Characterization of B3galt2 and Heg1 Expression in Dorsal Root Ganglia

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CHARACTERIZATION OF B3galI2 AND Heg1 EXPRESSION IN DORSAL ROOT GANGLIA

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

ALEXANDER H. NGUYEN
B.S.B.E, Wright State University, 2018

2020
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Alexander H. Nguyen ENTITLED Characterization of B3galt2 and Heg1 Expression in Dorsal Root Ganglia BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Nguyen, Alexander H. M.S. Department of Neuroscience, Cell Biology and Physiology, Wright State University, 2020. Characterization of B3galt2 and Heg1 Expression in Dorsal Root Ganglia

Proprioception provides unique sensory feedback about the body’s orientation in space and this sensation is essential for creating specific motor functions and coordinated movement. During neurogenesis, unique genetic markers are expressed and classified as early, transient/middle, and late markers depending on their timing of expression. This study focuses on the expression patterns of two late markers: Heg1 and B3galt2. We investigated how expression of these two markers respond to peripheral nerve injury (PNI). We cloned and generated riboprobes to detect expression of B3galt2 and Heg1 in DRGs using in situ hybridization after transection of the sciatic nerve. B3galt2 hybridization in control, sham, and injured DRG revealed in expression in all DRG neurons. Heg1 hybridizations showed positive specific staining on control tissue. Heg1 expression in lumbar DRG was normalized to 7.83 neurons per 50,000 µm². However, further investigation would need to be conducted to validate expression in injured DRG neurons.
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I. Introduction

Proprioceptors Introduction

Proprioception provides unique sensory feedback about the body’s orientation in space and this sensation is essential for creating specific motor functions and coordinated movement (Proske and Gandevia, 2012). This constant stream of information is relayed to the brain, integrated, and information of the body’s position is continuously updated.

The sensation of proprioception is derived from dedicated sensory receptors which relay information regarding the length of the muscle fiber. Proprioceptive sensory neurons (PSNs) detect changes in muscle length via two specialized endings in the skeletal muscle, the Golgi Tendon organs (GTOs) and muscle spindles (MS). The MSs are innervated by two subtypes of afferents, group Ia and group II afferents, which can relay information about the rate and the magnitude of changes in muscle length (Macefield and Knellwolf, 2018). GTOs are innervated by group Ib afferents which relay information about the contraction of the muscle fiber (Houk and Henneman, 1967).

Combining the sensory information of these afferents leads to the general perception of the body’s orientation in space based on the summation of mechanical and tensile forces within the body’s muscles.

Proprioceptor Development
PSNs work together with other sensory afferents to describe the mechanical actions occurring on/within the body. These collective afferents, including PSNs, are called mechanoreceptors. Differentiating PSNs from other mechanoreceptors can be described by looking at genetic lineages and markers. During sensory neurogenesis, mechanoreceptors in the dorsal root ganglion (DRG) separate into two genetic lineages expressing either the combination of TkB$^+$Shox2$^+$ or TrkC$^+$Rx3 (Ma et al., 1999; Kramer et al., 2006; Lallemand and Ernfors, 2012). The TkB$^+$Shox2$^+$ lineage will give rise to Meissner and Pacinian corpuscle afferents, which are described physiologically as rapidly adapting, low threshold mechanoreceptors (RA-LTMRs). Meissner and Pacinian Corpuscles encodes sensations of light tactile touch and deep pressure on the skin, respectively. In contrast, TrkC$^+$Rx3$^+$ neurons will develop into proprioceptive afferents and Merkel afferents, which can also be described physiologically as slow adapting low threshold mechanoreceptors (SA-LTMR) (Levanon et al., 2002; de Nooij et al., 2013). Merkel cells primarily encode tactile information regarding the shape and form of an object, while proprioceptive afferents encode muscle forces, as described above. PSN neurons can be further differentiated from other SA-LTMR afferents by expression of parvalbumin (PV$^+$). In addition to the intrinsic expression of transcription factors, the differentiation of PSNs depend on inductive signals transported along their respective sensory axons from the periphery (Tourtellotte and Milbrandt, 1998; Hippenmeyer et al., 2002).

Observing the development of these MS/GTO afferents during embryonic stages give insight to when proprioceptors are distinguished. Proprioceptors begin to establish
their distinct lineage at E12.5 and proceed to innervate their receptor targets at E14.5. By 
E17.5, establishment of their peripheral contacts is complete. Maturation of PSNs 
continue throughout the early weeks of post-natal development, P0-P21 (Hippenmeyer et 
el., 2002; Kramer et al., 2006; de Nooij et al., 2013). During these developmental periods, 
unique genetic markers are expressed. These markers can be classified as early, 
transient/middle, and late markers depending on their timing of expression (Wu et al., 
2019). Early markers are defined as transcripts that are expressed before the developing 
PSN has reached the target. Transient classification defines the transcripts that are 
expressed during the innervation and then down regulated. The final and latest 
classification, late markers, are first expressed during late embryonic and early post-natal 
stages of development. This study will focus on the expression patterns of two genes 
which are classified as late markers: Heg1 and B3galt2.

