=27; 250-350 g) were housed in a 12-hr-light: 12-hr-dark cycle and given free access to food and water. Procedures were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. All appropriate efforts were made to minimize the number of animals used in this study. Rats were randomly assigned to specific experiments, irrespective of their gender. Gender differences were not investigated in this study.

## 2.2. Surgery:

All surgeries were performed on animals anesthetized by 2%-3% isoflurane inhalation. Under deep anesthesia, the rat right sciatic nerve was exposed at mid-thigh level and cut with a sharp blade. The nerve was reapposed and sutured using ETHILON 8-0 black monofilament Nylon for alignment. Muscles and skin were sutured in layers. The animals were allowed to recover from the anesthesia in a warmed cage and then were housed individually until the day of perfusion. Naïve rats were used as control.

## 2.3. Tissue Preparation:

Rats were anesthetized with tribromoethanol [Avertin;2.5% (w/v)] and xylazine and perfused through the ascending aorta with 75 ml of calcium-free Tyrode's solution (pH 7.3), followed by 300 ml of fixative 0.2% paraformaldehyde (w/v), 0.8% (w/v) picric acid in 0.1M phosphate buffer, pH 7.3 [16]. Right L4 DRG and sciatic nerve were removed and placed for 4 hrs. in the same fixative used for perfusion at 4C. DRGs were transferred to 20% sucrose in 0.1M phosphate buffer, pH 7.3 for 24–48 hrs. at 4C.

## 2.4. Immunofluorescence:

Immunohistochemical localization of VGLUT 2 was performed for six different experimental time points: 1, 2, 7, 14, 28, and 70 days post right sciatic nerve transection (SNT) and resection, and naïve rats. The tissue was frozen, sectioned at 12 - 14  $\mu$ m with a Leica CM 1850 Leica (Microsystem Inc. Bannockburn, IL). Sections were thaw mounted to gelatin-coated SuperFrost slides (Fisher Scientific; Pittsburg, PA) and dried for 1 hr at 37C. Every fifth section was used to reduce the possibility of evaluating a neuron twice. Sections were washed three times for 10 min in phosphate buffered saline

(PBS) and incubated in 0.5% bovine serum albumin (BSA), and 0.5% polyvinylpyrrolidone (PVP) in PBS with 0.3% Triton X-100 (PBS-T) with primary antiserum for 4 days. For VGLUT 2 detection, rabbit antiserum (Sigma) against VGLUT 2 was used at 1:2000 dilution. The tissue was rinsed three times in PBS and incubated in AlexaFluor 488 goat anti-rabbit IgG secondary antibody in PBS-T (1:1500) for 1 hr at room temperature. Sections were rinsed two times in PBS following secondary antibody incubation and incubated in 4',6-diamidino-2-phenylindole (DAPI) for nucleus staining for 15 min. All sections were rinsed three more times in PBS and coverslipped with ProLong Gold mounting medium (Molecular Probes).

## 2.5. Image analysis:

Images were acquired using a 20X objective on an Olympus BX51 epifluorescence microscope (Olympus; Center Valley, PA) equipped with a SPOT RT740 quantitative camera (Diagnostic Instruments; Sterling Heights, MI). Three random fields of view were imaged for each DRG section. Images were saved as uncompressed TIFF files. The exposure time for all images was the same for all tissue sections from all animals. The exposure time was determined empirically so that weakly stained neurons could be distinguished for tracing, but that intensely stained neurons were not oversaturated [16,17]. This approach allowed images to be evaluated along the linear aspect of immunofluorescence intensity [17]. Captured images were 1600 X 1200 pixels with 2.69 pixels per micrometer. All neuron profiles with clearly visible nuclei that were not touching the edge of the image were traced with a Cintiq 21UX interactive pen display (Wacom; Kita Saitama-Gun, Saitama, Japan), using the freehand selection tool in Image J software (NIH). Nuclei were excluded from the regions of interest, making each



region of interest correspond to the cytoplasmic profile of a single DRG neuron [16, 17]. Images were acquired at a bit depth of 8, so pixel intensities ranged from 0 (darkest) to 255 (lightest) on a grayscale. The mean gray value and area in  $\mu\text{m}^2$  for each cytoplasmic profile were measured and copied to a spreadsheet. The values for area ( $\mu\text{m}^2$ ) were binned into small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ) sized categories to indicate three broadly defined neuronal subpopulations that corresponded to C, A $\delta$ , and A $\alpha/\beta$  fibers, respectively [18, 19].

For sciatic nerve analysis, images were obtained using the same methods as were used for DRG. To calculate mean gray intensity, three boxes were drawn near the end of the nerve transection on the side proximal to the DRG. Each box covered an area equal to  $10,000 \mu\text{m}^2$  and a mean gray intensity (MGI) for each was obtained. An average MGI for the sciatic nerve was calculated for each rat.

All data obtained were normalized to allow the evaluation of different time points in the same statistical test. The normalization was performed following this formula:

$$X = (\text{individual mean gray intensity per rat} \times 100) \div \text{the mean of total naïve mean gray intensity}$$

Where X is equal to the percentage change of mean gray intensity per injured rat in relation to the naïve rats.

## 2.6. Western Blot for total Vesicular glutamate transporter 2:

For immunoblotting, rats ( $n = 6$ ), ten weeks post sciatic nerve transection, control) were killed with  $\text{CO}_2$  and decapitated. Right L4 DRG was removed rapidly and homogenized [15]. DRG were homogenized individually with lysis buffer (50mM Tris

pH 7.4, 2 mM EDTA, 0.05% Triton-X 100) with phosphatase inhibitor cocktail I and II and protease inhibitor (Sigma). Homogenates were centrifuged (70,000 rpm, 20 minutes) and the supernatant were flash-frozen using liquid nitrogen for later use. All protein samples were kept at -80C. Protein concentration of the supernatant was measured (BCA Protein Assay Kit, Pierce, Rockford, Ill) to normalize the samples. Rabbit anti-VGLUT 2 antibody (Sigma) was bound to M-280 Dynabeads (Invitrogen) conjugated with sheep anti-rabbit antibody [15]. Equal amounts of total protein (75mg/mL) were exposed to rabbit anti-VGLUT 2 antibody beads (overnight, 4C) for VGLUT 2 purification [15]. Samples were exposed to a magnet to collect the bead antibody-protein complex. The purified protein was eluted using Laemmli buffer (10mM Tris, 1 mM EDTA, 2.5% SDS, 5%  $\beta$ -mercaptoethanol, 5% bromophenol blue, pH 8.0) and by heating the samples at 100C for three min. VGLUT 2 electrophoresis was performed on a 12.5% homogenous polyacrylamide gel (Phast-System, Promega) [15] along with molecular weight standards (Novagen). Proteins were transferred to a nitrocellulose membrane in a buffer of 25mM Tris, pH 8.0, 192mM glycine and 20% methanol at 25mA for 20 minutes. Immunoblotting was performed using the Protoblot II AP System (Promega) [15]. Membranes were dried at 37C, rinsed in 20mM Tris-HCL, 150mM NaCl, and 0.05% Tween 20, pH 7.5 (TBST), washed in 1% bovine serum albumin (BSA) in TBST, and incubated in rabbit anti-VGLUT 2 antibody (1/1000, TBST) for 1 hour at room temperature. Membranes were washed in TBST followed by incubation in alkaline phosphatase conjugated goat anti-rabbit antibody for 30 minutes. Membranes were washed twice in TBST and TBS. Membranes were incubated in Western Blue stabilized substrate for alkaline phosphatase (Promega; 5-bromo-4-chloro-3-indolyl-phosphate,

nitro blue tetrazolium, the membranes then dried overnight in room temperature. Dried membranes were scanned and the obtained images were analyzed by tracing the bands using the freehand selection tool in Image J software (NIH) to obtain a mean gray intensity.

