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Pannexin-1 Hemi-channel Expression and Influence on Regenerative Growth in Peripheral Nerves of Mice

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

in

Biology

by

Steven Horton

Committee in charge:

Professor Sameer B. Shah, Chair  
Professor Shelley Halpain, Co-Chair  
Professor Nicholas Spitzer

2016



The Thesis of Steven Horton is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2016

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## ABSTRACT OF THESIS

Pannexin-1 Hemi-channel Expression and Influence on Regenerative Growth in Peripheral Nerves of Mice

by

Steven Horton

Master of Science in Biology

University of California, San Diego, 2016

Professor Sameer B. Shah, Chair

Professor Shelley Halpain, Co-Chair

Pannexin-1 (PANX1) channels are expressed in multiple tissue types and have emerged as particularly attractive research topic based on their role in ATP release and purinergic signaling, which are responsible for multiple functional and developmental processes. However, the expression of Pannexin-1 channels and their contribution to the function of neurons in the peripheral nervous system have not been studied extensively. Therefore, we aimed to test whether or not Pannexin-1 channels are expressed in the axons of neurons, which are the functional units of nerves in the peripheral nervous system. We started by testing the overlap of Pannexin-1 with an axonal marker in cross sections of sciatic nerves of adult mice, and then followed up with a co-localization study in axon-



al projections of DRG cell culture explants where axons were more isolated and axonal expression of Pannexin-1 was confirmed. Once we confirmed that Pannexin-1 channels are expressed on peripheral nerve axons, we attempted to study their role in regenerative outgrowth of axonal neurites stemming from cultured DRG explants, which were harvested from adult mice. After analyzing the axonal growth in DRG explants from Pannexin-1 knockout mice (PANX1  $-/-$ ) in comparison to those from wild-type explants, our results suggest subtle, but not statistically significant, differences in growth rates between the two genotypes. More sophisticated analytical methods are being developed to further probe these potential differences.

## **Introduction**

### Peripheral Nerve Structure:

Peripheral nerves are categorized into two major fiber types: motor and sensory neurons. Sensory neurons are afferent and responsible for delivering signals from the periphery to the brain and spinal cord. Meanwhile motor neuronal fibers are geared towards delivering signals from the central nervous system to the periphery (efferent) to elicit a response. A given nerve of the peripheral nervous system contains a series of fasciculated bundles containing individual neuronal fibers. These fascicles can be grouped according to neuronal fiber type. The sciatic nerve in particular served as one of the model systems for our experiment, and integrates both motor and sensory neuronal fiber types. The sciatic nerve runs from the hip to the knee in mammals. Distally, the sciatic nerve trifurcates into three separate branches which extend from the knee down to the ankle. Of these three branches, one is strictly involved in sensory function (sural) while the other two are involved in both motor and sensory function.

Within a particular branch of a nerve, there are several layers of tissue and cell types aside from just axonal fibers to take into consideration when analyzing its structural components. When looking at the cross section of a mouse sciatic nerve, for example, there are several structural sub-sections to take into consideration. The outer connective tissue layer surrounding the nerve is known as the epineurium, while the perineurium refers to the matrix within the nerve that holds the integrity of the nerve together. Lastly, each perineurial sheath contains bundles of myelinated or unmyelinated axons surround

ed by an endoneurial layer of connective tissue. Blood vessels are also intermixed with axons to maintain vasculature in nerves.

Since the neurons themselves are the functional units of the nerve, we have analyzed the expression patterns of our protein of interest, Pannexin-1, throughout sciatic nerve cross sections and determined whether or not it is expressed on the axonal fibers. It is thus significant to determine whether or not Pannexin-1 channels are expressed axonally when investigating their role in nerve development and function. However, it is also likely that Pannexin-1 channel expression is not limited to just axons but is distributed throughout other components of nerves. Future directions may be able to explore the options of perineurium, myelin, or blood vessel expressing Pannexin-1 channels.

#### Pannexin hemi-channels

Pannexins are a subfamily of glycoproteins that have the ability to form non-specific transmembrane pores geared towards the exchange of ions, metabolites or signaling molecules from intracellular to extracellular space (Penuela et. al., 2013). Pannexin channels are classified as hemi-channels, meaning that they have the potential to form gap junctions for direct cell to cell communication, however this function has been more characteristic of their relative connexin channels. The group of Pannexin channels is comprised of subtypes Pannexin-1, 2 and 3 which all seem to serve separate functions across different species as well as within mammals (Penuela et. al., 2013). In mammals, Panx1 is the most prominent, being expressed ubiquitously in a wide variety of tissue types, where as Panx2 and 3 seem to be much more limited in expression and specific in

function. In comparison to connexins, pannexins are similar in terms of their structural lay-out and transmembrane domains, but are much larger in size which contributes to their ability to release large signaling molecules, particularly ATP (Penuela et. al., 2013). This aspect of Pannexin hemi-channel function has caused a recent surge in interest towards the proteins, as more regarding the expression patterns and functional role of Pannexin-1 in ATP signaling is being discovered. The release of ATP by Pannexin channels has already been linked to many functional processes in different tissues types, but in the grand scheme there is still much to learn.

#### Pannexin-1's Functional Role in Purinergic Signaling (Multiple Tissue Types)

Purinergic signaling is an instrumental mechanism in cell-cell communication and is responsible for triggering a plethora of downstream responses ranging from anti-inflammatory cascades to proper tissue development and function. The release of intracellular ATP into extracellular space often initiates purinergic signaling, enabling ATP to act on whatever tissue is being targeted via purinergic receptors (i.e. P2X7). For this reason, Pannexin-1 hemichannels have picked up a lot of relevancy in recent research. Pannexin-1 transmembrane pores are believed to signal by means of the release of intracellular ATP to extracellular space. This release of ATP has been identified as the source for purinergic signaling which acts on P2X7 receptors to initiate a multitude of responses. The relationship between Pannexin expression and ATP signaling has been connected to many different physiological processes in different tissue systems and cell types. When testing the influence of connexin and pannexin channels on normal skeletal muscle function, Cea and colleagues discovered that these channels were involved in the calcium in-

flux and depolarization necessary for contraction. Particularly, they inferred that ATP released from Pannexin-1 pores is a potential pathway for propagating a normal contractile response (Cea, et. al, 2012). A review written by Billaud et. al. highlighted the expression and role of Pannexin-1 channels in the smooth muscles lining arteries, and emphasized their role in maintaining vascular tone (Billaud, et. al. 2012). This pertains directly to our peripheral nerve model, since blood vessels are one of the structures complementing axonal fibers in nerves.