These genes show normal expression in various systems of the body. Most 
notable areas that B3galt2 and Heg1 can be found are in hippocampus and developmental 
heart in mice. B3galt2 products belong to a family of membrane-bound enzymatic 
proteins involved in galactosyltransferases (GalTs) (Amado et al., 1998). Various studies 
have shown Heg1 to be a marker and a target of interest for mesothelioma diagnoses and 
treatments (Tsuji et al., 2017). However, within the DRG, PSNs have been observed to 
uniquely express several genes, including Heg1 and B3galt2, which identifies them as 
some subclass of PSNs (Wu et al., 2019). Both genes begin to show expression at post-
natal day 0 (P0) in mice and have been observed to keep expression up to at least P21 
(Wu et al., 2019). As previously discussed, developmental gene expression follows a 
temporal pattern, divided into early, transient, and late markers. We will investigate how
expression of these two markers respond to PNI as these PSNs must regrow axons to MS and GTOs.

**Peripheral Nerve Injury (PNI)**

In this study, only peripheral nerve injuries (PNI) induced by application of kinetic energy to the nerve were studied. This is a common pathology underlying many PNIs (Robinson, 2000). PNIs of this kind can be seen in 1% of motor-vehicle accidents and are seen in 2% of the patients admitted into level 1 trauma centers (Noble et al., 1998). This is often the result of penetrating wounds, blunt or fall trauma, and work-related accidents (Kouyoumdjian, 2006; Missios et al., 2014; Kouyoumdjian et al., 2017). However, one problem when it comes to diagnosing these cases, is that patients are usually suffering from more imminent and life-threatening injuries. This leave diagnoses of a PNI days after admission (Noble et al., 1998; Robinson, 2000). Other causes of PNI that are not considered in this project include those induced by thermal, chemical, hypoxic, or disease-driven injuries.

**Acute Injury**

There is a cascade of events that the body will use to respond to a PNI. When the axon is severed, an immediate influx of extracellular cations flow into the cell (Ziv and Spira, 1993). This depolarization will send high frequency action potentials from the site of injury to the cell body, alerting that the cell membrane is compromised (Navarro et al., 2007; Raivich and Makwana, 2007). The cut axon can take up intracellular material from the surrounding Schwann cells that are also damaged. These contents enter the injury site before the membrane is resealed and are then transported back to the cell body in the
DRG (Ziv and Spira, 1993). This retrograde axonal transport is of foreign cellular material thought to initiate the process of axon degeneration (George et al., 1995). In rodents, axon degeneration begins 24-48 hours after an injury has occurred (Tsao et al., 1999; Beirowski et al., 2005). Humans can take up to 7 days until axon degeneration begins, and the process is dependent on the distance of the axonal injury from the cell body (Chaudhry and Cornblath, 1992).

During the acute injury phase, removal of debris and promoting neuron survival and repair are critical (Kang and Lichtman, 2013). Schwann cells will release cytokines to activate resident macrophages, who are the primary clearers of cellular debris, to the injury site (Gaudet et al., 2011). Macrophages that were activated by Schwann cells during the first days after the injury will remain at high levels for up to 7 days. Initially, these macrophages will release pro-inflammatory cytokines and then switch to anti-inflammatory cytokines to promote healing and regeneration (Chen et al., 2015).

Additionally, Schwann cells will release neutrophins to promote neuronal survival and regeneration (Scheib and Höke, 2013). These neutrophins are transported from the injury site to the soma and activate phosphorylation cascades (Raivich and Makwana, 2007) which in turn changes expression in over 60 proteins (Komori et al., 2007). Upregulated proteins, including antioxidant and metabolic proteins, protect injured neurons from oxidative damage. Some proteins are down-regulated, such as proteins that help with lipid-biosynthesis, due to an increase of free fatty acids from the injured axon and cellular debris (Fu and Gordon, 1997; Komori et al., 2007). Following the acute phase, the immune response is decreased, but healing and regrowth of axons will continue for months after the injury (Gaudet et al., 2011; Chen et al., 2015).
Regeneration

After acute injury phase of PNI, reinnervation of severed neurons to their target is time sensitive. Meaningful reconnection of axons to target tissues in humans can last up to 10-12 months and 35 days in adult mice (Ma et al., 2011). During this time frame, which can be phrased as the critical period for reinnervation, neurons undergo a transformation from “transmitting mode” to “growth mode” (Fu and Gordon, 1997; Komori et al., 2007). Urgency after the acute injury phase stems from the need for neurons to regrow axons before 3 factors: degradation of basal lamina tubes from neighboring Schwann cells, muscles atrophy due to loss of innervation, and an increase in expression of growth-inhibiting chondroitin sulphate proteoglycans (Zuo et al., 1998; Scheib and Höke, 2013).

Although regrowth of axons is time sensitive, the growth rate of axons only reaches up to 1-3 mm/day (Sunderland, 1947; Verdú and Navarro, 1997). Due to this slow regrowth rate and a short critical period, increasing this growth rate has been the focus of many studies. Both electrical stimulation and exercise have been shown to accelerate regeneration of both sensory and motor fibers (Elzinga et al., 2015; Gordon and English, 2016). Although these methods were able to accelerate axon regrowth, patients still exhibited disability and motor deficits (Wong et al., 2015). Regrowth rates differ between smaller diameter neurons and large diameter neurons (Kang and Lichtman, 2013), with smaller neurons demonstrating quicker regrowth rates and greater functional recovery. For smaller tactile-sensing neurons that innervate the skin, functional recovery is equivalent to reinnervation of the target tissue (Verdú and Navarro, 1997).
Meaning that when smaller neuron axons reinnervate the target, improvement in function can be seen at the same time.

