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A Pre-Clinical Assessment of Minocycline for Treatment of Chronic Neuropathic Pain after Spinal Cord Injury

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A PRE-CLINICAL ASSESSMENT OF MINOCYCLINE FOR TREATMENT OF CHRONIC NEUROPATHIC PAIN AFTER SPINAL CORD INJURY

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A

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By

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Dedication

I would like to dedicate my work to all my loved ones who passed away within the first few months of starting graduate school: my grandfather, Norbert Henning; my grandmother, Ruth Poteete; my aunt, Carol Henning; and my dear friend, Hal Smith. I would not be where I am today without all their love and support throughout my life.

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Patients living with a spinal cord injury (SCI) often develop chronic neuropathic pain (CNP). Unfortunately, the clinically approved, current standard of treatment, gabapentin, only provides temporary pain relief. This treatment can cause numerous adverse side effects that negatively affect the daily lives of SCI patients. There is a great need for alternative, effective treatments for SCI-dependent CNP.

Minocycline, an FDA-approved antibiotic, has been widely prescribed for the treatment of acne for several decades. However, recent studies demonstrate that minocycline has neuroprotective properties in several pre-clinical rodent models of CNS trauma and disease. Pre-clinical studies also show that short-term minocycline treatment can prevent the onset of CNP when delivered during the acute stage of SCI and can also transiently attenuate established CNP when delivered briefly during the chronic stage of SCI. However, the potential to abolish or attenuate CNP via long-term administration of minocycline after SCI is unknown.

The purpose of this study was to investigate the potential efficacy and safety of long-term administration of minocycline to abolish or attenuate CNP following SCI. A severe spinal contusion injury was administered on adult, male, Sprague-Dawley rats. At day 29 post-injury, I initiated a three-week treatment regimen of daily administration with minocycline (50 mg/kg), gabapentin (50 mg/kg) or saline.

The minocycline treatment group demonstrated a significant reduction in below-level mechanical allodynia and above- level hyperalgesia while on their treatment regimen. After a ten-day washout period of minocycline, the animals continued to demonstrate a significant reduction in below-level mechanical allodynia and above-level hyperalgesia. However, minocycline-treated animals exhibited abnormal weight gain and hepatotoxicity compared to gapabentin-treated or vehicle-treated subjects.The results support previous findings that minocycline can attenuate CNP after SCI and suggested that minocycline can also attenuate CNP via long-term delivery of minocycline after SCI (36). The data also suggested that minocycline had a lasting effect at reducing pain symptoms. However, the adverse side effects of long-term use of minocycline should not be ignored in the rodent model.

Gabapentin treatment caused a significant decrease in below-level mechanical allodynia and below-level hyperalgesia during the treatment regimen. Because gabapentin treatment has an analgesic effect at the concentration I administered, the results were expected. However, I also found that gabapentin-treated animals demonstrated a sustained reduction in pain ten days after treatment withdrawal. This result was unexpected because gabapentin has a short half-life of 1.7 hours in rodents and previous studies have demonstrated that pre-drug pain levels return shortly after withdrawal of treatment. Additionally, the gabapentin-treated animals demonstrated a significant and sustained increase in rearing events compared with all other treatment groups which suggested that gabapentin treatment was not only capable of reducing pain long-term but may also significantly improve trunk stability or improve motor function recovery.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

INTRODUCTION

A traumatic spinal cord injury (SCI) can produce devastating and life changing neurological deficits. According to an extensive survey conducted in 2009 by the Christopher Reeve Foundation known as, *One Degree of Separation,* an estimated 1,275,000 individuals in the United States suffer from the consequences of SCI (1). Aside from loss of motor function, chronic neuropathic pain (CNP) is one of the most prevalent problems reported among SCI patients. CNP is predominately defined as a state of long-term exaggerated pain that is brought about by alterations in the pain processing pathways within the central nervous system (CNS) and peripheral nervous system (PNS). This most often occurs as a result of disease or traumatic injury (2),(3),(4),(5). Neuropathic pain manifests into several types of evoked and non-evoked pain syndromes and significantly impairs the quality of life and functional recovery of SCI patients for the rest of their lives (6),(2). Unfortunately, CNP develops in 60%-80% of SCI patients (6),(7).

Currently, there is no cure for CNP and it remains very difficult to treat despite a growing effort among researchers (8). Unfortunately, drugs prescribed for the treatment of CNP also cause numerous side effects and, at best, only temporarily relieves symptoms. SCI patients greatly need a new therapeutic intervention to inhibit CNP and improve their quality of life. This pre-clinical study will investigate and analyze the FDA-approved drug minocycline and its potential to reduce and possibly abolish established CNP after SCI.

Spinal Cord Injury

The United States has the highest number of SCI incidents worldwide, with approximately 12,400 new cases each year (9). The leading causes of SCI in the United States are motor vehicle accidents (50%), falls (16%), violent acts (12%) and sports related injuries (10%) (9). Males are much more likely to acquire SCIs and account for nearly 80% of all new cases (9). After SCI, patients endure a lifetime of complications such as neuropathic pain and psychosocial issues in addition to permanent disabilities such as muscle spasticity and paralysis (7),(10),(11). Unfortunately, several incompletely understood mechanisms contribute to the wide array of problems associated with SCI. There are no FDA-approved treatments to restore function after SCI and treatments to relieve associated symptoms are often insufficient and temporary. Muscle spasticity, a common secondary problem after SCI may also be linked to a condition called autonomic dysreflexia which is reported most often when the insult occurs at the fifth and sixth thoracic segments (12),(13). Autonomic dysreflexia is a severe and debilitating condition characterized by a rapid attack that causes an enormous increase in blood pressure, shivering, sweating, anxiety and severe headaches (13).

In addition to enduring the life-changing physical consequences of SCI, the financial burden for treating the numerous chronic conditions is often overwhelming. Patients usually take medication, undergo years of physical rehabilitation and require caregivers and medical devices to help them perform daily functions. Healthcare costs for SCI patients during the first year of injury can range from $228,000 to $775,000 and cost up to three million dollars during their lifetime (1).

The consequences of SCI can vary depending on the severity and location of impact on the spinal cord. In moderate to severe cases, pathophysiology begins with a primary injury caused by direct mechanical insult to the spinal cord and is followed by a prolonged secondary injury phase where damage continues to occur. Primary injury is often due to contusion and compression forces which are caused by bone and/or disk fragments that bruise the spinal cord upon impact (14). During the first few hours after injury the patient may experience a variety of damaging pathological events such as edema, hemorrhaging, severing of white matter, shearing of blood vessels, and the formation of a necrotic epicenter (15).

The secondary injury phase occurs days to weeks after injury and can often last years (15). The pathophysiology consists of ischemia and disruption of blood flow, ionic imbalances and demyelination, as well as degeneration of axons. Apoptosis of neuronal tissue, infiltration of peripheral immune cells such as T-cells, neutrophils and macrophages, and formation of a glial scar also occur in response to injury (15). The pathology of secondary injury can spread far beyond the necrotic epicenter over many years causing permanent damage to the tissue surrounding the area of injury and leading to an environment within the spinal cord that is unfavorable to growth and repair (16),(17).

Figure 1, shown below, displays pathological events during the acute and chronic phase of injury. The events in the acute and chronic stage of SCI contribute to the permanent state of damage within the spinal cord that is highly unfavorable to growth and repair.

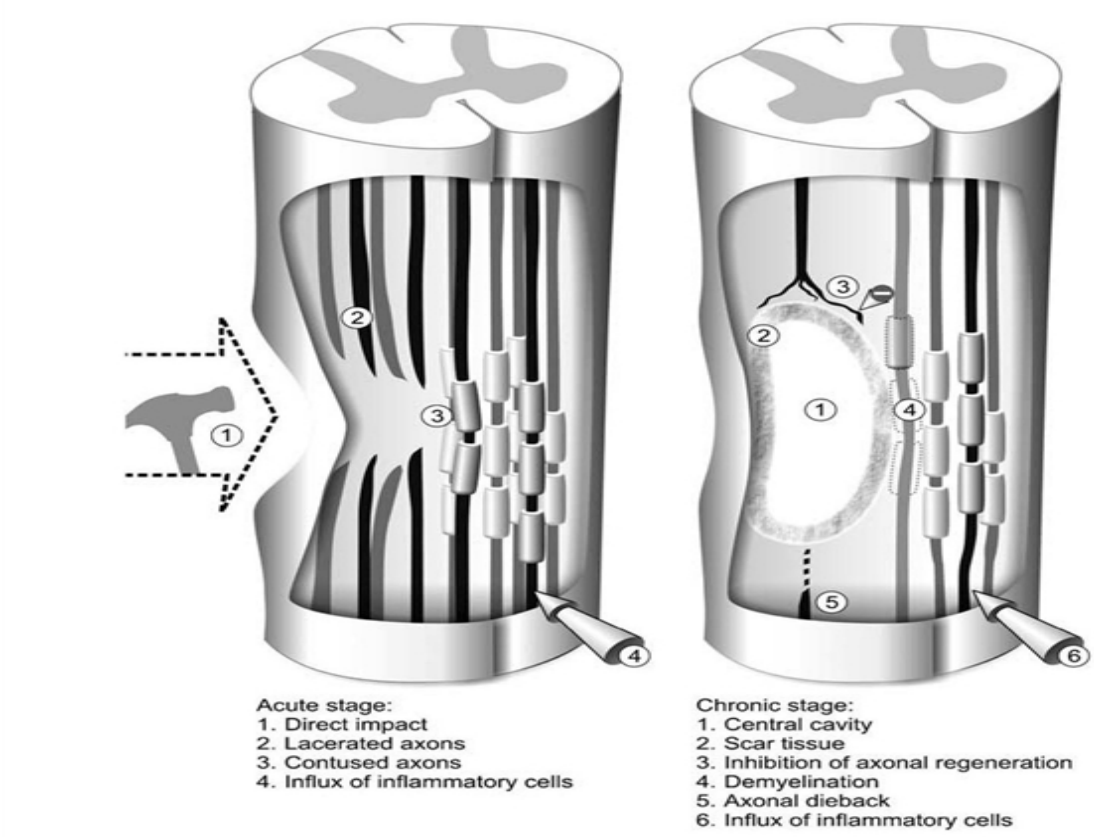


Figure 1: *Schematic presentation of the pathological events displayed during the acute and chronic phases of spinal cord injury. Adapted by permission from Macmillan Publishers Ltd: [Nature] copyright 2008 (18).*

One major consequence of SCI is a robust and prolonged inflammatory response. This complex response serves a dual role by providing both beneficial and deleterious effects during the acute and chronic phases of injury (15). This becomes a challenging issue for researchers working towards a treatment for the multitude of damaging consequences produced by SCI. Researchers work to provide a treatment or cure that will not only reduce neuronal death but will also promote regeneration of neurons and continue providing a neuroprotective environment for repair.

There are several cell populations involved in the process of inflammation after SCI including astrocytes, microglia, T-cells, neutrophils and monocytes (19). Cell populations function quickly to remove cell debris from the lesion site and attempt to create an environment suitable for re-growth of tissue. Meanwhile, other cellular populations counteract the response by advancing further tissue damage (20).

A major aspect of secondary injury is inflammation. This greatly contributes to the death of neuronal tissue within the spinal cord (21). For example, resident microglia cells within the spinal cord can play a beneficial role by becoming activated after injury where they act as macrophages, removing deleterious and foreign debris (22). However, activated microglia also begin to release an excessive amount of pro-inflammatory cytokines such as TNF-α and IL-1β, as well as reactive oxygen species, nitric oxide and proteases that continuously promote tissue damage within the spinal cord (21) Popovich and Jones, 2003; (22) Xu et al., 2001). Neurons and glial cell membranes produce arachidonic acid after CNS/PNS injury. This acid can release metabolic products such as prostaglandins, leukotrienes and free radicals that are largely associated with inflammation and neural toxicity (23),(24),(25),(26). Many recent studies stress the importance of targeting pro-inflammatory molecules in the acute stages of SCI to prevent tissue damage that can occur as a result of the immune response (27),(28),(20).

Fleming-Norenberg *et al.* found that activated microglia and macrophages were abundant up to one-year post-injury with analysis and histology of human cord tissue (20). Through microarray analysis of spinal cord tissue of rodents in the chronic phase of SCI, Nesic *et al.* discovered that there are many significantly up-regulated genes known to be involved in inflammation and increased expression of glial fibrillary acid protein (GFAP)

mRNA, a known marker of astrocytes (2). Therefore, inhibiting neuro-inflammation is an important yet controversial approach for treating patients not only in the acute phase of injury, but also patients living in the chronic phase of SCI.

Chronic Neuropathic Pain

CNP is a highly prevalent and permanent condition among the SCI population and is known to affect patients with a moderate injury to patients with total paralysis. In a study of pain after SCI, it was reported that six months after injury 64% of patients complained of pain and 21% described the pain as severe (29). In a recent pain review after SCI, 64%-82% of patients reported painful sensations five years after SCI (30). CNP can occur above, below and at the site of injury. The onset of at-level neuropathic pain most commonly occurs within weeks of the initial injury, while the onset of below-level neuropathic pain occurs months to years after injury (7). There is no clear definition of when above-level pain appears in the patient population. It is most often described as painful sensations when touching the face and an increase in sensitivity to cold and hot temperatures (31). Neuropathic pain can be characterized as spontaneous/non-evoked pain which is independent of peripheral stimuli or as peripherally-evoked pain which occurs when a stimulus is applied (7). Peripherally-evoked pain includes specific types of pain responses termed allodynia and hyperalgesia. Allodynia is described as a painful sensation in response to a normally non-painful stimulus (31). Hyperalgesia describes an exaggerated painful sensation in response to a stimulus that normally elicits a significantly reduced pain response (31). Phantom pain can also manifest in SCI patients with paralysis. Phantom pain is a type of spontaneous/non-evoked pain which causes patients to experience painful sensations

where all sensory input is gone (7). CNP syndromes are most often described by patients as persistent burning, cutting, stabbing, shooting, electrical and piercing pain (7),(31).

Pain is a challenging area to study in humans due to the numerous classifications and questionnaires developed by researchers and multiple descriptions of pain by patients. In animal models, the task of measuring pain becomes even more difficult in addition to being highly controversial. Von Frey hair filaments, thermal testing, and girdle response testing in a rodent model of SCI measure evoked pain. Researchers are currently unable to directly test spontaneous/non-evoked neuropathic pain in animals due to the absence of verbal communication. However, analysis of spontaneous exploratory behavior utilizing a photobeam activity box can provide some insight into the development of non-evoked pain in animal subjects.

The mechanisms of CNP are exceptionally complex and not entirely understood. However, recent studies have identified several contributing factors important for establishment and maintenance of neuropathic pain after SCI. Neuronal mechanisms contribute to pain by inducing long-term plastic changes along the pain sensory pathways (32). Nociceptive signaling begins at peripheral sensory neurons enters the spinal cord dorsal horn, acting as the first relay for pain transmission (32). In a recent study of pain behavior in a rodent model of SCI coupled with electrophysiology data, the authors indicate that the peripheral sensory neurons become hyper-excitable after injury and contribute to an increase in pain sensation (4),(33). At the level of the spinal cord dorsal horn, increased glutamate release and prolonged activation of NMDA receptors also play a critical role in long-term plasticity of pain signals (34). Electrophysiology studies show “central sensitization” of the spinal dorsal horn neurons correlates with an increased pain response

after SCI (35),(36). The combination of both sustained hyper-excitability of peripheral neurons and hyper-sensitization of central neurons sends a robust, amplified response to the thalamus (33). The intensified sensory interpretation by the thalamus of all the combined neuronal signals is pain (33).

Studies also suggest that synaptic reorganization such as sprouting in the dorsal horn occurs after SCI (37). Sprouting of dorsal horn neurons can lead to permanent neuronal changes and can also amplify pain signal pathways leading to the thalamus (33),(38),(36),(39). Evidence suggests that changes can also occur in third-order somato-sensory neurons (33),(40). Hyper-excitability of thalamic VPL neurons suggests that there is major reorganization of the synaptic circuits and neuronal pathways after SCI (40).

Furthermore, glial cells, which are normally in an inactive/resting state in healthy individuals, work to maintain the architecture and homeostasis of neuronal cells within the spinal cord (41). However, upon activation due to SCI, glial cells (astrocyte and microglia subtypes) increase in cell volume, become thickened and display characteristic "ramified branches" (41),(36). Permanent activation leads to increased proliferation of glial cells and a continuous up-regulation of inflammatory cytokine release (41). Once activated, astrocytes and microglia contribute to CNP through persistent hyper-excitability of the spinal dorsal horn neurons (2),(35). Chronically activated microglia produce pro-inflammatory mediators such as IL-1-β, TNF-α, IL-6, MMP-9, MCP-1, TIMP-1 and reactive oxygen species that contribute to the development and maintenance of CNP after SCI (42),(43),(36),(44).

