SURVEY OF DORSAL ROOT GANGLION NEURONAL SUBTYPES THAT SYNAPTICALLY CONNECT WITH TRANSPLANTED NEURONS IN THE INJURED SPINAL CORD

An Undergraduate Research Scholars Thesis

by

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This project required approval from the Texas A&M University Research Compliance & Biosafety office.

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TABLE OF CONTENTS

ABSTRACT

Survey of DRG Neuronal Subtypes That Synaptically Connect with NPC Transplants in the Injured Spinal Cord

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Spinal cord injury (SCI) is a devastating event that typically results in immediate and permanent loss of neurological due to massive disruption of neuronal circuits. This can lead to many complications including, but not limited to loss of voluntary motor function, sensory dysfunction, and autonomic dysreflexia. Currently, there are no effective treatment strategies that can successfully cure spinal cord injuries so primary care providers are forced to resort to treating symptoms and preventing further secondary conditions. A promising research avenue for the treatment of spinal cord injuries is neural progenitor cell (NPC) transplantation. In previous studies, NPC derived grafts have been shown to integrate into the hosts nervous system and improve sensory function in mice and primate models after SCI. However, there is no clear understanding of which specific subtypes of sensory neurons are forming synaptic connections with the neural progenitor cell graft. To understand this, we used Rabies helper/Syn1-cre grafts in C4 dorsal column lesions and the monosynaptic rabies tracing approach. By colocalizing rabies positive neurons with other biomarkers visualized through immunohistochemistry we will

be able to see which specific subtypes are forming these connections. Unfortunately, rabies infectivity in the spinal cord and neural progenitor cell graft was too low for rabies infection to reach the neurons of the dorsal root ganglion. Due to this fact, the goals of the project shifted towards identifying if nociceptive specific axons terminated in their subtype specific region of the spinal cords dorsal horn cytoarchitecture formed by the graft. This was accomplished by colocalizing peripherin intensity with calretinin positive neuron clusters. The results showed a small increase peripherin intensity within the calretinin positive subpopulation, but no statistical significance to it.

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

CalB	Calbindin
CalR	Calretinin
CGRP	Calcitonin gene-related peptide
CTB	Cholera Toxin Subunit B
DRG	Dorsal Root Ganglion
IB4	Isolectin B4
NPC	Neural Progenitor Cell
Parv	Parvalbumin
Prph	Peripherin
SCI	Spinal Cord Injury
SP	Substance P

1. INTRODUCTION

Spinal cord injury (SCI) is a traumatic injury that results in lifelong neurological dysfunction. There are approximately 300,000 people suffering from SCI in the United States and approximately 18,000 people receive SCI each year ("Traumatic Spinal Cord Injury Facts and Figures at a Glance," 2022). The most common causes of SCI in the United States are, in order, motor vehicle accidents, falls, and violence. Depending on the level and severity of injury, SCI can lead to a plethora of neurological dysfunctions including paralysis, neuropathic pain, loss of sensory function, orthostatic hypotension, and loss of bladder and bowel function (Sezer et al., 2015). These complications arise from the disruption of neuronal pathways that allow for homeostasis, or alternatively from maladaptive neural plasticity. There are no FDA approved, proven effective treatment options for improving neurological function after SCI, so care providers must focus on treating symptoms alone.

A promising therapeutic approach for the treatment of SCI at its source is the use of neural progenitor cell (NPC) transplantation. NPCs are a type of stem cell that are capable of giving rise to all populations of the cells found within the central and peripheral nervous systems NPC transplantation has been shown to promote neural regeneration after SCI in experimental studies. For instance, NPC grafts transplanted onto sites of SCI have been shown to differentiate and self-assemble in cytoarchitecture commonly found within the spinal cord (Dulin et al., 2018). With the use of the monosynaptic rabies tracing system, synaptic connections formed directly between the transplanted NPCs and the host can be visualized through fluorescent microscopy (Wall et al., 2010). This modified form of the rabies virus lacks the ability to infect and spread on its own and requires a transgenic cell that produces “rabies helper components”

which are the avian retrograde viral receptor protein TVA and viral glycoprotein. With these helper components the modified rabies virus can infect the primary neuron and spread to neighboring neurons that are directly synaptically connected to the primary neuron. Through the use of a Syn1-cre mouse model, rabies helper component expression can be ensured throughout the mouse embryo that NPCs are obtained from. Transplanting these rabies helper components expressing NPCs into the site of SCI, allows us to localize the initial infection of this modified rabies virus to exclusively those transgenic neurons which differentiate from the transplanted NPCs. Following administration of the modified rabies virus into the rabies-helper graft, the rabies will infect the graft neurons and also be transported in a retrograde fashion to the host neurons that are monosynaptically connected with primarily-infected neurons. Through this method, NPC transplants have been shown to form host-graft connections; they are innervated by host neurons within the brainstem, corticospinal tract, spinal cord, and dorsal root ganglion (Adler et al., 2017). This allows for signals from the central nervous system to pass through the graft on their way to caudal targets, potentially reestablishing communication and neural relays that were previously lost due to SCI. This may highlight a potential mechanism for how NPC grafts have been shown to improve motor, sensory, and respiratory function individually in rodent models of SCI (Fischer et al., 2020; Rosenzweig et al., 2018).