Reinnervation of specialized sensory organs, like MS and GTOs by PSN afferents, adds complexity to regeneration that smaller diameter neurons with free-nerve endings in the skin do not experience (Verdú and Navarro, 1997; Vogelaar et al., 2004). For example, the longest distance traveled by reinnervating PSN axons in the mouse is to reach the plantar muscles in the distal foot, a process that in mice can take up to 21 days after a nerve crush injury. However, the degree and complexity of reinnervation of stretch receptors in plantar muscles continue to increase even up to 40 days after the crush injury (Verdú and Navarro, 1997). This demonstrates that reinnervation of specialized organs extends several weeks past the time of injury, which is well past the critical period for axon regrowth (Ma et al., 2011). Once past the critical period for regrowth, additional time will not show improvement in function (Wang et al., 2015).

**Monosynaptic Reflex after Injury and Regeneration**

Studies that examine nerve regeneration after injury, observed that the monosynaptic reflex did not return to normal function even though motor and sensory fibers had reinnervated their respective tissues (Verdú and Navarro, 1997; Haftel et al., 2005; Bullinger et al., 2011; Prather et al., 2011; Wang et al., 2015). The monosynaptic reflex is a simple mechanism that demonstrates the connection between PSNs and motor output. The reflex is initiated when a muscle fiber stretches, and MS are stretched along with the muscles. The MS encodes this change in muscle length and fire action potentials. These signals are conducted along the afferent axon to the spinal cord where a synapse occurs between the afferent axon and a motor neuron. This motor neuron, which
innervates the same muscle fiber as the activated MS, receives excitatory input from the MS and fires its own action potential. This will lead to contraction of the previously stretched muscle fiber. Constant activity of this reflex maintains our posture and balance.

Deficits in the monosynaptic reflex after PNI are partially explained by structural changes within the spinal cord circuitry. After a PNI, the total number of synapses between PSNs and motor neurons is decreased (Bullinger et al., 2011; Schultz et al., 2017). However, even in experiments that exclude spinal circuitry from analysis, deficits within the monosynaptic reflex are still observed (Hyde and Scott, 1983). This demonstrates that abnormalities in this reflex cannot be fully attributed to changes in spinal cord circuitry. Persisting functional deficits in the monosynaptic reflex after regeneration are multifactorial and identifying all these factors is of high importance (Hyde and Scott, 1983; Vincent et al., 2015). However, no research has explored the intrinsic properties of PSN and the affects these properties have on nerve recovery.

**Transection vs Crush Injury Studies**

Effects of PNI and recovery has been observed and heavily researched using two different injury models. The first case of PNI is a nerve transection, in which all the axons in the nerve are completely severed and detached. The second case is a nerve crush where axons are still damaged, but basal lamina tubes are still intact. The nerve crush injury still allows neurons to follow the basal lamina tubes back to their original receptors (Hyde and Scott, 1983; Robinson, 2000). In transected nerves, axons no longer have a guided path during reinnervation and experience non-specific reinnervation, and can end up innervating the wrong muscle or receptor (Collins et al., 1986; Banks and Barker, 1989).
Non-specific reinnervation is a result of afferent axons reattaching to incorrect receptors. For example, a PSN that initially innervates a MS, reinnervates a GTO after PNI. After reinnervation, PSNs are thought to take on the properties of their new receptors. Now in this example, a PSN that once responded to activity from a MS, muscle stretch, now responds to activity from a GTO, muscle contraction (Collins et al., 1986; Banks and Barker, 1989). After non-specific reinnervation, the circuits in the spinal cord that process information from the PSNs often receive incorrect feedback signals (Pierrot-Deseilligny et al., 1981). This is due to the PSN reinnervating a new target receptor which encodes different activity, while retaining its original synapses in the spinal cord.

The consequences of non-specific reinnervation could provide explanation as to why there are persisting abnormalities in the monosynaptic reflex following regeneration. This theory has been researched by examining the monosynaptic reflex after a nerve crush and allowing regeneration to proceed (Prather et al., 2011). Results demonstrated that abnormalities were still observed even when injured axons retraced paths to their original receptors through intact basal lamina tubes. Additionally, input coming from PSN to motor neurons decreased by 30% (Prather et al., 2011). However, even though proprioceptive input was decreased, muscle contraction from the reflex had an overall increase. These changes provided evidence that changes in spinal cord circuitry is responsible for this increased motor response (Schultz et al., 2017). Specific mechanisms for causing these changes have still yet to be identified. Transecting a peripheral nerve showed a greater decrease in proprioceptive input onto motor neurons than a nerve that was crushed (Alvarez et al., 2011). Instead of an increase of motor output, regenerated nerves after a transections result in a decreased motor output (Haftel et al., 2005).
Failure of the monosynaptic reflex to return to normal has been primarily attributed to many factors, including non-specific reinnervation and changes in spinal cord circuitry. However, research regarding possible intrinsic changes to the PSN neurons that undergo axotomy, and then must regrow their axon and reinnervate muscle targets, has yet to be conducted. Investigating these properties and comparing observations between transected and crush nerve injuries could reveal additional mechanisms relevant to the failed recovery of the monosynaptic reflex.