Our focus is on how this process operates in the peripheral nervous system since it is not well understood to date. We are further narrowing the scope of our investigation of the functional roles of Pannexin-1 hemichannels in peripheral nerves to how they impact normal structural composition and regeneration. The establishment of interest in these topics stemmed from the premise set by a review written by Dr. Helen Makarenkova summarizing that the expression of Pannexin channels increases during the course of inflammatory and developmental pathways (Makarenkova, 2014). Through our research we attempted to expand on discoveries made in previous papers studying Pannexin-1 expression and function, and had two major goals in mind. We first wanted to set the premise for Pannexin-1 channels being involved in normal function or growth in peripheral nerves by testing whether or not Pannexin-1 is expressed in neurons (axons). Upon establishing such localization, we tested whether or not the expression of functional Pannexin-1 channels influenced the rate of regenerative growth in adult peripheral nerves.

### Pannexin-1 Expression Patterns in the Nervous System

In 2015 a study by Raslan and colleagues demonstrated that developing embryos of mice express Pannexin-1 mRNA throughout the Central Nervous System but also in sensory ganglia throughout the spinal cord, particularly in Dorsal Root Ganglia (DRG) which we have used as a model system when looking at specific expression patterns of Pannexin-1 channels and how they influence regenerative growth in adult mice (Raslan et. al., 2015). This paper referenced an additional study that recognized an increase in Pannexin-1 channel expression in response to neuropathic pain, specifically in Dorsal Root Ganglia (Zhang, et. al., 2015). Due to the aforementioned complexity of the structural components of nerves when analyzing cross-sectional images of a mouse sciatic nerve, using a cell culture model to confirm Pannexin-1 expression patterns is ideal. This experiment just adds further reinforcement to what we are searching for in the sciatic nerve tissue samples. Since DRGs have been acknowledged for expressing Pannexin-1 in other studies, using them as a model seemed advantageous. Previously, two studies by Stordeur et. al. also established that neural precursor cells *in vitro* from the Ventricular Zone of neonatal and adult mice expressed high-levels of Pannexin-1 channels during neurogenesis in the brain (Stordeur, et. al., 2012 and 2013). In these progenitor cells Stordeur and colleagues also discovered that Pannexin-1 channels interact with cytoskeletal proteins and that there is a decrease in Pannexin-1 expression after differentiation has taken place (Stordeur et. al., 2013). However, the expression pattern of Pannexin-1 channels within the structural components of an adult functioning nerve is not well understood and has not been extensively studied in the Peripheral Nervous System.

### Pannexin-1 Function in the Peripheral Nervous System

When looking at the structural and functional processes of a neuron, it is important to consider the concepts of myelination and cytoskeletal mobility. Whether or not an axon is myelinated determines how quickly a neuron can propagate signals. The process of determining which axonal fibers are myelinated within a nerve takes place during the development process. Neurons differentiate and some become myelinated while others do not. During this process in the sciatic nerve, purinergic (ATP) signaling onto P2X7 receptors has been identified as being responsible for this differentiation and the blocking of these receptors resulted in a reduction in the number of myelinated axons that develop in mice (Faroni et al, 2014). Ino et. al conducted a study in 2015 further revealing that purinergic signaling on P2Y2 receptors with extracellular ATP is responsible for the recruitment of Schwann Cells to initiate myelination by up-regulating the mitochondrial activity of SCs (Ino, et. al. 2015). These studies demonstrated that ATP release and signaling is actively involved in the myelination process of neuronal fibers, but the exact role of Pannexin-1 channels in this mechanism is not yet understood.

Cytoskeletal movement also plays a significant role during the differentiation process in neurons, as well as in the proliferation, growth and maturation of axons. In 2013, Stordeur and Swayne conducted a study on neural progenitor cells from the Ventricular Zone of the brains of developing mice testing the expression levels of Pannexin-1 before and after differentiation and determining whether or not Pannexin-1 channels interacted with the cytoskeleton during the development and proliferation of these cells. They found that during the development process Pannexin-1 levels were high, but after differentiation took place, Pannexin-1 expression decreased by about 60%. They also came to

the conclusion that decreasing the inhibition on Pannexin-1 channels (by removing siRNA) increased the amount of proliferation and cytoskeletal movement during development of these progenitors of the Ventricular Zone. Thus, they found that Pannexin-1 channels are very active in the development and differentiation processes. A study done by Boyce and colleagues in 2013 also proved that Pannexin-1 channels interact with specific cytoskeletal proteins during development to aid in differentiation and movement (Boyce et. al., 2014). However the Stordeur and Swayne study also recognized that levels of expression are reduced after differentiation is complete (Stordeur and Swanyne, 2013). This leaves the door open for further research on the role of Pannexin-1 channels in the peripheral nerves of adult mice, and gives us further reason to believe that there could be involvement of these hemi-channels in the regenerative proliferation of axons from DRG explants.

Previous publications have also explored the role of purinergic signaling and P2X7 receptors on the inflammatory response of tissue. This is of interest to us, because it set the premise for the idea that there may be Pannexin-1 channel involvement in the process of regenerative growth. Makarenkova et. al. revealed that the release of intracellular ATP leads to the recruitment of white blood cells which perform damage control on dying tissue via phagocytosis (Makarenkova, 2014). It has been documented that during trauma, there is an increase in pannexin expression on the injured tissue as well as a corresponding increase in extracellular ATP (Makarenkova, 2014). There is also evidence supporting the idea that pannexin makes direct contributions to the regeneration process in damaged tissue. Specifically, it has been documented that there are increased levels of



pannexin-1 expression on the damaged tissue following the inflammation response (Makarenkova, 2014). Since regeneration is the ensuing process succeeding this inflammation pathway, it can be inferred that pannexin-1 channels may be involved beyond the signaling for inflammatory control and contribute to regeneration although its exact role is not yet understood.

### Dorsal Root Ganglia (DRG)

The Dorsal Root Ganglion has proven to be an ideal model for measuring axonal outgrowth in peripheral nerves and looking at growth of axonal projections for a multitude of reasons. Since the DRG is a cell mass, it can sprout several projections from one culture at a relatively quick (3-5 day) growth rate. Also, the fact that all of the projections stem from the same mass and extend away from the DRG during outgrowth makes quantification of length more tractable. They can also be extracted in bulk from each sacrificed mouse (10-16 on average) optimizing resources, and finally, as DRGs are, in fact, explanted, thus requiring the severing of in vivo connections, they may be viewed as a regeneration model, even without subsequent injury in culture. Given the preceded studies on Pannexin-1's role in: the function of multiple different tissue types, the cellular inflammatory response, as well as the differentiation and outgrowth of developing neurons, we surmised that the DRG would be an excellent model to test the influence of Pannexin-1 channels on the regenerative growth rate of peripheral axons, free from confounding influences in vivo.

### Pannexin-1 ATP Signaling and Potential Negative Feedback

During neurogenesis and many of the other functional mechanisms which depend on the pannexin-1 related release of ATP, it is suspected that ATP interacts with P2X7 receptors to up-regulate Pannexin-1 channel expression on the cellular membrane. This promotes a positive feedback loop that initiates increased release of ATP corresponding with outgrowth, differentiation, calcium influx, and other physiological processes. However, a study carried out by Boyce et al. revealed that when extracellular ATP levels reaches a certain threshold it acts on P2X7 receptors to cause the endocytosis of the transmembrane Pannexin-1 channels into endosomal compartments (Boyce et al., 2015). Therefore, there is a dose-dependent negative feedback process that involves the down-regulation of Pannexin-1 membrane expression in the presence of high levels of ATP. This is likely to be a regulatory mechanism to control the physiological processes mediated by pannexin-mediated ATP release.