Adler et al. (2017) demonstrated that primary sensory neurons in the dorsal root ganglia (DRG) form synaptic connections with NPC grafts in sites of cervical SCI. However, the specific phenotypes of sensory neurons that form synaptic connections with NPC grafts has yet to be investigated. Therefore, we will perform a survey of neuronal subtypes within the DRG that can form functional synaptic connections with the NPC graft. We will focus specifically on DRG because the primary sensory neurons of the peripheral nervous system are housed there. We will

compare these rabies positive neurons in the DRG to where they terminate in the spinal cord from the cytoarchitecture formed by the NPC graft. We will further identify it's resemblance to the cytoarchitecture of the intact spinal cord.

In addition to identifying subtypes of rabies^{+} neurons in the DRG, we will evaluate the extent to which distinct subtypes of DRG neurons innervate NPC grafts with their central (afferent) axon branches. Previous work by Dulin et al. (2018) has shown that nociceptive DRG axons expressing calcitonin gene-related peptide (CGRP) innervate selective post-synaptic target zones in NPC grafts; specifically, zones that are populated with laminae I/II spinal cord neurons. These "dorsal horn-like regions" can be readily identified through their immunoreactivity for laminae I/II specific markers such as calretinin. Hence, we will take advantage of this approach to identify how distinct subtypes of sensory afferent axons innervate NPC grafts with regard to topography.

2. METHODS

2.1 Ethics Statement

Animal studies were performed in compliance with the NIH Guidelines for Animal Care and Use of Laboratory Animals. All experiments utilizing animals were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC).

2.2 Animals

A total of 32 adult male C57BL/6 mice were used for this study. All mice were obtained from Jackson Laboratories. Mice were housed 2-5 to a cage and allowed free access to food and water throughout the study.

2.3 Breeding and Neural Progenitor Cell Isolation

N=4 Rabies Helper (RabH) female mice (Jax #024708) and N=4 wild-type (WT) female mice (Jax #000664) which were within breeding age, received luteinizing hormone releasing hormone (LHRH) injections 4 days prior to pairing. RabH females were paired with N=4 Syn1-Cre males (Jax #003966), and WT females were paired with N=4 WT males. Mating pairs were separated the next morning. 12 days later, pregnant dams were euthanized by overdose with ketamine/xylazine/acepromazine cocktail and embryos were surgically removed from the dams. The dorsal aspect of embryonic spinal cords was collected and dissociated by trypsinization, as previously described (Pitonak et al., 2022). NPCs from WT/WT pairing served as a control (rabies helper negative grafts) while RabH/Syn1-Cre pairing served to make rabies helper positive grafts. Immediately after dissociation, NPCs were isolated and counted by following the procedure used in Pitonak et al., 2022 to ensure 2 million cells were transplanted into each SCI site.

2.4 Spinal Cord Injury/NPC Transplantation/Mono Synaptic Rabies Injection

A C4 dorsal column lesion was administered to N=16 WT female mice to ensure complete lesion of sensory afferent axons. N=12 mice received rabies helper positive grafts and N=4 received rabies helper negative/WT grafts. Each animal received 2 million NPCs per transplant, 58 days after transplantation, a 1.5 microliter solution of $1x10^7$ GC/ml monosynaptic EnvA-G-deleted rabies-GFP combined with cholera toxin subunit B (CTB) was injected evenly across 24 sites around grafts. The procedure form Pitonak et al. was followed for NPC graft creation and transplantation as well (Pitonak et al., 2022).

2.5 Tissue Collection

Seven days after the monosynaptic rabies injection, mice were overdosed with the anesthetic cocktail of ketamine, xylazine, and acepromazine. Next, mice were euthanized and perfused using 50 mL of 0.1 M phosphate-buffer saline injected directly into the heart transcardially. This was followed by 4% paraformaldehyde until fixed. The brains and spinal columns were dissected out and incubated in 4% paraformaldehyde overnight at 4°C to ensure fixation. The following day, samples were moved into a 30% sucrose solution for storage in 4°C. The cervical spinal cord containing the NPC graft was dissected from the mice along with DRGs at each spinal level. DRGs were stored in 96 well plates in 30% sucrose at 4°C and wrapped in aluminum foil to shield the tissue from light until further processing. Similarly, the dissected cervical spinal columns were stored within 1.5 mL tubes in 30% sucrose at 4°C and wrapped with aluminum foil to shield the tissue from light.

2.6 Sectioning and Immunohistochemistry

DRGs from C1 through C8 were embedded in an Optimum Cutting Temperature (OCT) medium and frozen using dry ice. The cells in frozen medium were then sectioned into 15

micrometer thick slices using a cryostat and directly mounted onto gelatin-coated slides. These slides were stored in -20 degrees Celsius. C1 through C8 was selected because these are the DRGs in closest proximity to the graft and would allow for visualization of rabies infection across the cervical region. If rabies positive cells are found the same procedure will be used at the thoracic and lumbar levels to visualize the extent of synaptic connections formed. Spinal column tissue was also frozen within the Optimum Cutting Temperature medium and frozen using dry ice. This tissue was sectioned in the sagittal plane into 30 micrometer slices and placed into a well of a 12 Well plate full of Tris buffered saline (TBS) for free floating storage at 4 degrees Celsius. DRG slides were thawed to room temperature before immunochemistry was performed. Slides were placed in coplin jars and washed three times in tris-buffered saline for 10 minutes. This was performed to remove residual Optimum Cutting Temperature medium from the slides. Once washed, slides were dried of excess TBS and a pap pen was used to outline the tissue on the slide. Next, slides were placed in to a humifying chamber and 150 microliters of blocking buffer (5% donkey serum in tris-buffered saline with 1% Triton-X-100) was added to each slide. Next, slides had 150 microliters of primary antibody and blocking buffer added to each slide. Slides were left in the humidifying chamber at 4 degrees Celsius for 24 hours. The primary antibodies being used are NeuN, Calretinin, Calbindin, CGRP, Peripherin, Substance P, IB4, and Parvalbumin. Tissue is then washed twice more in tris-buffered saline for 10 minutes and then placed back into the humidifying chamber with a 1:1000 dilution of tris-buffered saline with DAPI for 10 minutes. 150 microliters of 1:1000 dilutions of secondary antibodies and blocking buffer were then added to slides before incubating for 2 hours. Slides were given two more 10 minute TBS washes while being protected from light. Lastly, slides were dipped in water and allowed to dry before cover slips were affixed using Mowiol as the mounting medium.

