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A Potential Role of GSK-3beta in the Development of Pain Post-Spinal Cord Injury

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ABSTRACT

Chronic neuropathic pain is a very common consequence following spinal cord injury (SCI) and poses significant clinical challenge. Current treatments are largely ineffective and as a result, patients are often left with debilitating pain at and below the level of the spinal cord injury. After spinal injury, a cascade of events occurs within the nervous system. One important aspect of this cascade for investigation is the anatomical changes that occur post-SCI. There is evidence of maladaptive sprouting/growth in sensory nerve fibers that is thought to play a role in the development and amplification of pain signals. Sprouting of primary afferents in the cord, mediated by the dorsal root ganglia (DRG) may contribute to the sensory issues that patients experience after injury. The signaling cascade involving the protein glycogen synthase kinase- 3 beta (GSK-3β) may be important in this maladaptive growth. GSK-3β is highly expressed in the nervous system and is a known regulator of neurite growth through its interactions with cytoskeletal molecules. Recent evidence has shown a potential role for GSK-3β in contributing to pain post-SCI. The purpose of this study was to characterize alterations in GSK-3β in sensory processing regions of the spinal cord dorsal horn and DRG and correlate these expression changes with the presence of pain in a rat model of SCI. Long-Evans rats underwent intramedullary injection of quisqualic acid (QUIS) of saline (sham control) and were allowed to survive for 1 or 22 days. Animals in the 22-day group were examined daily for onset and severity of at-level pain related behaviors termed “overgrooming.” Spinal cord and DRG just below the level of injury were analyzed for alterations in GSK-3β activity. Immunohistochemical staining shows early and persistent changes in GSK-3β expression in the spinal cord dorsal horn and DRG. These findings further support a role for GSK-3β signaling in sensory afferent plasticity and provide insight into a molecular target in SCI-induced pain.

INTRODUCTION

Impact and demographics of SCI

There are approximately 17,000 new spinal cord injury (SCI) cases each year, and more than one million people living with SCI in the United States (NSCISC 2016). Patients may experience a variable loss of motor function depending on the level and severity of the injury. In addition to motor losses, patients are likely to experience muscle spasticity, urinary bladder dyssynergia, bowel dysfunction and widespread bodily disruption in the form of autonomic dysreflexia (Christensen 1997, Collins 2006, de Groat 2006, Johnson 2006, Mathias 2006, Nout 2006). In addition to these complications, up to 60% of these patients experience some form of chronic pain (van Gorp et al. 2015). The development of chronic pain post-SCI has a significant impact on the quality of life for these patients. It is estimated that in America alone chronic pain costs $560-635 billion annually due to medical treatment costs and lost work productivity (Institute of Medicine – US 2011).

Challenges of treating neuropathic pain

About 50% of patients experience chronic neuropathic pain after spinal injury (Burke 2017). Neuropathic pain is described by patients as a shooting, stabbing or burning pain and it is especially debilitating because of its persistent nature. Neuropathic pain post-SCI results due to lesions of damage to the somatosensory structures within the spinal cord interrupting sensory processing (Jensen 2001). The generation of pain due to damage of the nervous structures is not fully understood and is thus an important target of study as it is such a common outcome for patients. Neuropathic pain is especially difficult to treat, and this pain post-SCI can manifest both at or below the level of the injury (Figure 1)(Bryce 2012). At-level pain consists of pain that is

within the dermatome of the injury or three dermatomes below due to spinal cord or peripheral root damage. Below level pain exists more than three dermatomes below due to spinal cord or peripheral root damage and generates altered sensations. Both at and below-level neuropathic pain could be accompanied by pain from stimuli that are not normally painful (allodynia) and/or heightened pain sensitivity (hyperalgesia) (Bryce 2012). Patients are often prescribed anticonvulsants, antidepressants, opioids, and other pharmacological options such as botiunium toxin, baclofen, and cannabinoids to try to manage their pain. Additionally, patients are often told to try physical activity, acupuncture, and hydrotherapy to relieve some pain. Despite the multitude of options, these traditional analgesic methods are largely ineffective because they do not remedy the damage done to the nervous tissue (Franz 2019). As a result of failed pain management, chronic pain can have a serious debilitating impact on the lives of patients who struggle with it. Chronic pain post-SCI is linked to instances of psychological distress, poorer employment, depression, and an overall lower quality of life (Cairns 1996, Mariano 1992, Westgren 1998).

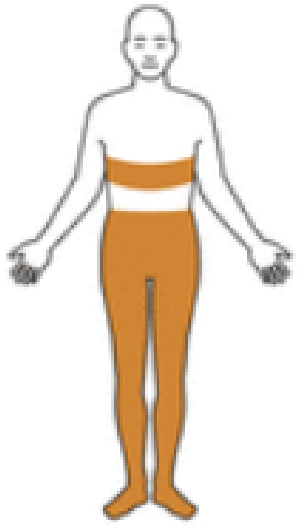


Figure 1: Schematic of At and Below-Level Pain

SCI patients can experience neuropathic pain at the level of an injury in a band of 1-3 dermatomes of the injury. Below-level pain or sensory disturbances including allodynia and hyperalgesia may manifest 3 dermatome levels below the injury.

Image via Finnerup 2021

Mechanisms of SCI pain

Spinal cord injuries are so debilitating because they can cause disruptions of sensory, motor, and autonomic systems that impact function at and below level of the spinal injury. When a person experiences an ischemic or traumatic injury to the spinal cord, the body immediately responds, initiating cascades to mitigate the damaged cord (Figure 2). Clinical and experimental models of SCI have provided evidence that there are four major components of the cascade which include: neurotransmitter/neurochemical changes, anatomical changes, excitotoxicity, and inflammation (Yezierski 2009). There are a multitude of alterations in neurotransmitter and neurochemical signaling post-SCI. Post-SCI, it has been shown that the Nav1.3 sodium receptor can allow higher than normal neuronal firing and is expressed at higher rates in the spinal cord after SCI (Hains 2003). Similarly, inhibition of GABA release or a loss of GABA producing cell post-SCI can result in less modulation of nociceptors. This can allow for spontaneous neuronal firing and amplified pain signals (Drew 2004). Excitatory amino acids are released post-SCI that are closely linked to cell death. These molecules create a hyperexcitable state with neurons shifting toward more depolarized potentials and increased persistent currents (Lampert 2006). These conditions cannot be maintained and thus can result in cell death and further damage to nervous tissue in addition to damage from the initial injury. Due to the presence of these excitatory amino acids, there is an upregulation of genes involved in inflammatory processes. Cytokines and peptides are released to cause inflammatory responses in addition to the activation of microglia and their migration to the injury site (Hains 2006). Anatomical alterations are an especially significant result of spinal injuries. Apoptosis, demyelination and cytoskeletal damage are all likely results of injury. Additionally, within the spinal cord and DRG, aberrant sprouting of afferent fibers can result. This sprouting in conjunction with the other aspects of the cascade

alters the connections between nociceptive and non-nociceptive neurons and contributes to amplification of pain signals. In addition, the early and ongoing hyperexcitable state of nociceptive neurons will generate the clinical outcomes of chronic pain, allodynia, and hyperalgesia that patients have to struggle with (Bedi et al. 2012, Yezierski 1998).