Evidence from authors of animal studies indicates a strong correlation between pain sensations and pro-inflammatory mediators released by microglia cells after SCI. For

example, TNF-α and IL-1β contribute to the development of mechanical allodynia after SCI, and IL-6 contributes to maintenance of mechanical allodynia (42). The macroglial cells, astrocytes, are also important in the maintenance and development of pain after SCI. Astrocytes, in an inactive state and healthy state, provide infrastructure and homeostatic control of neurotransmitters outside of the synaptic cleft (45). Activated astrocytes contribute to pain after SCI in numerous pre-clinical studies of CNP after SCI. For example, Crown *et al.* provided strong evidence that astrocytes, as well as microglia, have increased expression of p-38 MAPK enzymatic activity in a rat model of SCI (44). The increased expression of p-38 MAPK after SCI in the astrocytic cell population plays an important role in the development of CNP. Inhibition of p-38 MAPK correlates with a decrease in CNP as well as improved locomotor recovery after SCI (44).

Glial cells also prevent down regulation of GAD-65 in the spinal dorsal horn (35). This suggests that there is a decrease in endogenous inhibitory activity, which could contribute to hyper-excitability found in the neuronal pathways. The term "gliopathy" is now used to describe the many maladaptive responses of glial cells after neural injury (45). Recent evidence suggests that inhibition of glial activation in both astrocytes and microglia improves the outcome of chronic pain development with early intervention in animal models of SCI (28),(46),(44).

Treatment

As previously stated, there is no cure for CNP and treatment with common analgesic drugs is highly inadequate at reducing pain (8). The current, clinically approved treatment for CNP is gabapentin (47). Gabapentin was developed for the treatment of seizures and is

labeled as an anti-convulsant drug (48). The mechanism of action is still unclear. However, recent studies have demonstrated that gabapentin binds to a calcium channel subunit, alpha - 2-delta, and reduces calcium influx into cells (49). The reduction of calcium may contribute to a decrease in excitability by inhibiting transmitters such as glutamate and substance P in neurons and therefore provides an analgesic effect (50),(51). In a recent study, gabapentin treatment reduced symptoms of autonomic dysreflexia and muscle spasticity in a rodent model of SCI. The authors indicated that gabapentin administration (50 mg/kg), two to three weeks after injury reduced spinal motor reflexes and dysreflexic hypertension (12). The findings suggest that gabapentin may be producing an effect through more than one pathway. Therefore, in SCI patients it is beneficial for attenuating pain, but may also be able to attenuate muscle spasticity and autonomic dysreflexia. Unfortunately, clinical studies indicate that patients often discontinue use due to the numerous adverse side effects such as dizziness, confusion, sedation, cost and lack of efficacy (52),(53). Pregabalin, an alternative to gabapentin, is also currently being used for treatment of CNP (54). Recent clinical studies have shown pregabalin to be more effective at a lower dosing regimen than gabapentin (54). However, side effects such as dizziness and somnolence are reported in approximately 30% of patients (54). Side effects of drugs prescribed for CNP should be minimal because they can negatively affect patients' already difficult daily activities and further reduce their quality of life.

Previous drugs used for treatment of CNP, such as methylprednisolone, have proven to be ineffective in clinical trials (55). Opioids such as morphine and oxycodone are also used by patients to relieve pain symptoms (48). However, abuse of opioid drugs is common and most are considered highly addictive, both physically and psychologically (56).

Tolerance to opioid drugs occurs rapidly and withdrawal is often a challenge for chronic users (56). Therefore, opioid drugs are not a primary source of treatment for chronic pain syndromes associated with CNP. Based on the lack of efficacy and safety of current pain treatments, novel therapies with minimal or no adverse side effects need to be found for patients in the chronic stage of SCI who are living with CNP.

Interestingly, several pre-clinical SCI studies have not only shown that neuro-inflammation is an important mechanism in the development and maintenance of CNP; these studies have also demonstrated that there is a significant correlation between inflammation and neuropathic pain (33),(42),(43). A potentially promising drug to inhibit CNP in the chronic stages of SCI is an anti-inflammatory drug called minocycline, which has been FDA-approved since 1971, initially for the treatment of acne (57). Minocycline is a semi-synthetic, second generation tetracycline antibiotic (58). Importantly, minocycline is a highly lipophilic molecule allowing it to pass easily through the blood-brain barrier (58). Access to the blood-brain barrier may be necessary for targeting inflammation that arises within the CNS. Because CNP likely involves interconnecting pain-processing pathways in both the CNS and PNS, access to the blood-brain barrier may be imperative when developing a therapeutic intervention.

Minocycline as a therapeutic agent has become increasingly important due to efficacy in pre-clinical animal studies and clinical studies treating neurodegenerative diseases such as ischemia, multiple sclerosis, amyotrophic lateral sclerosis, acute and chronic spinal cord injury, Huntington's disease, Parkinson's disease and Alzheimer's disease (58). Minocycline exhibits the ability to be a potent anti-inflammatory and anti-apoptotic agent (58),(59). The anti-inflammatory action occurs by minocycline's ability to

inhibit activation of microglia and in turn inhibit the release of many pro-inflammatory cytokines (59). Minocycline also reduces inflammation by inhibiting matrix metalloproteinases (MMP), whose enzymatic activities are involved in the breakdown of extracellular matrix and tissue remodeling (60). In addition, minocycline has the capability of being neuroprotective by preventing apoptosis of neuronal cells through inhibition of cytochrome-c release from the mitochondria (61),(62).

Previously, clinicians prescribed minocycline for its known antimicrobial activity and effectiveness against gram-positive and gram-negative infections (63). In more recent years, minocycline has been used in clinical trials for neurodegenerative diseases such as acute stroke, acute SCI, and ALS (58). Minocycline is safe and effective for the treatment of rheumatoid arthritis. Currently, minocycline is prescribed by 18% of rheumatologists for rheumatoid arthritis (64). Minocycline use for treatment of diseases is becoming increasingly popular.

In several pre-clinical studies of acute CNS trauma and disease, minocycline has been proven to be effective in reducing pain symptoms and improving functional recovery (65),(36),(66),(67),(28),(46). Hains and Waxman *et al.* showed that minocycline treatment five days post-injury in a rat model of contusion injury improved pain thresholds using behavioral assessment over a four week time period (28). Hains and Waxman also found that minocycline can improve pain withdrawal thresholds after CNP has been established with a three day dosing regimen of minocycline in a one month post-SCI rat model (36). While the improvements in pain threshold were shown during the three day dosing regimen the pre-drug levels of pain returned immediately after withdrawal of minocycline (36). It is

currently unknown whether long-term administration of minocycline could be beneficial in reducing or abolishing symptoms of CNP after SCI in a rodent model.

The common side-effects of minocycline include skin complaints, nausea and dizziness (64). In general, minocycline has a reputation for being safe and well tolerated among patients who are administered the drug as an oral capsule. However, several case studies suggest that long-term use of minocycline have included rare, adverse side effects such as liver damage and autoimmune hepatitis that develop after an average of two years of use (68),(69),(70),(71),(72). Other studies have indicated rare side-effects such as black discoloration of the skin, bone and thyroid (73). Recent conclusions reported from a clinical study on the efficacy of minocycline in patients with amyotrophic lateral sclerosis were negative. Gordon *et al.* reported that ALS patients treated with minocycline for nine months declined more rapidly than those patients who received a placebo (74). Therefore, in future clinical studies it is important to be cognizant of the potential for adverse side effects that minocycline may cause, especially for long-term use in patients with CNS trauma or disease.

I performed this study to investigate the potential efficacy and safety of long-term administration of minocycline to attenuate established CNP and to determine if pre-drug pain levels return after withdrawal of minocycline in a rat model of early chronic SCI.

SPECIFIC AIMS

1. Determine whether minocycline can offer sustained relief of established CNP via long-term delivery.

Using an adult, male, Sprague-Dawley rat model of spinal contusion injury, I determined whether minocycline could reduce established CNP over a long-term treatment period. A severe spinal contusion injury was administered at spinal thoracic level ten using 200 kdyne of force. There was four treatment groups, (n=18); minocycline-treated, gabapentin-treated, vehicle- (saline) treated, and an uninjured, age-matched control group. Each group underwent direct and indirect neuro-sensory testing prior to SCI and every 2 weeks for 60 days after SCI (day 0). At day 29, administration of either minocycline (50 mg/kg), gabapentin (50 mg/kg), or vehicle (saline) began and continued daily for 3 weeks.

I assessed mechanical allodynia with von Frey hair testing of the hind limbs. Allodynia and hyperalgesia, above and below the level of injury were assessed with the girdle response test using von Frey filaments of non-noxious and noxious force.

A decrease in a variety of spontaneous behavioral activity can be a sensitive indicator of a non-evoked pain state. Therefore, I assessed exploratory locomotor behavior, which included fine and gross motor movement, time at rest, speed and distance using the photobeam activity system. The system was a completely objective and non-invasive tool. The animal subjects were allowed to move freely for 15 minutes, and then analysis of the software data identified alterations in the behavioral tasks.

2. **Determine whether CNP returns following withdrawal of minocycline administration.**

The potential of a minocycline treatment to cure CNP or to attenuate CNP via long-term delivery in SCI patients is currently unknown. At day 60, ten days after dosing was complete, I performed all direct and indirect neuro-sensory testing as previously described. I determined whether a sustained reduction of CNP had taken place beyond the conclusion of minocycline administration, which would indicate that minocycline treatment offered long-term relief of established CNP.

3. **Compare the potential efficacy and safety of minocycline against gabapentin, the clinically approved compound used for treatment of CNP.**

Finally, I compared the efficacy and safety of minocycline to that of gabapentin, the current treatment for CNP by analyzing and comparing the minocycline treatment group to the gabapentin treatment group in all behavioral assessments and any additional observations made during the experiment. Both treatment groups received the same dosing regimen (daily for 3 weeks) and same concentration of drug (50 mg/kg) to make an equal comparison between treatment groups.

METHODS

Animal Subjects

I used adult, male Sprague-Dawley rats (225-249 grams) purchased from Harlan Laboratories, Houston, TX) for this study. The University of Texas Health Science Center Institutional Animal Welfare Committee approved all animal protocols. All lab members strictly followed the procedures for laboratory animal care and use found in the Institutional, AALAC, and NIH guidelines. Animal subjects were housed with access to food and water in the rodent facility at the Center for Laboratory Animal Medicine and Care (CLAMC) at the University of Texas Health Science Center at Houston.

Surgery

The surgeon anesthetized the animal subjects with an intraperitoneal (IP) injection of ketamine 80 mg/kg, zylazine 10 mg/kg, acepromazine 0.75 mg/kg before the animals underwent a laminectomy at the tenth thoracic (T10) spinal segment. Next, the surgeon administered a "severe" contusion injury with the Infinite Horizons Spinal Impactor device (Precision Systems and Instrumentation) using 200 kdynes of force and zero dwell time. The injury model is a successful and well-validated model in the literature and defined by Dr. Nesic-Taylor at UTMB-Galveston. The surgeon completed the procedure by suturing the muscle layers together and used surgical staples to secure the skin closed. After surgery, the animal subjects recovered on heating pads overnight. I administered 3.0 mL subcutaneous saline injections to prevent dehydration. Animal subjects also received subcutaneous injections of Baytril (2.7 mg/kg) twice daily for ten days to prevent post-operative infection and subcutaneous injections of the analgesic buprenorphin (0.1 mg/kg) were administered

twice daily for five days post-injury. Animals were closely monitored and provided with the appropriate palliative care including manual bladder expression which was performed twice daily for approximately 15 days post-injury until reflex bladder function returned.

Drug Administration

Twenty-nine days post-SCI, I divided the injured animals into three treatment groups. One treatment group (n=18) received a 3 mL IP injection of 0.9% sodium chloride solution daily for 3 weeks. The second SCI group (n=18) received a 3 mL IP injection of minocycline (50 mg/kg) daily for 3 weeks beginning at day 29 post-injury. Stock solutions of minocycline hydrochloride (7 mg/mL; molecular weight (MW) of 538.98; TOCRIS Biosciences, Ellisville, MO) were prepared in 0.9% sodium chloride solution, gently warmed in a 60° C water bath for 15 minutes, vortexed, and stored at 4° C for use within 2 days. The third SCI group (n=18) received a 2 mL subcutaneous injection of gabapentin (50 mg/kg) daily for three weeks beginning at day 29 post-injury. A stock solution of gabapentin (10mg/mL; MW=171.2; Santa Cruz, Santa Cruz, CA) was prepared fresh daily by diluting in 0.9% sodium chloride solution and briefly mixing. The dose concentrations were based on previously published studies by Hama and Sagen, who described the effectiveness of gabapentin in reducing pain after SCI (75). In order to make experimental comparisons between the effectiveness of minocycline and gabapentin, I used the same concentrations for both treatment groups. A naïve control group (n=18) was also included in this study to compare to the injured animals and observe the normal behavior trends in non-injured, age-matched control animals.

Hind Limb Locomotor Functional Assay

Recovery of locomotor function following SCI was recorded using the BBB locomotor rating scale developed by Drs. Basso, Beattie and Bresnahan at Ohio State University (76),(77). This scale allows the observer to rate hind limb motor function on a scale of "0," no movement of hind limbs, to "21," coordinated gait, while the animal moved freely in an open field for four minutes. This test ensured reliability of the injury model and assessed functional recovery of the animal's hind limbs. Only animals that received a score of "0" or "1" at day one post-injury were included in the results of this study to ensure only animals of the same injury level were included.

Von Frey Hair Test

Mechanical allodynia was assessed by the application of von Frey filaments (3.61-5.18) with increasing stiffness that corresponded to a gram of force applied (0.4 g-18 g) to the glabrous surface of the hind limb paws *(refer to Figure 2B)*. The animals were acclimated to the cages for two consecutive days (one hour per day) before baseline testing began and 30 minutes prior to all testing sessions. The filament was applied perpendicular to the paw with enough force to bend the filament slightly and held on the hind paw for one second. A positive response was recorded if the paw was removed immediately or licked (78),(79). If a positive response occurred, I allowed 30 seconds to pass before the next filament application. The Dixon up-down mechanical threshold protocol was used to determine the value at which the animal withdrew its paw 50% of the time for the left and right hind paws (80),(78). The value corresponded to the mechanical withdrawal threshold for each animal. *Figure 2A,* shown below displays the template for recording results of the

von Frey hair test using the Dixon up-down mechanical threshold protocol. A "0" indicates no movement and an "x" indicates a positive response. I averaged the values for the left and right hind paw together for a measure of withdrawal response at each time point. I performed this test prior to injury for a measure of baseline response and after injury at day 14, day 28, day 42 and day 60.

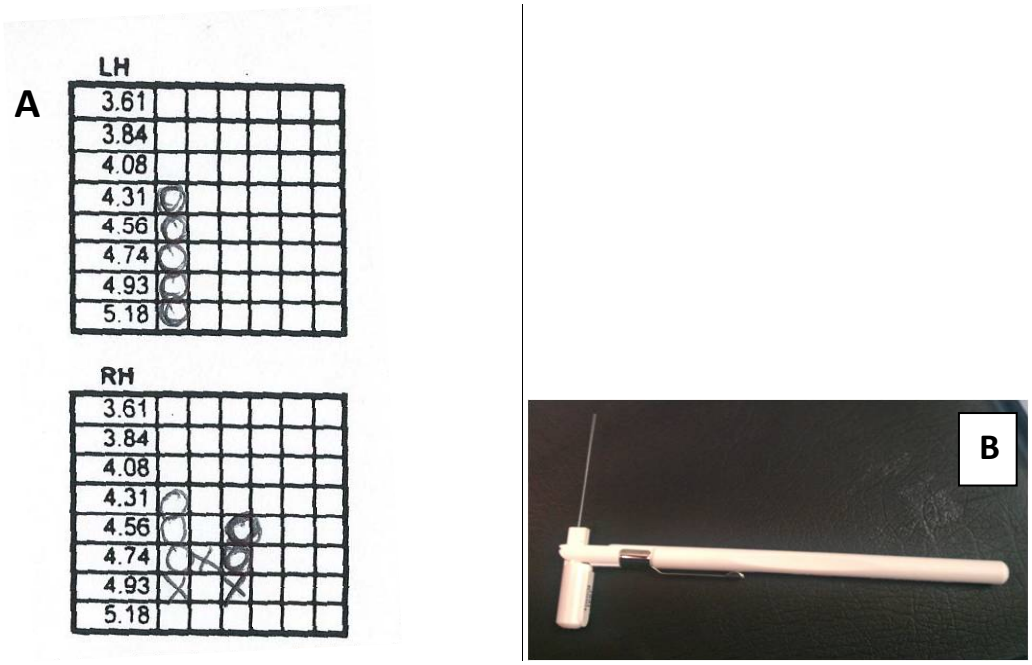
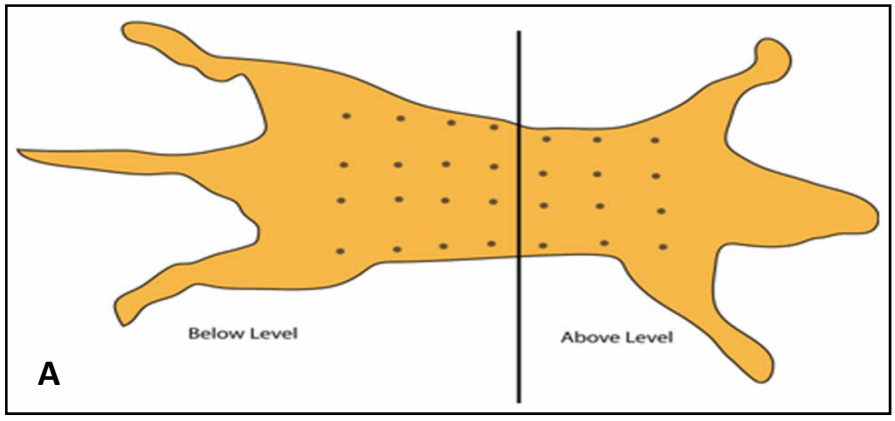


Figure 2: (A) *Example of a response recording for the left hind limb (on top) and right hind limb (on bottom) of one animal. Each number indicates an increasing stiffness and force of the filament from the lowest possible force of 3.61 to the highest force possible of 5.18;* ***(B)*** *Picture of a 4.13 von Frey filament*

Girdle response test

At-level mechanical allodynia and at-level mechanical hyperalgesia were assessed using a 2-gram non-noxious force (allodynia) and a 26-gram noxious force (hyperalgesia) with the application of von Frey filaments on the body (81),(82). Prior to the initial baseline testing session, I allowed the animals to acclimate to the restraint device for 30 minutes for two consecutive days. I placed the animal subjects in a restraint device and allowed them to

acclimate for 20 minutes before each testing session began. The restraint device consisted of seven slits that allowed for easy application of the filaments from left to right on the body at twelve different locations rostral to the level of injury and sixteen different locations caudal to the level of injury (*Figure 3A*). A positive response was an immediate flinching or vocalization after application of each filament (81),(82). I applied the 2-gram force first at each location on the body and the 26-gram force second. There was a 5 second delay between each application of the filament to allow the animal to relax between applications. The girdle response test was applied to each treatment group at baseline (before SCI), day 14, day 28, day 42 and day 60 and responses were recorded on a grid map *(Figure 3B*).



