

**INVESTIGATING MOLECULAR MECHANISMS OF DORSAL HORN
ASSEMBLY IN NEURAL PROGENITOR CELL GRAFTS**

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

	Page
ABSTRACT.....	1
DEDICATION.....	3
ACKNOWLEDGEMENTS.....	4
NOMENCLATURE.....	5
1. INTRODUCTION.....	6
1.1 Understanding the dorsal horn of spinal cord.....	7
1.2 Development of the dorsal horn of spinal cord.....	9
2. METHODS.....	13
3. RESULTS.....	17
4. CONCLUSION.....	19
REFERENCES.....	20

ABSTRACT

Investigating Molecular Mechanisms of Dorsal Horn Assembly in Neuron Progenitor Cell Grafts

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Spinal cord injury (SCI) is a form of damage to the spinal cord that dramatically impacts quality of life and can ultimately lead to severe effects such as loss of mobility and/or sensation. Neural progenitor cell (NPC) transplantation research has become a growing field of interest due to the therapeutic potential it has for SCI, as NPCs provide the foundation to generate different types of mature neurons that are then functionally and anatomically integrated into host circuitry after transplantation. That said, there are still many challenges with NPC transplantation that must be addressed before it becomes a viable treatment open to the market. One such challenge is truly understanding the mechanisms behind graft integration and formation of synaptic connections. The Dulin lab has previously demonstrated the remarkable ability of dissociated NPCs to self-assemble organized, multicellular domains resembling the native spinal cord dorsal horn. However, the mechanisms by which these accurately assembled tissue domains form in the dorsal horn has largely remained unknown. The goal of this study is to investigate the developmental mechanisms enabling the formation of these accurately assembled tissue domains. We hypothesize that signaling through the Notch/delta pathway, which normally

underlies the formation of dorsal horn laminae in the intact spinal cord, also contributes to the formation of these multicellular domains in dissociated NPC grafts.

To answer the research question, we obtained GFP and wildtype cells from E12.5 embryos to supply an in vivo and in vitro experiment. In vitro, 1%, 0.1%, and 0.01% dilutions of GFP to WT cells were cultured and stained with transcription factor markers. In vivo, 1% and 0.1% GFP to WT grafts were placed in the intervertebral space of 6 mice, which were then allowed to grow for 1 month. After sacrifice, sagittal sections of the cervical section of the spinal cords of the mice were obtained and stained for the same markers as the in vitro experiment. Future steps will involve determining when clustering occurs, depending on the clustering patterns of GFP to WT cells in vivo and in vitro seen in imaging. Once timing of this clustering is determined, inhibitors of Notch will be applied to determine if clustering is inhibited or not.

DEDICATION

To my friends, families, instructors, and peers who supported me throughout the research process and in university as whole. Their constant support has molded me into the person I am today.

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NOMENCLATURE

SCI	Spinal cord injury
NPC	Neural progenitor cell
E#	Embryo # days after conception
DRG	Dorsal root ganglion
CNS	Central nervous system
PNS	Peripheral nervous system
GFP	Green fluorescent protein
WT	Wild type
pd	Progenitor Domain

1. INTRODUCTION

Spinal cord injury (SCI) is a form of damage to the spinal cord that dramatically impacts quality of life and can ultimately lead to severe effects such as loss of mobility and/or sensation. The most common causes of SCI include motor vehicle accidents (36-48%), violence (5-29%), falls (17-21%), and recreational activities (7-16%) [1]. The National Spinal Cord Injury Association estimates that over 450,000 people in the United States are currently living with an SCI, with an estimated 17,000 new cases being added every year [2]. Unfortunately, there are currently zero effective treatments available that can improve quality of life for individuals with SCI.

Neural progenitor cell (NPC) transplantation research has become a growing field of interest due to the therapeutic potential it has for SCI. NPCs provide the foundation to generate different types of mature neurons that are then functionally and anatomically integrated into host circuitry after transplantation [3]. Additionally, NPCs can differentiate into glial cells (specifically astrocytes and oligodendrocytes), which can then support neural regeneration, produce lost myelin in injured host axons, support host and graft neurons, and reform the blood-brain barrier [4, 5, 6].

That said, there are still many challenges with NPC transplantation that must be addressed before it becomes a viable treatment open to the market. One such challenge is truly understanding the mechanisms behind graft integration and formation of synaptic connections. Previous research conducted by the Dulin lab has shown that spinal cord NPCs transplanted into injured adult spinal cords self-assemble into multicellular clusters similar to the intact dorsal horn of the spinal cord [7]. However, the mechanisms by which these accurately assembled

tissue domains form in the dorsal horn has largely remained unknown. The goal of this research project is to understand the mechanisms underlying layering of the superficial dorsal horn laminae and how these mechanisms are recapitulated in dissociated cells grafted in vivo to form “mini dorsal horn domains.”