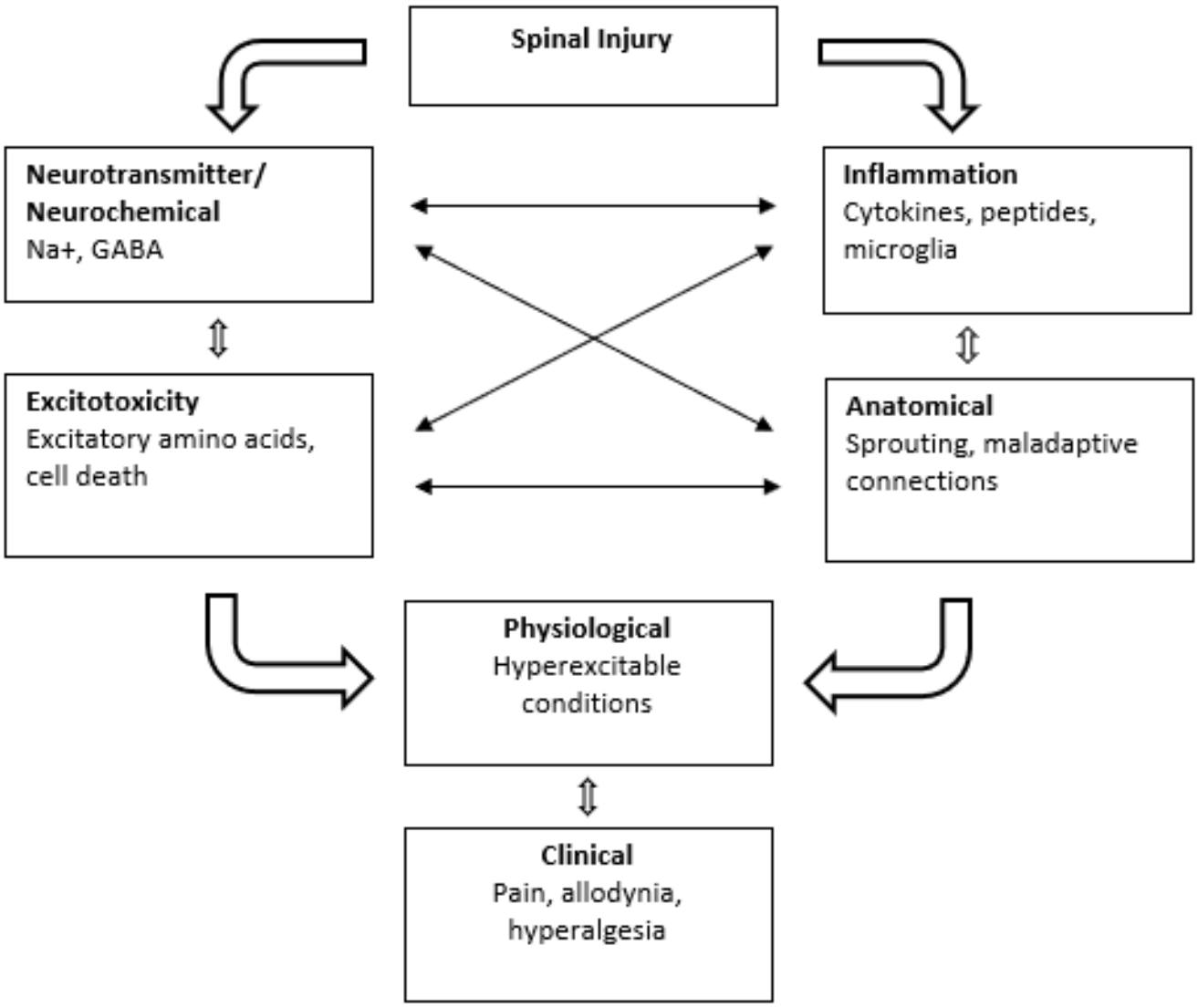


Figure 2: Spinal Cord Injury Cascade (Adapted from Yezierski 2009)

Spinal cord injury is extremely disruptive to the normal functioning of the body and results in alterations of many systems. The changes in neurotransmitters, excitotoxicity, inflammation, and anatomical alterations all interact to generate altered function of the nervous tissue. As a result of these cumulative effects the malfunctioning nervous tissue causes physiological changes that likely contribute to the clinical presentation of chronic neuropathic, allodynia, and hyperalgesia.

Peripheral Contributions to Central Neuropathic Pain

Treatments for pain post-SCI are largely ineffective, in part because the mechanisms that contribute to pain are not well understood. Historically, efforts to understand pain post-SCI have focused on the injury epicenter and supraspinal levels. Many studies focus on the descending input from higher nervous structures to try to attenuate pain signals coming in such as the serotonin system or altered plasticity in the thalamus which is a known sensory processing center (Hains et al. 2003, Lenz 2000). Newer evidence suggests that alterations in the periphery of the nervous system may additionally contribute to pain post-SCI through the alteration and amplification of pain signals. Focus on the alterations to the primary afferents post-SCI is providing more information to better understand how neuropathic pain develops. Several spinal cord injury models have been developed and studied to understand how chronic pain develops post-SCI specifically examining the aberrant growth and hyperexcitable conditions in primary afferents. There is evidence of maladaptive structural plasticity in rat SCI models. In a contusion model, an actual impact to the cord is created to create an injury at a specific location in the cord (Bedi 2012). In an excitotoxic model, injections are made to simulate the conditions in the cord after the ischemia and trauma of an injury (Yezierski 1998). Use of both of these models has shown evidence of morphological changes in the cord, and importantly, changes to the primary afferents in growth and excitability (Bareiss 2013, Bedi 2010). This has been demonstrated by increased nociceptive density as marked by Isolectin-B4 (IB4) showing evidence of growth of nonpeptidergic afferents (Stucky and Lewin 1999). Additionally, there is evidence of increases in growth promoting proteins such as GAP 43, which is upregulated post-SCI and is a known marker of neurite sprouting and growth. It has been found to be concentrated in nociceptive fibers after SCI in experimental models (Ondarza 2003, Christensen and Hulsebosch 1997). This

pattern of maladaptive growth has also been found to be present in humans, which has shown correlation of pain and afferent growth of nociceptive fibers. Specifically, calcitonin gene-related peptide (CGRP) is present in neurons that carry normal pain and temperature signals, especially in the dorsal horn of the spinal cord. CGRP-containing fibers in SCI patients have been demonstrated to be higher in patients with chronic SCI injuries than those without (Ackery et al. 2007). This growth of sensory fibers, specifically nociceptive ones, is a possible mechanism for the maintenance of chronic pain in SCI patients and an important target of study. SCI models have also demonstrated alterations in activity and growth in the DRG, (Bedi et al. 2010, 2012, Bareiss et al. 2013, 2015) making the primary afferent structures of the dorsal horn and DRG critical targets of study for the peripheral alterations that may contribute to chronic SCI pain.