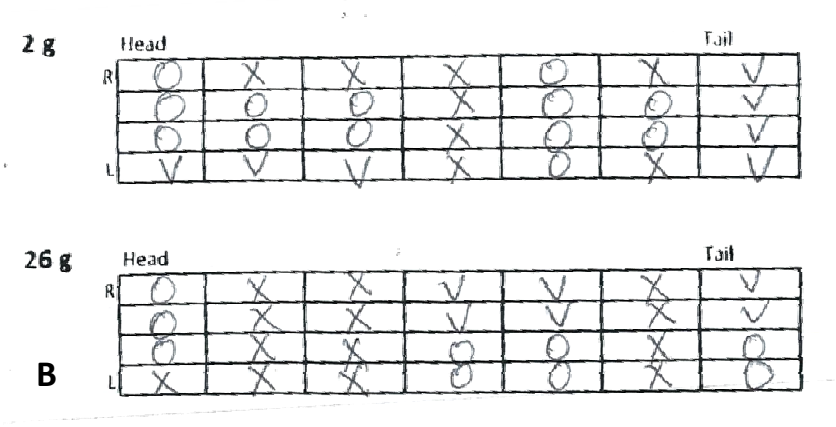


Figure 3: Girdle Test Grid Map: (A)*-A grid map of the body with black dots representing the location where the 2-gram (non-noxious) and 26-gram (noxious) von Frey filaments were placed above (12 locations) and below (16 locations) the level of injury.* ***(B)****Example of grid map recording session: "0" is no response, "X" is a flinch reaction, and "V" is a vocalization response.*

Photobeam Activity Box

The photobeam activity box (PAS; San Diego Instruments, Inc) was comprised of four, clear plastic chambers (40 X 40 X 40 cm) with a series of 32 photo beams that line the chamber in both the x-axis and y-axis. PAS has been widely utilized to examine exploratory behavior of rodents in research studies. The system is a completely objective and non-invasive tool for detecting fine and gross motor movements, rearing events, distance, speed and rest time of the animal subjects over a given period of time. Alterations in locomotor activity can be used as a sensitive indicator of a pain condition (83),(84). However, I had to take special precautions when analyzing the data. For instance, a decrease in the distance traveled by the animal subject could indicate discomfort, illness or drowsiness due to the drug or a pain state. Therefore, it was critical to be consistent when handling the animals, standardize all procedures and carefully observe trends in all treatment groups and for each

animal over time before drawing conclusions regarding PAS results. In this study, each testing session began at the same time each day (1pm) in a quiet room. I did not permit other animals or people in the room during active testing sessions. The chambers were cleaned with cavicide between each session which was important for removing "fear scents" that other animals could detect (85). I set each animal gently inside each chamber and allowed to move freely for a total of 15 minutes. The animal's movement in the x, y and z planes caused an obstruction in the beam breaks. A software database collected the various obstructions made by the animal's movement for analysis.

I assessed all treatment groups at baseline (before SCI), day 14, day 28, day 42 and day 60 post-SCI. I assessed the distance (centimeters) the animals moved, the average speed at which the animals traveled (cm/sec), the amount of time the animals were active (seconds), the total amount of ambulatory movements and the total amount of rearing events the animals performed during their 15 minutes in the chamber.

Histology

The animals were sacrificed using a 100 mg/kg IP injection of pentobarbital, and a trans-cardial perfusion was performed using approximately 150 mL of ice-cold buffered saline with heparin (20 mg/500 mL) followed by perfusion with approximately 300 mL of 4% paraformaldehyde solution. I then dissected the liver from several animal subjects and placed it in a 50 mL conical tube filled with 4% paraformaldehyde solution for 24 hours and placed the liver in a 50 mL conical tube filled with 30% sucrose solution for storage at 4° C. I cut liver sections on a cryostat machine with a thickness of 30µm per section and placed the sections into tris-buffered saline (TBS) solution. The tissue sections were carefully

mounted onto subbed glass slides and allowed to dry overnight. I then performed a hematoxylin-eosin (HE) stain by rinsing slides in distilled water for 2 minutes, staining in hematoxylin (cat#MHS-128; Sigma Aldrich) for 15 minutes followed by rinsing each slide in distilled water. The slides were decolorized in acid alcohol for 3 seconds followed by 15 minutes of rinsing in tap water. The slides were counterstained with 10 dips in eosin solution (cat# 152880250; Acros Organics), dehydrated with a series of ethanol solutions, cleared with Hemo-D solution, mounted with Cytoseal solution and cover slipped.

I used the Masson's trichrome for connective tissue kit (cat#26367; Electron Microscopy Sciences) for staining liver tissue sections. The protocol was performed according to Sheehan and Hrapchak: Theory and Practice of Histotechnology (St. Louis.1980, p. 190) with slight modifications to the incubation times to optimize color saturation.

Immunohistochemistry

Liver tissue sections of 30 μm thickness from the vehicle group (n=1) and minocycline group (n=2) were incubated for 1 hour at room temperature in a blocking solution made of TBS (tris-buffered saline) with 0.25% triton containing 5% normal donkey serum (Jackson Immuno Research 017-000-121). The sections were then incubated overnight at 4° C in a primary antibody solution. This solution consisted of TBS with 0.25% triton solution with goat anti- IL-1β (1:1000, R&D Systems) and mouse anti-ED-1/CD-68 (1:500, AbD Serotec). The sections were washed with three rinses of TBS and then placed in a secondary antibody solution for 3 hours at room temperature. The secondary antibody solution consisted of TBS with 0.25% triton, Alexa-Fluor 488 donkey anti-goat IgG, H+L

(1:500, Invitrogen), and Alexa-Fluor 568 donkey anti-mouse IgG (1:500, Invitrogen). The sections were washed with 3 rinses of TBS and mounted onto subbed glass slides and allowed to dry overnight. The dried tissue sections were then coverslipped with Fluormount-G (Fisher).

Statistical analysis

All behavior data was normalized to each animal's baseline score (% baseline) to reduce animal to animal variability. Statistical analysis was performed using Sigma Plot software with a repeated-measures two-way ANOVA test and Holm-Sidak pairwise-comparisons.

RESULTS

The Basso, Beattie and Bresnahan (BBB) locomotor test was performed on each animal at day 1, 2, 3, 5, 7 and 14 post-injury to verify that a severe contusion injury was properly administered (76). The results indicate the naïve-uninjured animals (n=18) scored a mean of 21 ± 0 at all time points tested. A score of 21 means the animals had normal locomotor function and gait, which was expected in naïve un-injured animals. Beginning one day post-injury BBB scores were significantly decreased compared to the naïve animals ($p=0.001$), confirming that a severe contusion injury was properly administered in the majority of the injured animals (*Figure 4*). The injured animals scored a mean of 0.6 ± 1.7 at day one post-injury, which means the animals had either no hind limb movement or slight movement of one joint. Over time, the injured animals developed slight locomotor recovery of the hind limbs indicated by an increase in score at day 14 with a mean of 11.1 ± 1.9. This score means the animals had frequent to consistent plantar steps with no forelimb, hind-limb coordination.

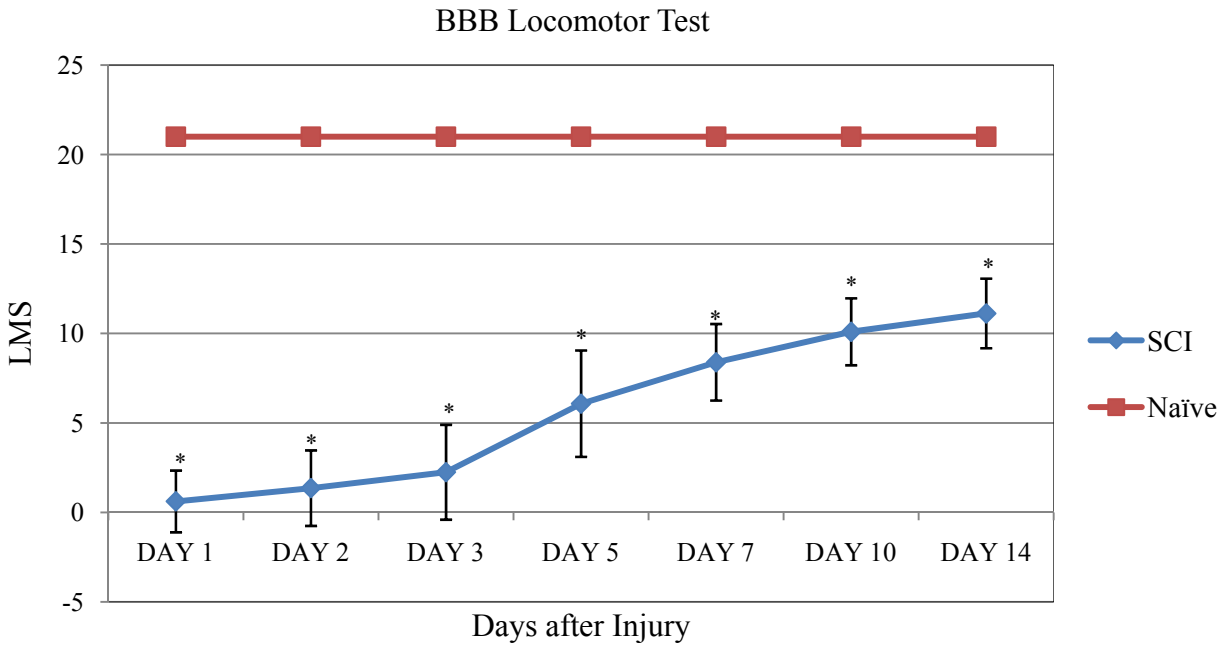


Figure 4: *The BBB locomotor rating scale shows there was no change in the un-injured naïve group (n=18) over time. The injured animals (n=56) show a significant decrease in hind limb motor function at day 1,2,3,5,7,10 and 14 after injury compared to the naïve animals (* p =0.001 vs. naive). Error bars represent mean ± standard deviation (SD).*

In order to establish the development of CNP after injury, the animals were subjected to a series of behavior testing sessions prior to SCI to determine baseline scores and then again at day 14 and 28 after injury. Approximately 70% of the animals developed pain by day 28 post-injury. SCI animals that did not develop pain were excluded from the study, meaning any animals performing as well or better than they did before injury were excluded. It was critical to assess only the animals that developed pain prior to treatment in order to assess treatment-dependent effects on pain. The results of all behavior data were normalized to each animal's baseline score following the exclusion of animals that did not develop pain to reduce animal-to-animal variability. Because of the exclusionary criteria, the number of animals per group was; naïve (n=18); vehicle group (n=12); minocycline group (n=13);

gabapentin group (n=13). The final exclusion criteria to eliminate non-pain developing animals are listed in *Table 1*.

Test	Reason	# of animals
BBB score	≥ score 2 at day 1	4
Von Frey Test Data	≥ 100% of baseline at day 14 and 28	2
Von Frey Test Data	≥ 200% of baseline at day 14 or 28	1
Von Frey Test Data	≥ 300% of baseline at any time point	4
Von Frey, Girdle and PAS Data	outlier at multiple time points in data	3
Von Frey and Girdle Data	outlier at multiple time points in data	2
Girdle and PAS Data	outlier at multiple time points in data	1
	Total Excluded From Study	17 of 56 animals

Table 1 Exclusion Criteria: *Animals that showed lack of pain development after examining all behavior data were excluded from this study. Seventeen of 56 injured animals were excluded, which resulted in pain development in 70% of all injured animals (n=56).*

Behavior Testing Results

A. Von Frey Hair Test

Below-level mechanical allodynia was measured by the von Frey hair test. The test was performed by touching von Frey filaments with increasing grams of force (0.4-15 g) to the glabrous surface of the left and right hind paw for one second. An immediate withdrawal of the paw or licking of the paw was recorded as a positive response. After injury (day 14 and day 28), there was a significant decrease in the force required to induce a positive response compared with baseline scores *($p< 0.003$; Figure 5).* Before injury, all of the injured animals demonstrated a relatively high withdrawal threshold

(group mean of 13.8 g ± 5.1 g). After injury, the withdrawal threshold decreased by day 28 *(group mean of 5.8 ± 3.6 g).* The sensitivity to a lower gram of force required to induce a positive response indicated the onset of below-level mechanical allodynia. The naïve un-injured group showed a slight decrease in withdrawal threshold at day 28 and day 42; however, these results were not significant. After two weeks of daily dosing, the von Frey hair test was performed again (day 42 testing). Minocycline and gabapentin significantly increased withdrawal threshold compared to day 14 and day 28 *($p<0.007$; minocycline group mean 10.2 ± 5.5 g; gabapentin group mean 9.7 ± 4.8 g).* The withdrawal threshold remained at the same significantly increased level by day 60 compared to day 14 and day 28 *($p <0.007$; minocycline group mean 10.6 ± 3.9 g; gabapentin group mean 9.9 ± 3.6 g).* The injured vehicle group did not display an increase in withdrawal threshold at any time point and clearly demonstrated significantly reduced withdrawal thresholds from day 14 to day 60 compared to baseline *($p<0.005$; day 42 vehicle group mean 4.5 ±1.6 g; day 60 vehicle group mean 6.9 ±3.6 g).*

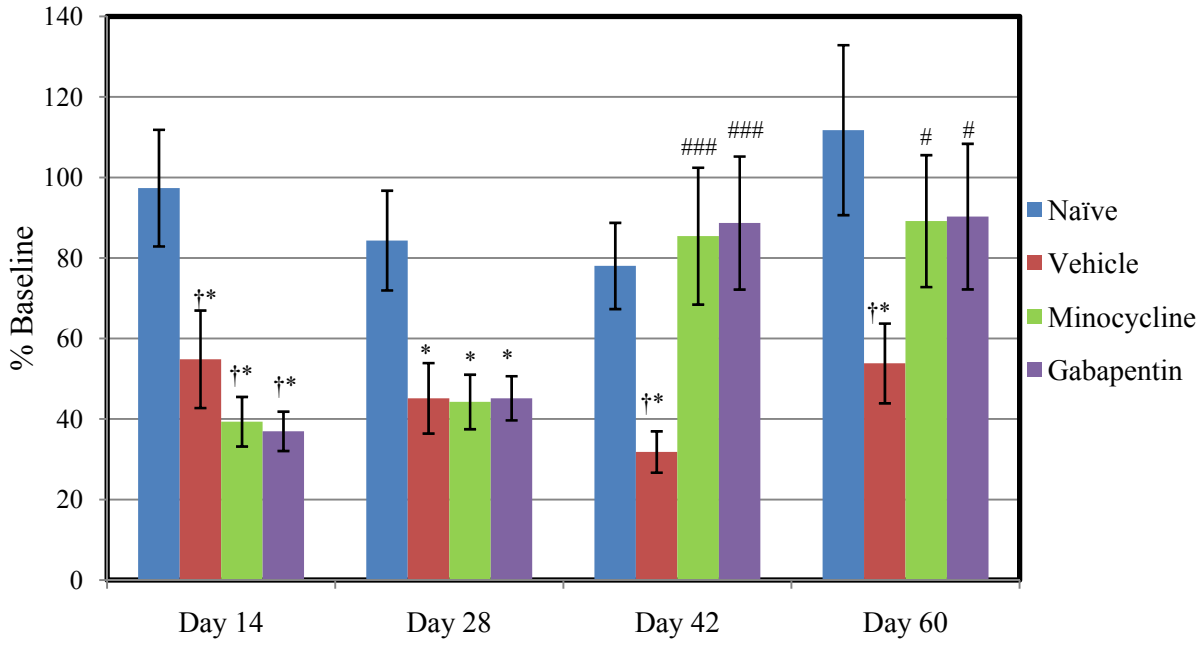


Figure 5 Below-level mechanical allodynia: *von Frey hair test calculated with the up-down Dixon method. Minocycline and gabapentin resulted in a significant increase in withdrawal threshold 2 weeks after treatment (day 42) and 10 days after cessation of the drug (day 60). Error bars display the mean ± SEM. [(*) p<0.005 vs. baseline; (†) p <0.015 vs. naïve; (#) p <0.007 vs. day 14/28; (##) p <0.005 vs. vehicle]*

B. Girdle Response Test

Hyperalgesia and allodynia were measured with the girdle response test on the torso of the animal below and above the level of injury while stabilized in a girdle restraint device. Hyperalgesia was measured with a 26-gram von Frey filament. The 26-gram filament was considered a noxious force to the animals and was applied to the body until the filament slightly buckled. Allodynia was measured with a 2-gram von Frey filament. The 2-gram filament is considered a non-noxious/non-painful force. First, the 2-gram filament was applied as previously described on the animal at sixteen locations below the level of injury (T10) from left to right and twelve locations above the level of injury

from left to right. Positive responses were noted as a flinch or vocalization and then recorded on a grid map. Following the 2-gram force the same procedure was repeated with the 26-gram force. An increase in the percentage of positive responses after injury indicated pain development.