1.1 Understanding the dorsal horn of spinal cord

The spinal cord, along with the brain, is an anatomical structure that makes up the central nervous system (CNS), the main processing center of the nervous system. The spinal cord can be divided into four main regions: cervical, thoracic, lumbar, and sacral. A transverse (horizontal) cut of the spinal cord shows the characteristic “butterfly” shape of the gray matter surrounded by white matter. The gray matter is largely composed of neuron somas and interneurons, while the white matter consists of myelinated axons that branch throughout the body. In a mature spinal cord, the dorsal horn (or “backside” of the spinal cord) is composed of afferent neurons, while the ventral horn (or “frontside” of the spinal cord) is composed of efferent neurons. This compartmentalization of the spinal cord into the dorsal and ventral horn is crucial and explains the flow of sensory input into motor output. Between the dorsal and ventral horns, millions of interneurons integrate information and either conduct direct pathways from afferent to efferent neurons (as seen in reflex arcs) or send information to the brainstem and thalamus, where information is then sent out to respective areas of the cerebral cortex for processing [8].

Specifically, the dorsal horn of the spinal cord is the primary central relay station for somatosensory perception and consists of ascending pathways that signal information to interneurons that connect either to the brain or to efferent neurons in the ventral horn (as seen in reflex arcs) [8]. Multiple major classes of neurons are found in the dorsal horn, some of which

include neurons that process touch, pain, and heat; neurons that are involved in the reflex-specific motor neuron output; and neurons that relay afferent information to the brainstem and thalamus, where the information can then be processed by different parts of the cerebral cortex [9]. Within classes of neurons, there is further segregation. For example, in somatosensory dorsal horn neurons, $A\alpha$ fibers are heavily myelinated and thick and propagate touch and pressure signals, $A\beta$ fibers are less myelinated and propagate temperature and sharp pain signals, while C fibers are thin and unmyelinated and propagate dull pain signals [10]. All of these fibers have their cell somas located in dorsal root ganglions (DRGs), and the axons synapse extensively with interneurons in the dorsal horn. Overall, two main types of neurons exist in the spinal cord that make up the sensory system: association interneurons and relay interneurons. Association interneurons form a closed system within the spinal cord itself and project along ipsilateral pathways (same side pathways), while relay interneurons send somatosensory information to the brain through contralateral pathways (opposite side pathways) [10].

The dorsal horn contains three prominent nuclei (clusters of cell bodies in the gray matter): the marginal zone (MZ), substantia gelatinosa (SG), and nucleus proprius (NP). The MZ relays pain and temperature sensation to the brain, the SG relays pain, temperature, as well as light touch, and the NP relays mechanical and temperature sensation [11]. An alternative way to group different sections of the dorsal horn is through Bror Rexed's laminae model, which identifies 10 total laminae, 4 of which line up with the above nuclei (lamina I corresponds to MZ, lamina II corresponds to SG, and laminae III and IV correspond to NP) [11].

1.2 Development of the dorsal horn of spinal cord

Despite the largely understood physiology of the dorsal horn, one major question that persisted for quite some time is whether or not neurons of the dorsal horn during development are locked into a specific physiological class upon generation or if neurons retain some plasticity – that is, all neurons are generated the same and different genes are activated by different transcription factors at different times that leads to neuron specialization. Recent studies have shown strong evidence that the presence of different transcription factors leads to development of different neurons.

Soon after the beginning of neural induction, the process by which cells in the ectoderm make a decision to acquire a neural fate, different transcription factors in the Hox family are expressed to create the aforementioned four main regions of the spinal cord (cervical, thoracic, lumbar, sacral) [12]. After this anterior-posterior axis of the spinal cord is developed, a dorso-ventral axis is developed based on different morphogenic cues secreted by the roof plate and floor plate of the neural tube. Studies have shown that the secretion of Sonic hedgehog (Shh) by the floor plate and bone morphogenic proteins (BMPs) and Wnt proteins by the roof plate in a gradient manner are among some of the morphogenic cues that lead to the specialization of progenitor cells [13]. Depending on the relative concentrations of Shh versus BMPs and Wnt proteins at a specific point along the dorso-ventral axis, progenitor cells specialize to produce different cell types. In the case of the dorsal horn, we see that the progenitors that give rise to dorsal neurons are influenced more by the cues from the roof plate than the floor plate [12, 13].