#### Summary of Experimental Objectives

Through this series of experiments, we have aimed to test the question as to whether or not Pannexin-1 channels are expressed axonally in peripheral nerves of mice, in hopes that it will elucidate the involvement of Pannexin-1 channels in axonal regenerative outgrowth in adult mice. Whether or not Pannexin-1 channels are expressed in axons was tested via immuno-histochemistry in sciatic nerves as well as explanted DRG primary cell cultures. The regenerative growth rate of neurites projecting from explanted DRGs was also measured in *Panx1*<sup>-/-</sup> knockout mice and compared to that of age-matched adult Wild-Type mice.

## **Methods**

### Animals

All of the animals used were housed and handled under conditions approved by University of California, San Diego and The Scripps Research Institute IACUC.

### Removal of Mouse Sciatic Nerves (Dissection)

Adult Wild-Type mice (8-12 weeks) were euthanized using gradual CO<sub>2</sub>-application to a sealed chamber. This process was carried out in a way that took the comfort of the mice into consideration. Sciatic nerves were harvested after removing the fur, fascia, and overlying skeletal muscle layers. Using forceps, the hamstring and quadriceps muscles were pulled in opposite directions to expose the sciatic nerve. Once exposed, the sciatic nerve was clipped using spring scissors at the proximal end adjacent to the hip of the mouse. The distal end was clipped just distal of the knee-cap in order to ensure that the trifurcation point (where the nerve branches into 3 separate distal nerves) would be included in optimal cross-sections containing the three branches. This allows for the opportunity to compare the expression of the proteins of interest in sensory vs motor nerves, since one of the three branches (sural) is sensory and the other two have motor neuronal fibers.

### Tissue Freezing (Freeze-Fix), Embedding and Sectioning:

For this fixation method, the sciatic nerves were immediately frozen after being extracted. The nerves were first pinned to cork and labelled for distal and proximal ends.

The tissue samples were then held in or above isopentane which was suspended in an canister of liquid nitrogen. After being exposed to the isopentane for 45 -60 seconds the pieces of cork holding the samples were immediately placed in a conical tube immersed in dry ice to prevent any thawing from taking place. The samples were then transported and kept in a freezer with a temperature of - 80 degrees Celsius to preserve them until the embedding process took place.

Prior to sectioning, the samples were transported from the freezer, to the cryostat chamber (-20 degrees Celsius) in dry ice. The nerves were then removed from the cork and embedded in a small square mold. A layer of “Optimum Cutting Temperature” (O.C.T.) was placed on the bottom of the mold only filling the mold to the half-way point. As the layer of O.C.T. began to freeze, the nerve was placed on top and a second layer of O.C.T. was added to fill the mold. This was done to ensure that the nerve would not thaw, and would be lying flat in one plane so that evenly cut sections could be made. Once the block containing the nerve solidified at - 20 degrees celsius in the cryostat chamber, the freezing procedure using liquid nitrogen and iso-pentane referenced above was applied to the mold containing the block for about 45 seconds. After the block was frozen it was either re-inserted into the cryostat chamber to continue with the sectioning process immediately, or stored in the -80 degree celsius freezer to be sectioned later.

To begin the sectioning process, the frozen block of O.C.T. containing the sciatic nerve was trimmed into a pentagonal shape and positioned on a metal platform in the cryostat chamber using more O.C.T. as an adhesive. The position of the block depended on whether cross or longitudinal sections were desired. The metal platform was then in-

serted and fastened into a holster in the cryostat positioned above a razor blade. The angle of the block was then adjusted to ensure that even cuts without folding or tears in the O.C.T. were made. The block of O.C.T. was trimmed until the nerve was visible. Then a shield was placed down behind the razor blade so that each sheet resulting the cuts would be held on a platform and not discarded. 10 micron slices were ideal for these sections, and when a sheet containing a sample of the nerve was successfully sliced and held on the platform a microscope slide would be placed on top of it to absorb the sample. Once the samples were placed on the slides they were kept in the cryostat chamber until they could be later transported to a box in the - 20 degree freezer on ice. The slides were also checked under a microscope to ensure that desirable samples were made before commencing with the staining process to optimize resources.

#### Tissue Fixing in Formalin and Sucrose (Alternative Method):

For this fixing procedure the steps of sciatic nerve removal and being pinned to cork were all done as mentioned above. However, as opposed to immediate freezing, the sample (and cork) was placed in a 50 mL conical containing 10% formalin or 4% paraformaldehyde solution and was left to soak overnight (24 hours). Once the initial soaking step was complete the sample was moved to a conical containing 15% sucrose in 1X PBS for 12 hours followed by a subsequent soaking phase in 30% sucrose in 1X PBS for an additional 12 hours. In between each soaking step including the initial fixation, the sciatic nerve was rinsed in 1X PBS to clear all residue from the previous solution. In the sucrose solutions, the tissue may have been removed sooner depending on the buoyancy of the

sample; if the sample sank before soaking for 12 hours, it indicated that it was fully saturated and the step was complete.

The embedding process required a square mold as indicated in the previous freeze-fix protocol. The OCT was applied in two layers as referenced before, with the nerve being placed after the first layer and the second layer covering the nerve and filling the mold. The only difference was that the cryostat chamber was held at a slightly warmer temperature than with the freeze-fix method (10-15 degrees Celsius). The sample was chilled in the chamber for 20-30 minutes before being frozen using liquid nitrogen and isopentane for 30 seconds. Once frozen, the block containing the nerve was thawed in the cryostat chamber for 20 minutes and either transported to the -80 degree Celsius freezer to be stored or immediately sectioned in the cryostat. The sectioning protocol other than a slightly warmer chamber temperature was the same as in the above methods section. Once the sections were made and added to microscope slides, the samples were kept in a humidity chamber until the Immunohistochemistry process began.