The results demonstrate that there was no significant increase in response above the level of injury when compared to the baseline using the 2-gram von Frey filament. Therefore, there was no verifiable allodynia demonstrated at day 14 and day 28 above the level of injury with this test (*Figure 6*). The naïve group had a significant decrease in response at day 42.

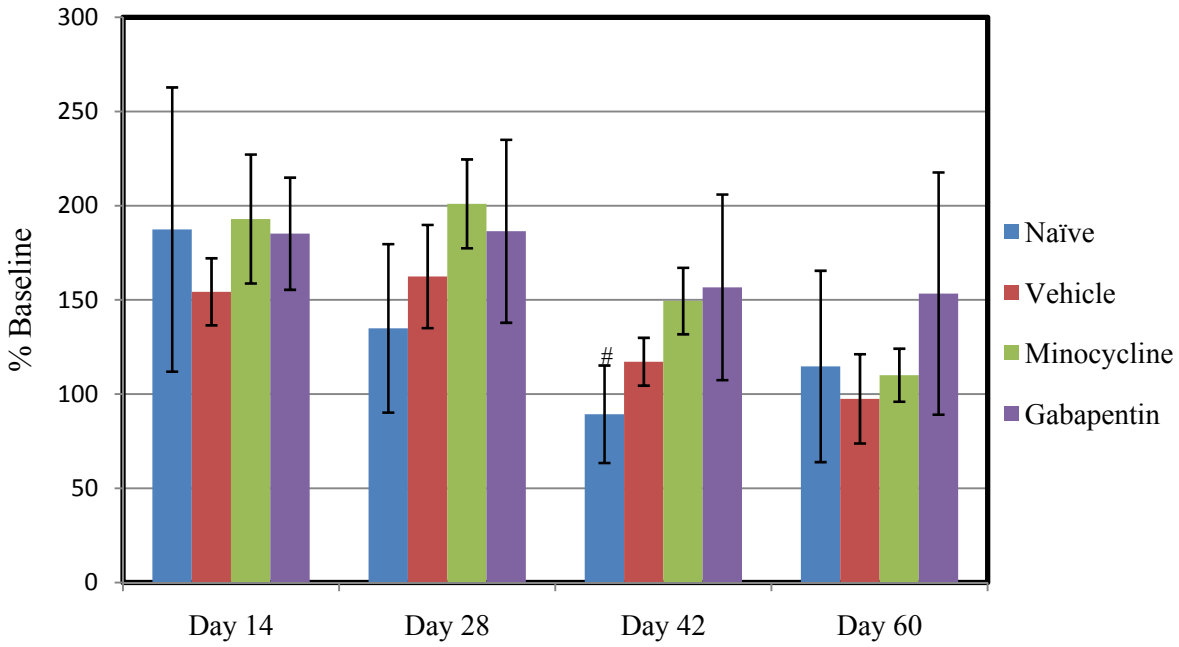


Figure 6 Allodynia above the level of injury*: There was no increase in positive responses with a 2-gram force applied above the level of injury in the vehicle, minocycline or gabapentin treatment groups. The naïve group's response significantly decreased compared to day 14. Error bars display the mean ± SEM. (# p =0.002 vs. day 14).*

There was no significant increase in positive responses below the level of injury using the 2-gram von Frey filament at day 14 and day 28 (*Figure 7*). Therefore, there was no demonstration of allodynia below the level of injury with the girdle response test. At day 60, the vehicle treatment group and gabapentin treatment group demonstrated a significant decrease in positive responses when compared to day 14 and day 28 *(p<0.004)*.

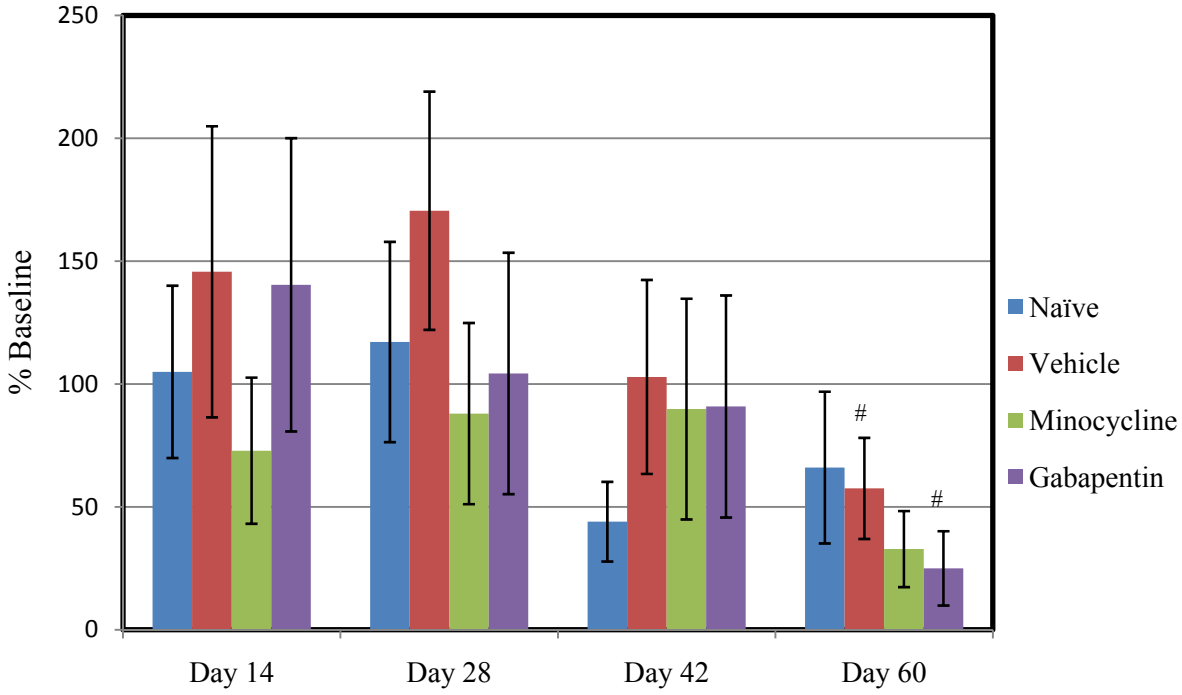


Figure 7 Allodynia below the level of injury: *There was no increase in positive responses below the level of injury with 2 grams of force. At day 60, all animal groups had decreased positive responses. There was a significant decrease in the vehicle and gabapentin treatment groups at day 60. Error bars display the mean ± SEM. (# p < 0.004 vs. day 14/28).*

There was a significant increase in positive responses after injury with application of the 26-gram von Frey filament above the level of injury at day 14 in all injured animal groups compared to baseline *(p <0.004, Figure 8).* The minocycline treatment group

also demonstrated a significant increase at day 28 *(p=0.003).* The vehicle and gabapentin group demonstrated an increased response at day 28; however, it was not significant. At day 42 of testing, 2 weeks of treatment with minocycline caused a significant decrease in positive responses when compared to day 14 *(p=0.005).*This result was not achieved in either the gabapentin or vehicle group. By day 60, ten days after dosing was complete *(*washout period*),* the minocycline-treated animals continued to demonstrated a significant decrease in positive responses when compared to day 14 and day 28 *(p<0.001).* The vehicle group also demonstrated a significant decrease in positive responses at day 60 compared to day 14 *(p=0.002),* the positive responses were still 50% greater than the baseline. No significant decreases in positive responses were demonstrated with the 26-gram force in the gabapentin-treated animals.

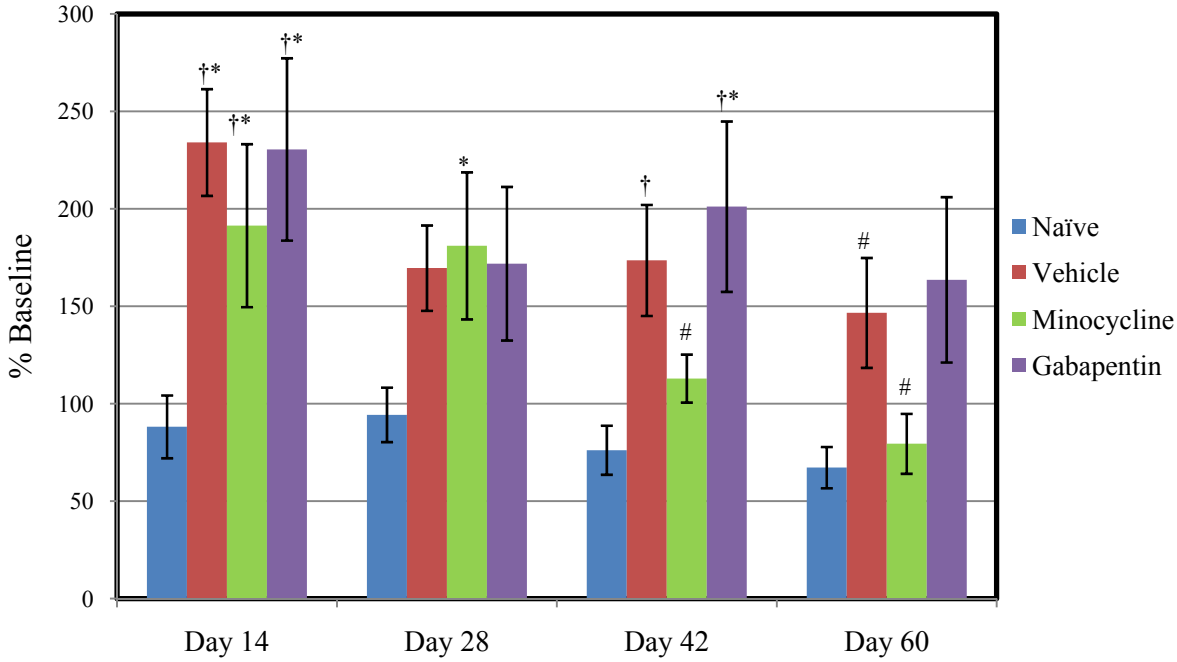


Figure 8: Hyperalgesia above the level of injury: *There was a significant increase in positive responses in the SCI animals after injury at day 14. Only the minocycline group had significantly decreased responses with treatment that remained through day 60. Error bars display the mean ± SEM. [(*) p<0.004 vs. baseline; (†) p <0.007 vs. naïve; (#) p <0.006 vs. day 14/28]*

Positive responses increased at day 14 and day 28 in all injured animals compared to baseline with application of the 26-gram force von Frey filament below the level of injury (*Figure 9*). However, only the vehicle and gabapentin treatment groups demonstrated significantly increased positive responses at day 14 and day 28 compared to baseline (*p<0.004*). After two weeks of treatment with minocycline (day 42), positive responses did not significantly increase. The vehicle group did not show a significant decrease in response at any testing session. However, two weeks of gabapentin treatment significantly reduced

positive responses at day 60, 10 days after treatment was complete (washout period) when compared to day 14, day 28 and day 42 testing sessions ($p<0.009$).

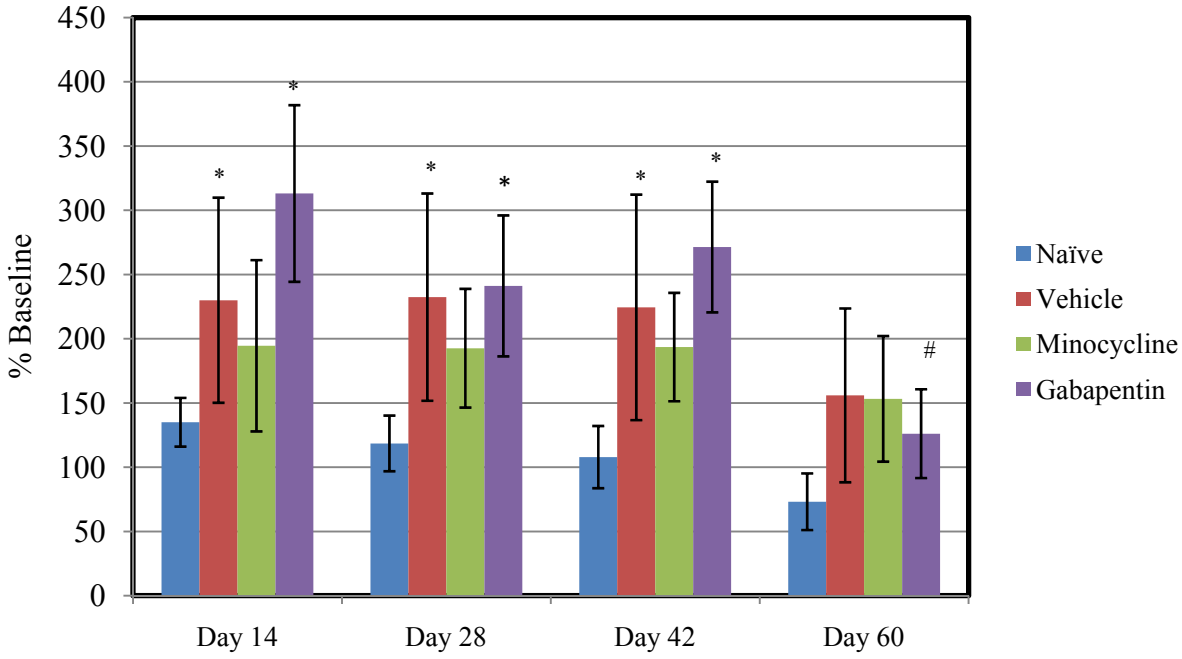


Figure 9 Hyperalgesia below the level of injury: *There was no significant increase in response in the minocycline group and no significant change after treatment. The vehicle and gabapentin groups demonstrated significantly increased responses to the 26-gram force after injury at day 14 and day 28. The gabapentin group had a significant decrease in positive responses at day 60. Error bars display the mean ± SEM. [(*) p<0.004 vs. baseline; (#) p <0.009 vs. day 14/28]*

C. Photobeam Activity Box

Animals were placed in a photobeam activity box for a total of 15 minutes during each behavior testing session. The activity box measured a series of locomotor behaviors in a software database for analysis of overall activity and fine and gross motor movements.

The distance that the animals moved throughout the chamber in 15 minutes was recorded in total centimeters. The naïve animals did not demonstrate any significant changes at any time point. However, all injured animals displayed significantly decreased distance in the chamber at day 14 *($p<0.002$, Figure 10).* At day 28, the vehicle and minocycline-treated animals continued to show a significant decrease in total distance *($p<0.002$),* while the gabapentin-treated animals did not demonstrate a significant decrease in total distance compared to baseline. Post-treatment (day 42 and day 60) minocycline did not increase total distance moved while gabapentin significantly increased at day 42 and day 60 compared to day 14 *($p<0.005$).* However, the difference in total distance moved at day 42 and day 60 in the gabapentin group was not significantly different compared to day 28. The vehicle group demonstrated a significant increase in total distance moved at day 60 compared to day 14 *($p=0.004$)*, however it was also not significantly different when compared to day 28.