GSK-3β as a modulator of growth

The process of normal neuronal growth is complex and involves many intra- and extracellular signals. Neurotrophins are extracellular factors specifically designed to act on nervous tissue and have been shown to induce axon growth *in vitro* (Patel 2000) through the action of signaling cascades (Landreth 1999). These extracellular signals act via receptor tyrosine kinases to connect the extracellular signals to actions and processes inside the cell. In the normal state, intrinsic signaling molecules have some control of the extension of the axons, but it has been shown that these extracellular signals are required for long axon extension as shown in mouse models where NGF signaling is removed and limited axon growth results (Lentz 1999, Markus 2002). The important intrinsic processes in the neuron for growth are those molecules that regulate cytoskeletal assembly. An important regulator of neuronal growth is glycogen synthase kinase-3β (GSK-3β). It is highly expressed in the nervous system and considered constitutively active, playing a role in neuronal growth and cell survival (Woodget

2001, Jiang 2005). GSK-3β regulates neuronal growth through its interactions with cytoskeletal associated proteins which contribute to changes in neural structural plasticity. Adenomatous polyposis coli (APC) is an important protein in microtubule assembly in neuronal growth by increasing their stability, but GSK-3β blocks this assembly at the growth cones (Zhou 2004, Takei 2000). δ-Catenin, a nervous system specific protein, is an important molecule for neuronal plasticity that is ubiquitinated and thus degraded after interaction is GSK-3β (Oh et al. 2009). Another substrate of GSK-3β, CRMP-2, is significant for axon and dendritic elongation. Its function has been shown *in vitro* to be important in microtubule and actin filament assembly and regulating transport of proteins to the distal growing axon (Kawano 2005, Arimura 2005). CRMP-2 and is inactivated by GSK-3β interaction at its residues (Tan et al. 2013). In the normal state, GSK-3β is constitutively active. Rat cell culture models have demonstrated that GSK-3β can be inactivated via phosphorylation in many pathways depending on the context in which GSK-3β is functioning and at which residue it is being phosphorylated at (Sutherland 1993, Wang 1994, Grimes 2001). It has been shown that GSK-β activity can be inhibited by phosphorylation on its Serine 9 residue by protein kinases, including Akt which is activated with phosphatidylinositol 3-kinase (PI3K) presence (Fang et al. 2000, Hannigan 2005, Grimes 2001). Phosphorylation of GSK-3β has been demonstrated to lead to collapse of the growth cone (Uchida 2009). In the SCI context, this GSK-3β inhibition would theoretically permit neuronal growth by the activity of APC, δ-catenin, and CRMP-2 molecules (Figure 3).

Role of GSK-3β in SCI

After injury, nervous tissue, particularly the CNS, has limited ability for regrowth. Studies have suggested that in order for neuronal regrowth to occur, neurotrophins are locally activated at the distal peripheral nerve after injury (Fu & Gordon 1997**,** Zhou 2004). The temporal activity of GSK-3β has been an important target of study of how to regulate neuropathic pain after nervous injury. In a peripheral nerve injury model, GSK-3β has been shown to be important in the development of neuropathic pain and the pain behaviors of hyperalgesia and allodynia (Weng 2014). GSK-3β was found to have a role in this pain development through its influence on glial cells and the cytokines they produce. This enhanced activity of GSK-3β was found to have an effect on initially increasing the glutamate transporters of glial cells and then suppressed this expression by day 10, possibly allowing more initial excitatory activity. The overproduction of pro-inflammatory cytokines was found to be increased in both the early and late stage of this peripheral nerve injury model. When GSK-3β activity was pre-emptively inhibited, the pain behaviors of hyperalgesia and allodynia were lessened (Weng 2014).

Interestingly, this pattern of initial GSK-3β inactivation then later expression as a mechanism for pain is reversed in other pain models. In a central pain model, the inactivation of GSK-3β has been demonstrated to contribute to generation of hypersensitivity in the form of thermal hyperalgesia and mechanical allodynia in rat models as opposed to the increased activity of GSK-3β allowing pain behaviors in a peripheral injury model (Obata 2004). This is an interesting variance of the development of neuropathic pain due to GSK-3β influence. However, we will be examining the influence of GSK-3β through only a central injury model where its role in the growth of neurites after injury is the focus of study. Molecules such as NGF activated

PI3K, which is an upstream inhibitor of GSK-3β, are locally activated after spinal injury at the distal tip and growth cone (Zhou 2004). This resulting local inactivation of GSK-3β at the growth cone will allow for distal axon growth in small neurons that convey nociceptive information (Zhou 2004). This could indicate that the activity of this signaling pathway in the cord and DRG post-SCI can trigger aberrant neuronal growth that contributes to pain and additional sensory dysfunction that patients experience after injury (Ackery 2007). Thus, the continual downregulation of GSK-3β in the sensory structures of the dorsal horn and DRG may be critical to the development of chronic neuropathic pain after injury to the spinal cord.