Slides are then placed in slides boxes and stored in the dark at -20 degrees Celsius to protect them from photobleaching. The procedure for immunohistochemistry of the free-floating spinal column sections was performed in a similar way but was washed in tris-buffered saline or incubated while free floating in wells of 12-well plates (for Tris-buffered saline washes) or 48-well plates (for blocking and antibody incubations). Tissue was mounted onto gel-coated slides after the immunohistochemistry process and cover slipped before being stored in the dark at -20 degrees Celsius to protect from photo bleaching. When performing the immunohistochemistry for the free-floating spinal cord tissue, the antibody dilutions used were the same as the dilutions that were optimized for the dorsal root ganglion tissue.

2.7 Antibody Optimization

Antibody dilutions used during immunohistochemistry were optimized through comparison at two different dilutions. First, the dilution recommended by the manufacturer, and a dilution of double that concentration. Whichever dilution produced a clearer image through the Nikon Eclipse upright fluorescence microscope with monochrome camera was chosen for use throughout the immunohistochemistry. Antibodies for calcitonin gene related peptide (CGRP) is commonly used to identify found within small diameter, nociceptive, C-fibers and high threshold mechanoreceptor A-δ fibers, both of the peptidergic variant. Antibodies for substance P are used to identify the same populations of nociceptive, small diameter, peptidergic C-fibers as CGRP. Peripherin antibodies identify the small diameter C-fibers of the nonpeptidergic variant. Biotinylated IB4 antibodies were used to identify small diameter, and nociceptive polymodal C-fibers and low threshold mechanoreceptor A-δ fibers, both of which are nonpeptidergic. Parvalbumin antibodies were used to identify large diameter A-α fibers (both Ia and Ib variants) as well as medium/large diameter A-βII fibers. Calretinin antibody is used to label small

diameter, nociceptive neurons within the dorsal root ganglia that terminate in laminae I or II. Calbindin antibody is used to identify the small diameter, nociceptive neurons of the dorsal root ganglia that terminate in laminae I or II. Lastly, NeuN is used to label the nucleus of all neurons present in the tissue. All secondaries used were in a 1:1000 dilution of secondary antibody to blocking buffer. Antibodies used and their dilutions can be seen in Table 1.

Table 1: Catalog number, RRID, and dilution for each antibody used.

Antibody	Catalog #	RRID	Dilution
Rabbit Substance P	Millipore Cat# AB5060	AB_2200636	1:250
Chicken Peripherin	EnCor Cat# CPCA-Peri	AB_2284443	1:1000
Rabbit Calretinin	EnCor Cat# RPCA-Calret	AB_2572244	1:5000
Chicken Calbindin	EnCor Cat# CPCA-Calb	AB_2572237	1:1000
Sheep CGRP	Abcam Cat# AB195387	AB_2515991	1:1000
Chicken Parvalbumin	EnCor Cat# CPCA-Pvalb	AB_2572371	1:1000
Biotinylated IB4	Sigma Cat# L2140	AB_2313663	1:300
Guinea Pig NeuN	Millipore Cat# ABN90	AB_11205592	1:1500

2.8 TSA Amplification

In an effort to increase visualization of rabies infectivity within the spinal cord, free floating tissue was removed from storage and placed into a 12-well plate filled with Tris-buffered saline (TBS) for 10 minutes while shaking. This was repeated in new wells of TBS two

more times. Tissue was then moved into a 48-well plate for blocking with TBS + donkey serum (5% donkey serum by volume) for sixty minutes while shaking. The primary antibodies chicken GFP (Aves, CAT# GFP – 1020, dilution of 1:3000), Guinea Pig NeuN (cat #, dilution of 1:1000), and goat anti-CTB (Biolegend, CAT# 802001, dilution of 1:2500) were then added to TBS (without donkey serum) and added to tissue within a new well of the 48-well plate. Tissue was incubated in primary antibodies overnight at 4 degrees Celsius. The next day, tissue was moved back into a 12-well plate filled with TBS for 10 minutes while shaking. Again, this was performed two more times in new wells of TBS. Tissue was then moved into a 48-well plate consisting of 3μL biotinylated Donkey anti-chicken secondary in 1mL of TBST (Tris-buffered saline with 1% Triton-X-100) and incubated for two and a half hours at room temperature. Tissue was moved back into a 12-well plate filled with TBS for 10 minutes while shaking. Again, this was performed two more times in new wells of TBS. 4μL of avidin and 4μL of biotin were added to 1mL TBST to create an avidin biotin complex (ABC) solution and was mixed for 30 minutes prior to be pipetted into a well of a 48-well plate. Tissue was then incubated in the ABC solution for 30 minutes while shaking. Tissue was then moved back into a 12-well plate filled with TBS for 5 minutes while shaking. Again, this was performed two more times in new wells of TBS. tissue was then incubated in a solution of biotnyl tyramide in dilutant buffer (dilution of 1:2500 for biotnyl tyramide in 6μL 30% Hydrogen peroxide in 20mL TBS) for 30 minutes while shaking. Tissue was then moved back into a 12-well plate filled with TBS for 5 minutes while shaking. This was performed three more times in new wells of TBS. After washes, tissue was incubated in secondary antibodies (Streptavidin 488 in dilution of 1:800, donkey anti-guinea pig 555 in 1:1000 dilution, and donkey anti-goat 657 in a 1:1000 dilution) to TBST for two and a half hours while shaking at room temperature and shielded from light. Tissue was