### Immunohistochemistry

Immunohistochemistry was used to tag the sciatic nerve tissue samples with protein-specific fluorescent labels after they were placed on microscope slides. A hydrophobic pen was utilized to ensure that certain reagents were localized to the tissue sample on each slide. For tissue samples that were “freeze-fixed” there was an initial fixation step consisting of the slides being soaked in 10% formalin solution for 45 minutes. The samples were then soaked in a reagent containing 0.2% Triton-X in PBS for 10 minutes

in order to permeabilize the cell and tissue membranes for antibody binding. Blocking Buffer composed of 3% BSA/10% fetal goat serum in PBS was applied to each individual tissue sample and sat at room temperature for 20 minutes. For mouse sciatic tissue samples being stained with an anti-mouse primary antibody (B- tubulin) it was necessary to have an additional over-night blocking phase with an unconjugated AffiniPure F(ab) fragment (goat anti-mouse IgG (H+L) at 100ug/mL of PBS) to eliminate non-specific binding and background noise. Primary anti-bodies used for the experiment included: monoclonal anti- $\beta$ -Tubulin Isotype III antibody produced in mouse as an axonal marker, and a polyclonal anti-Pannexin-1 antibody produced in rabbit as a marker for Pannexin-1 channels. For optimal specificity the samples were left with the primary antibodies overnight in a refrigerator kept at 4 degrees Celsius. For optimal results, red secondary fluorescent antibody (anti-rabbit) was used to label the Beta-Tubulin, while green secondary antibody (anti-mouse) was used to label Pannexin-1 channels. The samples were soaked in the secondary antibodies for an hour, while being kept in a dark chamber because the antibodies are light-sensitive. At any point that the samples containing secondary antibody were removed from the chamber, all lights were turned off to avoid any depreciation of fluorescence. Lastly, Vectashield was applied to each sample and they were coverslipped and sealed using clear nail polish. Intermittent PBS washing was used in between reagent steps to ensure the removal of any residual solution. The PBS contained 0.5% Tween-20 detergent to further eliminate background noise during the imaging process.

### Confocal Microscopy

Stained slides and culture dishes were transported to the confocal microscope (Leica DMI 6000 B) in aluminum foil or in a closed container to ensure limited exposure to light, since the samples are now light-sensitive. Once transported, the samples were imaged using the confocal microscope. DRG explant samples were imaged at a 20X objective lens within a range of gains from 600 to 850. The sciatic nerve cross-section samples were imaged under a 63X objective lens with glycerol for fluid immersion within a set gain range from 600 to 850. Line-averages and z- stacks are used to get complete images with limited background noise. All z-stacks were taken with 25 steps and a line average of 5 scans.

#### Negative Control

For each set of samples being stained using the Immunohistochemistry protocol listed above, a control slide was used containing only the secondary antibodies. All of the other steps for the protocol are included for the control, with exception to the primary antibodies. This is to demonstrate that the fluorescent binding is not non-specific, and to determine what fluorescence can be attributed to background noise as opposed to specific binding. These slides were imaged using Confocal Microscopy like the other samples, and also use to set the baseline for optimal gain / focus settings to avoid background noise.

#### Panx1-/- Imaging Using Confocal Microscopy

See “Objective 1 Methods: Part A V” for general confocal microscopy protocol. For this particular experiment, an additional negative control was used. A slide of tissue



from a PANX1<sup>-/-</sup> mouse was stained with pannexin-1 primary antibody as well as a fluorescent secondary to make sure that the antibody did not bind non-specifically to anything and that pannexin-1 channel expression was completely knocked out. This will eventually be used comparatively with the experiment in objective 1 Part A to analyze the expression of Pannexin-1 channels in tissue sections taken from mouse sciatic nerves.

### Removal of Dorsal Root Ganglia

Prior to removing DRGs from mice, Wild-Type mice (8-12 weeks) were euthanized using gradual CO<sub>2</sub>-application to a sealed chamber. This process was carried out in a way that took the comfort of the mice into consideration. Often, sciatic nerves were harvested from the same mice following DRG removal (DRG culturing is time-sensitive).

Once the mouse was sacrificed, the layers of fur and fascia were removed first. Once the muscles and organs were exposed, all of the organs were removed leaving the head, limbs, and tail. In order to expose the spinal canal, the head was then removed (the extremities and tail remained intact to preserve the orientation of the spine). Next, an incision was made from the rostral to caudal end of the spine on the dorsal side to access the spinal cord. If necessary, a following incision was made on the ventral surface of the spine to further reveal the cord (however the following steps of the protocol all took place on the dorsal surface). The lateral edges (lamina) of the spine were pushed apart using forceps and the intervertebral branching of nerves from the spinal cord could be visualized. This visualization marks the point of transitioning to a dissecting microscope to carry out the remainder of the procedure since the dorsal root ganglia are difficult to distin-

guish with the naked eye. These branches indicated the position of the Dorsal Root Ganglia even though the DRGs themselves could not be seen. The laminae were clipped using bone-cutting spring scissors exposing the DRGs. The dorsal root and associated nerves on the proximal and distal sides of each DRG were clipped carefully and the DRGs were extracted using forceps, and placed in a cell-culture dish containing Hank's Saline Solution. DRGs were separated according to which anatomical region of the spine they were removed from (cervical, thoracic, lumbar).

### Culturing of DRG Explants

Prior to the extraction of DRGs, cell culture plates were coated with two separate steps. 35mm glass micro-well plates were used for the DRGs. They were first coated with Poly-D- Lysine Hydrobomide (PDL) at a concentration of 5mg in 50mL of sterilized millipore water. The PDL was applied until the glass circles within the plates were completely covered and the plates were left to incubate for 2 hours or overnight. After being coated with PDL, the supernatant was removed and the dishes were left to incubate again for 20 minutes. Then laminin at a 1:50 dilution in PBS was applied in 120  $\mu$ L to amounts each dish and the plates were left to incubate for 2 hours or overnight.

The best results were experienced if the DRGs were plated and supplemented with media within 15 minutes of being removed. For this reason, the supernatant from the laminin coating was removed prior to DRG removal and the plates were prepared for the culturing process. After the cells were transported to the cell culture hood and all contents entering the hood were sprayed with 70% ethanol, the DRGs were transported into sterile

cell culture containing sterile Neurobasal-A media and were rinsed twice with new sterile Neurobasal-A. Once the DRGs were ready to be plated, a Matrigel droplet of about 1  $\mu$ L was added to one of the glass plates and one DRG was applied to the Matrigel using sterile forceps. A maximum of 1-2 DRGs were added to each plate to ensure that the projections stemming from the cell mass would have enough room to grow without interference and could be easily quantified. After a DRG adhered to the Matrigel, 1-2 droplets of DRG Cell Culturing Media were applied and the cells were left to incubate for 20 minutes before more media was applied. This was to ensure that the cells did not break away from the Matrigel. Following incubation, media was applied until the glass region of the plate was filled. The DRG Cell Culturing Media was comprised of Neurobasal-A media (Invitrogen, Carlsbad, CA) supplemented with 1% 8 Penicillin/Streptomycin (Invitrogen) 1% L-glutamine (Invitrogen) and 5% 50x B-27 (Invitrogen). Neural Growth Factor (NGF) was applied fresh before a new batch of cells were plated. NGF was aliquoted in amounts of 2  $\mu$ g in 200  $\mu$ L of DMEM media and stored in the -80 degree Celsius freezer. 50  $\mu$ L of this solution was applied to 50 mL of DRG Cell Culturing media that would be immediately applied to a new batch of cells. Media was changed every 2-3 days and the cells stopped growing and were fixed after 10-15 days.