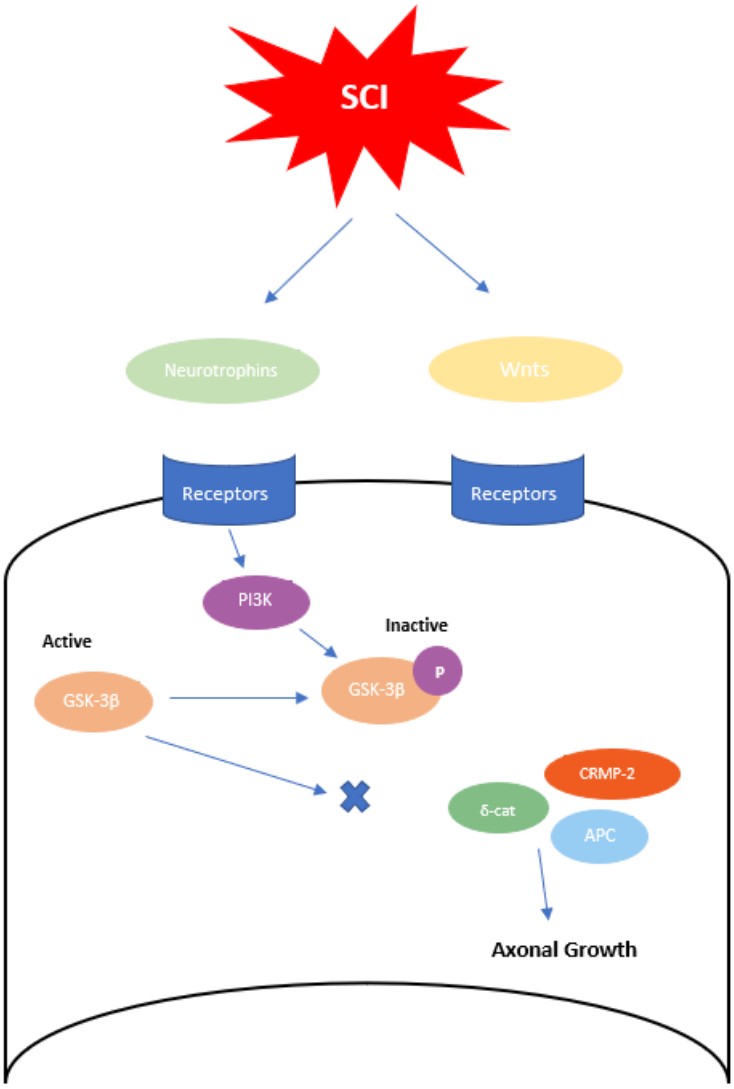


Figure 3: Hypothesis: Post-SCI GSK-3β signaling cascade

The signaling cascade that results after spinal injury may cause inhibition of GSK-3β activity through phosphorylation by PI3K activity, which is increased after SCI. Decreased GSK-3β activity can allow for cytoskeletal protein assembly and activity that leads to axonal growth.

HYPOTHESIS

There is a tremendous need to develop treatments for patients living with SCI pain. In this study we will examine the peripheral contributions to SCI pain and examine a signaling molecule potentially important to the process. We hypothesize that post-SCI pain results from aberrant primary afferent growth responses induced by inhibition of GSK-3β signaling. The purpose of this project was to characterize alterations in GSK-3β and in sensory processing regions of the spinal cord dorsal horn and dorsal root ganglia (DRG) and correlate these expression changes with the presence of pain in a rat model of SCI. To test this hypothesis, we will use a well-established rodent model of spinal cord injury that reliably results in development of at and below-level pain related behaviors. Spinal cord and DRG tissue will be extracted and immunostained with GSK-3β-p to characterize the activity changes post-SCI. We predict that SCI will induce changes in GSK-3β-p that correlate with the presence of at and below-level pain post-SCI.

MATERIALS AND METHODS

Spinal Cord Injury

Experiments were approved by the Institutional Animal Care and Use Committee of East Carolina University. Male Long Evans rats were given an intramedullary injection of 1.2 μL of quisqualic acid (QUIS) into the gray matter of the dorsal horn between T12 and L1 as previously described (Yezierski 1998, Yezierski & Park 1993). Quisqualic acid is an AMPA-metabotropic receptor agonist and thus acted to produce an excitotoxic spinal cord injury and lesions. The vertebral column was then exposed with a posterior midline incision and a laminectomy was performed at approximately T11-L2. The dura was incised and retraced. A glass micropipette

with a 5-10μm diameter tip was attached to a 10μL Hamilton syringe mounted on a microinjector (Kopf 5000, Tunjunga, CA). Injections of 125mM quisqualic acid were made between the midline vessel and lateral aspect at 1000μm below the surface of the cord at the T12 or T13 spinal level. Animals received 1.2μL of quisqualic acid or phosphate buffered saline (PBS) in the sham group over a 60 second time interval in a manner of 3 injections of 0.4μL separated by 0.3mm parallel to the long axis of the cord. Quisqualic acid triggers an elevation of excitatory amino acids that would be present after SCI (Simpson et al. 1990). This injection SCI model produces targeted neuronal loss to spinal tissue resulting in sensory deficit (Yezierski 1998). Animals were allowed to survive for either 1 or 22 days (QUIS =10, SHAM n=6) then spinal cord and dorsal root ganglion tissue were collected.

Animal behavior

The animals' skin was inspected daily for signs and severity of overgrooming or spontaneous removal of the fur and damage to the underlying skin at the level of the injection. This behavior is indicative of at-level pain experienced after the QUIS injury. Animals that did not exhibit overgrooming behavior were classified as non-groomers (NG). Animals that had less severe overgrooming behavior of fur removal and damage to superficial layers of skin classified as less-severe groomers (G1/2), and animals that the most extensive damage of fur removal and severe damage to the dermal layers of the skin classified as severe groomers (G3). Any animals progressing past the G3 stage and producing damage into the subcutaneous tissue were euthanized and removed from the study.

Immunohistochemistry and Microscopy

At varying time points the spinal cord (L1) and adjacent DRG tissue from QUIS and sham group animals were removed and fixed in 4% paraformaldehyde for 24 hours then transferred to 30% sucrose in PBS for 24 hours. Tissue was cut using the Lecia 2400 sledge microtome at a thickness of 8μm for the DRG and 16μm for the spinal cord tissue then prepared for immunohistostaining. Tissue sections were blocked with 1% BSA, 3% donkey serum, and 3% Triton in PBS for one hour at room temperature. Then tissue was incubated at 4◦F overnight in primary antibodies of GSK-3β-P (abcam ab9336) and IB4 conjugated to fluorescent FITC (1:30 dilution) (Jackson Immuno Research) in a humidity chamber. The following day, primary antibody was removed and tissue rinsed 3 times with PBS and incubated with secondary antibody (Cy3, 1:300 dilution)(Jackson Immuno Research) for 1 hour at room temperature in a humidity chamber and dark environment. After incubation, tissue was again rinsed 3 times with PBS, then slides were coverslipped using DAPI mounting media (Vectashield, Burlinegame, CA USA). Slides were left to dry overnight in a dark environment at room temperature. Image capture was done the next day using a Nikon Eclipse Ti inverted microscope at 10x for spinal cord samples and 10x and 20x for DRG samples. Analysis of the images was performed using ImageJ NIH software.

Analysis

Data were expressed as mean ± SEM. Paired t-tests or one-way analysis of variance (ANOVA) were used for statistical significance followed by Tukey's post hoc test for between group comparisons. Significance was set at $p \leq 0.05$. All data was analyzed and presented using Graph-Pad Prism software.

RESULTS

To test our hypothesis if there was a relationship between the alterations in GSK-3β activity in the dorsal horn and DRG and the presence of pain we first asked if alterations in GSK-3β occurred early post-SCI and if these changes correlated with the presence of at and below-level pain related behaviors in this model. Previous work from our laboratory has shown that QUIS induced SCI results in early (1 day) peripheral nerve growth responses that correlate with pain related behaviors in this model (Bareiss 2015, 2013). We and others have also demonstrated that GSK-3β-p is expressed in the superficial laminae of the spinal cord and in the DRG (Bareiss 2015, Goold 2014, Weng 2014).