All cells that make up the dorsal spinal cord originate from six neuronal and glial progenitors that inhabit the ventricular zone, an embryonic layer of tissue that lies the ventricular system that connects the four ventricles to the central canal of the spinal cord and circulates

cerebrospinal fluid secreted by ependymal cells that make up the choroid plexus [8, 9]. These six progenitors are numbered from dI1 to dI6, and they are separated into two classes based on the expression of different transcription factors. Class A progenitors (dI1 to dI3) are the dorsal-most progenitors (closest to the roof plate of the neural tube), and each progenitor domain expresses the basic helix-loop-helix (bHLH) factor Olig3 [8, 9]. Class B progenitors range from dI4 to dI6 and do not exclusively express a common transcription factor (all Class B progenitor domains express the homeodomain factors Pax3, Pax6, and Pax7, but these are also shared by some Class A progenitor domains). [8, 9].

To be specific, at around E10.5, six distinct early born neuronal subtypes in the dorsal spinal cord can be distinguished, and the post mitotic cells that these progenitors give rise to settle in characteristic parts of the spinal cord [12]. This is shown in Table 1.1. Starting from the most dorsal progenitor, dI1 progenitors give rise to excitatory dI1 early born neurons that settle in the intermediary spinal cord and are involved in proprioceptive circuits. Similarly, dI2 early born neurons are excitatory and settle in the intermediary spinal cord, but a small subpopulation migrates to the ventral horn. Excitatory dI3 neurons settle in the deep and intermediate dorsal horn. In the class B domain, dI4 early born neurons are instead inhibitory and migrate to the lateral deep dorsal horn. While the precise locations of dI5 and dI6 have yet to be determined, studies have shown that dI5 neurons are excitatory and dI6 neurons are inhibitory.

Table 1.1: Transcription factors expressed in early born neurons emerging from the 6 dorsal progenitor domains of the spinal cord [12].

Class	Type of Early Born Neuron	Transcription Factors
A	dI1	Pou4f1, Barh1, Lhx2, Lhx9, Lh2a, Lh2b
	dI2	Pou4f1, Lhx1, Lhx5, Foxd3, Foxp2
	dI3	Pou4f1, Tlx3, Prrxl1, Isl1, Phox2b, Lmx1b
B	dI4	Lbx1, Pax2, Lhx1, Lhx5
	dI5	Lbx1, Tlx3, Lmx1b, Prrxl1, Pou4f1
	dI6	Lbx1, Pax2, Lhx1, Lhx5, Wt1, Dmrt3

Note that all Class B early born neurons express *Lbx1*. While all Class A early born neurons express *Pou4f1*, it is not enough to distinguish class A neurons as dI5 early born neurons express *Pou4f1*.

The importance of *Lbx1* in the development of the dorsal horn was demonstrated by Gross et al. and Muller et al. Specifically, mice without the *Lbx1* gene lost specification of dI5 neurons and actually respecified into dI3 neurons, while dI4 neurons respecified into dI2 neurons due to similar transcription factors (dI6 neurons were not observed in this mutant spinal cord) [8, 9]. Additionally, removal of the *Lbx1* gene in mice led to a flattening of the dorsal columns in E14.5 spinal cords, and at E18.5, a defined curvature of the dorsal horn was not observed [9]. On the other hand, misexpression of *Lbx1* in class A neurons in chick embryos suppressed the differentiation of certain neurons. Specifically, *Lbx1* appeared to antagonize the expression of transcription factor *Lh2a/b* in dI1 neurons [8, 9]. Overall, these studies show the plasticity of dorsal neuron differentiation, meaning neurons are not locked into a fate after mitosis from a progenitor domain.

In addition to an early neurogenic phase, progenitor domains expressing *Gsx1/2* and *Ascl1* (dI4 and dI5) expand in the ventricular zone at around E11.5 and begin a late neurogenic phase [12]. Specifically, dI4 progenitors expressing *Ptf1a* give rise to inhibitory dILA neurons that settle in the superficial laminae of the dorsal horn, while dI5 progenitors without *Ptf1a* give rise to excitatory dILB neurons and settle in the same region [8, 9]. Interestingly, these dILA and dILB neurons emerge in a salt and pepper manner, most likely due to the alternating nature of *Ptf1a* expressing and non-expressing progenitor domains in the ventricular zone [12]. Given that both dILA and dILB neurons come from class B progenitor domains that have simply expanded, both neurons express *Lbx1*.