again washed thrice in TBS for 10 minutes as described earlier. Lastly, tissue was mounted onto slides and had coverslips affixed using Mowiol as a mounting medium.

2.9 Antigen Retrieval

Antigen retrieval was used in an effort in to increase the visualization of rabies and CTB infectivity within DRG tissue. Sodium citrate buffer was created by mixing 2.94 g of trisodium citrate dehydrate in 1 liter of dH_2O until dissolved for a final concentration of 10 mM. A final pH of 6 for the solution was achieved through the addition of 1 N HCl and ensured through the use of pH test strips. Once slides were thawed to room temperature, they were placed in coplin jars and washed in TBS for 10 minutes, while shaking, three consecutive times. Slides were then incubated in a coplin jar of sodium citrate buffer within an 80°C water bath for 30 minutes. Tissue was then washed three more times in TBS while shaking for 10 minutes. Once washed, slides were dried of excess TBS and a pap pen was used to outline the tissue on the slide. Next, slides were placed in to a humifying chamber and 150 microliters of blocking buffer (5% donkey serum in tris-buffered saline with 1% Triton-X-100) was added to each slide. They were allowed to incubate within the chamber for an hour at room temperature. After blocking, slides had 150 microliters of primary antibody (GFP, NeuN, and CTB) diluted in blocking buffer was added to each slide. Slides were left in the humidifying chamber at 4 degrees Celsius for 24 hours. Tissue is then washed twice more in TBS for 10 minutes and then placed back into the humidifying chamber with a 1:1000 dilution of TBS with DAPI for 10 minutes. 150 microliters of 1:1000 dilutions of secondary antibodies and blocking buffer were then added to slides before incubating for 2 hours. Slides were given two more 10 minute TBS washes while being protected from light. Lastly, slides were dipped in water and allowed to dry before cover slips were affixed

using a Flouromount mounting medium. Slides are then placed in slides boxes and stored in the dark at -20 degrees Celsius to protect them from photobleaching.

2.10 Imaging and Analysis

A Nikon Eclipse upright fluorescence microscope with a monochrome camera and Nikon NIS-Elements software was used for capturing images. 10X and 20X images were exported as TIFF files and analyzed using ImageJ software. Rabies positive neurons could be visualized due to the GFP tag on monosynaptic rabies allowing for GFP positive neurons were colocalized with primary antibodies to see which subtypes of neurons are forming these connections with the NPC graft. Peripherin analysis was performed using the Fiji software. Regions of interest were drawn around the graft as whole using an image of all channels (NeuN, CalR, and Peri). Next this region of interest was overlayed onto a calretinin image of the same graft and the area outside of the graft was removed. Contrast and brightness were then increased to improve visualization of the calretinin clusters. New regions of interest were then draw around each individual cluster. Lastly, on a peripherin only image of the same section the whole graft region of interest was overlayed, and a measurement of area and integrated density was taken. Next the calretinin cluster regions of interest were overlayed and measurements of area and integrated density were taken. This was performed for all sections containing graft tissue across all animals. The sum of the area and sum of the integrated density were taken across all sections for each individual animal for both the whole graft and calretinin clusters independently. Values for intensity were normalized by dividing the summed integrated density by the summed area, allowing for the intensity by pixel area to be found for both the graft and the calretinin clusters. Using this data, the normalized peripherin intensity graph was created using GraphPad Prism.