SCI Induced Pain Behaviors

QUIS induced spinal cord injury reliably results in the development of at and below-level pain behaviors which appear at temporally different timepoints after injury. During out study, at level grooming behaviors had onset between 7 and 14 days after QUIS injury and below-level pain behaviors of thermal hyperalgesia and mechanical allodynia developed at day 14 (Figure 4, 7), this is consistent with the development of pain that is well-characterized in this SCI model (Yezierski 1997). There were 6 total animals used for 1 day after injury tissue collection including sham (n=3) and QUIS (n=3) animals. Ten total animals were used for 22 days post-injury including QUIS non-groomers, QUIS groomers, and sham (Figure 5). Within this 22 day group, 4 of the 7 animals that received QUIS injection developed at level pain related behaviors or overgrooming (QUIS G). Previous reports demonstrate that QUIS induced SCI reliably results in all animals demonstrating hind paw (below level) mechanical and thermal heat hypersensitivity with onset between 14 and 22 days. Tissue samples were taken of both the dorsal horn of the spinal cord and the DRG at day 1 and day 22 (Figure 4).

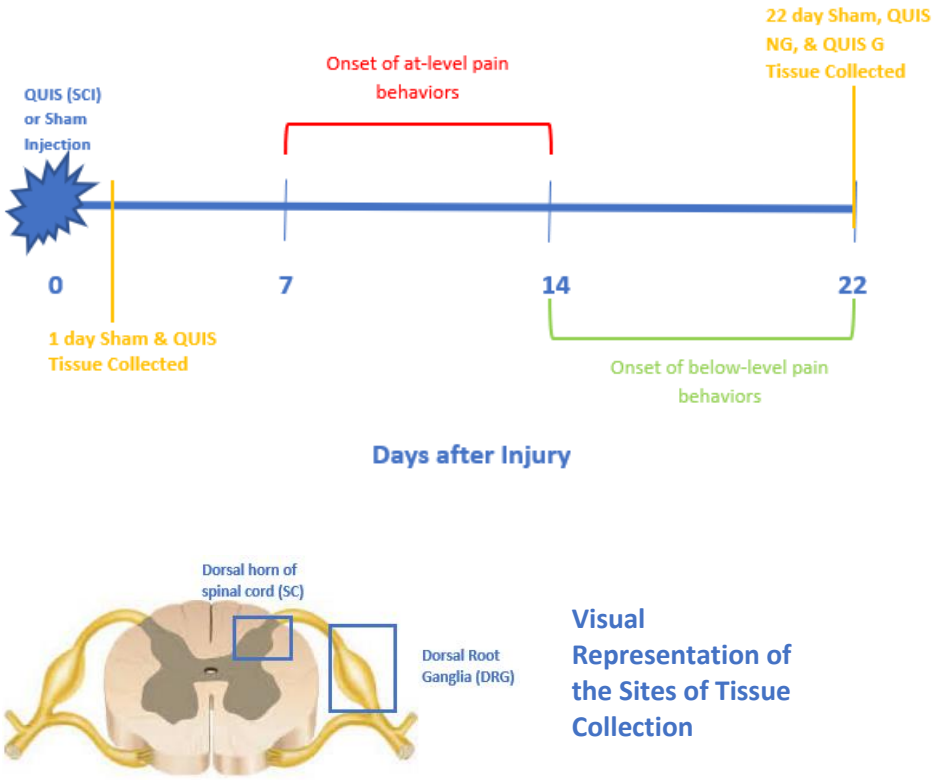


Figure 4: Timeline of study showing time of injury, survival periods, and onset of pain related behaviors

At 1 day after injury, 6 animals were selected for tissue harvesting (Figure 5). The onset of at level grooming behaviors indicative of pain was observed from day 7 to day 14. Below level thermal and mechanical hypersensitivity had onset from day 14 to 22. Then at 22 days, 10 animals were selected for tissue harvesting.

1 day	Sham: n = 3	QUIS: n = 3	
22 day	Sham: n = 3	QUIS non-groomers: n = 3	QUIS groomers: n = 4

Figure 5: Number of animals in each group

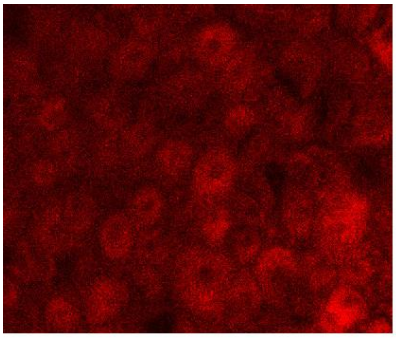
There were two groups: 1 day survival post injury or sham injection and 22 day survival. One day was chosen to demonstrate if there were early alterations that preceded the development of pain related behaviors. At 22 days, both at and below-level behaviors are present. QUIS/SCI animals were observed daily for presence of at-level pain behavior (overgrooming) with n=3 classified at nongrooming (NG) and n=4 classified as groomers.

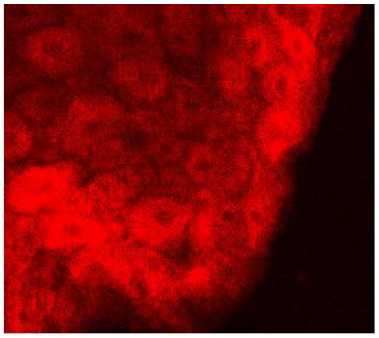
Correlating GSK-3β-p Expression post-SCI in the Spinal Cord and DRG

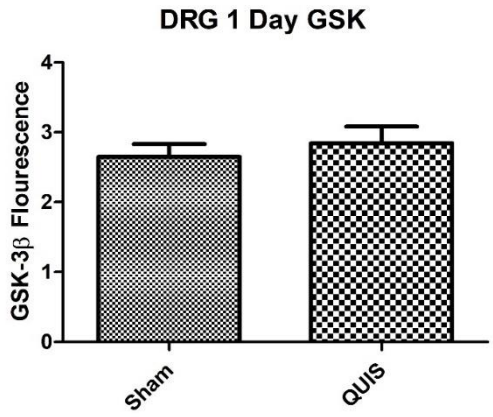
1 day post-SCI

Six animals were selected for tissue harvesting 1 day after injury to examine the early changes of GSK-3β in the dorsal horn and the DRG. The presence of GSK-3β-p was quantified to determine if there were changes in activity in these primary afferent structures that started before the onset of below-level pain behaviors. Analysis of the mean fluorescence of DRG samples 1 day after injury indicated that there was not a significant difference between the Sham (2.649 ± 0.1832; n=3) and the QUIS (2.843 ± 0.2731; n=3) groups (Figure 6a). However, in the spinal dorsal horn samples, the difference between the GSK-3β-p expression in the Sham (2.340 ± 0.1536) and QUIS (3.630 ± 0.5473) groups was determined to be significant (*$p<0.05$) with an increased presence of GSK-3β-p in the QUIS 1 day post-SCI animals' dorsal horn (Figure 6b).