### Immunocytochemistry

Cells were fixed in 10% formalin for 10 minutes, permeabilized in Triton-X100 for 2 minutes, and then blocked in 3% BSA/10% fetal goat serum in PBS for 30 minutes. For mouse DRG cell culture samples being stained with an anti-mouse primary antibody ( $\beta$ -tubulin III) it was necessary to have an additional over-night blocking phase with an

unconjugated AffiniPure F(ab) fragment (goat anti-mouse IgG (H+L) at 100ug/mL of PBS) to eliminate non-specific binding and background noise. For optimal results, the samples were then stored in a 4 degree Celsius refrigerator over night with the primary antibodies. Primary anti-bodies used for the experiment included: monoclonal anti- $\beta$ -Tubulin Isotype III antibody produced in mouse as an axonal marker, and a polyclonal anti-Pannexin-1 antibody produced in rabbit as a marker for Pannexin-1 channels. Cells were then incubated in AlexaFluor-488 goat-anti-mouse secondary antibody (Invitrogen) and AlexaFluor-594 goat anti-rabbit antibody (Invitrogen) for one hour at room temperature. Intermittent PBS washing was used in between reagent steps to ensure the removal of any residual solution. The PBS contained 0.5% Tween-20 detergent to further eliminate background noise during the imaging process. After the process was complete the cell- plates were kept in the refrigerator wrapped in foil to avoid light exposure until they were imaged using confocal microscopy.

#### Imaging the Progression of Projection Growth in DRGs from Wild-Type and PANX1<sup>-/-</sup> Mice

During the growth process, the living DRG cell culture plates were imaged under a brightfield microscope with a camera. Images were taken of every explanted DRG as individual samples every other day for 11 days. These images would be analyzed later to quantify the growth rate / progression of projection growth during the 11 day span.

#### Data Analysis

Fluorescent images taken using confocal microscopy were analyzed using the ImageJ program and “Just Another Co-localization Plug-in” (JACoP) was used to quantify the overlap of fluorescent signal expressing Pannexin-1 and  $\beta$ -Tubulin III. We chose to use the Mander’s 1 and 2 Coefficients to represent our data because they measure the percentage overlap of fluorescent pixels from each image, separating how image 1 overlaps with image 2 and visa versa respectively. For the sciatic nerve cross-sections there was a total of 3 samples from 3 different mice, while for the DRG explants there were 9 samples from 3 different mice. All samples were taken from Wild-Type Mice.

Once the bright field images for the growth rate analysis were taken of a particular explanted DRG sample over the 11 day span, the amount of axonal growth taking place during that time was quantified using ImageJ. A perimeter was drawn around the cell mass as well as a separate perimeter around the edges of the furthest projecting axons outlining the axonal region at each day. In addition, the most prominent or longest 10 axons (or as many as could be identified) were measured at each day for each sample. Once all of these perimeters were measured we chose to record the following for Wild-Type and PANX1<sup>-/-</sup> mice representations of axonal growth: maximum axon length (longest), average axon length, perimeter of axonal region, major axis, and average axis. The averages, standard deviations and standard errors were taken for each set of data and each measurement was plotted showing Wild-Type vs PANX1<sup>-/-</sup> throughout the 11 day span. For Wild-Types there was a total of 9 individual samples from 3 different mice, while for the PANX1<sup>-/-</sup> genotype we had 8 samples from three different mice.

#### Statistical Analysis:

For the co-localization studies we determined that the M1 and M2 coefficients demonstrating the correlation of overlap between the  $\beta$ -Tubulin III and Pannexin-1 signals required no further statistical analysis. However, for the projection results we conducted a series of 2-tailed, unpaired T-tests for each measurement at each time point to determine if there was a significant difference between PANX1<sup>-/-</sup> knock-out mice and Wild-Types in terms of axonal growth.

## **Results**

### Determining Local Expression Patterns of Pannexin-1 Channels in Peripheral Nerves of Mice:

In order to determine the exact role of Pannexin-1 hemi-channels in the function of peripheral nerves we started by looking into what structures within particular nerves express Pannexin-1. Since we hypothesized that Pannexin-1 may play a role in axonal growth and function based on previous literature, we addressed the specific question as to whether or not Pannexin-1 is expressed on the axons of peripheral nerves in mice. To test this question we first performed immuno-histochemistry on cross sections of the sciatic nerves of mice using antibodies for Pannexin-1 channels as well as Beta-Tubulin III, which we used as a neuronal marker. This allowed us to image the cross-section samples using confocal microscopy and analyze the overlap of Pannexin-1 with Beta-Tubulin III using co-localization analysis. Using “Just Another Co-localization Plug-in” with ImageJ we were able to quantify the overlap of Pannexin-1 and Beta-Tubulin III by generating Mander’s Coefficients 1 and 2. We were able to determine a strong correlation between the two fluorescent signals (Table 1), demonstrating that Pannexin-1 hemi-channels are indeed expressed on axons within the Peripheral Nervous System.

Due to the fact that sciatic nerves are comprised of multiple cell types which may also express Pannexin-1 (blood vessels, epithelial tissue, myelin), we performed a similar co-localization study in Dorsal Root Ganglion primary explants from adult mice (8-12 weeks). We were able to isolate axons in this system since neurite projections growing from the explanted DRGs are strictly unmyelinated axons (no dendrites or other neuronal structures). Our observations confirmed that the co-localization of Pannexin-1 with  $\beta$  -

Tubulin III (neuronal marker) was associated with the axonal expression of Pannexin-1. Using the same Pannexin-1 and  $\beta$ -Tubulin III primary antibodies as well as Alexafluor 488 and 594 secondary antibodies we were able to fluorescently label and identify the Pannexin-1 and  $\beta$ -Tubulin III proteins via Immunocytochemistry and confocal microscopy. We then generated Mander's Coefficients 1 and 2 (Table 1) for the co-localization of Pannexin-1 channels with the  $\beta$ -Tubulin III neuronal marker demonstrating a strong correlation of the expression of the two proteins in the axons of the DRG explant model.

While analyzing the expression patterns of Pannexin-1 in the DRG explants we had reason to believe that there is Pannexin-1 expression in the cell bodies of the sensory ganglia (Figure 1D). We did not quantify this expression, but see it as a future direction of our research. We also took images of sciatic cross-sections from the PANX1<sup>-/-</sup> knock-out mice stained with  $\beta$ -Tubulin and PANX1<sup>-/-</sup> (Figure 1C) which we did not quantify (such quantification is a future direction). Qualitatively, there appeared to be expression of Pannexin-1; however, the pattern appeared different from that in wild-type nerves, perhaps since the mice were functional knock-outs that contained truncated Pannexin-1, which was still expressed.