3. RESULTS

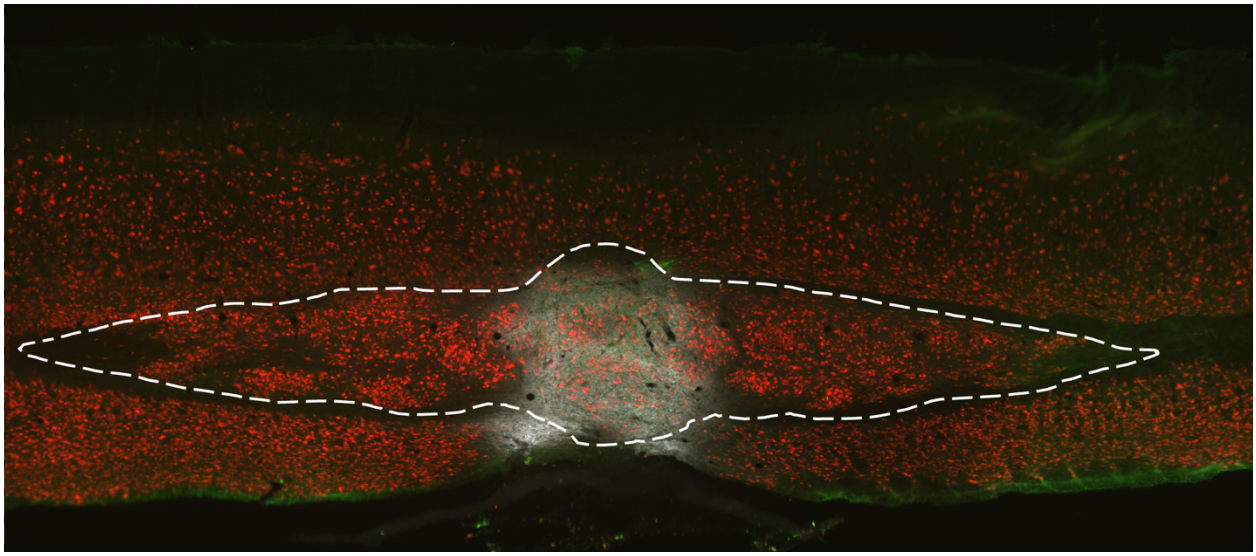

Figure 1: NPC graft outlined by the dashed line. NeuN in red), GFP in green, and CTB in white.

In figure 1 a typical graft can be seen immunostained with NeuN, GFP, and CTB. We found that rabies expression within the grafts was extremely low or nonexistent across all animals (between 1 and 3 positive neurons when infection occurred) despite CTB labeling showing that injections were properly placed within the grafts. TSA amplification did work to improve the visualization of rabies infected neurons, showing an increase from 3 to 28 total infected neurons within the single sample of tissue used, but this extent of infection was still insufficient. Due to this minimal increase, amplification was not performed on tissue samples for the rest of the rest of the tissue samples. Images comparing the visualization of infection between samples of the same animal are shown in figure 2.

Figure 2: GFP visualization in the tissue samples from the same animal before (A) and after (B) TSA amplification.

Although staining for subtype specific markers in the DRG was successful, as seen in figure 3, we did not identify any neurons that were rabies+. This is presumably because it failed to gain adequate infection in the spinal cord. Although CTB seemed to infect neurons on the spinal cord to a greater extent than rabies, it had similarly nonexistent expression in the DRG. Even if CTB had gained infection, it would not have been possible to confirm that CTB positive neurons in the DRG were forming connections with NPC derived neurons since CTB is not selective enough.