A - DRG 1 Day:

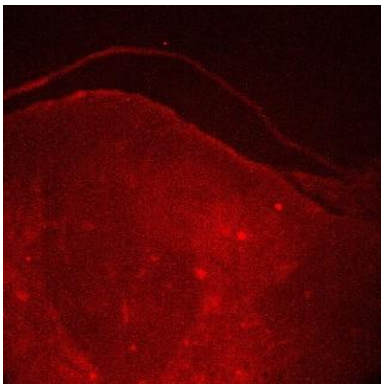


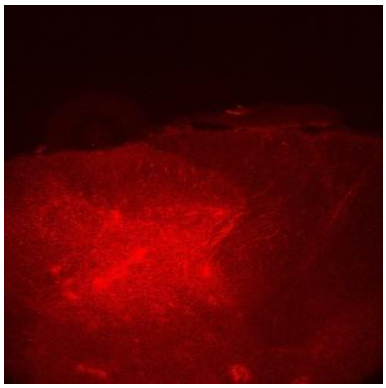


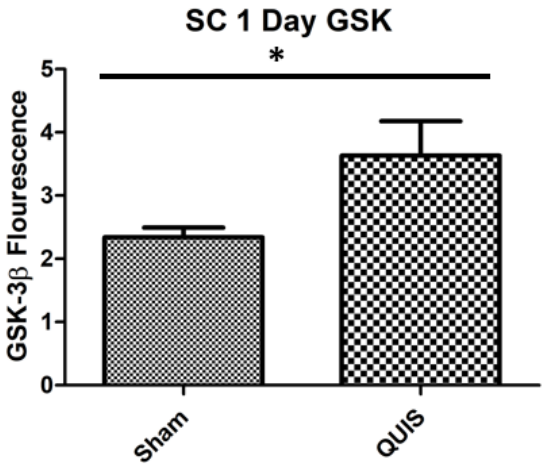


Sham QUIS

B – SC 1 Day:







Sham QUIS *

Figure 6: GSK-3β-p Intensity 1 day post-SCI

(A) GSK-3β-p is not significantly increased in the DRG 1 day post-SCI. Data presented as Mean ± SEM.

*(B) There is significant early presence of GSK-3β-p 1 day post-SCI in the spinal dorsal horn. Data presented as Mean ± SEM.(*p<0.05)*

22 days post-SCI

Ten animals were allowed to survive until 22 days post-induced SCI or sham surgery to examine the persistent changes in GSK-3β-p in the dorsal horn and the spinal cord as well as observe the onset of at and below-level behaviors through the study. Starting 14 days after QUIS injury, 4 of 7 animals developed at-level grooming behaviors (Figure 7) (QUIS G group) while the remaining QUIS non-groomers and Sham surgery animals did not. All QUIS animals (NG and G) developed thermal hyperalgesia and mechanical allodynia by day 22. At 22 days tissue was harvested and the dorsal horn and DRG were analyzed for GSK-3β. In the DRG at 22 days there was a significant ($*p<0.05$) difference in mean GSK-3β-p fluorescence between the Sham (7.008 ± 0.8840; $n=3$) and QUIS NG (10.72 ± 0.8838; $n=3$) groups. Additionally, there was a significant ($*p<0.05$) difference between the Sham and QUIS G (10.79 ± 0.7515; $n=4$) groups at 22 days after injury. There was no significant difference between the QUIS NG and QUIS G groups, but both were elevated in comparison to the Sham group at 22 days (Figure 8a). Thus, the elevation of GSK-3β-p by 22 days in QUIS NG and G animals aligns with the later onset of the development of at-level pain behaviors. When examining the spinal cord samples 22 days post-SCI it was found that there was no significant difference between the Sham (7.609 ± 0.3912; $n=3$), QUIS NG (6.819 ± 0.6101; $n=3$), and QUIS G (7.900 ± 0.4677; $n=4$) groups (Figure 8b).

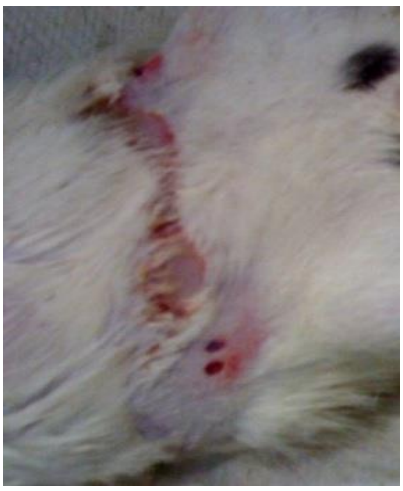
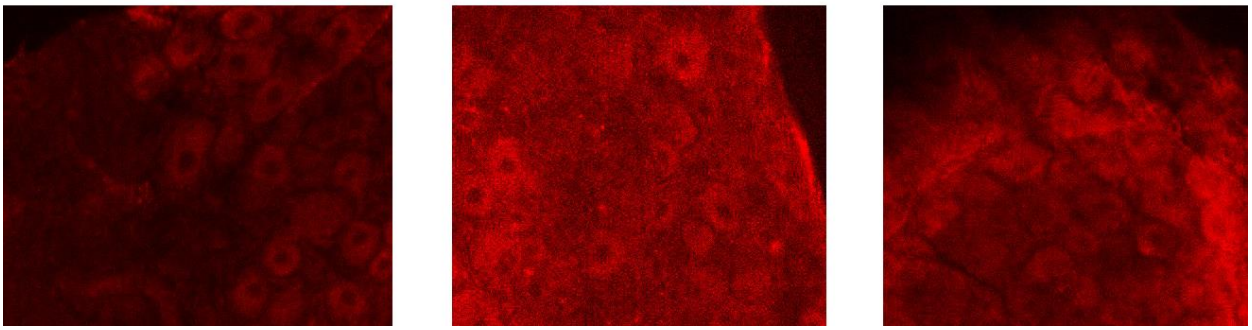


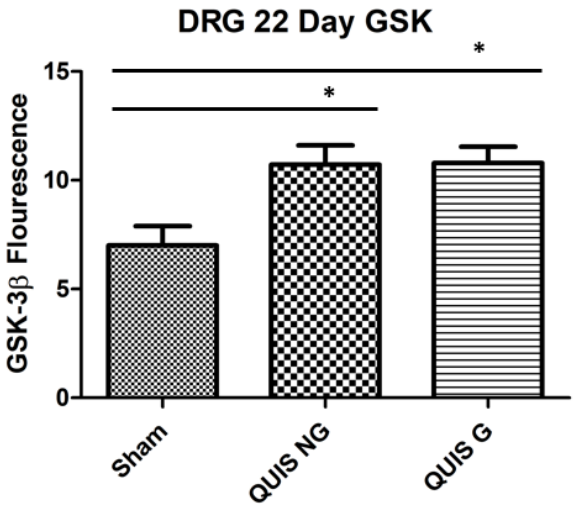
Figure 7 – Example of At-Level Overgrooming Behaviors

Animals developed overgrooming behaviors at the level of the injury site indicative of pain and sensory disturbances within 1-3 levels of the injury.. Grooming became severe in some cases as above such that fur and skin were removed. This onset of at-level disturbances began between 7 and 14 days.