In addition to a dILA and dILB segregation, individual subtypes of dILA and dILB neurons appear to segregate based on the expression of different transcription factors. Upon birth, all dILA neurons express *Lbx1*, *Pax2*, *Lhx1*, and *Lhx5*, while all dILB neurons express *Lbx1*, *Tlx3*, *Lmx1b*, *Pou4f1*, and *Prrxl1*. However, at E18.5, staining for *Lbx1*, *Tlx3*, and *Lmx1b* in dILB neurons show that the most superficial layer of the subpopulation of dILB neurons in the dorsal horn express *Tlx3*, while middle neurons express *Lbx1* and deep neurons express *Pou4f1* [13]. In dILA neurons, interactions between *Ptf1a* and *Rbpj*, a transcriptional mediator of Notch, appears to play a role in the diversification of these inhibitory neurons as *Lbx1*, *Pax2*, and *Lhx1/5* factors are not uniformly expressed during maturation [14]. What was once thought to be a homogenous group of dILA neurons has now been assumed to contain as many as 50 subclasses [15]. Due to the presence of this *Rbpj* mediator in dILA neurons (and as an extension dILB neurons due to similar neurogenic phases), Notch signaling is hypothesized to also be involved in the formation of these mini dorsal domains of dILA and dILB neurons in cell grafts.

2. METHODS

Adult female and male mice were used for all experiments. All animal experiments were approved by the Texas A&M University Institutional Animal Care & Use Committee (Animal Use Protocol #2018-0156). National Institutes of Health guidelines for laboratory animal care and safety were strictly followed.

First, 8 WT female mice were injected with 100 cc of luteinizing hormone releasing hormone (LHRH) on Day 0 to prepare them for mating. On Day 4, the female mice were paired with either WT or GFP males (specific mating shown below in Table 2.1). Females and males were separated on Day 5. Female mice were then checked for pregnancy and allowed to grow until Day 17.

Table 2.1: Female and Male Mice Pairing on Day 4

WT-WT Pairing		WT-GFP Pairing	
Female #	Male #	Female #	Male #
490	87827	493	736
496	87822	491	740
495	87824	494	742
497	87821	492	743

E12.5 embryos from females 490, 495, 496, 494, 492, and 493 were harvested on Day 17. In total, 11 WT spinal cords and 7 GFP spinal cords were dissected from the embryos (some of

the embryos from the WT-GFP pairing were WT embryos as the GFP males were heterozygous, leading to less GFP spinal cords obtained). Care was taken to remove the dura mater and the dorsal root ganglia (DRGs) of the spinal cord as these would contaminate the cells. The GFP and WT cords were then placed in separate 50 mL conical tubes containing Hank's Balanced Salt Solution (HBSS) and then were dissociated for 8.5 minutes each using trypsin. To quench trypsinization, a 10% solution of fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) was added to both the WT and GFP solutions. Both solutions were centrifuged, and the pellet of cells were resuspended in 1:50 solution of B27 vitamin serum to neurobasal medium (NBM). This centrifugation/resuspension process was repeated once more, and the NBM/B27 + cell solutions were filtered with a 40 μ L cell strainer and transferred to new conical tubes.

1:10 dilutions of both WT and GFP cells using 10 μ L of cells, 40 μ L of NBM/B27 solution, and 50 μ L of Trypan Blue were made to count the total number of cells obtained from both WT and GFP embryos. Cells were counted using a hemocytometer and an EVE automated cell counter. Alive cells were denoted by a ring of Trypan Blue encasing the cell membrane, while dead cells were filled with Trypan Blue. Alive cells were counted in four distinct quadrants, and the number of cells was averaged and multiplied by the dilution factor and by 10^4 (to account for the fact that cells were counted in a 10^{-4} mL suspension in each quadrant).

After cell counts were obtained, 1% and 0.1% GFP grafts were made to graft into 6 mice (shown in Table 2.2). No laminectomy was induced, but rather cells were injected in the intervertebral space of C4 and C5.

Table 2.2: 1% and 0.1% GFP Grafting in Mice

1% GFP	0.1% GFP
793	796
794	797
795	798

Each well in a 48 well plate was then coated with 125 μL of 50 $\mu\text{g}/\text{mL}$ of poly-D-lysine (PDL) in sterile dH_2O (shown in Figure 2.1). Afterwards, 1%, 0.1%, and 0.01% dilutions of GFP cells in WT cells were created. For each of the three conditions, 8 wells were filled with 42 μL of cell mixture and 200 μL of NBM/B27 mixture. Every day for a week, 125 μL of the NBM/B27 was replaced with fresh NBM + B27 + Penicillin-Streptomycin-Glutamine (PSG) solution and incubated at 37 $^\circ\text{C}$ to allow cells to grow. After one week, cells were fixed using paraformaldehyde (PFA).