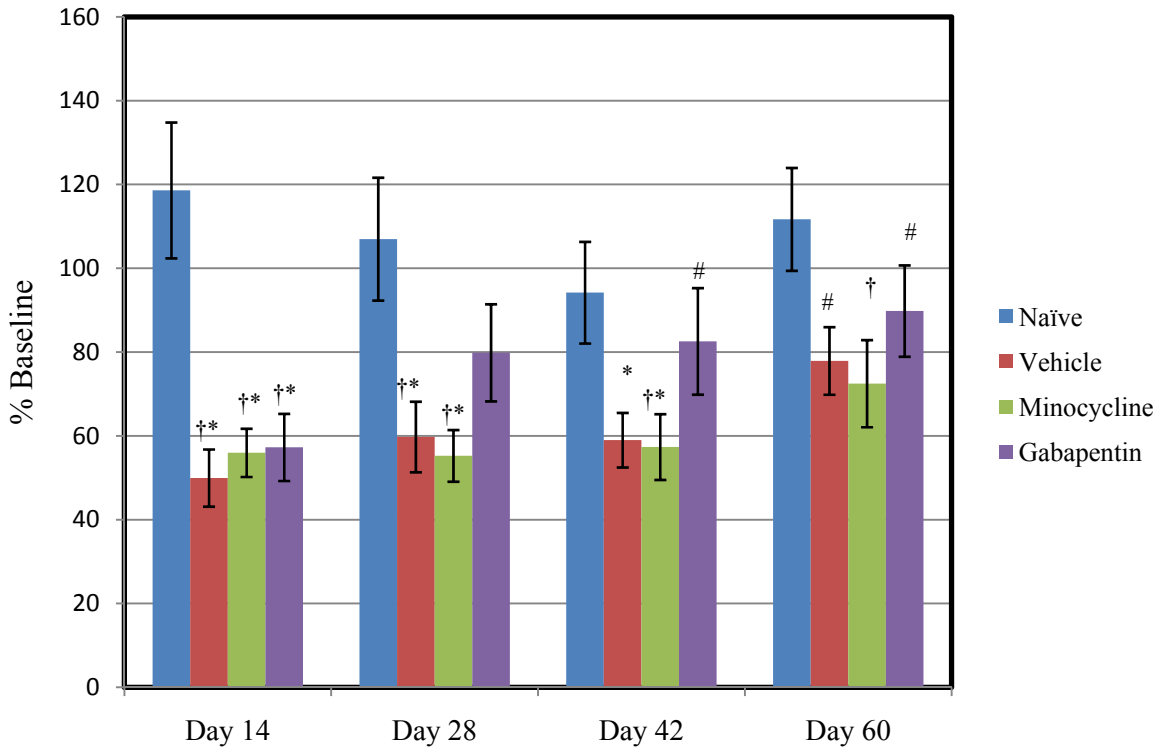


Figure 10 Total Distance: *There was not a significant increase in total distance in the minocycline group post-treatment. The gabapentin and vehicle group significantly increased total distance at day 60 compared to day 14. Error bars display the mean ± SEM. [(*) p<0.002 vs. baseline; (†) p <0.007 vs. naïve; (#) p <0.005 vs. day 14/28]*

The average speed (cm/sec) the animals traveled in the activity box chamber was recorded throughout the 15 minutes. All injured animal groups significantly reduced their average speed by day 14 *(p<0.004; Figure 11)*. At day 28, all the injured animals showed a reduction in average speed. However, the vehicle and minocycline treatment groups were the only groups that demonstrated a significant decrease in average speed compared to baseline *(p<0.001).* At day 42, two weeks post-treatment, the vehicle and minocycline groups still demonstrate a significant decrease in average speed compared to baseline *(p<0.004)*. At day 60, ten days after the dosing regimen ended, all injured animal groups

significantly increased in average speed compared to day 14 *(p<0.003)*. The average speed of the naïve group was consistent over time and had no significant changes.

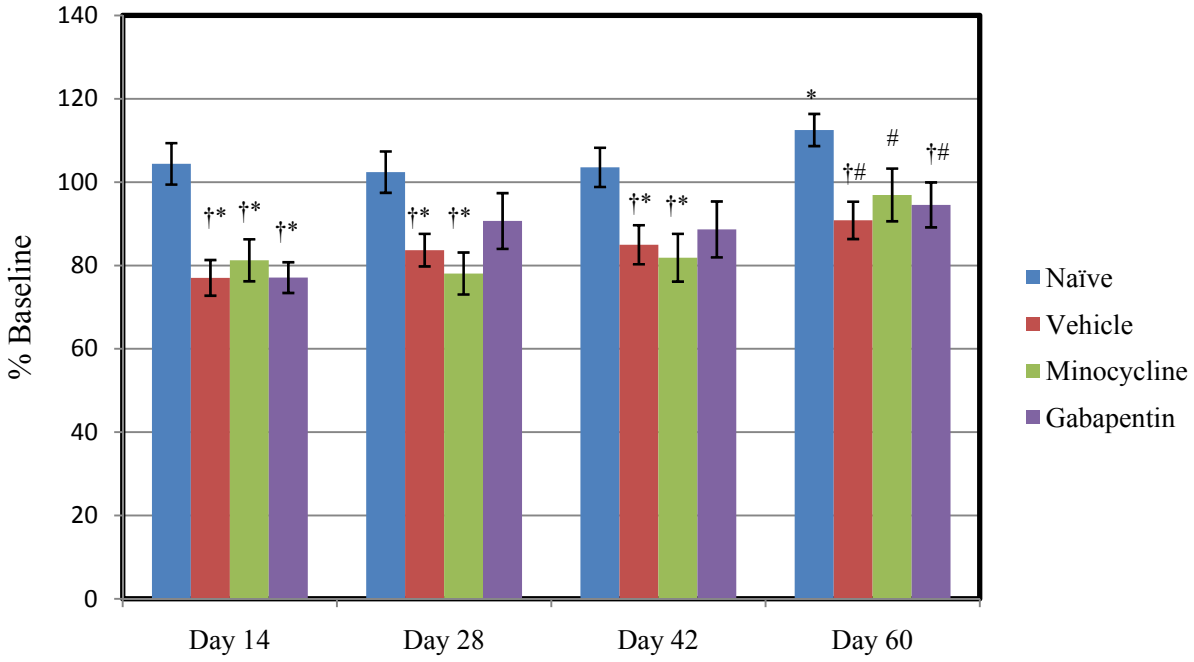


Figure 11 Total Average Speed: *All injured animals had significantly reduced average speeds after injury and all groups significantly improved their speeds by day 60. Error bars display the mean ± SEM. [(*) p<0.004 vs. baseline; (†) p <0.005 vs. naïve; (#) p <0.003 vs. day 14/28]*

During the 15 minutes that the animals were in the activity box chamber, the software calculated the total amount of time the animals were at rest in seconds. The amount of time that each animal was active was then calculated. The naïve animal group displayed no significant changes throughout each of the testing sessions. All injured animal treatment groups were significantly less active at day 28 *(p<0.002; Figure 12)*. By day 60, all injured animal treatment groups were significantly more active when compared to day 28 *(p<0.003)*.

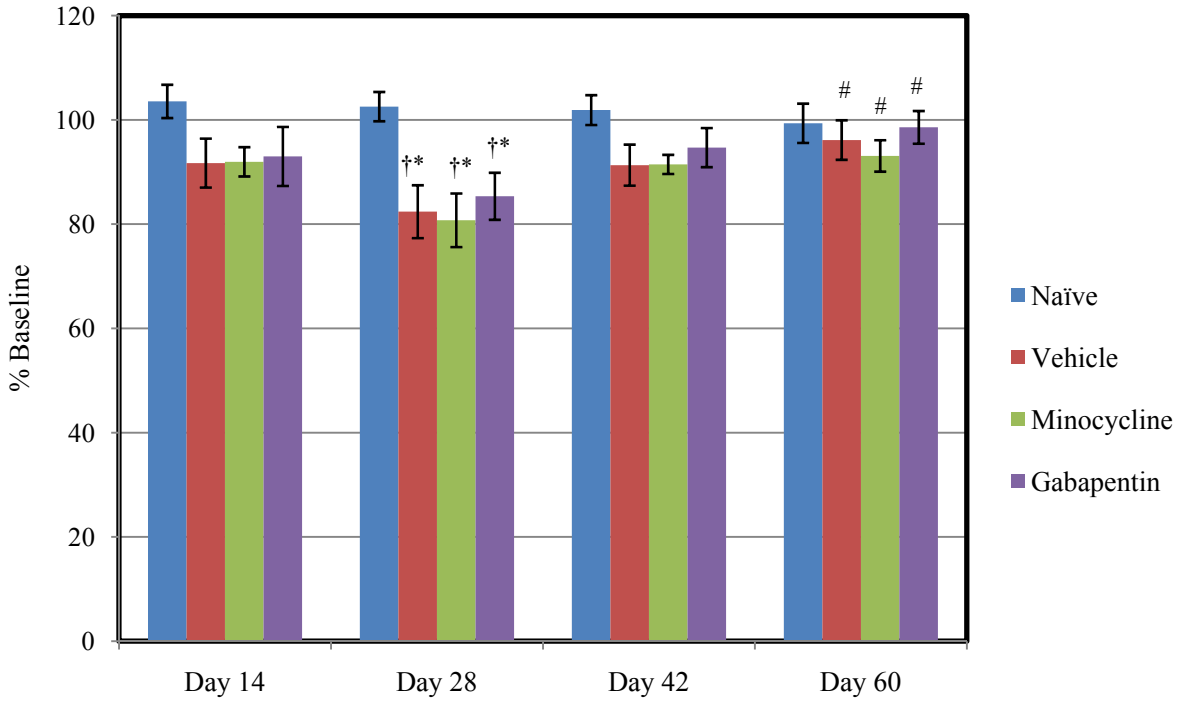


Figure 12 Total Active Time: *All injured animals displayed a significantly decreased amount of time spent active post-injury at day 28. At day 60, ten days after treatment was stopped, all injured animals significantly increased their time spent in an active state compared to day 28. Error bars display the mean ± SEM. [(*) p<0.002 vs. baseline; (†) p <0.005 vs. naïve; (#) p <0.003 vs. day 14/28]*

The PAS software calculated the total amount of ambulatory movements or total gait made by each animal within the 15 minutes. The naïve un-injured animals displayed consistent gait at each testing session and no significant changes were found, as expected. After injury, all SCI animal groups significantly decreased the total amount of ambulatory movements at day 14 and day 28 *(p<0.002; Figure 13).* After treatment (day 42 and day 60), there were no significant improvements in the amount of ambulatory movements made by any treatment group.

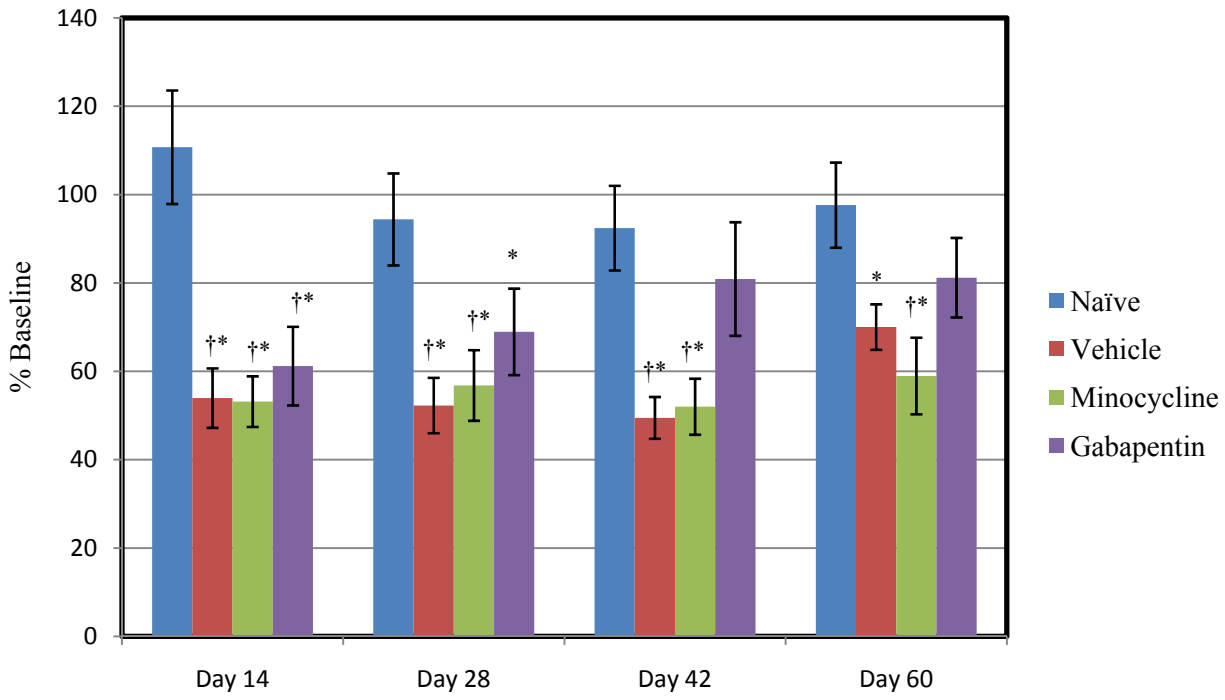


Figure 13: Total Ambulatory Movement: *All injured animals had significantly reduced ambulatory movement after injury at day 14 and day 28. There were no significant improvements in any SCI animal group post-treatment. Error bars display the mean ± SEM. [(*) p<0.002 vs. baseline; (†) p <0.003 vs. naïve]*

The PAS software calculated the total amount of rearing events or amount of times the animals stood on their hind limbs. The naïve un-injured animals had a significantly decreased amount of rearing events at day 28, 42 and 60 *(p<0.005; Figure 14).* All injured animal groups displayed a significantly decreased amount of rearing events after injury at day 14 and day 28 *(p<0.005).* The minocycline group maintained a significantly reduced amount of rearing events throughout day 60 *(p<0.005)*. At day 42, after 2 weeks of treatment, the gabapentin group significantly increased the total amount of rearing events compared to day 14 *(p<0.001).* At day 60, ten days after gabapentin treatment was complete

(washout period), the total number of rearing events remained significantly higher compared to day 14 *(p<0.001).*

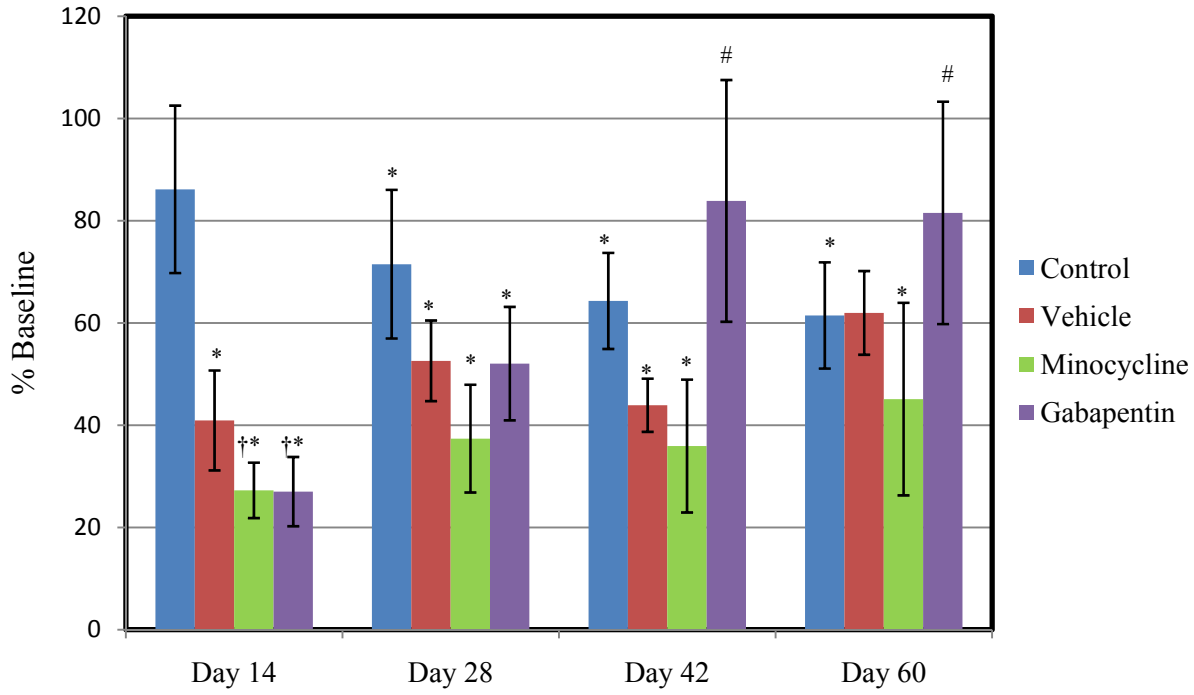


Figure 14 Total Rearing Events: *A significant decrease in rearing events occurred after injury in all SCI animal groups at day 14 and 28. There was no significant improvement in the vehicle and minocycline group post-treatment. Gabapentin significantly increased the total number of rearing events at day 42 and day 60. Error bars display the mean ± SEM. [(*) p<0.002 vs. baseline; (†) p =0.001 vs. naïve; (#) p =0.001 vs. day 14*

Adverse Side Effects

The original study paradigm included a 4-week dosing regimen of 50 mg/kg via i.p. injections of minocycline. However, minocycline produced concerns approximately two weeks after treatment began. Minocycline treatment via i.p. administration began producing sores around the injection sites even with sufficient rotation of appropriate injection sites in the left, right and center abdominal areas. There were no visible sores in the vehicle-treated animals receiving daily i.p. injections of 3 mL of saline. Therefore, I decided to conclude treatment of minocycline after three weeks of administration instead of the full 4 weeks as laid out in our original study paradigm. This resulted in the development of aim 2 of this study, using the ten day washout period before day 60 testing to determine if any lasting effects at reducing pain were maintained.