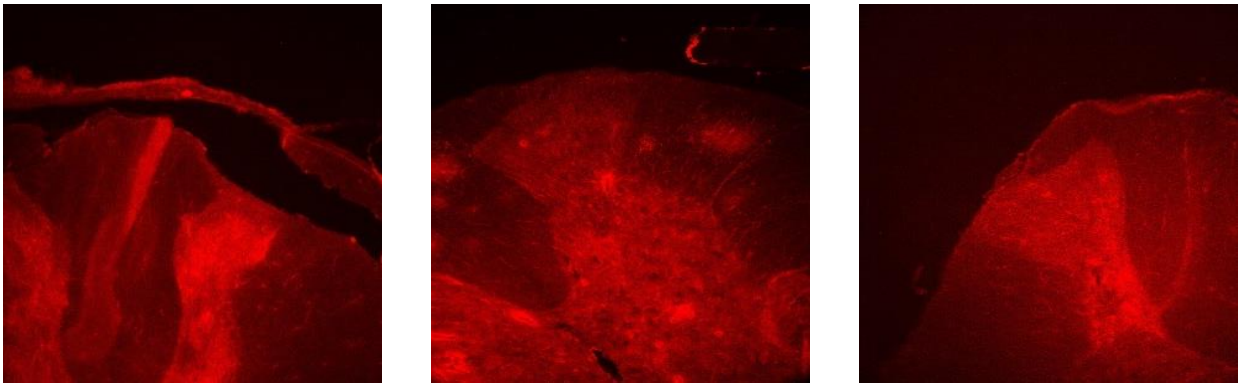
A – DRG 22 days



Sham QUIS NG * QUIS G*



B – SC 22 days



Sham QUIS NG QUIS G

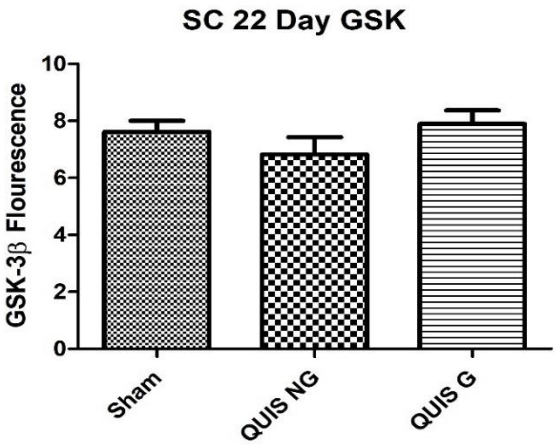


Figure 8: GSK-3β-p Intensity 22 days post-SCI

*(A) There is persistent activity of GSK-3β-p in the DRG at 22 days. There is a significant increase in both the QUIS NG and QUIS G groups in comparison to the Sham groups. There was no significant difference between the QUIS NG and QUIS G groups. Data presented as Mean ± SEM. (*p<0.05)*

(B) There is not significant continued GSK-3β-p presence in the dorsal horn at 22 days. Data presented as Mean ± SEM.

Discussion

This work serves to characterize the expression of GSK-3β in the spinal cord and dorsal root ganglion and correlates this expression to events following spinal cord injury. In these studies, we found that GSK-3β is expressed in the spinal cord and the DRG of rats. We further characterized the expression of GSK-3β-p in both tissue types and found that it is expressed in the DRG and in the sensory processing laminae of the dorsal horn of the spinal cord. Furthermore, we show that SCI-induced changes in GSK-3β-p expression and we observed early spinal cord dorsal horn changes and long term (22 day) DRG changes that were also associated with the presence of at-level pain as demonstrated by overgrooming behavior.

In this study we demonstrated that there were alterations of GSK-3β activity induced by spinal cord injury. The inactivated form of GSK-3β (GSK-3β-p) that results after QUIS-induced SCI was isolated in both primary afferent structures of the dorsal horn of the spinal cord and DRG at both 1 and 22 days. Here we found that one day after injury, there was an early significant increase of GSK-3β-p in the dorsal horn of the spinal cord, but this significance did not persist through 22 days. This early presence of GSK-3β-p in the dorsal horn was followed by the onset of at level behaviors by day 7 post-SCI. At 22 days after injury, there was a significant increase of GSK-3β-p in the DRG which also temporally aligned with the onset of below-level mechanical allodynia and thermal hyperalgesia which began at day 14 and persisted through day 22. This difference of significance between the days could possibly attributed to the small sample size as only 3 of 6 animals received the QUIS injury in the 1 day group, and 7 of 10 animals were QUIS of the 22 day group. In a larger sample size, the effect of GSK-3β-p may be observed to be more significant across DRG and the dorsal horn samples for both 1 and 22 days post-SCI. Conversely, this effect of early significant GSK-3β-p in the dorsal horn, then later

significant presence in the DRG could reveal a pattern of GSK-3β-p activity after injury that starts in the dorsal horn and then progresses to the DRG as time passes. This late inhibition of GSK-3β-p in the DRG may possibly allow for the persistence of pain behaviors, both at and below-level as both are not present at only 1 day after injury.

Alterations in GSK-3β following injury are supported by other studies. Previous work demonstrates that GSK-3β shows alterations in nervous tissue by 14 days after QUIS injury. A higher proportion of GSK-3β-p than GSK-3β was identified in both the spinal cord and DRG of animals that received QUIS than Sham animals at 14 days, indicating that biochemical changes occur following central injury to the spinal cord (Bareiss 2015). GSK-3β-p is the inactive form of the protein that becomes prevalent post-SCI, which would allow for neurite growth such as in Bareiss 2015 to happen. In our study, the early significant presence of GSK-3β-p in the dorsal horn of the spinal cord shows evidence of growth happening in this afferent region that may have allowed for the earlier onset of at-level overgrooming behaviors by day 14. Additionally, the late increase (day 22) in GSK-3β-p in the DRG was temporally correlated with the delayed onset of below-level mechanical allodynia and thermal hyperalgesia that was also demonstrated in to be correlated with neurite growth as in Bareiss 2015.

Prior studies show that DRG neurons collected from animals 14 days post-QUIS have extended neurite growth both at and below the level of injury (Bareiss 2015). This growth was associated with more at-level overgrooming behaviors as well as below-level mechanical allodynia and thermal hyperalgesia. In addition to demonstrated changes in growth due to GSK-3β-p activity in primary afferent structures there are additional peripheral sensory consequences that may result following SCI. It has been shown that SCI can induce a long-lasting spontaneous hyperexcitable state in DRG neurons (Bedi 2010). This state causes DRG neurons to increase

their responsiveness to depolarization by lowering their resting membrane potential (RMP) thus possibly accounting for heightened sensitivity to stimuli such as in mechanical allodynia and thermal hyperalgesia following SCI. Spontaneous activity was also more likely in small diameter soma fibers that were identified as nociceptive in both dissociated and *in vivo* DRG neurons. This prolonged spontaneous activity has been identified as an early trigger of persistent pain in two peripheral neuropathic pain models as well (Xie 2005). Chronic spontaneous activity of the afferent structures in conjunction with alterations of GSK-3β activity seen in this study may both be important in the development and maintenance of chronic neuropathic pain post-SCI.