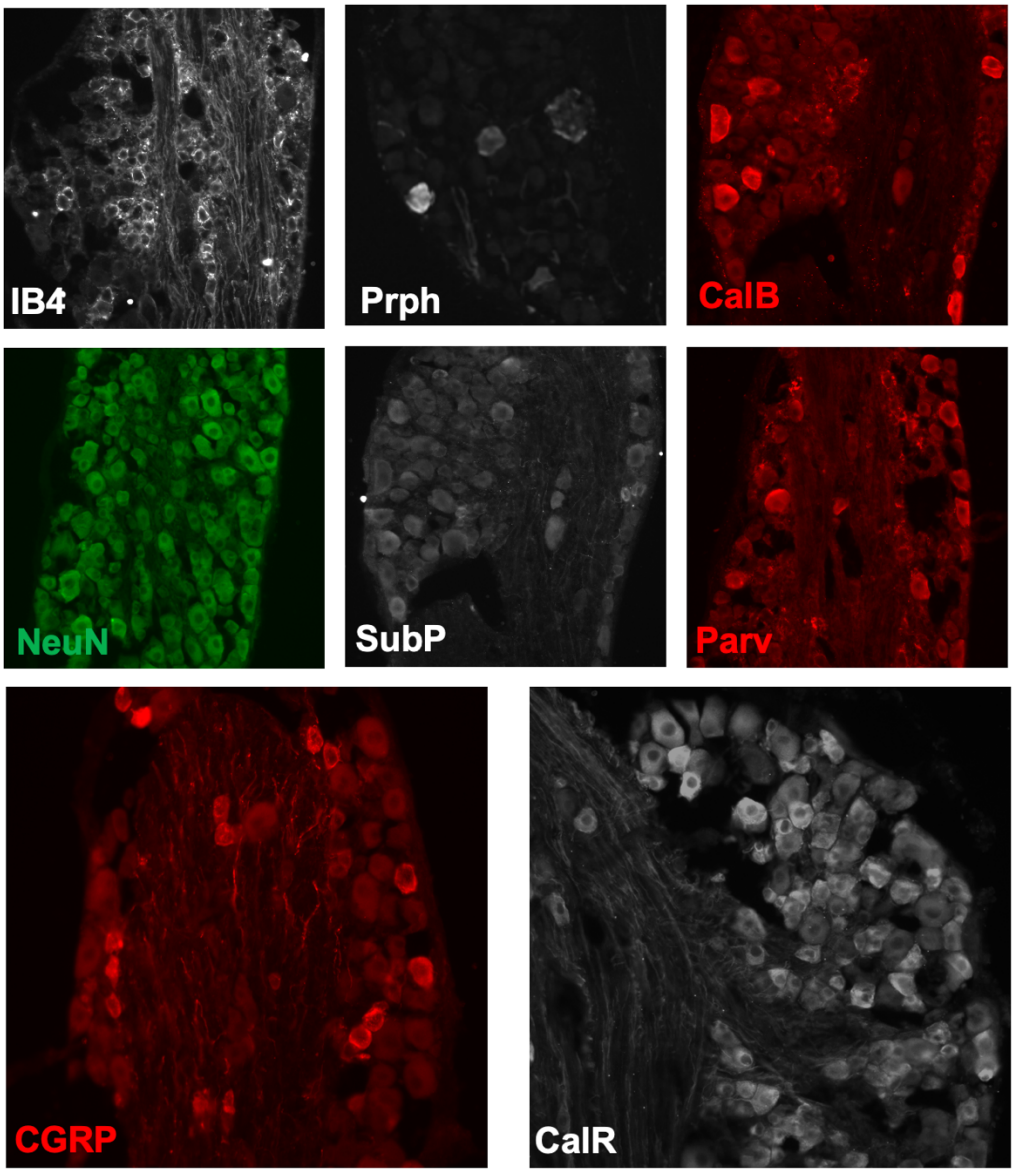

Figure 3: subtype specific stains for neurons within the DRG. All images taken from optimization.

Many factors can play a role in rabies infectivity, and infectivity can vary greatly between batches, even when purchased from the same manufacturer. Another factor that can negatively affect the experimental outcomes of rabies use include improper storage during transit. Since CTB was found within the grafts, it should not be assumed that the injection method used was at fault for the low infectivity. As for lack of results from dorsal root ganglion

tissue, human error may be to blame. Over fixation can cause DRG tissue to have poor binding from the blocking buffer, primary, and subsequently secondary antibodies leading to little or no visualization of the desired neuronal markers (Arber, 2002). In an effort to reverse the effects of over fixation, antigen retrieval was attempted, but it unfortunately failed to work. Comparisons of DRG tissue from the same animal with and without antigen retrieval can be seen in figure 4.

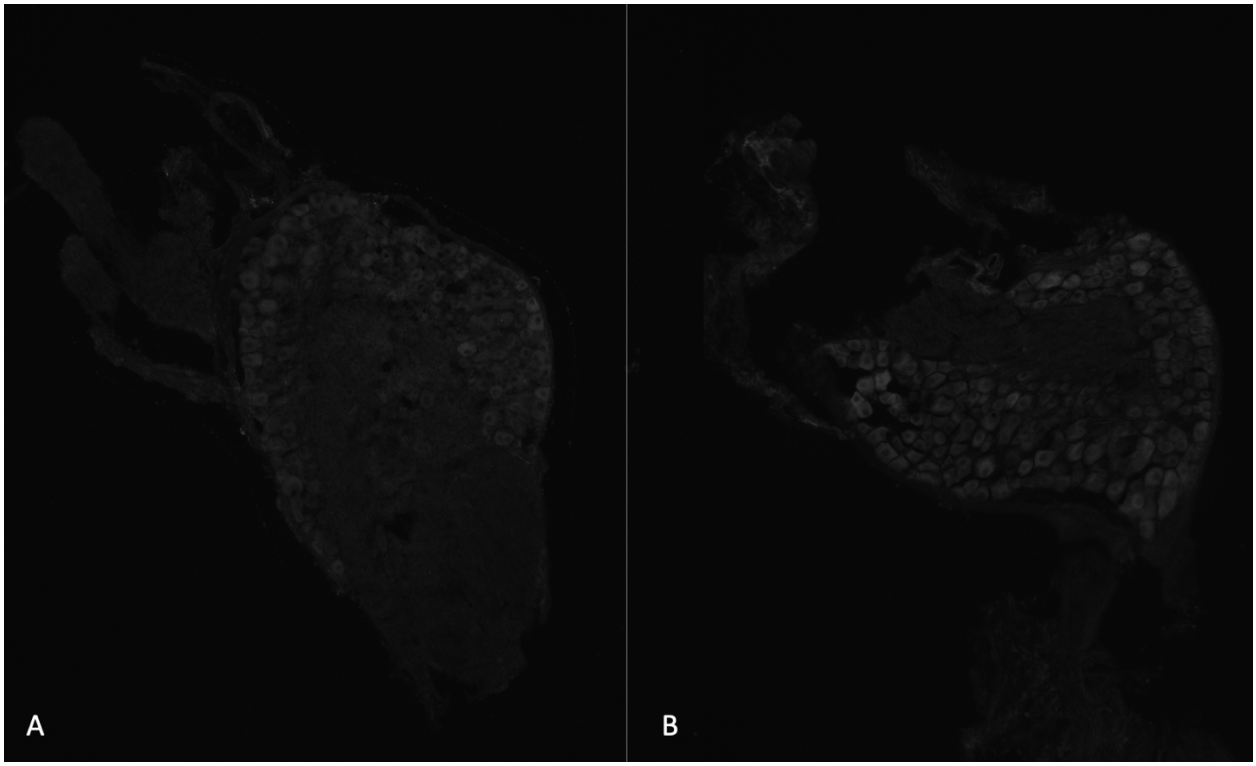

Figure 4: DRG Neurons of the same animal stained for GFP after antigen retrieval (A) and without antigen retrieval (B).

Following our failure to observe significant rabies infection within the grafts, the goals of the project were then shifted towards seeing subtype sensory neuron termination and innervation within the NPC graft itself. To date, there have only been a handful of studies that have investigated the termination of distinct subtypes of axons within NPC transplants (Dulin et al., 2018; Kadoya et al., 2016; Lu et al., 2012). Using the spinal cord issue collected, a new goal of the project will be to focus on the termination of these subtype specific markers within our NPC

grafts. Some markers, such as CGRP, have been shown to terminate in their subtype specific laminae. Other antibodies being used in this project, such as IB4, peripherin, and Substance P, have not been investigated. This knowledge could give the scientific community using NPC transplantations for SCI treatment a greater understanding of if these subtype specific axons terminate in their expected regions and create a greater base for future research to start from. If certain subtypes can or cannot terminate in the NPC graft, then that could potentially be a direct reason for certain behavioral outcomes following NPC transplantation in the injured spinal cord.