How will expression of PSN genetic markers change in response to PNI? Will the developmental sequence reset while the nerve regenerates? If so, would we see genes that are usually expressed in embryonic stages resurface in more mature subjects? Another alternative could be that PSN marker genes that are normally expressed in adult mice may be downregulated while other functions take over during recovery. To answer these questions, this study focused on the effect of PNI on two genes, *Heg1* and *B3galt2*, which have been stated to be expressed uniquely in proprioceptors during post-natal stages of development (Wu et al., 2019).

We studied if gene expression in adult wild type mice differs from experimental mice undergoing transection surgeries. This study required the development of tools necessary to detect changes in these intrinsic properties of PSNs. Specifically, we cloned and generated anti-sense RNA probes to detect expression of *B3galt2* and *Heg1* in lumbar DRGs using *in situ* hybridization after transection of the sciatic nerve.
II. Materials and Methods

Animals

Experimental procedures with animals were conducted according to National Institutes of Health guidelines and with the approval of the Institutional Animal Care and Use Committee at Wright State University. Animals used in this study were C57BI/6J mice of both sexes. Tissue was collected from one animal for each condition: control, sham surgery, and transection. To collect tissue for control experiments to test in situ hybridization protocols, animals were euthanized at postnatal day 28 (P28). Experimental animals that underwent either nerve transection or sham surgeries were 8-10 weeks of age at the time of surgery. All surgeries were performed by the same veterinary technician at Wright State to minimize experimental variability. For each surgery, mice were anesthetized by inhalation of 2-5% isoflurane. The left sciatic nerve was exposed by separating the hamstrings using blunt dissection. For experimental animals, transection injury was applied by completely transecting the left sciatic nerve. The cut nerve was then rejoined with epineural sutures. For sham surgeries, the nerve was simply exposed. After all surgeries, injured and sham, the skin was reclosed, and the animals were allowed to recover for 10 days before being euthanized for tissue collection.
**Tissue Samples**

The tissue samples were first fixed with 4% paraformaldehyde for 24 hours, then equilibrated in a cryoprotectant solution of 30% sucrose in PBS. Relevant tissue samples were then dissected and frozen in compound (Fischer Scientific) and frozen at -80°C until sectioning on a cryostat. All tissue was sectioned at a thickness of 20 µm, mounted on slides, and frozen at -80°C until being used for *in situ* hybridizations. Control tissue sections included both sagittal and coronal sections of brain and transverse cross-sections of L4, L5, L6 spinal cord with DRG on both sides. Experimental and sham surgery tissue samples were embedded so that longitudinal sections of L4, L5, L6 spinal cord with DRG on both sides were visible on each section.

**Creating anti-sense RNA Probes**

Probes were made by subcloning regions of full-length cDNA clones obtained from Dharmacon (B3galt2: 6392262; Heg1: 30645937). PCR primer sequences to amplify regions of the cDNA clone were taken from Wu, et al. (2019) or novel primers were designed to amplify 500-900 bp regions. Primer sequences used were: *Heg1* (forward: ACTTCCAAATGTCCCCATACAC; reverse: CCAGCCCAATCTATTAAGTGCA), B3galt2 (forward: TCACAGGGCTGCAGAACA; reverse: TGCCTGCTTTTCTTCCAC). PCR fragments were then ligated into TA Cloning pCR™II Vectors. Resulting colonies were screened using PCR to determine orientation in the pCR™II vector using each respective forward primer paired with Sp6 or T7 primers. Thermocycler temperatures were adjusted for each marker to account to different melting temperatures (Tm) of primers. If two primers had differing Tm, the lowest Tm of the two was used. Insert size was confirmed using agarose gel.
electrophoresis with ethidium bromide dye on agarose gel and orientation was confirmed by sequencing (GeneWiz). Subcloned plasmids were then linearized and purified using phenol/chloroform/isoamyl alcohol (PCI) Extraction. Finally, digoxigenin-labeled riboprobes were made using SP6/T7 DIG RNA labeling mixture and were confirmed using agarose gel electrophoresis. Concentrations and purity of all PCR products and RNA probes were analyzed using a spectrophotometric Nanodrop device (Thermo Scientific).

**In Situ Hybridization**

In situ hybridization analysis for Heg1 and B3galt2 were performed on 20 µm cryostat sections using digoxigenin-labeled cRNA probes and a three-day protocol adapted from (Arber et al., 2000). The general outline of the protocol is given below.

**Day 1**: Tissue sections on slides were passed through a series of solutions including: 4% paraformaldehyde fix, phosphate buffered saline (PBS), proteinase K solutions, an acetylation buffer, and formamide solution to prepare the tissue for hybridization with DIG-labeled RNA probe. Probes were applied to the tissue samples and incubated overnight (16 hours minimum) at 65°C.

**Day 2**: After incubation, slides were washed in solutions of: 5X sodium citrate buffer (SSC) and .2X SSC at 65°C. Afterwards, slides were soaked with 1 mL of PBST (PBS + Tween20) and allowed to rest in a moist chamber for 5 minutes. The PBST solution was poured off and replaced with 1 mL PBST with 10% Normal Goat Serum (NGS) for 1 hour. After 1 hour, PBST/NGS solution was poured off and replaced with 200 uL 1:5000
ratio of anti-DIG antibody in PBST/1% NGS. This would incubate overnight in a moist chamber at 4°C.