#### Influence of Pannexin-1 Channel Expression on Regenerative Growth of Neurites in explanted Dorsal Root Ganglion Cell Cultures:

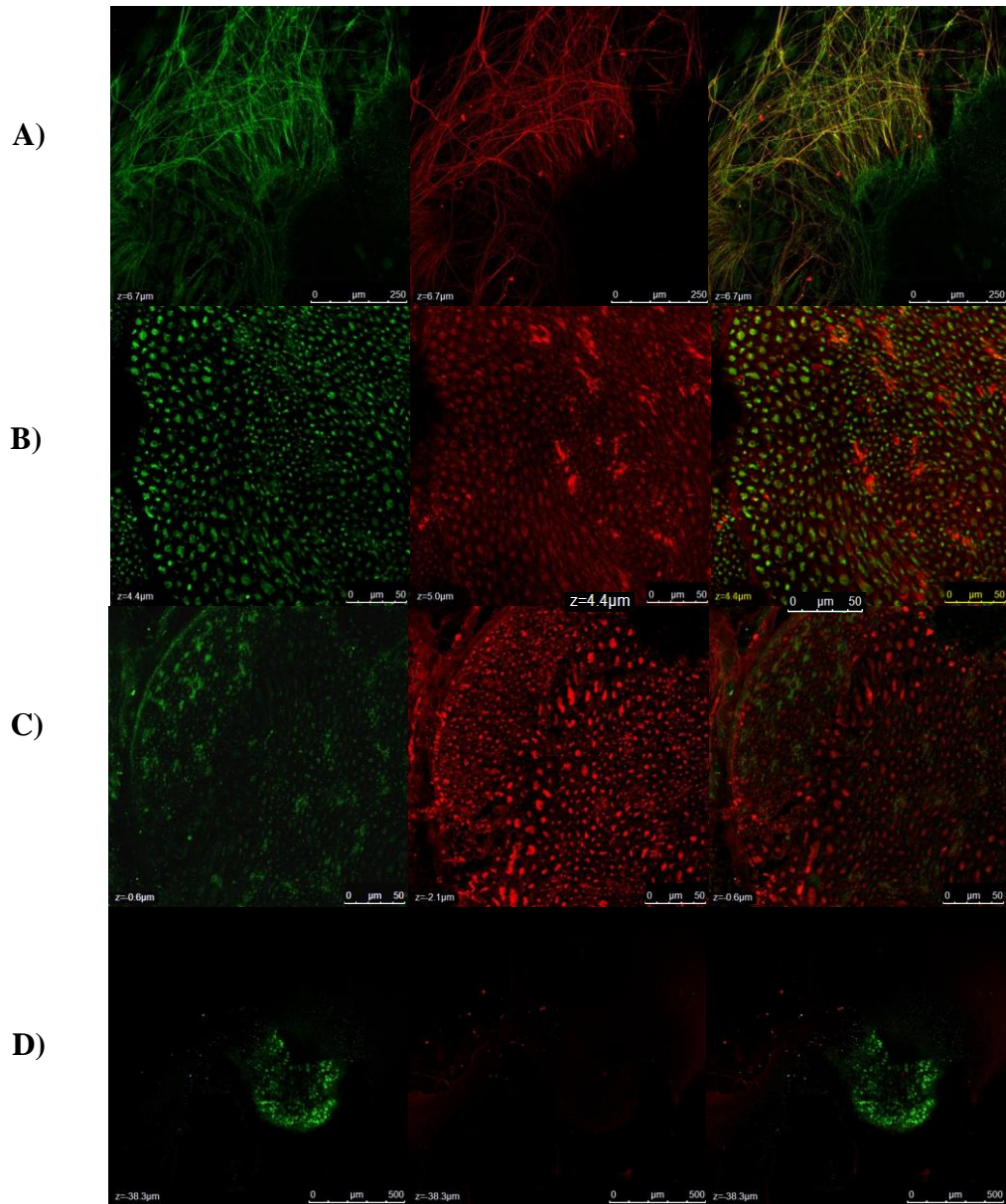
In order to evaluate whether or not Pannexin-1 channel expression and functionality influences the rate of growth in regenerating peripheral neurites of adult mice, we compared two experimental groups separated by genotype: Wild-Type and PANX1<sup>-/-</sup>



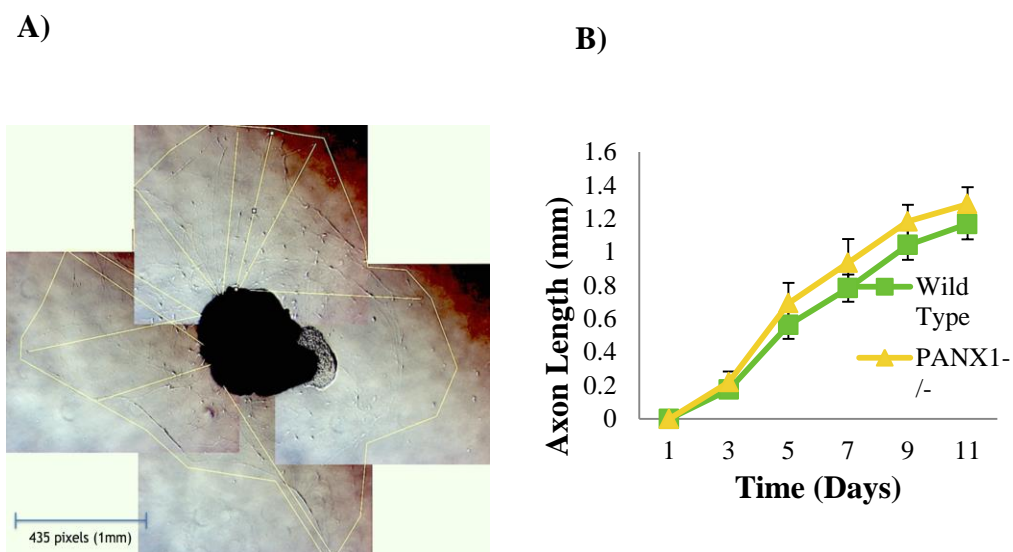
mice. The PANX1<sup>-/-</sup> mice still expressed Pannexin-1 channels, but they were functionally inhibited.