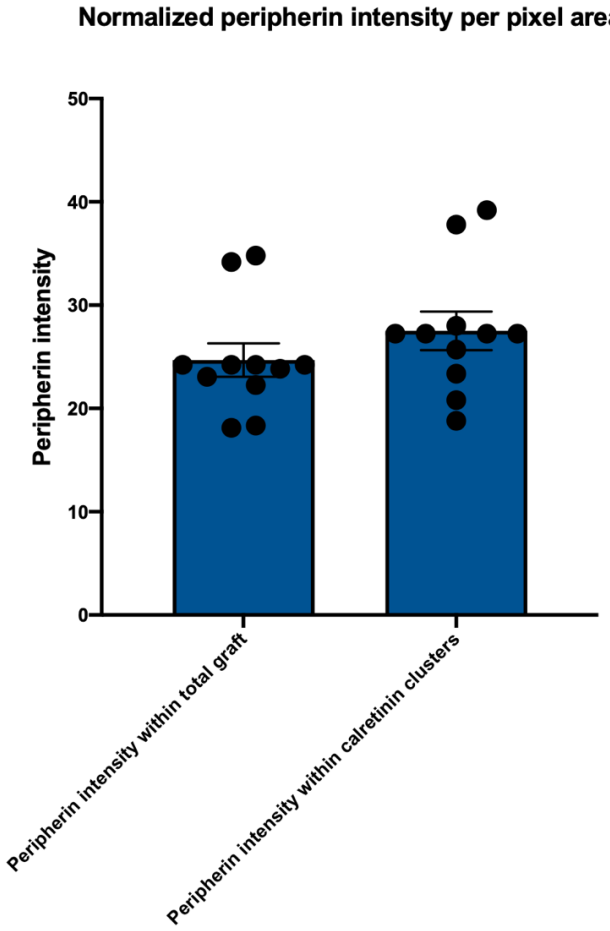

Figure 5: Normalized peripherin intensity per pixel area for the graft as a whole vs the calretinin clusters.

To investigate the presence of these markers (IB4, peripherin, and Substance P) in relation to laminae specific markers within the NPC grafted neurons Image J was used. Since IB4, Peripherin, and substance P are all nociceptive markers and, as such, they are known to terminate in the first and second laminae of the uninjured spinal cord. Calretinin positive neurons are known to be in laminae II of the dorsal horn. Subsequently, colocalization of NPC graft derived calretinin positive neurons with the intensity of IB4, peripherin, or substance P axon intensity will paint a picture of whether or not these subtype specific connections can form in NPC grafts. The peripherin intensity of calretinin positive clusters was slightly higher than that of the whole NPC graft, but not to a statistically significant extent. Unfortunately, the staining for Substance P and IB4 were both unsuccessful, so results for these antibodies are absent.

In theory, colocalization of various biomarkers visualized through the primary antibodies of immunohistochemistry will allow us to see which possible subtypes of neurons can make connections with the NPC grafts. Since the initial rabies infection has been localized to the neurons that differentiate from the transplanted progenitor cells, if GFP is present in a host cell containing antibodies indicative of a particular subtype of sensory neuron, then that would be proof of the graft derived neurons forming a connection with that subtype. Within the DRG, if no rabies positive cells are present, but Cholera Toxin B is, then the rabies infectivity was simply too low for it to make it to the DRG. If neither Cholera Toxin B nor rabies is present, then our injection method failed to work. If rabies and cholera toxin B are present but no primaries are visible, then immunohistochemistry failed to work. If rabies positive neurons are found throughout DRGs from the cervical, thoracic, and lumbar regions, then we will be able to know that these graft derived neurons can form synapses across the entire nervous system. In this case, by looking into which DRG levels contain rabies positive neurons, we can visualize the extent

that these newly formed connections can be made across the host's nervous system. By relating the cytoarchitecture of the graft to which interneurons or subtypes are positive, we will be able to know which newly formed laminae the connection originated from. If rabies infectivity is low within the grafted cells but is still present within the DRG then synaptic connections formed by the graft can be confirmed, but at a low rate. In this scenario it may be impossible to confirm the formation of subtype specific synapses from graft derived laminae. CTB will also have to be used as the sole reporter for possible connections with the graft derived neurons, since confirmation with rabies will be impossible.