The body weights of all treated animals were monitored throughout the dosing regimen. The minocycline-treated animals displayed abnormally low weight gain when compared with the gabapentin and vehicle-treated animals *(Figure 15).* By the end of the 3rd week of daily dosing the gabapentin-treated animals had gained an average of 23 grams compared to their weight at the end of week one dosing and the vehicle group had gained an average of 16 grams. However, the minocycline-treated animals had only gained an average of 2 grams over the same time period.

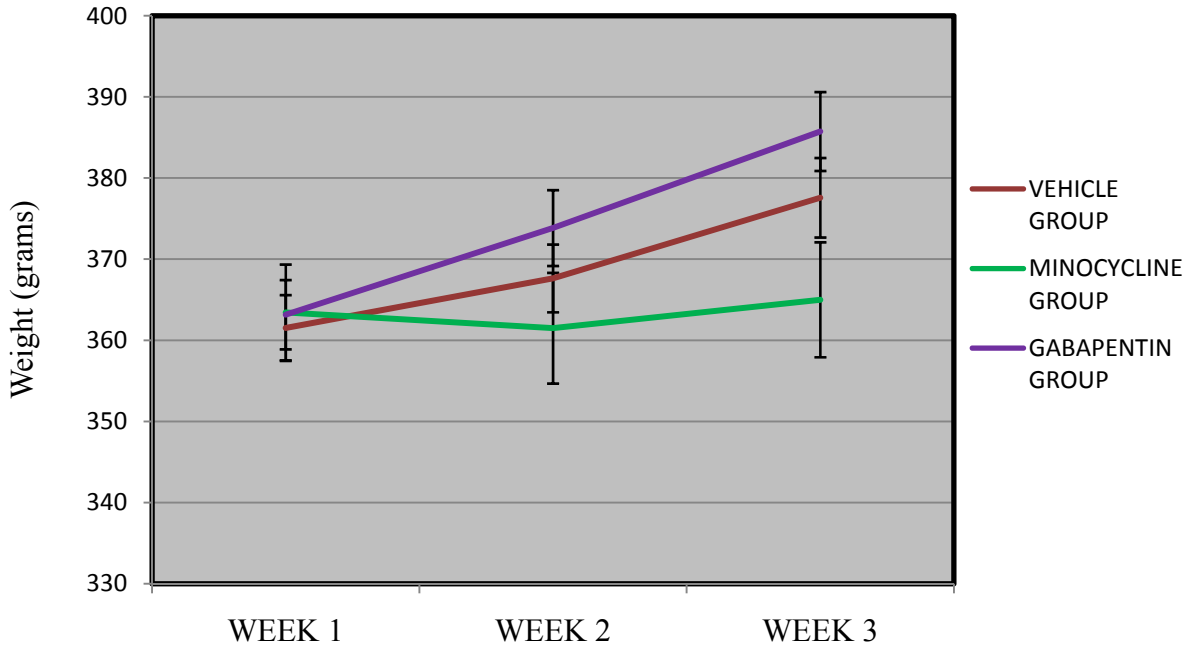


Figure 15 Average weight gain *After 3 weeks of monitoring weight and daily dosing, minocycline treatment caused abnormally low weight gain (average= +2 grams) compared to the saline treatment (average= +16 grams) and the gabapentin treatment (average=+23 grams).*

After completion of all behavioral assessments, the animals were sacrificed and a trans-cardial perfusion was properly performed using buffered-saline with heparin followed by 4% paraformaldehyde. During this procedure, I noticed a remarkable difference in the livers of the minocyline-treated animals compared to the gabapentin and saline-treated animals (*Figure 16A, 16B and 16C*). Therefore, the livers were collected (n=1, per treatment group) and properly stored. Histology and immunohistochemistry techniques were performed for analysis.

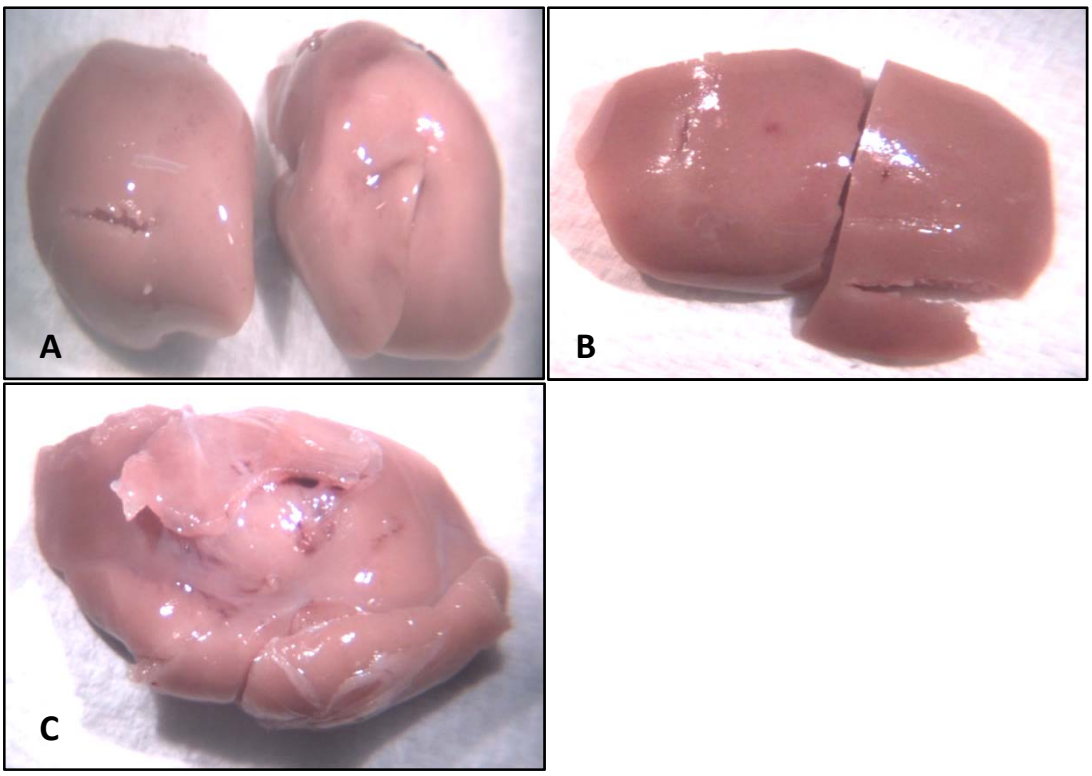


Figure 16: ***(A)*** *Gross anatomy of a vehicle treated animal liver;* ***(B)*** *Gross anatomy of a gabapentin treated animal liver;* ***(C)*** *Gross anatomy of a minocycline treated animal liver displaying an obvious increase in fibrous tissue.*

Because of the obvious increase in fibrous tissue on the exterior surface of the minocycline treated animal liver, 30 µm sections of the liver were cut on a cryostat for subsequent staining procedures. To determine if minocycline caused liver damage I first performed a hematoxylin and eosin (H & E) stain. Hematoxylin stained the nuclei blue, and eosin stained the cytoplasm and extracellular proteins pink *(Figure 17A, 17B, 17C).* The livers of the saline and gabapentin-treated animals appear to have equally spaced cellular structure whereas the liver of the minocycline-treated animal appears to have abnormally increased cellular density and thickening near the exterior surface in the upper right quadrant of the tissue section.

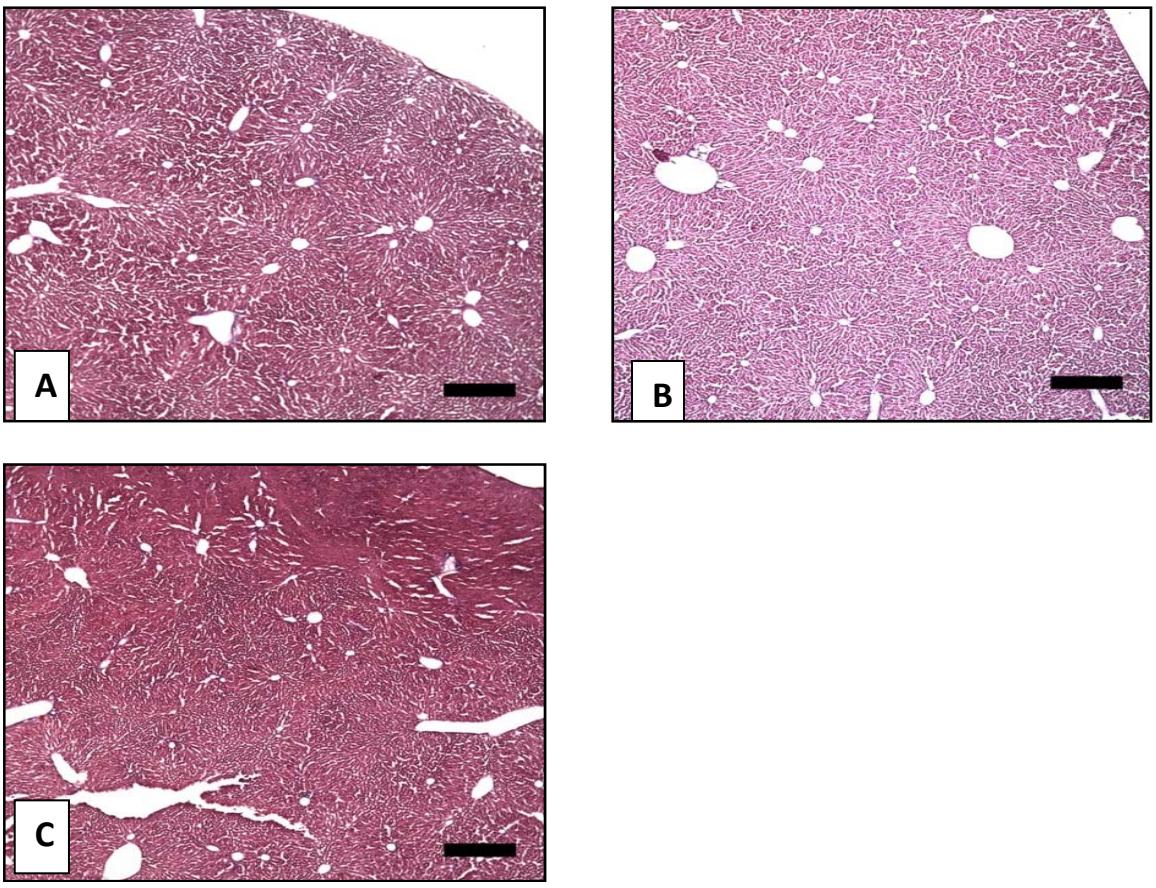


Figure 17: *4x image of H&E stain* ***(A)*** *saline-treated animal liver;* ***(B)*** *gabapentin-treated anima liver;* ***(C)*** *minocycline-treated animal displaying increased cellular density and thickness toward the exterior surface in the upper right quadrant. Scale bars= 500µm*

In order to gain a better understanding of what was developing in the minocycline-treated rat liver, I decided to stain sections with a trichrome stain. The trichrome stain provides more detail of structures by staining nuclei black, staining cytoplasm of hepatocytes red and staining collagen and mucin bright blue. The extent of liver fibrosis in patients can usually be determined after a biopsy with a trichrome stain coupled with H&E stain (86). The results of the trichrome stain indicate there is a massive amount of collagen on the exterior surface of the liver in the minocycline-treated animal *(Figure 18C)*. The collagen, indicated in blue, also appears to be infiltrating deep into the liver tissue. There

was no abnormal collagen expression found on the exterior of the liver in the saline and gabapentin-treated animals *(Figure 18A and 18B)*.

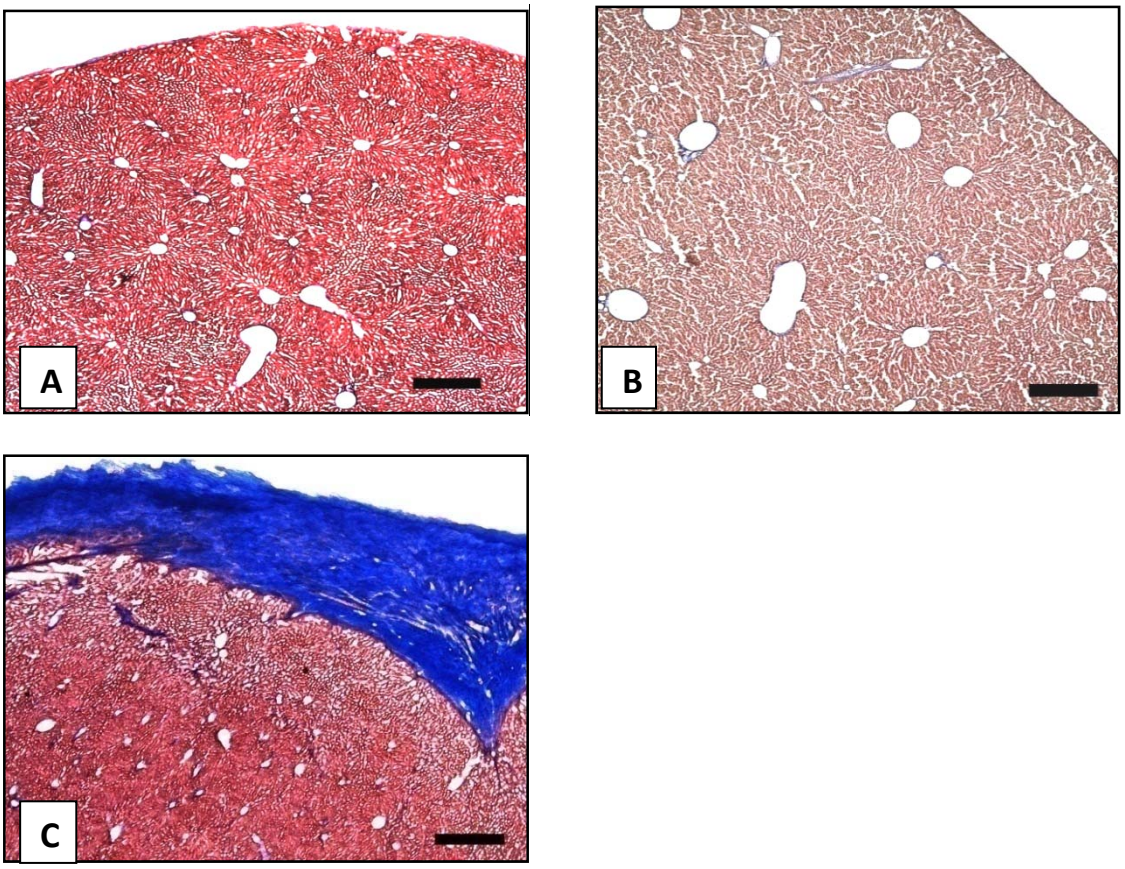


Figure 18: *4x image of Trichrome stain* ***(A)*** *saline-treated liver;* ***(B)*** *gabapentin-treated liver;* ***(C)*** *minocycline-treated liver displaying large collagen deposits on the exterior surface and infiltrating into the tissue. Scale bar=500µm*

The minocycline-treated animal liver showed obvious signs of health problems characterized by abnormally low weight gain during treatment and liver fibrosis. In order to determine if minocycline caused inflammation in the liver I decided to perform immunohistochemistry with a known pro-inflammatory cytokine, Interleukin 1-beta (IL1-β). During acute and chronic stages of inflammatory response caused by many types of injury and disease, monocyctes and macrophages release IL1-β (87). The results of the IL1-β

immuno-fluorescence labeling indicated that there was no IL1-β expression found in the saline and gabapentin-treated animal livers *(Figure 19A and 19B)*. However, I identified a substantial up-regulation of IL1-β expression that was widespread in the minocycline-treated animal liver section visible by labeling with bright green fluorescence in Figure 19C.