## 2.7. Statistics:

Data from the analyses are reported as mean value of the changes in mean gray intensity with standard deviation. A one-way ANOVA test, followed by Tukey test was used to determine differences between nerve injury and control groups (Prism version 5.01, GraphPad Software Inc., San Diego, CA). In all analyses, P values less than 0.05 were considered significant.

## 3. Results

### 3.1. Vesicular glutamate transporter 2 IR in Dorsal Root Ganglia:

At day 2 and 28, VGLUT2 immunoreactivity (IR) in L4 DRG neurons post SNT rats appeared to decrease compared to DRG neurons from naïve rats (Figure 3.1). Therefore, the VGLUT2 IR intensities of the total immunoreactive neurons and the three different sizes of DRG cell bodies were analyzed (Figure 3.2). On Day 2, total neuron VGLUT2 IR percentage intensity of L4 DRG cell bodies (Figure 3.2 A) from the SNT rats ( $68.14 \pm 4.4/\mu\text{m}^2$ ) was less ( $P < 0.05$ ) than naïve rats ( $100 \pm 7.79/\mu\text{m}^2$ ). By day 7 and 14 post injury, VGLUT2 IR returned to normal intensity levels. Day 28 post SNT, VGLUT2 IR of L4 DRG cell bodies showed another decrease in the percentage intensity similar to day 2 ( $68.01 \pm 4.38/\mu\text{m}^2$ , day 28 post SNT vs.  $100 \pm 7.79/\mu\text{m}^2$  control). For both day 2 and 28 post SNT, DRG neurons size subgroups also showed significant

decrease in VGLUT2 IR compared with naïve DRG neurons (Figure 3.2 B, C, and D).

Day 70 post SNT, only small sized neurons subgroups (figure 3.2 B) continued to have a significant decrease ( $P < 0.05$ ) in VGLUT2 IR intensity of L4 DRG cell bodies from naïve rats ( $100 \pm 7.79/ \mu\text{m}^2$ ) to SNT rats ( $79.9 \pm 9.22/ \mu\text{m}^2$ ).

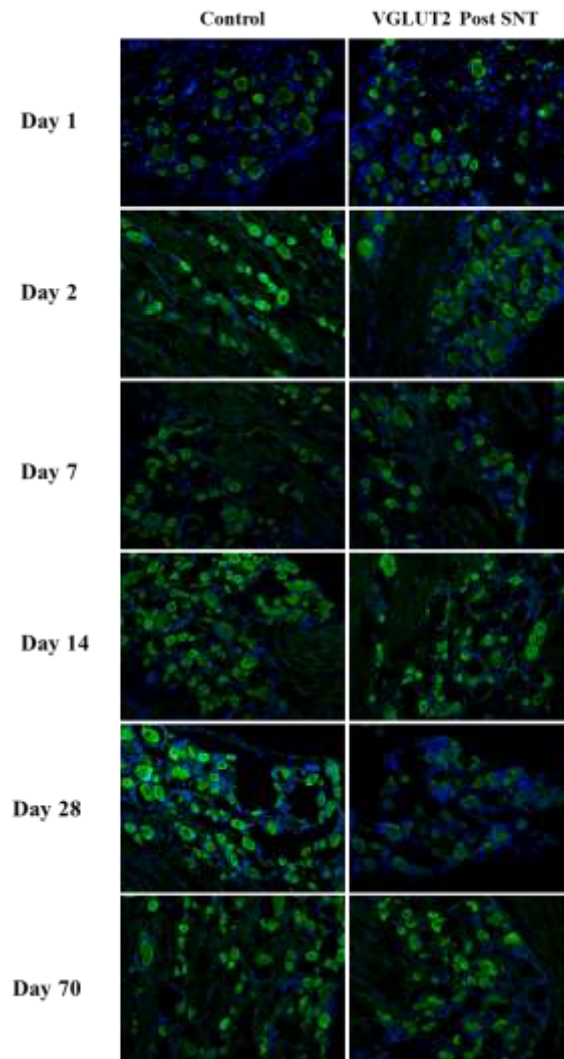


Figure 3.1: Vesicular glutamate transporter 2 (green) immunoreactivity (IR) and , DAPI (blue) nuclear staining in rat L4 dorsal root ganglia (DRG) following 1, 2, 7, 14, 28, and 70 days post right sciatic nerve transection (SNT). DRG sections were processed with a rabbit VGLUT2 antiserum and photographed under identical conditions. In day 2 and 28 sections, VGLUT2 IR was decreased in all neuronal cell sizes compared to control sections. No observable change in VGLUT2 IR in any of the neuronal cell sizes occurred in day 1, 7, 14, and 70 post SNT.

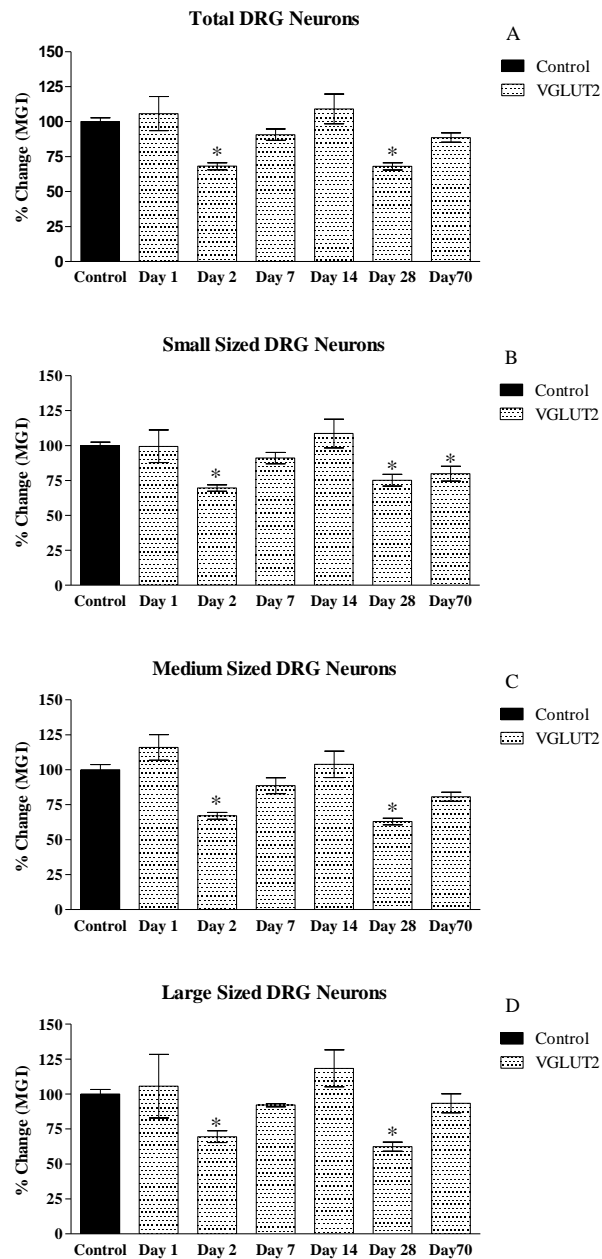


Figure 3.2: Image analysis of vesicular glutamate transporter 2 (VGLUT 2) immunoreactivity (IR) in L4 DRG neurons after 1, 2, 7, 14, 28, and 70 days post SNT. Data are presented as percentage change in mean gray intensity. DRG neurons were categorized into three area size groups: small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ). Total VGLUT2 IR (A) decreased at 2 and 28 days post SNT compared to control rats. This overall decrease was similar in small (B), medium (C), and large (D) cell size subgroups. In addition, small sized neurons continued to show a decrease in VGLUT 2 IR 70 days post SNT compared to control. (\*  $P < 0.05$ ). One-way ANOVA followed by Tukey test were used.