CGRP+ cells come in a variety of diameters and conductivities (Bae et al., 2015). The vast majority of CGRP+ cells within the DRG are peptidergic, small diameter, unmyelinated C fibers responsible for nociception and thermal sensation (Lai et al., 2016; Price & Flores, 2007). A small subset of these CGRP+ cells are medium diameter, myelinated, high threshold mechanoreceptor (HTMR) A-δ fibers that are also responsible for nociception and heat sensation. Being that CGRP+ neurons participate in pain sensation, they terminate within laminae I and II of the dorsal horn and can be used as a marker for this type of subtype specific laminae connection (Dulin et al., 2018). SP+ neurons share the same type of C fibers with CGRP+ neurons, making their colocalization indicative of peptidergic, small C fibers (Zylka et al., 2005). Similarly, Prph+ neurons also fall under the C fiber category. Interestingly, peripherin is nonpeptidergic, and can been seen in peptidergic C-H fibers, like SP and CGRP, as well as coexpressed with TRPM8, a maker for the nonpeptidergic C-C fibers that play a role in nociception and cold perception (Black et al., 2012; Morenilla-Palao et al., 2014). This may suggest that Prph+ small diameter C fibers that are CGRP-/SP- may be of C-C fiber subset. IB4+ cells are also nociceptive cells, the majority of which are small unmyelinated C fibers, with some

research showing them to polymodal C-fibers, as well as low threshold mechanoreceptor (LTMR) a-δ fibers responsible for the sensation of pain, gentle touch, and cold temperatures (Fang et al., 2006). Previous research has also shown small amounts of colocalization of CGRP and IB4 (~10%) and moderate amounts of SP and IB4 colocalization (~40%) (Price & Flores, 2007). CalR+ and CalB+ neurons are also nociceptive and terminate in the superficial aspects of laminae II, and the interior aspect of laminae II (and possibly laminae III) respectively (Todd, 2010). Being that neurons positive for all the aforementioned markers are nociceptive, these neurons are expected to terminate in laminae I, II, or both. Lastly, Parv+ neurons can indicate the proprioceptive neurons of both A-α fibers (Ia and Ib) and A-βII fibers, terminating in laminae III through V as well as Clarke's Column.

For example, if a cell is positive for rabies, CGRP and Substance P it can be considered a nociceptor, while if it is rabies and CGRP alone it may be a C-fiber or a HTMR A-δ fiber for. If it is positive for peripherin alone it may be nonpeptidergic C fiber, while if it also positive for CGRP or SP then it is most likely a peptidergic C fiber. Cells that are positive for IB4 alone may be a polymodal C fiber if they are of a small diameter or a LTMR A-δ fiber is of a medium diameter. If a cell is positive for IB4 and Substance P it is most definitely a small diameter C fiber. A neuron that is rabies positive and parvalbumin positive is indicative of the graft derived neurons being able to form connections with proprioceptive circuitry. By relating the cytoarchitecture of the graft to which interneurons or subtypes are positive, we will be able to know which newly formed laminae the connection originated from.

4. CONCLUSION

With low rabies infectivity, the main goals of the project were unattainable. Following the unsuccessful TSA amplification to increase the visualization of rabies infectivity within the spinal cord, and antigen retrieval to increase visualization of rabies infected neurons within the DRG, the goals of the project had to be shifted towards the broader goal of identifying laminae specific connections within the NPC graft. IB4, substance P, and peripherin were investigated. The immunohistochemistry for Substance P and IB4 were unsuccessful. Peripherin on the other hand was successful and a slight increase in peripherin axon intensity was seen within calretinin clusters, but it was statistically unsignificant. These results may be more significant through the use of other laminae I and II markers.

By being able to see which subtypes of neurons can form functional synaptic connections with NPC grafts, which portions of the cytoarchitecture they originate from, and determining which levels of DRGs are rabies positive, we can gain a greater understanding of NPC transplantation as a potential treatment for SCI through the regrowth of neurons and subsequent reformation of neural networks. We can see which laminae of the newly formed cytoarchitecture can mature enough to form branching connections with the peripheral nervous system. Learning which levels of DRGs possess rabies positive neurons can show the extent to which these new connections spread through the nervous system. Knowing which subtypes form these new connections could potentially explain the increased sensory recovery seen after NPC transplantation.

Depending on which subtypes form these connections, these results could strengthen NPC transplantation as a means for improving sensory function after SCI. Conditions like

orthostatic hypotension, loss of feeling, allodynia, and hyperalgesia could have a new means of treatment. For future research, retrying the experiment with a different batch of rabies may yield positive results so true classification of these subtypes can be conducted. Subtype specific antibodies being used could be expanded to include others, such as NF200 to show connections between graft derived neurons and another subpopulation of nociceptor and proprioceptor, investigate colocalization between TrkB and IB4 may confirm IB4 as a possible marker for nonpeptidergic LTMR a-δ fibers, investigate is rabies could infect the subpopulation of MAS related GPR family member D and IB4 positive (Mrgprd+/IB4+) C fibers, the MAS related GPR member B4 positive (MrgprB4+) subpopulation of C fibers, or transient receptor potential cation channel subfamily M member 8 positive (TRPM8+) cold sensing C-fibers (Dhaka et al., 2008; Liu et al., 2007). NPC transplantation into other regions of the spinal cord may form connections with other sensory types and should be investigated as the composition of neuronal subtypes within various DRG levels may be different. Allowing the NPC graft to mature for longer may lead to greater innervation, communication, and rabies infectivity. As for the unsuccessful IB4 and Substance P analysis, these portions of the experiment should be repeated as well, with the inclusion of other subtype specific markers that terminate in calretinin positive populations of the dorsal horn. With the use of other laminae specific markers, the same type of analysis could be performed for neurons terminating in a wide variety of laminae, anterior horn included. Although this project focused on specifically sensory neurons, another experiment could be created with a focus on rabies tracing within the corticospinal tract, to investigate the biological basis for any motor recovery seen with NPC transplantation. Repeating this experiment using various other injury models at different spinal levels may also help the scientific community better understand

how NPC transplantation could possibly lead to improved outcomes following a wider variety of SCIs.

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