Once images were taken of explanted DRG samples from both groups of mice every other day over an 11 day span, the growth of axonal projections was quantified using ImageJ. A series of parameters were measured at each time interval to quantify the length of axons at that particular time. Our primary measurement of axon length was by taking the individual lengths of the 10 longest or most prominent axons and taking the average at each time period. We then plotted the distribution of average axon lengths for Wild-Types with PANX1<sup>-/-</sup> samples (Figure 1B). We also established a perimeter around the cell mass as well as a separate perimeter around the edges of the furthest projecting axons outlining the axonal region at each day, and recorded a series of supplementary measurements demonstrating the expansion of that region including: length of the perimeter of the axonal region, major axis of this perimeter, average of major and minor axis of the perimeter, and the length of the longest recorded axon (Figure 3). A sample of how these measurements were recorded using ImageJ is shown in Figure 1A. For each measurement we recorded Standard Deviations and Standard Errors to test the variability in the data from different explants. A series of T-tests (2 tailed, unpaired) were used to determine whether or not the differences in axonal growth were significant. P-values were generated for each measurement at each time point showing the relationship between the PANX1<sup>-/-</sup> and Wild Type groups. Though no significant differences were observed, there were trends indicating faster growth of PANX1<sup>-/-</sup> axons. More sophisticated future analysis will be used to further probe these trends

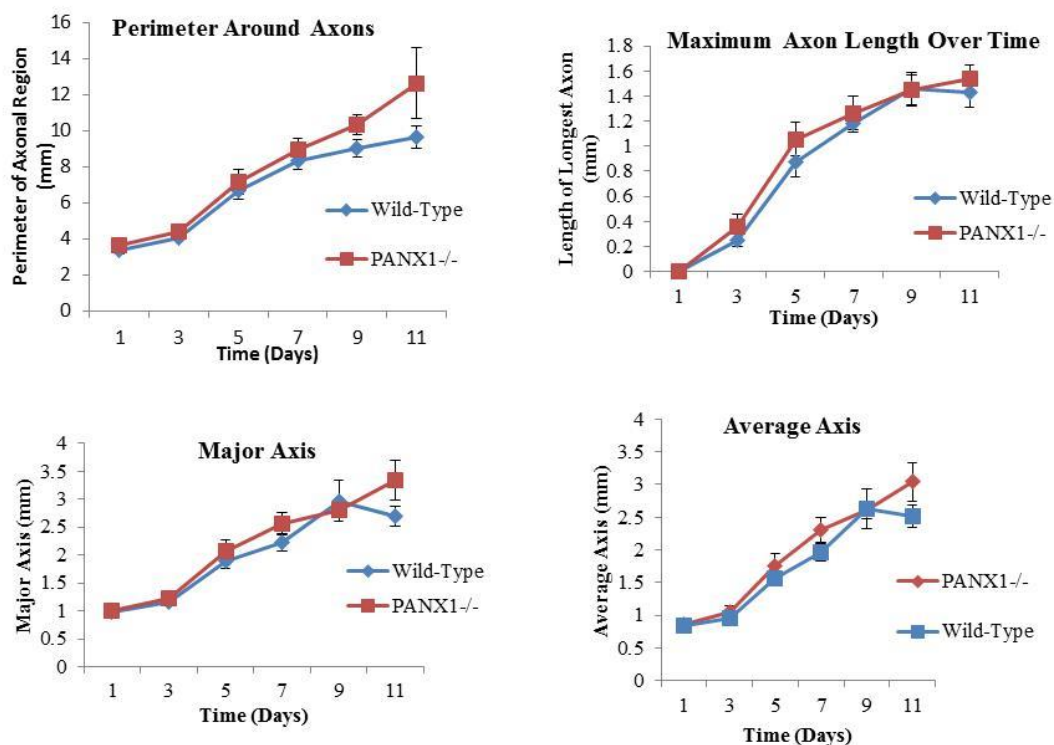
## Figures



**Figure 1: Co-localization of  $\beta$ -Tubulin III and Pannexin-1 in multiple tissue types.** Pannexin-1 is stained green (Alexafluor 488; left) while  $\beta$ -Tubulin III is stained red (Alexafluor 594; middle). The overlaid images (right) shows the overlap of the two signals indicated by yellow, showing axonal expression of Pannexin-1. A) Images taken of the projections from a Wild-Type DRG explant. Images were taken at 20X magnification using confocal microscopy. B.) Wild-Type sciatic nerve cross sections taken at 63X magnification with glycerol immersion. C) PANX1<sup>-/-</sup> mouse sciatic nerve cross sections, stained with Pannexin-1 (red) and  $\beta$ -Tubulin III (green). D) Wild-Type DRG explant demonstrating expression of Pannexin-1 in cell bodies (green), while  $\beta$ -Tubulin III had no signal on the cell mass



**Figure 2: Quantification of axonal projection length in Wild-Type and PANX1-/- mice from plated DRG explants.** A) Methods for determining length of axons at each time point. Bright-field image of a Wild-Type DRG explant with projections after day 11 (max) of growth. Growth of axons every other day were measured by drawing a perimeter around the edges of the axonal region, and also tracing the length of the 10 most prominent or longest axons. B) Average axon length at each time point (every other day) in Wild-Type and PANX1-/- mice. This was calculated by averaging the 10 traced axons and plotting them over time for both genotype groups. Error bars were generated using the standard error of all of the samples at each time period for each group.



**Figure 3: Other measurements indicating axonal projection growth in Wild-Type and PANX1<sup>-/-</sup> DRG explants.** Data was taken over an 11 day span, every other day. Error bars were generated using the standard error of all of the samples at each time point for both experimental groups. A) The average length of the perimeter drawn around the edges of the axonal projections stemming from the DRG cell-mass for each experimental group. B) The average length of the longest axon traced in each sample for the two genotype groups over the 11 day span. C) The average major axis of the perimeter measured around the outer edge of the axonal projections. D) The mean average axis (of major and minor) of the perimeter measured around the outer edge of the axonal projections in the two groups.

## Tables

**Table 1: Distribution of Mander's Coefficients 1 and 2 demonstrating co-localization of Pannexin-1 with  $\beta$ -Tubulin on axons in sciatic nerve cross sections and DRG explants of Wild-Types.** The M1 coefficient indicates the overlap of  $\beta$ -Tubulin III on Pannexin-1, while M2 shows the overlap of Pannexin-1 on  $\beta$ -Tubulin III. Both correlations are relatively strong for both model systems analyzed, however sciatic nerve cross sections showed stronger M2 coefficients while the DRG explants had higher M1 values. The sample sizes, averages, standard deviations and standard errors for both tissue types are indicated at the bottom of the table.

Animal Number	Sciatic Nerve			DRG Ex-plant		
	Sciatic Sample Number	Mander's 1 Coefficient	Mander's 2 Coefficient	DRG Sample Number	Mander's 1 Coefficient	Mander's 2 Coefficient
8	1	0.652	0.898	—	—	—
9	1	0.597	0.961	—	—	—
10	—	—	—	1	0.935	0.793
	—	—	—	2	0.951	0.956
11	—	—	—	1	0.993	0.912
	—	—	—	2	0.988	0.887
	—	—	—	3	0.991	0.885
	—	—	—	4	0.979	0.792
12	1	0.872	0.943	1	0.949	0.726
	—	—	—	2	0.963	0.955
	—	—	—	3	0.855	0.706
Average	3 Samples	0.707	0.934	9 Samples	0.956	0.846
Standard Deviation		0.145	0.0324		0.0407	0.0890
Standard Error		0.0840	0.0187		0.0129	0.0281

**Table 2: Projection P-values.** The P-values were generated from 2-tailed unpaired T-tests taken for each measurement of axonal projection growth between Wild-Type and PANX1<sup>-/-</sup> DRG explants at each indicated time point. Lower P-values indicate stronger statistical differences between the two distributions. We see this most when looking the length of the axonal perimeter at Day 9 and Day 11 of growth.