### 3.2. Vesicular glutamate transporter 2 Western blot:

Western blot of L4 DRG showed one characteristic band for VGLUT2 at a molecular weight of 65 kDa (Figure 3.3 (a)). Analysis of the rat L4 DRG showed no significant change between naïve and day 70 post SNT rats (Figure 3.3 (b)).

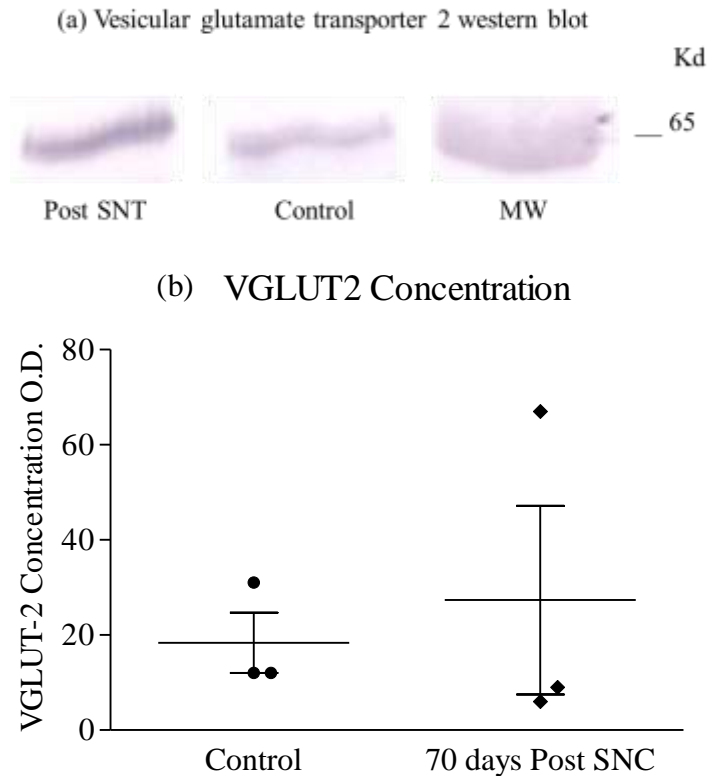


Figure 3.3: Western blot analysis of vesicular glutamate transporter 2 (VGLUT2) within the L4 DRG from 70 days post SNT (n=3) and controls (n=3) rats. (a) Western blot shows VGLUT2 immunoreaction (65 kDa) from L4 DRG of 70 days post SNT and controls rats. (b) Statistical analysis of VGLUT 2 immunoreactivity between post SNT and control rats. No significant change was found.

### 3.3. Vesicular glutamate transporter 2 IR in Sciatic Nerve:

At day 1, 2, and 28, VGLUT2 immunoreactivity (IR) in right sciatic nerve segments proximal to the area of transection (pre transaction) were increased compared to

intact right sciatic nerve from naïve rats (Figure 3.4). Therefore, the VGLUT2 IR intensities of the immunoreactive nerve fibers were analyzed (Figure 3.5).

At day 1 post SNT, VGLUT2 IR percentage change in mean gray intensity ( $185.83 \pm 24.66/\mu\text{m}^2$ ) at the right sciatic nerve segment (pre transection) increased significantly ( $P < 0.05$ ) compared to control rats ( $100 \pm 15.28/\mu\text{m}^2$ ). This increase persisted at day 2 post SNT, SNT rat  $204.13 \pm 34.99/\mu\text{m}^2$  vs. Control rats  $100 \pm 15.28/\mu\text{m}^2$ (figure 3.5). VGLUT2 IR percentage change in mean gray intensity returned to normal levels at day 7 and 14 post SNT. However, at day 28 post SNT VGLUT2 IR percentage change in mean gray intensity ( $145.89 \pm 20.21/\mu\text{m}^2$ ) again showed a significant increase ( $P < 0.05$ ) compared to control rats ( $100 \pm 15.28/\mu\text{m}^2$ ), only to return again to normal levels 70 days post injury (figure 3.5).