**Day 3:** Slide were washed with PBST for 5 minutes twice. Afterwards, 1 mL pf B3 solution was applied for 15 minutes twice. The developing solution (B3/BCIP Reagents) was made and applied to the tissue sample. After application of the developing solution, the moist chamber containing the tissue samples was covered in aluminum foil to prevent exposure to light. The developing solution was left on the slides for several hours at room temperature and moved to 4°C overnight. After staining was complete, cover slips were applied to dry slides using Dako glycergel mounting medium heated at 55°C.

**Analysis**

All images were taken using brightfield microscopy with an Olympus BX51 microscope and cellSens software. Individual DRGs were isolated on the field of view using either 10x or 20x objective lenses. Images were analyzed using Image J software. The area of the DRG was noted utilizing the software, as well as counting the number of positive neurons in the DRG. Positive neurons were counted if the cell within the DRG showed strong expression (dark staining) for the gene of interest.
III. Results

Cloning

One goal of this project was to produce anti-sense DIG-labeled RNA probes from full-length cDNA clones of $B3galt2$ and $Heg1$. The full-length clones were obtained from Dharmaco and partial sequences from these clones were identified for PCR amplification to serve as the probes. Figures 1 and 2 illustrate the full cDNA sequence of $B3galt2$ and $Heg1$, respectively, as well as the regions used as template for the probe. Regions of interest were amplified using PCR with the forward/reverse primers that are highlighted in the figure. The amplified insert needed to be ligated into TA-cloning pCR™II vectors to create the subclones with necessary restriction enzyme cut sites and T7/SP6 primer sites. This cloning strategy takes advantage of the fact that Taq polymerase leaves insert 3’ – Adenine (A) overhangs at the end of each DNA molecular synthesized during PCR. The pCR™II vectors come with 5’ – Thymine (T) overhangs on both strands. These 5’-T overhangs are complimentary to the 3’-A overhangs of the PCR and addition of T4 DNA ligase fuses the inserts into the empty plasmid vector. The orientation of the insert after ligation is random, however it is critical to know the orientation in order to create an anti-sense riboprobe.

Producing the probe is dependent on the orientation that the insert was ligated into the pCR™II vector, so several steps were taken to determine the orientation of the
inserted PCR product. The first step of screening was to treat bacterial colonies with X-Gal, which transform empty colonies, those that ligated together without the insert blue. Colonies that had taken up the insert remained a white color. To confirm that the blue colonies (X-Gal treated) did not contain the insert, those colonies went through PCR with the T7/SP6 primers and revealed very small bands, on the order of 200 base pairs in length, which corresponds to the distance between the primer sites on the empty plasmid vector, as shown in Figure 3. Orientation of both probes were screened by using PCR with two different combinations of primers (forward/T7 and forward/SP6) on several bacterial colonies containing the insert. Figures 4 and 5 visualize the agarose gels that confirmed the orientations of B3galt2 and Heg1 respectively. Both subclones required forward/SP6 primers to be amplified and required SP6 RNA polymerase to create the DIG-labeled RNA probe. Plasmid vector maps for both B3galt2 and Heg1 are shown in Figures 6 and 7, respectively, and illustrate the insert orientation, primer sites, and restriction enzyme (NotI) cutting site used to linearize the plasmid before polymerizing the RNA probe.

Linearization was confirmed using agarose gel with the linearized plasmid adjacent to their respective open-circular/supercoiled plasmids (Figure 8). The open-circular DNA traveled across the gel at the slowest speed and showed a higher band size than its actual length. The supercoiled DNA traveled the fastest, and instead showed a smaller band size than its actual length. The linearized DNA then traveled at an intermediate speed and revealed a band below the open-circular plasmid and above the super-coiled plasmid.

**In Situ Hybridization on Control Tissue**
To validate the efficacy of the DIG-labeled riboprobes, we first performed *in situ* hybridization on sections from control tissue from P28 uninjured, wild-type animals. Sections of sagittal and coronal brains and cross-sections of spinal cord and adjacent DRGs were used. Staining on the brain was used as confirmation for the specificity of both $B3galt2$ and $Heg1$ individually. $B3galt2$ hybridization showed specific staining in the hippocampus and DRG, shown in Figures 9 and 10, respectively. In mouse hippocampus, $B3galt2$ has been found to be expressed specifically in the dentate gyrus and the entorhinal cortex.

$Heg1$ hybridization showed specific staining on the lumbar DRGs (Figure 11). $Heg1$ was expressed by only a fraction of the neurons in the DRG of control tissue. In an attempt to quantify the number of cells expressing $Heg1$ for future studies and comparison, cell counts were normalized by the area of the DRG within the focal plane sectioned onto the slide (Table 1). Each DRG was analyzed using Image J software to ascertain the area of the DRG cross-section and to count the number of positive neurons that show expression of $Heg1$. From the 7 DRGs sections analyzed, there were an average of 7.83 neurons per 50,000 $\mu$m$^2$.