It has also been shown that SCI can cause an intrinsic growth promoting state that causes growth of DRG neurites both at and below-the level of injury (Bedi 2012, Bareiss 2013). Taking into consideration spontaneous activity and the role of GSK-3β activity on neurite growth in the dorsal horn and DRG, this previously shown growth at and below-level is only further supported by the findings of this study. SCI may induce pain by decreasing GSK-3β activity in the DRG and dorsal horn, as we have shown, thus allowing growth that has been shown both at and below-level of injury. Finally, chronically increased electrophysiological activity at these sites may allow for the amplification and persistence of pain signals being sent due to the alterations in GSK-3β activity and resulting growth in afferent structures.

While the connection between reduced GSK-3β activity, neurite growth, and spontaneous activity may be a mechanism for the development and maintenance of chronic neuropathic pain, the pattern of GSK-3β activity is not consistent across all neuropathic pain models. In this study, a central QUIS-induced injury model was used to create damage to the central structure of the spinal cord. This resulted in an inactivation of GSK-3β activity resulting in a higher proportion of the inactive version, GSK-3β-p, early in the dorsal horn of the spinal cord, and late in the

DRG. However, evidence in a peripheral injury model has demonstrated differences in the pattern of GSK-3β activity post-SCI (Weng 2014). This model used a partial sciatic nerve ligation to study the formation of neuropathic pain after nervous injury. This group found that post-SCI, early (day 3) GSK-3β activity was suppressed but then had increased by day 10. Additionally, mechanical allodynia and thermal hyperalgesia were present in the late stage in accordance with increased GSK-3β presence. This is in direct contrast to the evidence found in this study that suggests that GSK-3β is inhibited across a longer timeframe post-SCI. These differences only further emphasize the pivotal role that GSK-3β has as an intracellular signaling protein that has many functions in different systems. One possible reason for the variability between the pattern of GSK-3β activity may be that it serves a different function after a central or peripheral injury to nervous structures. Furthermore, the regenerative capacity of neurons is greater in the peripheral nervous system, so potentially growth mediated by GSK-3β inactivity may not be needed. GSK-3β could potentially serve in a different function after peripheral injury where it may mediate alterations in the inflammatory response of cytokines and glial cells which could separately contribute to neuropathic pain instead of the growth of afferent structures as in central injury.

As many treatments for spinal cord injury are largely ineffective, it gives added importance to the role of GSK-3β as a potential therapeutic target for drug treatment. GSK-3β has already been elevated to an important signaling molecule in other diseases such as Alzheimer's, multiple sclerosis, and AIDS dementia complex where it plays a role in neuroinflammation that causes the clinical issues with these nervous system disorders (Maixner 2013). There has been evidence of improvement of learning and memory in an Alzheimer's model when GSK-3β was able to be targeted using an antisense oligonucleotide to specifically

target the kinase activity of GSK-3β (Farr 2013). In addition to specifically targeting GSK-3β itself, there has been some evidence that targeting the signaling pathway of GSK-3β may be a potential method to prevent and alleviate the development of neuropathic pain post-SCI. PI3K is an upstream inhibitor of GSK-3β that increases inhibition of GSK-3β post-SCI through its increased activity from neurotrophins released after nervous injury (Figure 3). Injection of a PI3K inhibitor (LY294002) directly after QUIS-injection has been shown to significantly reduce injury induced DRG neurite outgrowth and reduced the incidence of at-level pain indicated by lessened overgrooming behaviors in a rat model (Bareiss 2015). PI3K inhibitors have been approved for treatment of breast cancer as this pathway is also aberrantly activated in cancer, as post-SCI. The PI3K's kinase ability becomes malignant and hyperactive in cancer, and using a targeted inhibitor has shown success in slowing its activity (McPhail 2020, Vanhaesebroeck 2022). If a targeted PI3K inhibitor such as LY294002, that is beginning to be tested, can be applied to spinal cord injuries to halt maladaptive afferent growth, it may be the start of effective treatment of chronic neuropathic pain of which patients have still not found effective relief.

In summary, this study demonstrated that there was a temporal relationship between the alterations of GSK-3β activity in the afferent structures and the development of at-level pain and below-level sensory disturbances. Changes in GSK-3β-p were characterized via immunohistochemistry in both the dorsal horn of the spinal cord and the dorsal root ganglia, indicating that GSK-3β activity is reduced after central QUIS-induced SCI. The presence of GSK-3β-p was found to be significant in the early stage (1 day) in the dorsal horn of the spinal cord and in the late stage (22 days) in the DRG. The early presence of GSK-3β-p in the dorsal horn precedes the development of at-level pain displayed by overgrooming, possibly contributing to its development by day 7 post-injury. The late and persistent presence of GSK-3β-p in the

DRG likely contributed to the onset of below level disturbances of mechanical allodynia and thermal hyperalgesia that had onset starting at day 14 and continued until day 22 when late GSK-3β-p tissue presence was determined. Thus, the early pain behaviors may be due to alterations first in the dorsal horn of the spinal cord and then delayed onset of below-level pain behaviors due to later changes in the DRG.

Our work further characterizes the importance of GSK-3β activity changes in the primary afferent fiber (DRG – cell bodies and the central process in the dorsal horn) and its potential contribution to the development of neuropathic pain at and below the level of injury in this SCI model. The potential of manipulating the control of GSK-3β activity in these sensory structures may open up new possibilities for the treatment of neuropathic pain post-SCI for patients who have found no relief in other analgesic methods. However, additional study is still needed on the consistency of GSK-3β activity across injury models before any clinical treatment can be developed for patients. Determining the mechanism behind the variability of activity of GSK-3β in a central and peripheral nerve injury model will prove important if treatments are to be made using GSK-3βas a target. Whether GSK-3β activity varies in the development of central or peripheral neuropathic pain will prove important for determining treatments depending on the injury location. Additionally, the activity of GSK-3β in varying nervous injury types will need to be determined. GSK-3β activity in a central contusion SCI model needs to be studied to examine if the role of GSK-3β is similar in comparison to this excitotoxic model, as a contusion model will result in both sensory and motor consequences, instead of solely the sensory and pain consequences seen in this model. Finally, all further work in targeting GSK-3β as a potential treatment will have to take into consideration other side effects that may happen in the nervous system. It will be important to be able to target GSK-3β in these sensory structures without

causing further disturbance to them to increase nociceptive activity and growth as well as not interfere with normal functioning. Treatment will also have to be able to have analgesic function without producing additional unintended motor consequences. Finding the balance between halting maladaptive sensory plasticity in the dorsal horn and the DRG that may contribute to neuropathic pain and promoting motor fiber growth will prove crucial in the future. This study suggests that GSK-3β is certainly an important target for future study to develop balanced treatments.

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