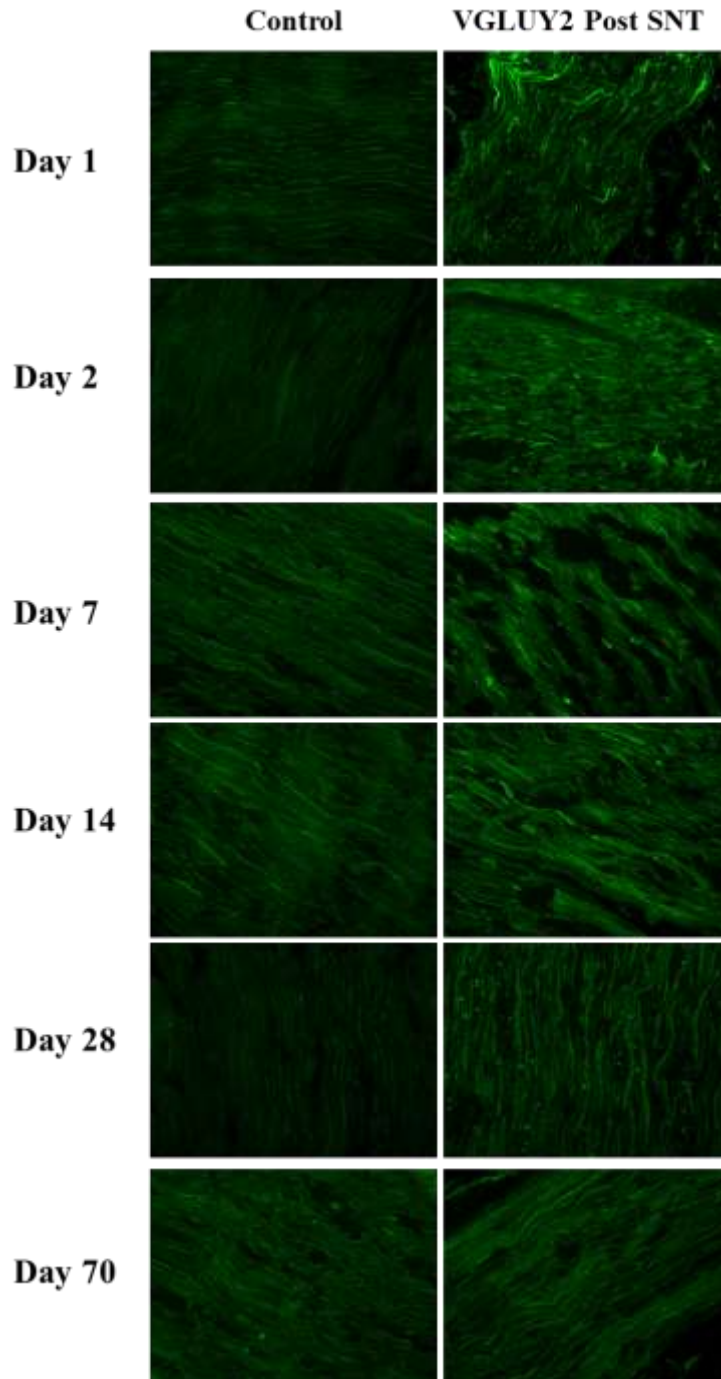


Figure 3.4: Vesicular glutamate transporter 2 (green) IR in rat right sciatic nerve following 1, 2, 7, 14, 28, and 70 days post right SNT. Sciatic nerve sections were processed with a rabbit VGLUT2 antiserum and photographed under identical conditions. In day 1, 2, and 28 sections, VGLUT2 IR were relatively high compared to control sections. No changes in VGLUT2 IR occurred in day 7, 14, and 70 post SNT.



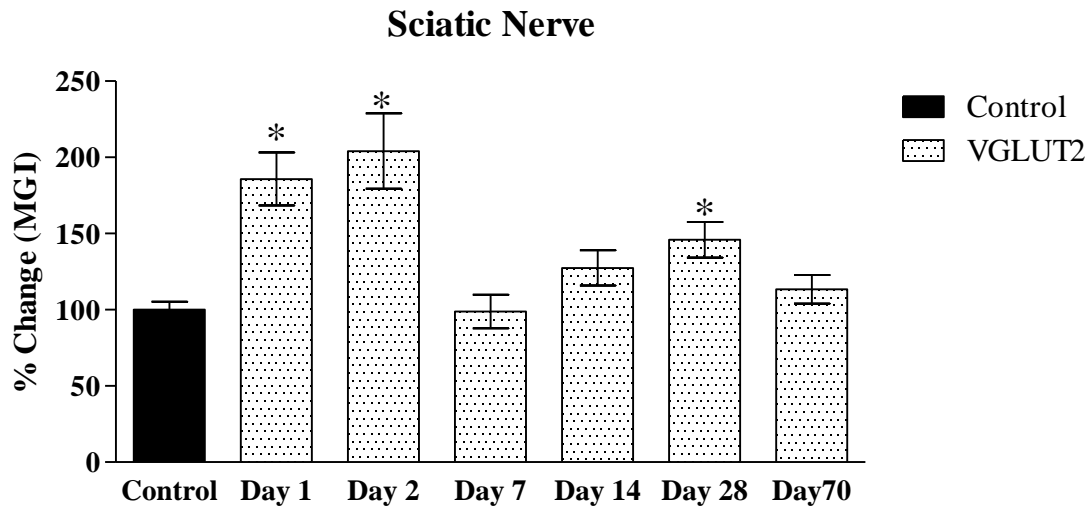


Figure 3.5: Image analysis of vesicular glutamate transporter2 (VGLUT2) immunoreactivity (IR) in right sciatic nerve after day 1, 2, 7, 14, 28, and 70 post sciatic nerve transection (SNT). Data are presented as percentage of mean gray intensity area from pre sciatic nerve transection site. VGLUT2 IR increased at 1 and 28 days post SNT compared to Control rats. (\*  $P < 0.05$ ). One-way ANOVA followed by Tukey test were used.

#### 4. Discussion

In this study, we evaluated vesicular glutamate transporter 2 immunoreactivity in DRG neuronal bodies and in the sciatic nerve proximal to the site of transection and resection in six different time points. Peripheral nerve injury can produce a chronic painful state of neuropathy [23]. This painful state leads to multiple changes in DRG neuronal cell bodies and axons. In chronic pain conditions, DRG neurons constantly modify their neurotransmitter production and concentration [15]. DRG neurons primarily use glutamate as their excitatory neurotransmitter [9] and VGLUT2 is an essential protein for normal pain expression [25]. Furthermore, DRG neurons with transected axons establish a regenerative process to reinnervate their target tissue. During the regenerative phase, glutamate can be used as an exocytosed chemical mediator for determining

directionality in axonal growth cones [26, 29]. Alterations in VGLUT2 levels observed in the current study, therefore, could be influenced by one or both processes.

Sciatic nerve crush is a common model used in peripheral nerve axonotmesis. Complete motor and sensory recovery is expected to be complete by ten to twelve weeks post injury [27]. The validity of nerve crush model in producing replicable nerve injury is still debatable. This is evident by different morphological and functional results obtained at similar time points by different researchers [27, 28]. To avoid any uncertainty in our results, we used a complete sciatic nerve transection and reposition model. Because the severed nerve was resected this provides a conduit for regeneration. Furthermore, we studied the regenerated nerve at six different time points to observe VGLUT2 changes in acute (day 1 & 2), sub-acute (day 7 & 14), and chronic (day 28 & 70) phases.

In the acute phase, VGLUT2 IR levels decreased remarkably in DRG neuronal cell bodies at day 2. This decrease is associated with a significant increase in VGLUT2 IR level in the sciatic nerve at the site of transection. In contrast, VGLUT2 IR levels in both the DRG and sciatic nerve return to normal levels in the sub-acute phase. The chronic phase, however, exhibited a similar pattern as the acute phase. There is a lack of similar research in rat models, but a number of explanations can be drawn from our results. First, similar to what happens in the case of acute phase of peripheral inflammation [15] DRG neurons increase the trafficking of VGLUT2 synaptic vesicles into the axons. This is an inflammatory response due to the injury. In addition, there is an increased amount of VGLUT2 IR at the transection site due to an accumulation of synaptic vesicles. In the sub-acute phase, as the inflammation subsides, VGLUT2 IR returns to normal levels. In this phase, axonal regeneration is occurring, but it appears to

have no significant effect on the amount of the VGLUT 2. In beginning of the chronic phase, where the axonal regeneration process peaks, there is a decrease in DRG VGLUT2 IR and an increase in sciatic nerve VGLUT2 IR. This may indicate an increase in production of VGLUT2 in the neuronal cell body with a concurrent elevation of trafficking of VGLUT2 synaptic vesicles. Eventually, as DRG neurons have completed the regenerative process, the DRG neuronal cell bodies adjust their VGLUT2 production to accommodate the newly regenerated axonal needs for vesicular glutamate.

Further studies are needed to establish VGLUT2 activity changes in DRG, sciatic nerve, and spinal cord following longer time period or until full recovery is achieved. In addition, functional assessment studies are needed to determine if VGLUT2 morphological changes is correlated with functional recovery.

## CHAPTER IV

### VESICULAR GLUTAMATE TRANSPORTER 1 AND GLUTAMINASE EXPRESSION IN RAT DORSAL ROOT GANGLION NEURONS DURING THE REGENERATIVE PHASE FOLLOWING SCIATIC NERVE TRANSECTION

#### 1. Introduction

In previous chapters we discussed the changes in vesicular glutamate transporter 2 (VGLUT2) in dorsal root ganglia (DRG) and sciatic nerve after sciatic nerve crush (SNC) or transection and resection. We conclude that, after SNC, VGLUT2 IR in DRG neuronal cell bodies increases at day 7. In the sciatic nerve transection and resection (SNT) model, VGLUT2 IR decreases at day 2 and 28 post injury in the DRG neuronal cell bodies.