$B3galt2$ hybridization showed specific staining in the hippocampus and DRG, shown in Figures 9 and 10, respectively. In contrast to $Heg1$, it appeared that every neuron in the control DRG samples showed expression for $B3galt2$. The lack of staining in adjacent tissues (spinal cord, peripheral nerves, and muscles suggested that this staining was not non-specific. A fraction of the spinal cord on Figure 10, in the bottom left, shows that no spinal cord neurons were hybridized. While all DRG neurons appeared
to express *B3galt2*, there were a select few neurons, per DRG, that showed greater expression than others.

**In Situ Hybridization with Sham and Transection-injured Tissue**

Having confirmed the specificity of the *Heg1* and *B3galt2* probes, we examined expression on 10-week-old animals that had undergone sham-surgery or transection surgery. From literature, *B3galt2* and *Heg1* were known to be expressed up to at least P21 in the DRG, and our control experiments confirmed expression in P28 animals, but we wanted to test if they still showed expression in even older animals. Both *B3galt2* and *Heg1* have been classified as late markers, meaning they are first expressed after successful innervation during normal development (Wu et al., 2019). We reasoned that expression may be down-regulated at the 10-day post-injury time point we investigated because the majority of PSNs will not have regrown to their target muscles.

*B3galt2* showed similar staining to control hybridizations, where every neuron showed expression (Figure 12 and 13). However, general intensity of expression was stronger in control versus sham/injured tissue, but differences may be due to different lighting fixtures used on the microscope when taking the picture. Again, there are select cells that show higher expression than others, however data is inconclusive if these cells are PSNs or some other sensory neuron. Replication of this procedure would need to be done on other tissue. *Heg1* expression was very weak and found in only a few scattered cells in DRGs from either the sham or injured DRG tissue (Figure 14 and 15). So, few cells were labeled that it was impossible to confidently quantify and normalize expression as was done for the P28 control DRGs. One possible explanation for this non-result is
that *Heg1* is not expressed in older animals; alternatively, it may be the result of a technical issue during hybridization.
Table 1: Measurements of each *Heg1* expressing neuron in several DRG section from P28 wild-type tissue. The cross-sectional area (in µm²) of the DRG was measured on each tissue section. This was used to normalize the expression data to determine that, on average, 7.83 neurons expressed *Heg1* per 50,000 µm² of DRG area in this tissue.

<table>
<thead>
<tr>
<th>Heg1 DRG</th>
<th># of Expressing Neurons</th>
<th>DRG Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG 1</td>
<td>8</td>
<td>68,470</td>
</tr>
<tr>
<td>DRG 2</td>
<td>12</td>
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<td>DRG 3</td>
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<tr>
<td>DRG 10</td>
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B3galt2 cDNA sequence (BP: 1 -2041)

1 ggcaaagcct tttttttccc ccaatgcaa ctgaaacact aaaccacagc tctgctgctt 61
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cattgtccag cggggaatgc agcagatgta aagagcttca atgcatacaac tgtgcgaagaag 181
agtcaactgt gcacccaaata caacagacag ctacagctct tttggtatact gtgaaaagca atcacaagag 241
aatgaagaag aagaaaaaat ctctgaaagac tataagatat agacatagtc tcaagaaagag 301
taactttgaag acacccgcag agatggacac ttttcatgctt gtaaaaagca atcacaagag 361
gcagactgtt gggggtatgt cgcatgttgc atagcatcgt tttttgtgta agtgatggcg 421
tgcaaaaaag attttcaagta gacataatac ttcctcatct atggtctgac caagaaagaa 481
agaatacatgc cagggagcct gatcctggaac cagaagacag tgaatcaagag gcaagtattga 541
tcggaccccc aacaacatgt tttttggccaa cagacataac ttactgtcat tggataccta 601
cacaatgtct cagttgagaa gacgacactg ctgctttgccca aaatgatcct ggacctcataa 661
gaggtctctctg ccctggaacc tccctacgctct cttgcttctct ctggttcttat tctttctatat 721
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tatcaatgag cctgaaatgc caagcagaaag ctctttcattt ttaatactat taataagctgc 1081
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ttctggggat ctggcagaga agatatagttt ggttttcttta ggtatatgtctgctt 1621
Figure 1: Full \textit{B3gal2} cDNA sequence spanning more than 2 kilo-bases Dharmacon (NCBI accession number: BC046322). Highlighted sequences indicate the forward primer (green) and the reverse compliment of the reverse primer (blue) respectively. The 918 base pair region between the two primer sites (yellow) was amplified by PCR and subcloned in order to create the anti-sense RNA probe.
Heg1 cDNA sequence (BP: 2341 – 3241)