Measurement	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11
Average Axon Length	—	0.5568	0.3936	0.3733	0.3121	0.3919
Max Axon Length	—	0.3913	0.3578	0.5802	0.9519	0.5146
Perimeter Around Axons	0.5041	0.4516	0.5307	0.4270	0.09534	0.1840
Major Axis	0.9227	0.5766	0.4762	0.2029	0.6997	0.1364
Average Axis	0.8400	0.4698	0.3990	0.1663	0.9639	0.1522

## Discussion

Confirming that Pannexin-1 channels are expressed axonally in peripheral nerves of adult mice was the first test in evaluating whether or not Pannexin-1 channels contribute to axonal and neuronal function. In the DRG explant model, we also clearly observed overlap in Pannexin-1 expression in axons. Morphologically, projections from the DRG explants are purely axonal and no other cellular types interfere with the projecting axons. In sciatic nerves, while there was definitely axonal expression of Pannexin-1 channels there was also fluorescent signals indicating Pannexin-1 expression in other components of the nerve. In particular, there were large circular structures intermixed with the labelled axons that had strong signal for Pannexin-1 expression and were not neuronal in structure. Morphologically and in terms of expression pattern, these structures appear to be blood vessels. This is consistent with previous literature indicating the expression of Pannexin-1 in the smooth muscles lining blood vessels and that the channels are heavily involved in the maintenance of vascular tone. Due to the labelling of other structures in the sciatic nerve model, the DRG explant labelling was ideal to confirm and further establish that Pannexin-1 channels are expressed on axons of peripheral nerves in adult mice. We chose to use the Mander's 1 and 2 Coefficients to represent our data because they measure the percentage overlap of fluorescent pixels from each image, separating how image 1 overlaps with image 2 and visa versa. This was helpful since PANX1 seemed to bind to many non-neuronal structures and it was interesting to compare how  $\beta$ -Tubulin III overlapped with Pannexin-1 to how Pannexin-1 overlapped with  $\beta$ -Tubulin

III expression. In the DRG explant model, the correlation of overlapping  $\beta$ -Tubulin III on Pannexin-1 indicated by Mander's Coefficient 1 was incredibly strong while the Mander's 2 coefficient was strong but not quite as high. We attribute this to the fact that Pannexin-1 expression was also apparent in the cell masses of the explanted ganglia. We plan to follow up with this by confirming expression of Pannexin-1 in cell bodies (and also plan to analyze their expression patterns within the sensory ganglia) by staining cross sections of DRG cell masses and looking at the distribution of Pannexin-1 channels. Nonetheless, this study was successful in demonstrating that Pannexin-1 channels are expressed on axons of adult mouse peripheral nerves.

A major drawback of the DRG model was that the dorsal root is strictly associated with sensory function, meaning that expression patterns could vary if the same study were conducted in motor neurite projections since they are different fiber types. A potential follow-up experiment that we can conduct moving forward is comparing the strength of co-localization in the sensory branch of the sciatic to that of motor or mixed branches. This will allow us to determine whether or not there is a difference in the Pannexin-1 expression density or pattern between sensory and motor fiber types.

Upon establishing axonal expression of Pannexin-1, we were then able to explore the potential involvement of these channels in regenerative outgrowth of the projections of explanted ganglia, which were severed from their original anatomical connections *in vivo*. When comparing the measurements used to quantify axonal growth over time, we found that analyzing the average length of the 10 longest or most prominent axons was the most accurate. Therefore when comparing the axonal growth at each time period be-



tween DRGs explanted from Wild-Types to those from PANX1<sup>-/-</sup> mice, this was the best measurement for describing the data. These curves suggest the amount of growth taking place in the PANX1<sup>-/-</sup> mice is higher than the Wild-Types at every recorded time point. While we have calculated error bars using standard error, to indicate the strength of the data we are yet to draw a comparison between the two groups using a series of T-Tests. The way we will evaluate this data is by making an individual curve showing axon length over time for each sample of each genotype, and take the average slope between time points for each group. A T-test will then compare the growth rates for each time interval (day 1 to day 3, etc.) of Wild-Types and PANX1<sup>-/-</sup> DRGs to test if there is a significant difference in growth rate between the two groups. This statistical analysis will tell us whether or not PANX1<sup>-/-</sup> DRGs indeed have a faster growth rate than Wild-Types as suggested by the current graph of average axon length over time (Figure 1).

If we can determine that the growth rate of axonal projections of explanted DRGs is higher in PANX1<sup>-/-</sup>, or even that there is no significant difference between the two groups there are a few theories that we can test through further experimentation. It has been documented that in several Pannexin Knock-Out mice models, a single knock-out of Pannexin-1 alone is not enough to see phenotypical or physiological changes. A possible explanation for this is that Pannexin-2 and 3 are able to compensate for the functional responsibilities in the absence of Pannexin-1 (Penuela et. al., 2012). This can be tested moving forward via Western Blot analysis to see if there is an increase in the expression of PANX2 and 3 in the absence of functional PANX1. This can be an accurate explanation as to why mice knocked out for Pannexin-1 see an increase or no change in growth rate in comparison to Wild-Types if the Western indeed shows an increase in PANX2 and

3 expression. Another aspect of Pannexin-1 expression behavior that could contribute to this unexpected trend involves the aforementioned negative feedback loop. If extracellular ATP released by Pannexin-1 channels reaches a certain level, there is endocytosis of Pannexin-1 channels to regulate ATP release and subsequent signaling (Boyce, et. al. 2015). It is possible that functionally knocking-out transmembrane Pannexin-1 channels eliminates this negative feedback loop meaning that Pannexin-1 channels (or potentially Pannexin-2 and 3) that are normally internalized have an increase in expression on the cell membrane leading to an increase in purinergic signaling and outgrowth.

Following up with this outgrowth study, we can determine whether or not the changes (if any) in growth rate are attributed purely to a lack of Pannexin-1 by degrading all extracellular ATP in the medium using the enzyme apyrase. This will allow us to see whether or not ATP is being released solely by Pannexin-1 or if there are other sources contributing to purinergic signaling.

This study has revealed that Pannexin-1 channels are expressed axonally in the peripheral nerves of adult mice, implying that they are most likely involved in axonal or neural function. Moving forward with this research we can unveil more regarding the exact influence of Pannexin-1 channels on regenerative growth of axonal projections, and hopefully gain a more complete understanding of the role of Pannexin-1 in normal axonal function in peripheral nerves. In the big picture, having an improved understanding of the cellular mechanism driving regeneration in recovering peripheral nerve tissue may contribute to regenerative medicine. Once the exact mechanism involving Pannexin-1 in regenerative outgrowth is understood, regulation of Pannexin-1 channels may be able enhance the regrowth process and lead to more complete neural recovery.

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