These studies are consistent with the changes in glutamate metabolism cycle.

In addition to VGLUT2, vesicular glutamate transporter 1 (VGLUT1) and glutaminase (GLS) are markers for glutamate metabolism. GLS is the primary enzyme in the glutamine cycle for the conversion of glutamate from glutamine. Glutamate is transported into vesicles by the vesicular glutamate transporters 1 and 2 [24]. VGLUT1 in DRG neuronal cell bodies is mainly localized in medium to large neurons whereas VGLUT2 is localized in small to medium neurons [12]. Because glutamate is the most abundant excitatory neurotransmitter in the adult central nervous system (CNS) [10]

and has critical roles in CNS function, it is crucial to understand its role in different injury models in order to have a clearer understanding of regeneration and neuropathic pain.

Previous studies indicate that glutamate metabolism is altered in DRG neurons after axonal injury. Following SNT in rats, glutamate levels double in the nerve at 15 days post-injury [13]. After dorsal root crush in cats, glutamate levels in the DRG decrease from 2-4 days, but regain normal levels from 10-25 days [14]. Glutamate levels are diminished 4-25 days in dorsal roots following dorsal root crush [14]. Sciatic nerve transection in mice at 7 days decreases the overall number of DRG neurons for vesicular glutamate transporters 1 & 2 [12]. Despite an overall decrease, VGLUT 2 increases in a subpopulation of small diameter neurons after 7 days of sciatic nerve transection [12]. The effect of peripheral nerve injury or regeneration on DRG glutamate metabolism, however, has not been studied systematically.

In the present study, I evaluated vesicular glutamate transporter 1 and glutaminase as additional markers of glutamatergic function in DRG cell bodies during the regenerative phase following sciatic nerve transection and resection in the rat. VGLUT1 and GLS were evaluated in DRG with immunohistochemistry at day 1, 2, 7, 14, 28, and 70 after transection and resection of the sciatic nerve (SNT).

## 2. Materials and Methods

### 2.1. Animals:

Adult Sprague Dawley rats (n=27; 250-350 g) were housed in a 12-hr-light: 12-hr-dark cycle and given free access to food and water. Procedures were approved by the

Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. All appropriate efforts were made to minimize the number of animals used in this study. Rats were randomly assigned to specific experiments, irrespective of their gender. Gender differences were not investigated in this study.

## 2.2. Surgery:

All surgeries were performed on animal anesthetized by 2%-3% isoflurane inhalation. Under deep anesthesia, the rat right sciatic nerve was exposed at mid-thigh level and cut with a sharp blade. The nerve was reapposed and sutured using ETHILON 8-0 black monofilament Nylon for alignment. Muscles and skin were sutured in layers. The animals were allowed to recover from the anesthesia in a warmed cage and then were housed individually until the day of perfusion. Naïve rats were used as control.

## 2.3. Tissue Preparation:

Rats were anesthetized with tribromoethanol [Avertin;2.5% (w/v)] and xylazine and perfused through the ascending aorta with 75 ml of calcium-free Tyrode's solution (pH 7.3), followed by 300 ml of fixative 0.2% paraformaldehyde (w/v), 0.8% (w/v) picric acid in 0.1M phosphate buffer, pH 7.3 [16]. Right L4 DRG were removed and placed for 4 hrs. in the same fixative used for perfusion at 4C. DRGs were transferred to 20% sucrose in 0.1M phosphate buffer, pH 7.3 for 24–48 hrs. at 4C.

## 2.4. Immunofluorescence:

Immunohistochemical localization of GLS and VGLUT 1 was performed for six different experimental time points: 1, 2, 7, 14, 28, and 70 days post right sciatic nerve

transection (SNT) and resection, and naïve rats. The tissue was frozen, sectioned at 12 - 14  $\mu\text{m}$  with a Leica CM 1850 Leica (Microsystem Inc. Bannockburn, IL). Sections were thaw mounted to gelatin-coated SuperFrost slides (Fisher Scientific; Pittsburg, PA) and dried for 1 hr at 37C. Every fifth section was used to reduce the possibility of evaluating a neuron twice. Sections were washed three times for 10 min in phosphate buffered saline (PBS) and incubated in 0.5% bovine serum albumin (BSA), and 0.5% polyvinylpyrrolidone (PVP) in PBS with 0.3% Triton X-100 (PBS-T) and primary antiserum for 4 days. For GLS detection, polyclonal rabbit antiserum against whole GLS (gift from Dr. N. Curthoys, Colorado State University, Fort Collins, Colo) was used at 1:20,000 dilution. For VGLUT 1 detection, rabbit antiserum (Sigma) against VGLUT 1 was used at 1:2000 dilution. The tissue was rinsed three times in PBS and incubated in AlexaFluor 488 goat anti-rabbit IgG secondary antibody in PBS-T (1:1500) for 1 hr at room temperature. Sections were rinsed two times in PBS following secondary antibody incubation, and then incubated in DAPI for nucleus staining for 15 Min. All sections were rinsed three more times in PBS and coverslipped with ProLong Gold mounting medium (Molecular Probes).

## 2.5. Image analysis:

Images were acquired using a 20X objective on an Olympus BX51 epifluorescence microscope (Olympus; Center Valley, PA) equipped with a SPOT RT740 quantitative camera (Diagnostic Instruments; Sterling Heights, MI). Three random fields of view were imaged for each DRG section. Images were saved as uncompressed TIFF files. The exposure time for all images was the same for all tissue sections from all animals. The exposure time was determined empirically so that weakly stained neurons

could be distinguished for tracing, but that intensely stained neurons were not oversaturated [16,17]. This approach allowed images to be evaluated along the linear aspect of immunofluorescence intensity [17]. Captured images were 1600 X 1200 pixels with 2.69 pixels per micrometer. All neuron profiles with clearly visible nuclei that were not touching the edge of the image were traced with a Cintiq 21UX interactive pen display (Wacom; Kita Saitama-Gun, Saitama, Japan), using the freehand selection tool in Image J software (NIH). Nuclei were excluded from the regions of interest, making each region of interest correspond to the cytoplasmic profile of a single DRG neuron [16, 17]. Images were acquired at a bit depth of 8 and pixel intensities ranged from 0 (darkest) to 255 (lightest) on a grayscale. The mean gray value and area in  $\mu\text{m}^2$  for each cytoplasmic profile were measured and copied to a spreadsheet. The values for area ( $\mu\text{m}^2$ ) were binned into small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ) sized categories to indicate three broadly defined neuronal subpopulations that corresponded to C, A $\delta$ , and A $\alpha/\beta$  fibers, respectively [18, 19].

All data obtained were normalized to allow the evaluation of different time points in the same statistical test. The normalization was performed following this formula:

$$X = (\text{individual mean gray intensity per rat} \times 100) \div \text{the mean of total naïve mean gray intensity}$$

Where X is equal to the percentage change of mean gray intensity per injured rat in relation to the naïve rats.