2341 ctgttgccaga aagagttaaa atgacataag taaaactc ac ttcaaagtg gggaacttcca
2401 aatgtccca tacaag atg ctcctgctcg caagagtggg ggcgaagaagc
2461 cattgaatg caagcagacc gaagcacaac aaacaacctg cagatgacac acgtgtacta
2521 ctcgccccac aacgttaag caaacagctcgtgacagcgaccctcacac
2581 tggactgc ggtcagcgac actcttgcgt catctccccagac ggttataac ccctctctctat
2641 cagcgcagag agcaggagga gagaactactt ctgagtccag gacagaggg gaggccccccq
2701 ttctcttgac ccaacctccc gcacatctgg ctcagacac tacacccccaa aagggagaagca
2761 ccatgcacta gctgctcttg ggaactccgc gacagacagt taggcccgt ggcacacagtq
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3001 gcaaccttat agatggggtt ggcacacttc cacactgtgt ctagaggcccc ctaggggcgc
3061 tcgagtcggg tgcagagagct ttaaacgatt ctgcggtttgc cgtgctcatt tccagccccq
3121 tcgacaatg cacagctgtg catttcgaag gctcaacacaa aacgcttttt atgtcagagtq
3181 gcacagtttg cttcaaatgt atagttttta cttccccag caaggtgaa tcgggcccqa
3241

**Figure 2:** Partial Heg1 cDNA sequence (from base pairs 2341 to 3241) obtained from Dharmaco (NCBI accession number: BC076596). Highlighted sequences indicate the forward primer (green) and the reverse compliment of the reverse primer (blue) respectively. The 629 base pair region between the two primer sites (yellow) was amplified by PCR and subcloned in order to create the anti-sense RNA probe.
Figure 3: Agarose gel of PCR screening of three bacterial colonies that turned blue when treated with X-Gal, signifying they did not contain a PCR insert. Lanes 1, 2 and 3 used T7/SP6 primers as the forward/reverse primers in this PCR. This experiment was run as a control test to determine if treatment with X-Gal, which turns colonies without the insert of interest blue and colonies with the insert white, was viable. While all three colonies displayed bands of the expected size (about 200 bp), colony number 2 (indicated with *) showed the strongest band. The PCR control on the right shows the expected size of the Heg1 insert and is bigger than the small bands produced from the three colonies, further evidence that these blue colonies were empty plasmid vectors.
Figure 4: Agarose gel of $B_{3galt2}$ visualized to screen for the orientation of the subclone. Lanes in the top image used the $B_{3galt2}$ forward primer in combination with the SP6 primer as the reverse primer for PCR. The same colonies were also screened using the $B_{3galt2}$ forward primer in combination with the T7 primer as the reverse primer in the bottom lanes. A 100 base pair DNA ladder is shown to estimate the band sizes. Reactions with a clear, single band results (indicated with *) were used to create the probe.
**Figure 5**: Agarose gel of *Heg1* visualized to screen for the orientation of the subclone.

Lanes in the top image used the *Heg1* forward primer in combination with the SP6 primer as the reverse primer for PCR. The same colonies were also screened using the *Heg1* forward primer in combination with the T7 primer as the reverse primer in the bottom lanes. A 100 base pair DNA ladder is shown to estimate the band sizes. Reactions with a clear, single band results (indicated with *) was used to create the probe.
Figure 6: A vector map of the final subcloned insert for the B3galt2 probe. Not1 restriction enzyme digest was used to linearize the plasmid, followed by SP6 primer to create the antisense riboprobe.
Figure 7: A vector map of the final subcloned insert for the Heg1 probe. NotI restriction enzyme digest was used to linearize the plasmid, followed by SP6 primer to create the antisense riboprobe.
Figure 8: Agarose gel with linearized plasmids of B3galt2 and Heg1 adjacent to their open circular and supercoiled plasmids which are in the same lane. The open circular DNA will travel the least along the gel and the supercoiled DNA, although the same base pair length, will travel the fastest. The linearized DNA will run at an intermediate speed and end up in between the two other bands.
**Figure 9: B3galt2 Expression in the Hippocampus in 4-week-old WT Mice** – Hybridization on 4-week-old WT mice using B3galt2 riboprobe. The hippocampus is a known landmark where B3galt2 is expressed and was used to confirm both hybridization techniques and use of the probe. The hippocampus is visualized by the dark purple staining neurons, which express B3galt2. Scale bar equals 200 µm.
Figure 10: B3galt2 Expression in the Lumbar DRG in 4-week-old WT Mice –

Hybridization on 4-week-old WT mice using B3galt2 riboprobe. This experiment was used to confirm that hybridization techniques and riboprobe could accurately visualize neurons in the DRG. Expression in WT mice at 4-weeks show that every neuron in the DRG express B3galt2. Scale bar equals 50 µm.
**Figure 11:** Heg1 Expression in the Lumbar DRG in 4-week-old WT Mice - Hybridization on 4-week-old WT mice using Heg1 riboprobe. This experiment was used to confirm that hybridization techniques and riboprobe could accurately visualize neurons in the DRG. Expression in WT mice at 4-weeks show select neurons express Heg1 and were observed to be PSN. Scale bar equals 100 µm.
**Figure 12:** $B3galt2$ Expression in the Lumbar DRG in 10-week-old Mice with Sham Surgery – Hybridization on 10-week-old mice using $B3galt2$ riboprobe. Visualization of longitudinal DRG show that all neurons in the DRG express $B3galt2$. Scale bar equals 100 µm.
**Figure 13:** *B3galt2 Expression in the Lumbar DRG in 10-week-old Mice with Injury* – Hybridization on 10-week-old, injured mice using *B3galt2* riboprobe. Visualization of longitudinal DRG show that all neurons in the DRG express *B3galt2*, but at lower intensities than control and sham surgery tissue. Scale bar equals 100 µm.
Figure 14: *Heg1 Expression in the Lumbar DRG in 10-week-old Mice with Sham Surgery* – Hybridization on 10-week-old mice using *B3galT2* riboprobe. No neurons in the DRG showed positive results for *Heg1* hybridization. Scale bar equals 100 µm.
**Figure 15:** *Heg1 Expression in the Lumbar DRG in 10-week-old Mice with Injury* – Hybridization on 10-week-old mice using *B3galt2* riboprobe. There are results of faint staining the on the DRG tissue, but no positives for neurons expressing *Heg1*. Scale bar equals 100 µm.
IV. Discussion