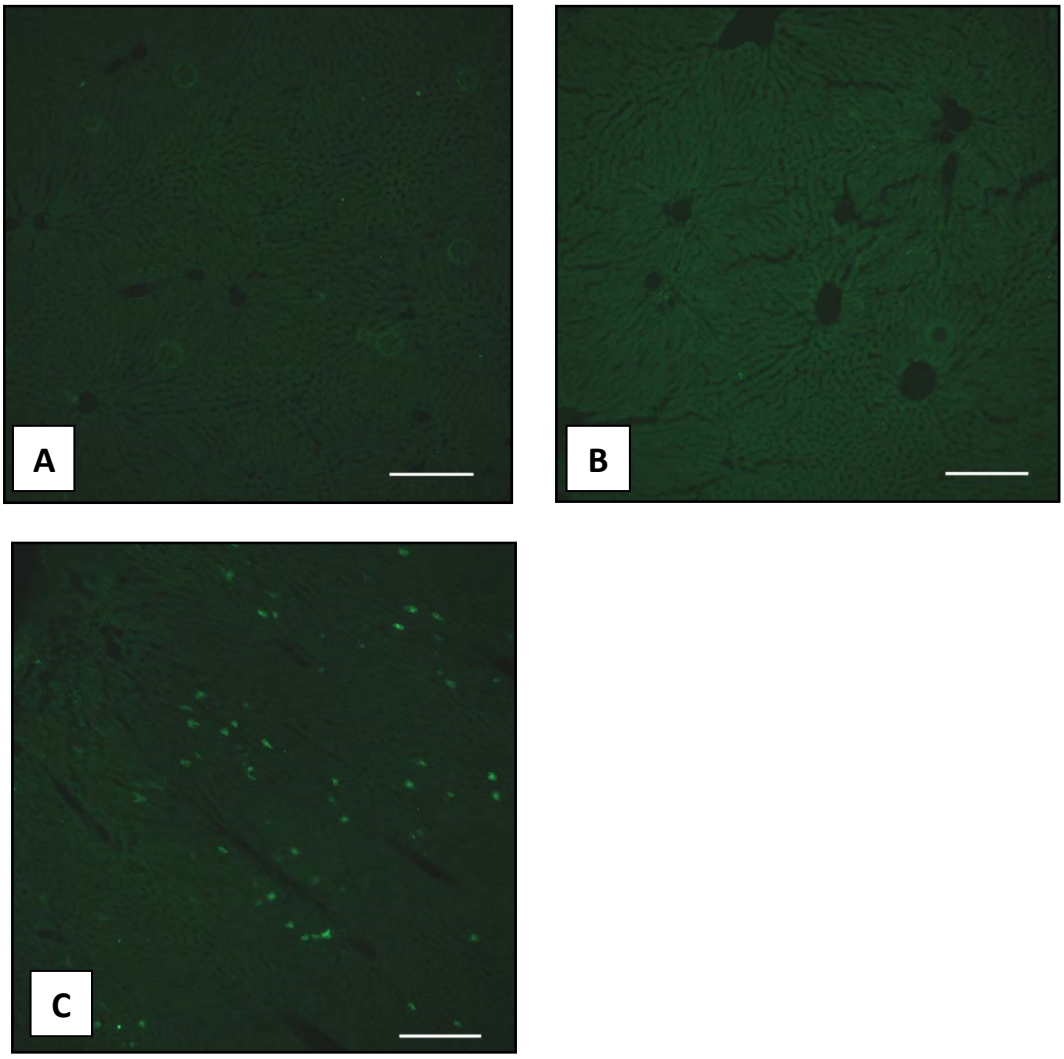


***Figure 19**: 10x image of immuno-labeling with pro-inflammatory cytokine, IL1β **(A)** saline-treated animal liver; **(B)** gabapentin-treated animal liver; **(C)** minocycline-treated animal liver displaying substantial expression of IL1-β. Scale bar=250 µm*

In order to determine if the positively labeled IL1-β cells in the minocycline-treated animal liver infiltrated due to an inflammatory response or simply resident cells, I decided to label the tissue with markers for IL1-β and ED-1. ED-1 is a marker of activated macrophages. Macrophages are known to migrate to sites of inflammation where they activate and release a host of cytokines and toxic radicals to remove debris and kill invading pathogens (88). IL1-β expression co-labeled with ED-1 would further explain that minocycline caused an infiltrating, inflammatory response leading to hepatoxicity. The results indicated that IL1-β was highly co-localized with ED-1 in the minocycline-treated animal liver *(Figure 20)*.

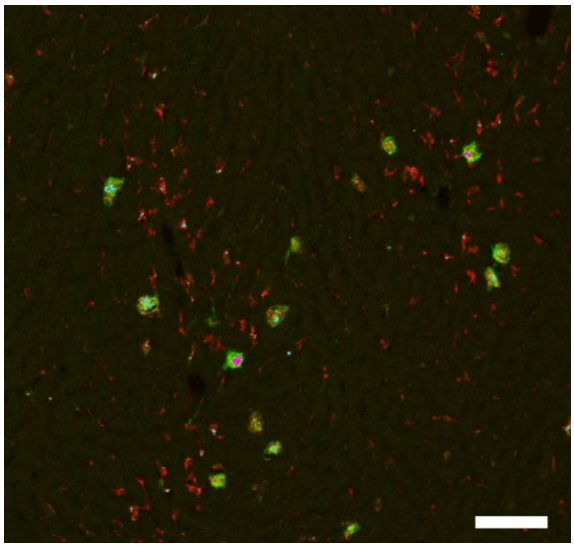


Figure 20: *20x image immuno-label using ED-1, a marker of activated macrophages, in liver of minocycline-treated animals. ED-1 (red) is highly co-localized with immuno-labeling of IL1-β a pro-inflammatory cytokine in green. Scale bar=100 µm*

DISCUSSION

The purpose of this study was to investigate the potential therapeutic benefit of long-term minocycline administration to attenuate or abolish established CNP after SCI. I performed a series of pre-clinical pain behavior assessments in a rodent model of SCI. First, I focused on determining if a two week dosing regimen of minocycline (50mg/kg) could attenuate established CNP. I then determined if a ten day washout period of the drug would reduce CNP long-term. Finally, I compared the efficacy and safety of minocycline with the current "gold standard" of treatment, gabapentin. I hypothesized that long-term administration would decrease established CNP following a severe contusion SCI in a rodent model.

Minocycline

I found that systemic minocycline treatment attenuated established neuropathic pain in rats following spinal cord injury. This treatment is in accordance with the precedent set by several pre-clinical studies that showed an attenuation of SCI-induced pain with acute treatment of minocycline administered shortly after injury (46),(65),(66),(67),(62),(28),(39). Previous studies have focused on early intervention for neuropathic pain after SCI with minocycline treatment administered shortly after the SCI. While the ability to suppress CNP before it can become established will provide hope to those who encounter an SCI in the future, it should be noted that there are many individuals living in the chronic phase of SCI and approximately 65% have established CNP (89).

This study focused on finding a therapeutic intervention for reducing or stopping neuropathic pain long after establishment. The study focused on providing knowledge that could potentially benefit thousands of individuals who are enduring CNP many years after a

traumatic SCI event. A pre-clinical SCI study by Hains and Waxman showed that a brief, three day treatment regimen with minocycline could attenuate established neuropathic pain in rats 30 days post-SCI. However, the pain returned immediately after treatment was stopped (36). This study is the first to show that it was possible to reduce established CNP via long-term administration of minocycline in both below-level mechanical allodynia of the hind paws and hyperalgesia above the level of injury.

I observed no significant reduction in below-level hyperalgesia after two weeks of minocycline treatment. The animals may have experienced pain on the torso area below the level of injury. However, an alternative explanation is that pain levels remained high due to application of a noxious force near the sites of irritation caused by i.p. injections.

I also observed that there were no significant improvements in exploratory behavior such as distance, speed and active time after two weeks of minocycline administration. Spontaneous, exploratory behavior can be used to measure pain-like behaviors in an indirect, non-evoked manner because animals in pain move less (84). Measuring pain in animals is a difficult task not only because of the complexities of CNP development, but also because of the inability to define pain in animal subjects that correlates to all the pain modalities experienced by SCI patients. The photobeam activity box software may help us to identify symptoms of non-evoked pain or pain where no peripheral stimulus exists. Non-evoked pain is a major contributor of severe CNP in humans (89),(90),(91).

After injury, the animal subjects displayed a significant decrease in spontaneous behavior such as distance, average speed, active time, ambulatory movement and rearing. Therefore, any significant improvements in spontaneous behavior after treatment could be associated with a decrease in overall pain. The minocycline treatment group followed the

same trend as the vehicle treatment group when comparing distance moved, average speed moved and amount of time spent active over time. I found a significant decrease in ambulatory movement and rearing events compared to the baseline that also followed the same trend of the vehicle treatment group. This data suggests that overall pain may still have affected the minocycline and vehicle-treated animals post-treatment.

The lack of improvement in exploratory behavior after two weeks of minocycline treatment could be due to gut irritation from repeated i.p. injections and possible toxicity problems as previously described. The sores caused a great restriction in hind limb motor movement that did not allow the animal to have proper locomotor function while on the dosing regimen. It was unclear whether the observations were due to a continuing phenomenon of CNP, from irritation of the i.p. injections or from toxicity due to the treatment schedule. In future studies with long-term minocycline administration it will be important to use a different mode of administration and an alternative schedule for long-term dosing to rule out issues caused by i.p. injection and toxicity.

Despite the discrepancy in the activity box data most of the suppressed behavioral trends while on the minocycline dosing regimen improved by day 60 testing. The sores caused by repeated i.p. injection healed after the ten day washout period of the drug and the trends in distance, speed, active time, ambulatory movement and rearing events improved compared to day 42. This suggested that the animals may have recovered a portion of their locomotor function that was previously restricted due to the i.p. injections. It was also possible that the animals were experiencing less pain because the wounds were healed. However, due to improving trends in all of the same behavior assessments in the vehicle treatment group, this remained unclear. Overall improvements in locomotor function and behavior activity in the

vehicle treatment group suggested that there was an obvious, naturally occurring improvement in the Sprague-Dawley rat model of SCI, 60 days post-injury.

This study was the first to discover that minocycline had a lasting effect in reducing CNP after SCI. I found that even after the ten day washout period of minocycline, below-level mechanical allodynia and above-level hyperalgesia significantly diminished. The half-life of minocycline in rodents is approximately two to three hours which means the drug is completely out of their system by day 60 testing following a final dose at day 50 (92). I found that minocycline produced a sustained reduction in CNP, and this contradicts previous findings by Hains and Waxman who showed that pain returned immediately following withdrawal of minocycline (36). Hains and Waxman tested minocycline with a three day regimen of twice daily injections (100 ug/5 ul) intrathecally while I administered minocycline a total of three weeks intraperitoneally (36). The two different modes of administration vary greatly. An intrathecal dose involves an intrathecal catheter placed in the lumbar enlargement of the animal subject and correlates to an intrathecal injection in humans where a drug can be delivered into the fluid cavity surrounding the spinal cord (93). This method has the disadvantage of being an extremely invasive procedure. However, the intrathecal delivery method eliminates the concern that the drug will not pass the blood-brain-barrier by making the injection directly into the spinal cord. For example, SCI patients received continuous administration of baclofen, a drug that specifically targets Gamma-aminobutyric acid (GABA) receptors within the spinal cord, by an implanted intrathecal pump (94). This method was not only safe; it was significantly more effective than an oral dose and required a dose amount that was 100-fold less than an oral dose (94).

Intraperitoneal injections place the potential therapeutic directly into the intraperitoneal space in the lower abdominal area with caution to avoid contacting any organs. The advantage of i.p. administration is that it is the most widely used method in rodents for safe, systemic delivery of drugs (95). However, it does not correlate well to humans. Currently, clinicians are only using intraperitoneal injections for delivery of several chemotherapy agents(96). However, they are highly controversial because of the risk of complications and toxicity (96). Overall, the differences in the mode of administration in animal subjects can vary greatly and can cause differences in outcome, efficacy and side effects. It is most important to deliver the drug in a safe and tolerable manner in the animal subjects to ensure that the drug is able to target the intended mechanisms of action effectively.

The possible mechanisms by which minocycline administration could attenuate CNP is still unclear, however there are several interesting CNP mechanisms that it may target. A robust immune response followed by inflammation of the tissue surrounding the injury is a prolonged pathological event after SCI that contributes to CNP (42),(21). Minocycline is considered a multi-target or "dirty" drug because aside from antibiotic properties, it also targets the inflammatory response through the inhibition of microglia activity (36),(39),(97) (98). Microglia cells within the spinal cord normally remain in an inactive/resting state to maintain architecture and homeostasis of neuronal cells (41). After SCI, microglia cells activate, display "ramified branches" and release pro-inflammatory cytokines (41),(36). Fleming-Norenberg *et al.* reported that activated microglia were abundant up to one-year post-SCI in humans (20). Activated microglia cells produce an ongoing inflammatory response that correlates with maintenance of CNP (42),(43),(36),(44). Inhibition of microglia activation by minocycline administration reduces the production of pro-

inflammatory cytokines such as TNF-α, IL-β, IL-6 and prostaglandin-E2 (PGE2) that contribute to the continuation of pain (98). Minocycline inhibits matrix metalloproteinases (MMPs) activity, which reduces inflammation and prevents tissue remodeling (60). After SCI, MMPs become activated; exhibiting enzymatic activity that breaks down extracellular matrix components. While a crucial activity for permitting angiogenesis, MMP activation also contributes to inflammation and tissue damage within the spinal cord (60). Minocycline also inhibits apoptosis by inhibiting cytochrome c release from the mitochondria, an initial step in the signaling of the apoptosis cascade (61),(62). Inhibition of apoptosis prevents cell death, spares tissue around the spinal cord and contributes to an overall neuroprotective environment (67),(62),(66),(65).

Due to minocycline's ability to target the immune response, I must also be aware that the wounds from repeated i.p. injections may have been prevented from healing. The wounds depend upon the immune system's ability to heal efficiently. However, minocycline may inhibit the immune cells necessary for wound healing which, in this model, may have delayed the healing process. Overall, the finding suggested that long-term i.p. administration of minocycline at 50 mg/kg had a lasting effect of reducing some symptoms of CNP after SCI. While this study is not mechanistic in nature, I am intrigued by the possibility that sustained minocycline treatment may exert a sustained reduction in pain through modulation of known inflammatory mechanisms. This question will be addressed in subsequent studies.

Minocycline vs. Gabapentin

Gabapentin (Neurontin ®) is the current "gold standard" drug prescribed for the treatment of CNP in chronic SCI patients. The use of gabapentin includes multiple adverse side effects that include dizziness, somnolence and nausea (52). It has been well-established

that gabapentin has the ability to transiently reduce symptoms of CNP (99). Yet many clinicians are seeking alternatives to gabapentin due to adverse side effects. Patients who use gabapentin as a treatment for CNP often complain that it provides inadequate pain relief and also causes a multitude of adverse side-effects that make it difficult to function in daily life (53),(52). In order to determine the potential of minocycline to replace gabapentin as a standard of treatment for CNP I focused on comparing the efficacy and safety of both drugs.

The results indicated that gabapentin reduced below-level mechanical allodynia after two weeks of daily dosing with 50 mg/kg via subcutaneous injections. This concentration of gabapentin was chosen based on a previous study by Hama and Sagen who found that 50 mg/kg effectively reduced CNP after SCI in a rodent model (75). Minocycline is equally as effective at reducing below-level allodynia at this time point, which provided further evidence that minocycline could be a potential new therapeutic intervention for CNP.

Gabapentin treatment reduced below-level mechanical allodynia at day 60 testing, ten days after withdrawal of gabapentin. The data suggested that the reduction in pain was long-term because the effects were still significant ten days after gabapentin treatment ceased. Gabapentin has a very short half-life in rodents of 1.7 hours (100). Therefore, I expected pain levels to return by day 60 testing.

I observed no reduction in above-level hyperalgesia in the gabapentin treatment group using the girdle response test. This is in contrast to my finding that minocycline treatment significantly reduced pain at this level. The data suggested minocycline may effectively reduce symptoms of hyperalgesic pain above the level of injury that gabapentin cannot and that there may be different mechanisms responsible for above level pain compared to below level pain which should be further explored.

Gabapentin treatment significantly diminished below-level hyperalgesic pain whereas minocycline treatment did not. Interestingly, the reduction of pain was observed during day 60 testing, ten days after dosing was complete. I did not expect to see a significant decrease in below-level hyperalgesia after a ten day washout period because of gabapentin's short half-life. Therefore, long-term gabapentin treatment may have a lasting effect at reducing below-level hyperalgesia in a rodent model of SCI.

I found a significant increase in pain at day 42, while the animals were on the dosing regimen of gabapentin. The significant increase in below-level hyperalgesia could be explained by the mode of administration. Subcutaneous injections were made in the same areas where the application of the filaments was placed on the torso. Therefore, the significant increase in pain at day 42 could be explained by hypersensitivity caused by the mode of administration causing pain or irritation on the animal's lower torso area. This may also explain why there was no reduction in above-level hyperalgesic pain using the girdle response test after gabapentin treatment. The results suggested that gabapentin may have had a lasting effect at reducing below-level hyperalgesia and below-level allodynia. The data is in contradiction to the current literature that demonstrates that neuropathic pain levels return shortly after withdrawal of gabapentin treatment in a rodent model of SCI (101).