## 2.6. Statistic:

Data from the analyses are reported as mean value with standard error of the mean. A one-way ANOVA test, followed by Tukey post-test was used to determine



differences between nerve injury and control groups (Prism version 5.01, GraphPad Software Inc., San Diego, CA). In all analysis, P values less than 0.05 were considered significant.

### 3. Results

#### 3.1. Glutaminase IR in Dorsal Root Ganglia:

At day 14, GLS immunoreactivity (IR) in L4 DRG neurons post SNT rats qualitatively were increased compared to DRG neurons from naïve rats (Figure 4.1). Therefore, the GLS IR intensities of the total immunoreactive neurons and three different sizes of DRG cell bodies were analyzed (Figure 4.2). At Day 14, the total, medium and large sized neurons have an increase in GLS IR in L4 DRG cell bodies (Figure 4.2 A, C, and D) from the SNT rats ( $140.38 \pm 40.01/\mu\text{m}^2$ ) compared to naïve rats ( $100 \pm 6.76/\mu\text{m}^2$ ;  $P < 0.05$ ). This increase in GLS intensity was not seen in day 1, 2, 7, 28, and 70 post SNT. In addition, day 14 post SNT DRG neurons small sized neurons subgroup did not show significant change in GLS IR compared with naïve DRG neurons (Figure 4.2B).

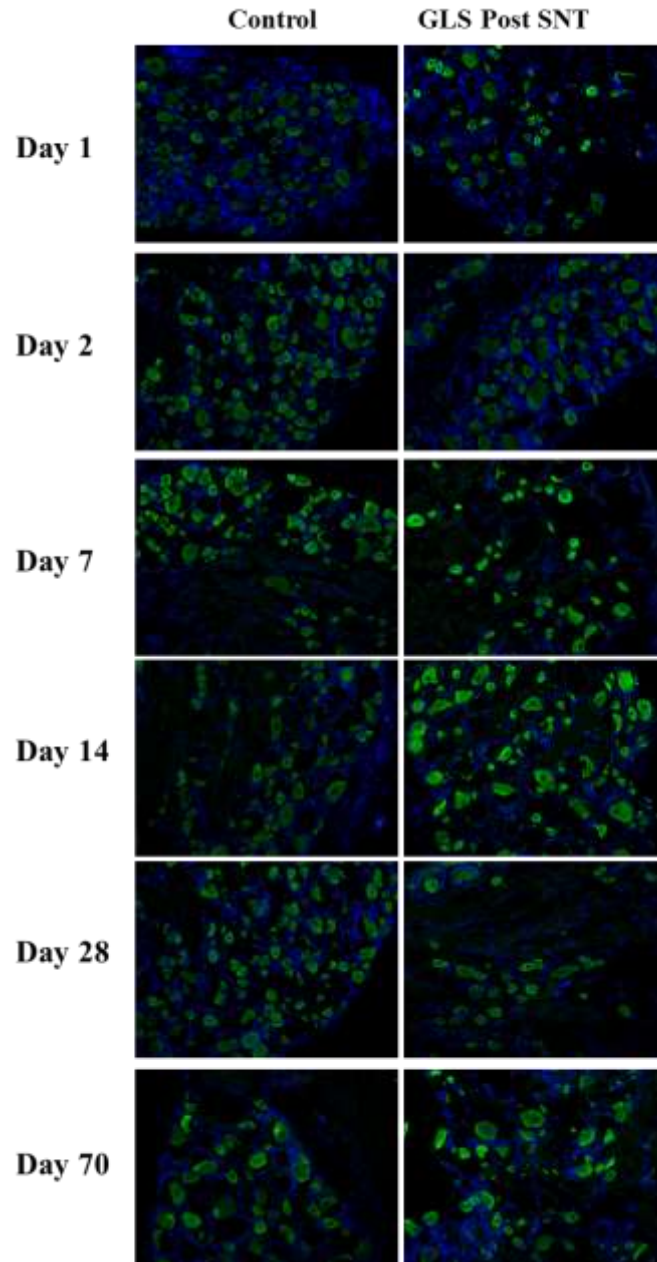


Figure 4.1: Glutaminase (green) immunoreactivity (IR) and DAPI (blue) nuclear staining in rat L4 dorsal root ganglia (DRG) following 1, 2, 7, 14, 28, and 70 days post right sciatic nerve transection (SNT). DRG sections were processed with a rabbit VGLUT2 antiserum and photographed under identical conditions. At day 14, GLS IR was more intense in neurons compared to control sections. No noticeable change in GLS IR occurred in day 2, 7, 28, and 70 post SNT.

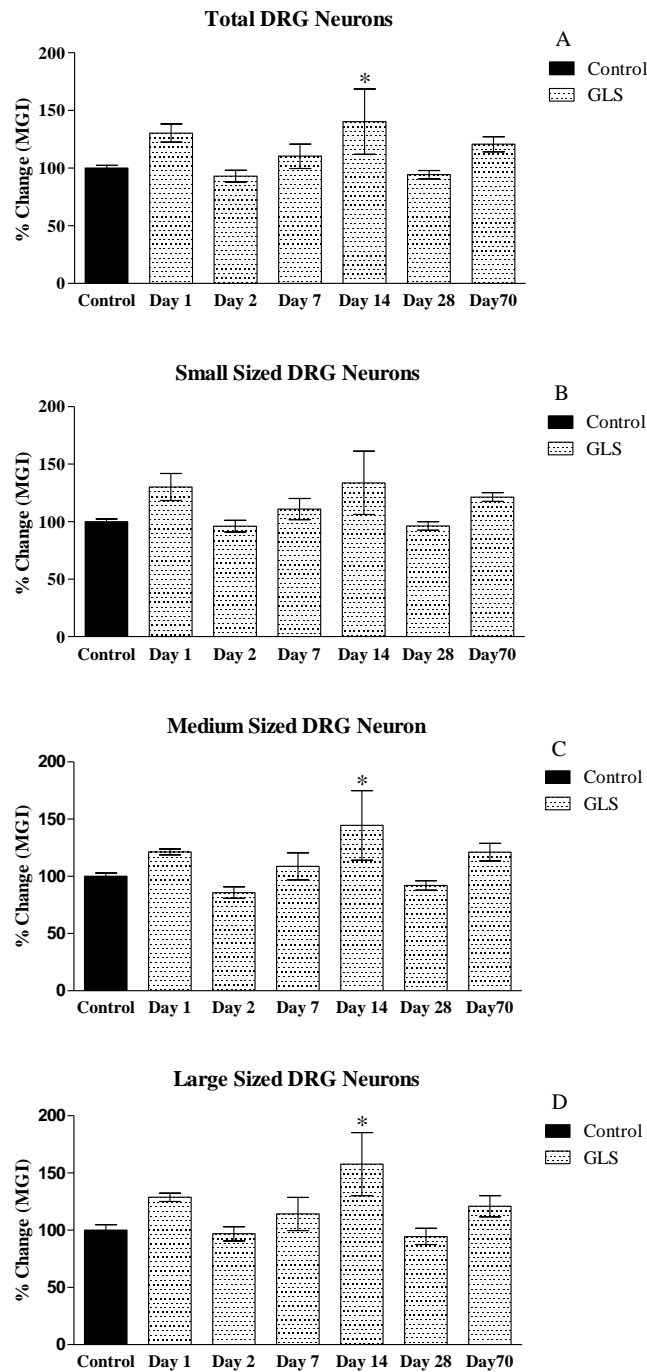


Figure 4.2: Image analysis of Glutaminase (GLS) immunoreactivity (IR) in L4 DRG neurons after 1, 2, 7, 14, 28, and 70 days post sciatic nerve transection (SNT). Data are presented as percentage change in mean gray intensity. DRG neurons were categorized into three area size groups: small ( $<400 \mu\text{m}^2$ ), medium ( $400\text{--}799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ). Small sized neurons GLS IR (B) increased at day 70 post SNT compared to control rats. (\*  $P < 0.05$ ). One-way ANOVA followed by Tukey test were used.