This study developed DIG-labeled RNA probes for the purpose of \textit{in situ} hybridization to investigate the levels of expression of two genes, \textit{B3galt2} and \textit{Heg1}, in lumbar DRG. Development of these probes confirmed that this tool can be used to investigate expression of various markers of PSN subclasses. Further investigation of other PSN markers, including those that are only expressed transiently early during development will be able to shed light on the currently unknown genetic changes experienced by PSN during regeneration after PNI.

\textit{Creation of RNA Probes}

cDNA clones of \textit{B3galt2} and \textit{Heg1} were ligated into pCRT\textsuperscript{TMII} vectors and confirmed using agarose gel electrophoresis. These gels confirmed the correct length of the inserts of both \textit{B3galt2} and \textit{Heg1} which were 918 and 629 base pairs respectively. Visualizations of both inserts on the gel also validates the use of our forward and reverse primers adapted from Wu et al, 2019. PCR-based screening approaches confirmed with DNA sequencing, were used to determine the orientation of the insert, and using SP6 RNA polymerase to synthesize the probe was successful. A critical step in generating the DIG-labeled probe is the linearization of the plasmid, to prevent the RNA polymerase from transcribing DNA sequences in the plasmid beyond the cloned insert. The use of restriction enzymes followed by visualization on agarose gels were used to confirm the presence of linearized DNA plasmid to create the probe. Three forms of plasmid DNA
are possible after restriction digest. If the digest is successful, the single restriction site on
the plasmid sequence will cut the normally circular plasmid into a single, linear DNA
molecule. If the digest is unsuccessful or incomplete, any remaining circular plasmid will
travel through the agarose gel in one of two forms: open-circular and super-coiled. Each
of these three forms of DNA will travel through the gel at different speeds with open-
circular, linearized, and super-coiled ranked from slowest to fastest, respectively (Cole
and Tellez, 2002). When we ran the linearized product in one lane of the gel, a single
band was observed, suggesting a complete digest of the plasmid DNA. In an adjacent
lane, a small amount of uncut plasmid was run as a comparison. The linearized product
band was in the middle of two bands, which we observed to be the supercoiled (lower
band) and open-circular (higher band) (Figure 8).

**B3galt2 In Situ Hybridization**

Hybridization on control tissue showed positive results, in which specific staining
for *B3galt2* showed on the slide. *B3galt2* hybridizations was able to clearly visualize the
mouse hippocampus (Figure 9) and the olfactory bulbs (not shown) which is where it has
been known to be expressed. *B3galt2* hybridization in control, sham, and injured DRG
showed that all sensory neurons expressed *B3galt2*. This finding was unexpected, as
*B3galt2* was known to be a identifier of PSN subclasses (Wu et al., 2019). Wu et al.
acknowledged that there was a high amount of staining in surrounding cells while
probing for *B3galt2*. Hybridization images from mouse spinal cord atlas at P4 show very
few cells that express *B3galt2*. The difference, between these animal models, is that
animals in our experiments were older. The observed trend is that as an animal continues
through post-natal development, $B3galt2$ begins to be expressed by other DRG neurons in addition to PSNs.

While staining for $B3galt2$ was widespread in the DRG, the staining was still specific, due to lack of staining in spinal cord neurons or other cell types (including glia) found on the tissue section. Although all DRG neurons expressed some levels of $B3galt2$, there were a few neurons that showed much heavier expression of $B3galt2$ than other neurons in the same field of view. However, further investigation would need to be conducted to determine if these darker staining neurons were PSNs or abnormalities with hybridization techniques. It would also be interesting to further investigate the changes of $B3galt2$ expression in animals of different ages, pre- and post-PNI.

**Heg1 In Situ Hybridization**

$Heg1$ hybridizations showed positive specific staining on control tissue in the cerebral cortex neurons and DRG, as reported in the literatures (Wu et al., 2019). $Heg1$ expression in lumbar DRG was normalized to 7.83 neurons per 50,000 $\mu$m$^2$, an abundance consistent with the fact that PSN account for approximately 20% of the number of neurons in the DRG. An unexpected result was that expression of $Heg1$ was not detected in the DRG of sham and injured animals in this study. This was in contrast to the strong staining observed in control tissue, and no neurons showed sufficient staining for confident cell counting. This may be due to a technical abnormality in hybridization techniques. For example, specific probes may require different incubation periods for sufficient staining to occur. Another factor is that these mice are 8-10 weeks of age compared to 4 weeks of age of control mice. During this difference in development, it is possible that $Heg1$ expression levels are downregulated in DRG sensory neurons.
However, it is inconclusive if either of these speculations are valid. Further investigation of expression in older animals, both pre- and post-PNI would need to be conducted.
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