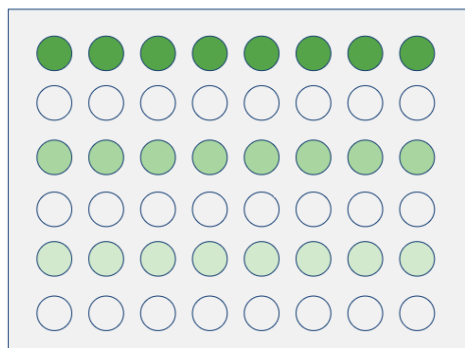


Figure 2.1: Schematic of 1%, 0.1%, and 0.01% dilutions of GFP in WT cells in a 48 well plate. Cells were cultured for 7 days.

The first two wells of each condition were then blocked in 5% Donkey serum in Tris-buffered saline + triton (TBS-T) and then stained with 1:2000 dilutions of Chicken calretinin and Rabbit GFP in TBS-T + 5% Donkey and 1:400 Guinea Pig Lbx1 in TBS-T + 5% Donkey primary antibodies. Afterwards, cells were stained with 1:1000 dilutions of Donkey Rabbit 486, Donkey Guinea Pig 647, and Donkey Chicken 555 in TBS-T + 5% Donkey secondary antibodies for imaging.

For one month, both 1% and 0.1% GFP graft mice were monitored, after which the mice were sacrificed, and the spinal cords were obtained. Spinal cords were dissected from the rest of the perfused mice, and cervical sections of the spinal cords were taken. Sagittal sections of each cervical section were taken using the cryostat, and these sections were stored in 24 well plates filled with TCS. For staining, the first column of each 24 well plate was used, ensuring that each spinal cord sample remained separate. Sections were washed with TBS and then blocked in TBS-T + 5% Donkey. The same staining patterns used for the cell cultures were once again used for the sagittal sections.

3. RESULTS

Unfortunately, proper images of from the staining of the in vitro cells and sagittal sections of the in vivo experiment were not obtained. The experiment is currently under the retooling phase to determine which steps went wrong and why by the time of imaging most cells were dead. Well before the staining process for the in vivo grafts, mouse 798 was found dead, despite no weight loss or visible complications. As per in vitro cells, cells were fed every day for 7 days, yet by the time of staining, most cells were dead as staining was very poor and the only signal to appear on the microscope was the endogenous GFP signal of those specific cells. One possible explanation for this may be the fact that during feeding, some cells lifted off the PDL coating on the bottom of the wells. The PDL coating ensures that cells stick to the bottom of the well, as swirling cells in solution die off very quickly. Additionally, despite feeding the cells every day at consistent times, there were instances where the pink media appeared yellow, indicating a large concentration of metabolic wastes. As per the in vivo grafts, possible reasons have yet to be determined.

Nonetheless, certain hypotheses can be made on what the images could have shown. We would have expected to see mini dorsal horn domains in these grafts, but the composition of these grafts could be of two options - clusters of only GFP or only WT cells could be observed or a mix of both GFP and WT cells in the same cluster could be observed. If a segregation of cell types was visible, this would indicate that cells of the same origin have a communication pathway (assumed to be Notch) that allows them to congregate into these mini dorsal horn domains with characteristic layer of cells. On the other hand, if both GFP and WT cells were

found in individual clusters, this would indicate that cells regardless of origin share a similar communication pathway (assumed to be Notch) that allows them to congregate.

4. CONCLUSION

After repeating this experiment and determining the nature of cluster formation, future steps will include determining when clustering formation begins in grafts. One possible way to go about this would be to stain for specific dILA and dILB markers at different time points after plating different dilutions of cells. Understanding when this formation begins is critical for another step – determining if Notch signaling is involved in cluster and laminae formation. If it is determined when specification and clustering begin, we can use Notch pathway inhibitors such as the gamma-secretase inhibitor DAPT. Studies have shown that the treatment of zebrafish embryos with DAPT produces severe neurological phenotypes similar to Notch signaling deficiencies, but whether DAPT inhibits this laminar formation in this mini dorsal horn domains would be tested in this case. If laminar formation is indeed inhibited with DAPT, we would then want to see the balance between different types of neurons in the graft. Without communication between cells, what would be the default state of these neurons? What balance would be expected between dILA and dILB neurons? Such questions would guide future steps of this project.

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