### 3.2. Vesicular glutamate transporter 1 IR in Dorsal Root Ganglia:

At day 2, VGLUT1 immunoreactivity (IR) in L4 DRG neurons post sciatic nerve transection rats qualitatively decreased compared to DRG neurons from naïve rats (Figure 4.3). Therefore, the VGLUT1 IR intensities of the total immunoreactive neurons number and three different sizes of DRG cell bodies were analyzed (Figure 4.4). Day 2, total neuronal VGLUT1 IR intensity of L4 DRG cell bodies (Figure 4.4 A) from the SNT rats ( $74.17 \pm 4.22 / \mu\text{m}^2$ ) was less ( $p < 0.05$ ) than naïve rats ( $100 \pm 8.23 / \mu\text{m}^2$ ). At day 7, 14, 28 and 70 post injury, VGLUT1 IR returned to normal intensity levels. In addition, neurons size subgroups from day 2 post SNT DRG showed a significant decrease in VGLUT1 IR compared with naïve DRG neurons except for medium sized DRG neurons (Figure 4.4 B, C, and D).

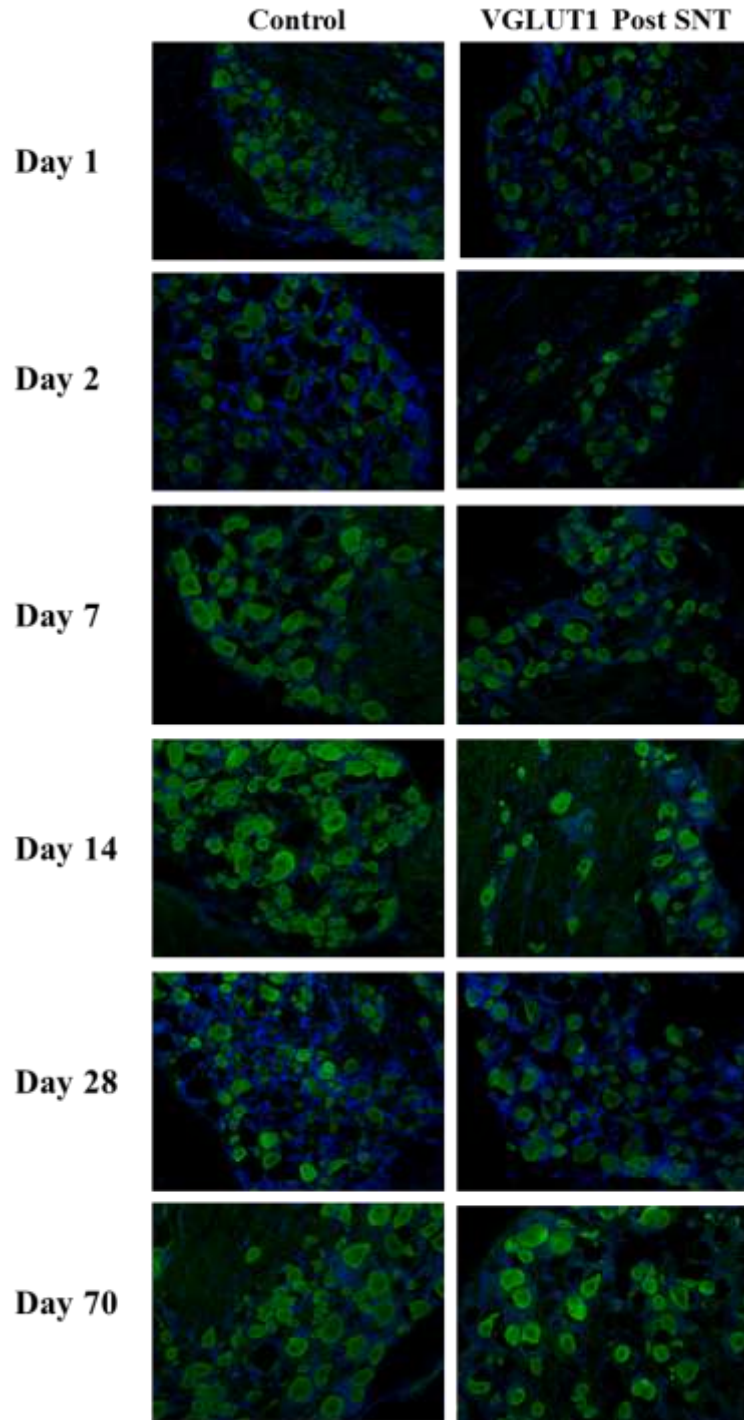


Figure 4.3: Vesicular glutamate transporter 1 (green) immunoreactivity (IR) and DAPI (blue) nuclear staining in rat L4 dorsal root ganglia (DRG) following 1, 2, 7, 14, 28, and 70 days post right sciatic nerve transection (SNT). DRG sections were processed with a rabbit VGLUT1 antiserum and photographed under identical conditions. In day 2 sections, VGLUT1 IR was diminished compared to control sections. No noticeable changes in VGLUT2 IR occurred at day 1, 7, 14, 28, and 70 post SNT.