In addition to the unexpected findings after the washout period, I also observed a lasting improvement in the behavioral activity data. Total distance significantly increased at day 42 and day 60 in the gabapentin treatment group whereas the minocycline group continued to decrease. The vehicle treatment group also significantly increased total distance at day 60 indicating that the natural improvement over time most likely accounted for the improvements with total distance, average speed and active time.

Interestingly, gabapentin treatment significantly improved the number of rearing events within the activity box over the 15 minute period. I did not observe this in any other treatment group. Gabapentin-treated animals demonstrated a significant increase of rearing events at day 42 and after the ten day washout period at day 60. This is the first study to show that long-term gabapentin treatment could increase the number of rearing events after an extended washout period in a rodent model of SCI. This suggested that gabapentin provided long-lasting relief at reducing below-level pain, which allowed the animals to feel more comfortable standing up on their hind limbs. However, the increase in rearing events could also indicate that the animals had more homeostatic control over their muscles and therefore, improved trunk stability. It could also mean that perhaps gabapentin treatment reduced secondary injury and improved motor function recovery after SCI in the animal model.

Gabapentin treatment may improve trunk stability or provide neuroprotection and prevent secondary injury by an unknown mechanism. Recently, a single dose of gabapentin (200mg/kg or 30mg/kg) administistered immediately after injury in a rabbit model of spinal cord ischemic injury significantly protected the ultrastructure of the spinal cord (102). This study suggested that a single dose of gabapentin immediately after ischemic injury could have neuroprotective effects. Likewise, a pre-clinial study demonstrated that a single dose of gabapentin (200mg/kg or 30mg/kg) in a rat model of SCI demonstrated significant neuroprotective characteristics by examination of spinal cord tissue and plasma levels (103). Perhaps long-term gabapentin treatment was administered at an oportunistic time window post-injury in my study that provided neuroprotective effects and reduced secondary injury and increased motor recovery of the hind limbs.

Additionally, a recent study also demonstrated that gabapentin significantly reduced symptoms of muscle spasticity and autonomic dysreflexia with a single dose of gabapentin (50 mg/kg, i.p.) in an SCI rodent model when measured between 2 and 3 weeks after injury (12). Autonomic dysreflexia is a severe and painful condition characterized by a rapid attack that causes an enormous increase in blood pressure, shivering, sweating, anxiety and severe headaches (13).

Muscle spasticity and intense, painful muscle spasms are common complaints among SCI patients (12). Abnormal somatic and autonomic motor reflexes are thought to be the cause of symptoms associated with muscle spasticity and autonomic dysreflexia after SCI (12). In 1997, Gruenthal *et al.* performed a clinical trial to test if (2,400 mg over 48 hours) gabapentin treatment could reduce muscle spasticity in SCI patients. The authors concluded that gabapentin treatment significantly decreased muscle spasticity, which consequently improved the patients' overall quality of life (104). Gabapentin may diminish the somatic and autonomic motor reflex pathways by inhibiting glutamate release at presynaptic terminals in neurons (12). In my model of SCI, long-term gabapentin treatment may have produced similar effects. By reducing muscle spasticity with gabapentin treatment, this could explain why the animals had an increase in rearing events and perhaps long-term gabapentin treatment could contribute to more homeostatic control of muscles or increased muscle tone.

The currently accepted mechanism of gabapentin is its ability to binds to a calcium channel subunit, alpha-2-delta, and reduce calcium influx into cells (49),(50),(51). This decreases excitability of neurons (49),(50),(51). Decreasing the excitability of neurons by

inhibiting transmitters such as glutamate and substance-P provides an analgesic effect (50),(51). However, I propose that gabapentin treatment and its mechanisms of action should be further investigated for its potential as an early intervention after SCI to prevent or reduce symptoms that develop into chronic conditions such as neuropathic pain, muscle spasticity and autonomic dysreflexia. Taking into account all of the unexpected improvements demonstrated after the washout period and the significant increase in rearing events, not only does long-term gabapentin-treatment reduce pain; it may also beneficially target additional mechanisms that lead to permanent changes and muscle tone and recovery after injury.

Additionally, I measured pain by application of von Frey hair filaments. However, I cannot rule out the possibility that an intense flinch reaction above or below the level of injury using the girdle test may instead be an intense reflex reaction in our animal model. Likewise, I cannot rule out the possibility that a quick withdrawal of the hind paw using the von Frey hair test may instead be an intense reflex reaction. Provoking a reflex reaction instead of pain would indicate the animals are experiencing increased muscle spasticity and application of filaments on the hind limb and hind paw were simply inducing a severe muscle spasm. Recent studies suggest that this may be the case when measuring hypersensitivity below the level of SCI. Baastrup *et al*. reported that when measuring below-level pain in a rat model of SCI there was no indication of an increase in brainstem reflexes or unpleasant behavior which would be indicative of pain development as it correlates to humans (105). They concluded that they were simply measuring spinal reflexes and that measuring spinal reflexes is most likely not an appropriate measure for studying below-level pain development in rodents (105). Likewise, Urban *et al.* reported in a recent pre-clinical

study on mice that measuring mechanical hypersensitivity may not adequately measure chronic pain because they did not find any significant behavior changes such as increased anxiety, depression or changes by examining home cage behaviors which would indicate a decrease in quality of life compared to control animals (106). They indicate that depression and an alteration in daily activities in human patients living with chronic pain is a relevant and critical feature of chronic pain and that the mice did not develop these qualities which would indicate chronic pain development (106).

Therefore, my data could also suggest that gabapentin and minocycline may specifically target mechanisms underlying muscle spasticity and motor recovery after SCI instead of pain. Several studies indicate that acute minocycline treatment improves hind limb motor recovery weeks after injury using the BBB test and that minocycline has a neuroprotective effect after injury (66), (62),(67),(65). Therefore, minocycline could also improve motor recovery and decrease muscle spasticity with long-term administration and needs to be explored with additional, more rigorous assessments of motor function in subsequent studies.

Adverse Side Effects

I found several unexpected, negative side effects caused by a three week daily dosing regimen of 50 mg/kg minocycline administration via i.p. injections. The minocycline treatment group demonstrated abnormally low weight gain when compared with the other treatment or vehicle groups. Because this was the first assessment of a delayed, long-term dosing regimen of minocycline in an early chronic rat model of SCI, the toxicity levels were uncertain. One explanation for the low weight gain could be due to the antibiotic properties of minocycline. However, it is unclear whether antibiotics such as minocycline can cause

decreased appetite. In contradiction to our findings recent evidence suggests that extensive use of antibiotics including minocycline, may actually cause weight gain in patients (107). Therefore, the abnormally low weight gain in the minocycline treatment group could be indicative of signs of toxicity. I must consider that the dosing-schedule, concentration (50 mg/kg) and/or mode of administration (i.p.) were the cause. A wide range of pre-clinical studies of CNS trauma and disease have used concentrations of minocycline ranging from 10-100 mg/kg (28),(62),(67),(66),(65),(46). However, pre-clinical studies in rats using the i.p. delivery route have shown efficacy in improving functional recovery and attenuating pain with only acute treatment of minocycline. These acute studies range from a single dose to a maximum 5 day dosing regimen with concentrations ranging from 45 mg/kg to 180 mg/kg (28),(62),(67),(66),(65),(46). This was the first study to show complications of long-term minocycline administration via i.p. injections in a Sprague-Dawley rat model of SCI.

In addition to abnormally low weight gain, long-term i.p. injections caused extensive chemical burns and sores around the sites of injection in the abdominal area. This is consistent with the findings by Nessler *et al.* who discussed a concern that the effects of minocycline treatment causing abdominal irritation with repeated dosing of 100 mg/ml i.p. injections in a rat model of autoimmune encephalomyelitis (108). Additional research would be advantageous for understanding the proper mode of administration and correct concentration of minocycline in a rat model of SCI as an important step to assess efficacy prior to clinical translation. A pre-clinical assessment utilizing several modes of administration such as intrathecal, oral gavage and intravenous would provide further evidence as to which method is the most efficacious in the SCI rat model. Discovering the proper mode of administration in animal subjects is critical for effective translation into

clinical trials. Intraperitoneal injections (i.p.) have little relevance to translation in humans except for chemotherapy delivery, whereas intrathecal, oral gavage and intravenous injections are not only relevant, they can help determine the safest and most effective delivery method for patients. In addition, I further recommend performing a pre-clinical dose-response test to determine the optimal dose of minocycline that would effectively decrease CNP and minimize the negative side effects. Finally, my data suggested that a reduction in pain may be possible beyond the cessation of treatment. If this is the case, it will be important to determine the shortest possible time period for delivery of minocycline to elicit a long-term reduction in CNP while preventing or minimizing the adverse side effects observed with long-term minocycline treatment.

I observed a pronounced cellular inflammatory response in the livers of minocycline-treated subjects that suggested the onset of minocycline treatment-dependent hepatoxicity. Hepatoxicity is defined as drug-induced liver injury and is often difficult to diagnose in patients (109). My observations indicated that hepatoxicity may have occurred in the minocycline-treated subjects. This may have been due to the observed increase in collagen production as well as the presence of macrophages, many of which co-localize with the pro-inflammatory cytokine, IL1-β. There are several reports of hepatotoxicity in pre-clinical SCI studies using minocycline. It is unclear if the liver damage observed in my study was due to long-term administration or because of the route of minocycline administration. Some damage to the liver could be due to the close proximity of the liver to the area in which the drug was administered. Minocycline is a drug soluble in a weak acidic solution (pH 5) that may have affected not only the abdominal epidermis but also organs near the injection sites (92). Interestingly, my results are in agreement with many human case studies that found

evidence of liver damage with long-term use of minocycline. This suggests that the liver damage I observed may not have been caused by i.p. injections alone. Autoimmune hepatitis and liver damage from tetracycline antibiotics such as minocycline are reported in patients after an average use of two years (69),(68). Additionally, SCI patients could be predisposed to autoimmune hepatitis or liver disease due to their injury alone and minocycline could greatly increase their chances of developing hepatoxicity. Researchers and clinicians are discovering that SCI may cause decreased metabolic function, or function of the liver, due to recent evidence found in pre-clinical models of SCI and in patients with SCI (110),(111). Research studies focusing on the degree of metabolic dysfunction and drug-induced liver damage in a rodent model of SCI would be very helpful in determining a patient's benefit-risk ratio of taking drugs known to cause hepatoxicity. In future studies it will also be important to identify the minimal dose of minocycline required to reduce pain long term while also minimizing toxicity.

Limitations

The study of pain in animals is very difficult because pain is a highly subjective sensory interpretation. The tests developed and used in my study to measure pain were developed based on the assumption that the stimulus applied to an animal will evoke pain much like it does in humans. I measured mechanical hypersensitivity of the hind paws with the use of von Frey filaments with increasing force which correlated to peripherally-evoked mechanical allodynia in humans. I measured hypersensitivity of the torso using the girdle test which correlates to peripherally-evoked hyperalgesia and allodynia of the torso in humans. Finally, I measured spontaneous behavioral activity of the animal subjects to

perhaps provide better insight into the development of non-evoked pain or spontaneous pain which correlated to the development of spontaneous/non-evoked neuropathic pain in humans. SCI patients experience spontaneous neuropathic pain with sensations described as burning and pricking usually at or below the level of injury (90). Phantom pain is spontaneous pain that does not require a peripheral stimulus and occurs where there is total sensory loss (90). I also cannot rule out the possibility that the von Frey hair filaments are simply evoking a reflex response instead of pain as previously described. Determining whether the pain behavior tests used in rodents acccurately measure pain or instead measure muscle spasticity would greatly impact the field of study on neuropathic pain.

I did not measure thermal sensitivity of the hind paws in the animal subjects. Thermal sensitive testing in animal subjects correlates to thermal sensitivity in SCI patients who often experience a significantly increased response to hot and cold temperatures, most often above the level of injury (112). I also did not use the Randall-Selitto test which measures nociceptive withdrawal threshold of the dorsal and plantar surfaces of the paws by applying an increasing amount of force with an electronic algesimeter until the animal withdrawals the paw (113). Recently, Santos-Nogueira *et al.* used the Randall-Selitto test to measure neuropathic pain in animal models of both spinal cord contusion and transection injury (114). They found that the test was a sensitive and adequate measure of neuropathic pain in the animal subjects and that earlier time points could be tested compared to the von Frey hair test because weight support was not required (112). In future studies, it would be beneficial to combine behavior testing with developed diagnostic and physiological measures of pain in animals. The lab of Dr. Raymond Grill is currently developing an approach to discern evoked verses non-evoked pain using non-invasive functional MRI.

Based on my results there was high variability in the amount and types of neuropathic pain each animal experienced that I believe correlated well with studies on neuropathic pain in humans. Pain prevalence data after SCI is difficult to gather and analyze due to the many classifications and ways of identifying neuropathic pain in humans (7). In my study, some animals developed increased sensitivity to the mechanical allodynia test while other animals only developed sensitivity in the hyperalgesia test. Using several testing procedures such as von Frey hair test, girdle test and behavior activity can be beneficial in detecting a wide array of pain symptoms. However, it also makes it difficult to decide which animals animal subjects to include in the study based on which ones developed significant levels of CNP. I developed strict criteria to eliminate animals that did not appear to develop pain after injury, which decreased the total number of animals for analysis. In future studies that use only behavior tests for outcome measures, it may be useful to develop a strategy to analyze each test individually, instead of eliminating animals from all tests that did not show pain in only one or two tests. It may be beneficial to use one behavior test to measure overall pain and continue to develop better diagnostic strategies for analyzing neuropathic pain in rodent models.

The animals are extremely sensitive to the testing environment. It was important to test animals in a quiet room with no other animals or people allowed in and to clean the testing equipment between each testing session. I moved the location of behavior testing in the middle of our study and upgraded the von Frey hair testing apparatus. I did not notice any significant differences in behavior due to these changes.

Additionally, I observed hyper-sensitization in the naïve un-injured group. Animals are known to produce a "fear scent" if they are in pain, startled or afraid which could cause

other animals to become stressed (85). The naïve animals may have detected a fear scent released by the injured animals due to pain and distress from their injuries. In future studies, it would be beneficial to test the naïve un-injured animals separately from the injured animals to prevent hypersensitivity in the naïve group.

The girdle test was used to measure sensitivity of the torso area. However, the animals were rapidly growing in length and girth over the course of the study and changes in the size of the restraint devices were made over time. This made it difficult to apply the von Frey filaments in a consistent location at each testing session. Additionally, some animals became noticeably anxious even after being allowed to acclimate to the device before each testing session. Therefore, some animals may have flinched or vocalized due to the stress and anxiety of being secured in a small restraint device instead of actually experiencing provoked neuropathic pain or muscle spasms.

This was not a completely blind study. I divided the animals into treatment groups at day 29, which means that baseline, day 14 and day 28 testing were blind and day 42 and day 60 were not. After dosing, animals were divided into treatment groups, and behavior testing and dosing was performed by one person. It is beneficial to have only one person performing all behavior tests because some testers will be more stringent than other testers. In future studies it would be ideal to have two people perform behavior tests and then average the results of both raters.

Finally, another limitation of this study is that I was only able to look at one concentration of minocycline for long-term treatment. The dose concentration of 50 mg/kg used in this study is an average dose commonly used in rodents, as the dosing ranges from 10 mg/kg to 180 mg/kg in most pre-clinical studies of minocycline. The dose concentration

in humans is approximately 2-3 mg/kg (58). Therefore, minocycline administration is at a much higher concentration in pre-clinical rodent studies than the common human dose concentration. This makes translation into clinical trials difficult due to the adverse side effects I observed with the dosing regimen tested, despite the fact that it is an FDA approved drug. Perhaps a lower dose of minocycline would be sufficient to reduce pain without causing liver damage. I suggest further pre-clinical testing with minocycline to develop a safe mode of administration for long-term use while also performing a dose response test to identify the minimal dose required to achieve significant pain relief. This is particularly important in the SCI population as well as other CNS trauma and disease populations that may be experiencing decreased metabolic function.

CONCLUSIONS

I have demonstrated that minocycline can attenuate CNP during a long-term dosing regimen. Further, the anti-nociceptive effects are sustained following cessation of treatment, suggesting that minocycline may have potential as a clinical therapeutic in the treatment of SCI-induced CNP.

However, long-term use of minocycline in with the treatment schedule caused negative side effects such as gut irritation, low weight gain, and hepatotoxicity that need to be addressed with further pre-clinical studies. Using a lower concentration, different dosage schedule and different modes of administrations such as oral gavage may reduce the side effects I observed.

Gabapentin treatment caused a significant and sustained reduction in CNP ten days after withdrawal of treatment. This result was unexpected because gabapentin has a short half-life of 1.7 hours in rodents and previous studies have demonstrated that pre-drug pain levels return shortly after withdrawal of treatment. Additionally, the gabapentin-treated animals demonstrated a significant and sustained increase in rearing events compared with all other treatment groups. The data suggested that gabapentin treatment was not only capable of reducing pain long-term but it may significantly improve trunk stability and muscle tone or provide neuroprotection which decreased secondary injury.

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CURRICULUM VITAE

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