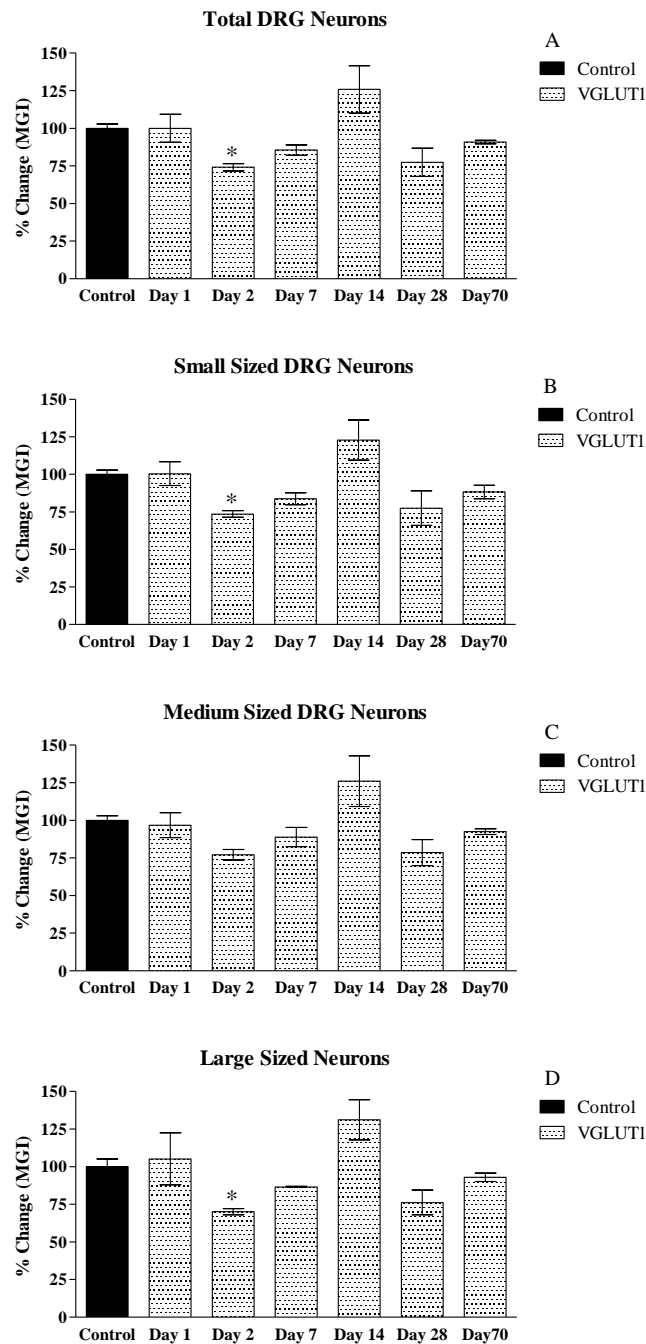


Figure 4.4: Image analysis of vesicular glutamate transporter 1 (VGLUT1) immunoreactivity (IR) in L4 DRG neurons after 1, 2, 7, 14, 28, and 70 days post sciatic nerve transection (SNT). Data are presented as percentage change in mean gray intensity. DRG neurons were categorized into three area size groups: small ( $<400 \mu\text{m}^2$ ), medium ( $400-799.99 \mu\text{m}^2$ ), and large ( $\geq 800 \mu\text{m}^2$ ). Total VGLUT1 IR (A) decreased at day 2 post SNT compared to control rats. This overall decrease was similar in small (B), and large (D) cell size subgroups. (\*  $p < 0.05$ ). One-way ANOVA followed by Tukey test were used.

#### 4. Discussion

In pathological conditions, DRG neurons constantly modify their neurotransmitter concentration and production [15]. DRG neurons primarily use glutamate as their excitatory neurotransmitter [9]. In addition to VGLUT2, glutaminase and vesicular glutamate transporter 1 are markers of the glutamate cycle [11]. In this study, I evaluated glutaminase and vesicular glutamate transporter 1 immunoreactivity at DRG neuronal bodies after sciatic nerve transection and resection at six different time points.

The sciatic nerve transection and resection model was used allowing for analysis of changes of VGLUT1 and GLS under the same conditions as my previous studies. In addition, the same six time points of the regenerative process was analyzed for changes in the GLS and VGLUT1 IR in the acute (day 1 & 2), sub-acute (day 7 & 14), and chronic (day 28 & 70) phases. This allowed for comparisons between changes in GLS and VLGLUT 1 with changes observed for VGLUT 2.

Similar to changes observed for VGLUT 2, VGLUT 1 IR decreases in DRG neurons at day 2 then returns to normal levels. This decrease in VGLUT1 IR may be due to an inflammation response or an early regenerative response. This decrease in VGLUT1 immunoreactivity may indicate increased trafficking of synaptic vesicles for elevated glutamatergic neurotransmission in the spinal cord. Alternatively, vesicular release of glutamate may be occurring at the regenerating peripheral process.

Furthermore, GLS exhibits the same general pattern of change in IR observed in VGLUT 1 & 2. Although the level of GLS IR was unchanged significantly at day 2, GLS IR was significantly increased at day 14 post SNT in DRG neurons. This indicate that

glutamate cycle follow the same pattern in glutamate synthesis as in glutamate packaging. There are no previous studies on GLS after sciatic nerve transection. However, in inflammation models, GLS increases 7 days post injury [15]. Thus, we can assume that GLS IR does not follow the same path in inflammation during nerve injury as in peripheral inflammation.

Further studies are needed to establish GLS and VGLUT1 activity changes in DRG, sciatic nerve, and spinal cord following longer time periods or until full recovery is achieved. In addition, functional assessment studies are needed to determine if GLS and VGLUT1 morphological changes are correlated with functional recovery.



## CHAPTER V

### SUMMARY AND FUTURE WORK

Neuropathic pain is a common complication of peripheral nerve injuries. This is due to the prolonged time of recovery and misguided directional axonal growth. In pain studies, it has been revealed that glutamate neurotransmission has a critical role in the noxious stimulus sensation. The glutamate cycle has several well established markers: vesicular glutamate transporters 1 & 2 and glutaminase. However, systemic studies for changes in vesicular glutamate transporters 1 & 2 and glutaminase after peripheral nerve injuries have not been done. Thus, in this thesis project I studied the changes in vesicular glutamate transporters 1 & 2 and glutaminase systemically at time points that represent the acute (day 1 & 2), subacute (day 7 & 14), and chronic (day 28 & 70) phases in peripheral nerve regeneration. The peripheral nerve injury models were used imitate two types of peripheral nerve injury in human. The first model, the sciatic nerve crush (SNC), represents the axonotmesis injury type. The second model, the sciatic nerve transection (SNT), represents the neurotemsis injury type. Additionally to avoid the formation of neuroma in the transection model, the severed sciatic nerve was sutured together to bridge the gap between the two ends of the transected nerve. This provides the best environment for the nerve to grow to its original target.

In summary, my results showed an interesting alteration for vesicular glutamate transporters 1 & 2 and glutaminase immunoreactivity that differ in each model. For the SNC model, I examined VGLUT 2 at the subacute phase in conjunction with Gap43. VGLUT 2 at day 7 post injury showed significant increases compared to sham operated rats. No significant change was seen at day 14. Gap43 immunoreactivity levels examined to confirm that our model produced a nerve injury. Gap43 immunoreactivity level is increased in the regenerative process following nerve injury. Consistent with the SNC model, Gap43 levels were elevated at day 7 and 14 post injury.

In the sciatic nerve transection and resection model, examination of VGLUT 2 immunoreactivity in the DRG neurons showed a decrease at day 2 and 28 post injury in all cell sizes. This decrease occurs only in small sized neurons at day 70 post injury. VGLUT 2 immunoreactivity was increased at the proximal end of the transected sciatic nerve at day 1, 2, and 28 post injury. On the other hand, studies of VGLUT 1 immunoreactivity at the DRG neurons showed a decrease in neuronal cell bodies only at day 2 post sciatic nerve transection injury. Furthermore, studies of glutaminase immunoreactivity of the DRG neurons revealed an increase at day 14 post SNT injury in the neuronal cell bodies.

In conclusion, these immunohistochemistry studies for VGLUT 1 & 2 and glutaminase provide insight into part of the story of neuronal response to injury. To more fully understand the underlying mechanisms of changes in the glutamate metabolism cycle after peripheral injury, further studies need to be done. For example, western blot studies of VGLUT 1 & 2 and GLS for the acute, subacute, and chronic phases need to be done in conjunction with *in situ* hybridization studies to compare VGLUT 1 & 2 and

GLS proteins amount with their mRNA levels. Furthermore, behavioral studies are needed to see if the molecular change observed in glutamate cycle is comparable with the alteration in pain sensation following peripheral nerve injury. Lastly, an understanding of the molecular and physiological changes after peripheral nerve injuries is a necessary step in the development of a treatment to improve the functional outcome of those injuries.

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## VITA

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