Salisbury UNIVERSITY

Honor College at Salisbury University

Honors Thesis

An Honors Thesis Titled

The migration of sensory neurons following chemical-genetic B-cell ablation in Danio rerio larvae

Submitted in partial fulfillment of the requirements for the Honors Designation to the

Honors College

of

Salisbury University

in the Major Department of

Biological Sciences

by

Amanda Backer

Date and Place of Oral Presentation: April 2018 at NCUR

Signatures of Honors Thesis Committee

	Signature	Print
Mentor:	JClark	Jessica Clark
Reader 1:		Jennifer Nyland
Reader 2:		Kristen Walton
Dean:		James Buss

Abstract

Peripheral nerves act as a vital bridge between the environment and the brain; environmental information is received by sensory neurons and transported, through electrical impulses, along axons to the central nervous system (CNS). The brain processes the information and sends a response back to target organs or muscles through motor axons. When any component of the peripheral nerve is damaged a condition called peripheral neuropathy can develop. This condition often presents in the form of numbness, aching, and burning in the extremities as a result of the nerve damage. One specific cause of peripheral neuropathy is hyperglycemia, an excess of blood glucose which is often associated with diabetes. Damage may be present in both the sensory and motor nerves that make up the peripheral nervous system (PNS), diminishing the ability of those affected to interact with their environment. Preliminary data from the Clark lab suggests that sensory neurons migrate away from their origins at the dorsal root ganglia (DRG). Based upon this work and the knowledge that nerve changes occur in correspondence to hyperglycemia, I hypothesize that the onset of hyperglycemia by pancreatic β-cell ablation results in peripheral neuropathy. Future work will include a small molecule drug screen that may be able to provide insight on the molecular mechanisms underlying hyperglycemia-induced degeneration of the peripheral nerve. Additionally, I plan to study how hyperglycemia presents in the CNS utilizing the same model system.

Introduction:

Peripheral nerves are a major component of the PNS and include the motor and sensory nerves. The motor and sensory nerves possess similar structures, and both play a role in receiving and passing information between the peripheral and central nervous system. Sensory nerves take in afferent, sensory information about the environment. They send this information along their axons in the form of electrical impulses to the CNS, namely the brain, for interpretation. This allows for the perception of the touch sensations from stimuli though texture and object recognition[1]. Once received, the CNS integrates this information and sends an appropriate response back through motor axons to the target organ or muscle[2]. This sensory-motor feedback, comprised of afferent and efferent information coming from the peripheral nerves, is vital for perception and reaction to the environment

A peripheral nerve is composed of many axons from multiple neurons. Axons function to carry afferent and efferent messages between the CNS and periphery in the form of electrical impulses which are generated by action potentials following stimulation. Most axons are enveloped by myelin for insulation, allowing for speed and efficiency of the electrical impulses. Axons are bundeled together into fascicles by a protective blood-nerve barrier, the perineurium, and multiple fascilces are further packaged together by the epineurium to form one complete nerve[2]. All of these layers protect the axons from environmental insults that would otherwise damage the axon itself or prevent the nerve's proper function.

Damage to any of these components can result in a condition known as peripheral neuropathy. This is caused by a number of factors including chemotherapy, hyperglycemia, autoimmune disease, alcoholism and genetics; however, the most common cause of the condition is hyperglycemia. Hyperglycemia is often associated with Diabetes Mellitus (DM), as it is a

common cause for the condition[3]. DM comes in two forms, Type I DM and Type II DM. Type I DM is characterized by the loss of insulin-producing pancreatic β-cells, while Type II DM is characterized by dysfunction of insulin signaling.

Approximately 50% of people in the United States presenting with diabetes are afflicted by diabetic peripheral neuropathy (DPN)[4]. Although DPN affects both the motor and sensory nerves, damage to the sensory nerves is believed to occur earlier in disease progression due to differences in location and protective barriers. Sensory neurons are pseudounipolar, meaning that they have two axons branching off from the same point on a neuron's cell body, or soma[2]. While one axon leads to the CNS and one leads to the periphery, the soma is located at the DRG adjacent to the spinal cord. This is the location where all sensory input, or afferent information, from one axon is collected prior to being sent to the CNS along the second axon. Motor neurons; however, are unipolar with a single, long axon extending from the soma, which is located within the spinal cord of the CNS[5]. Unlike the PNS, the CNS contains more protection including the three layers of meninges tissue (dura mater, arachnoid layer, and pia mater), the blood-brain barrier, cerebrospinal fluid, and bone[6]. Therefore, components in the PNS are more susceptible to damage than those in the CNS, suggesting that motor neurons may be more heavily protected due to their location within the spinal cord compared to sensory neurons that lie outside of the spinal cord.

In the Clark lab, changes following the induction of hyperglycemia have been observed in the PNS, including defasciculation of peripheral nerve axons, axonal blebbing, diminished and disrupted perineurium, and migrating sensory neurons (Fig. 1). Such damage would suggest diminished efficiency of the PNS in receiving and sending afferent information, which would

have debiliating consequences for those afflicted. In fact, patients typically present with pain, aching, numbness, tingling, or burning prior to muscle weakness or loss of reflex[7].

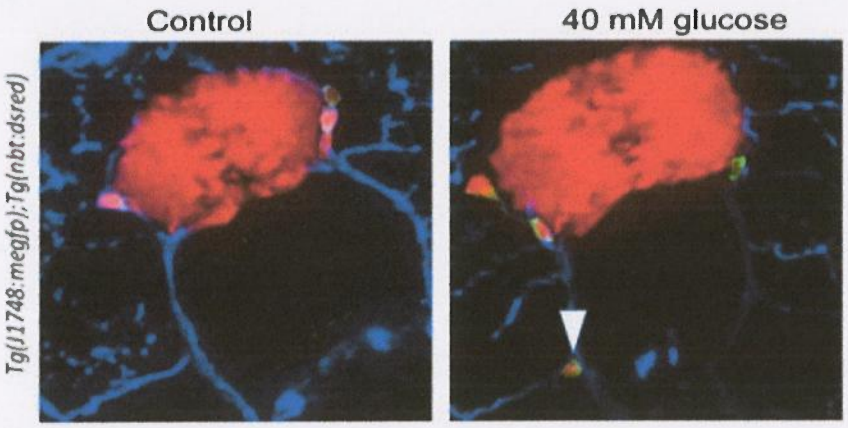

Figure 1. Sensory neurons migrate away from the DRG in hyperglycemic zebrafish at 8dpf. The green transgene, Tg(J1748:megfp) indicates the sensory neurons. These are clustered appropriately around the spinal cord in the control, but have migrated along an axon (TUB; blue; arrowhead) in the hyperglycemic fish in 40 mM glucose solution. Tg(nbt:dsred) is a non-specific neuronal marker.

Although DPN affects a large portion of the population and contributes tremendously to the cost of health care, treatment is limited to pain management. Until an animal model exists that can determine and manipulate the underlying molecular mechanisms causing the onset of DPN, this will continue. Here, I propose using zebrafish to model the acute changes in sensory neurons following the onset of hyperglycemia. Unlike other animal models, zebrafish are small, transparent vertebrates and are genetically malleable. This, along with their high fecundity, lower cost, and genetic similarity to humans makes them better candidates to model DPN than other animal models[8].

For this study, I will utilize an established chemical-genetic ablation model to induce hyperglycemia. This model utilizes a nitroreductase system to ablate pancreatic β-cells in zebrafish larvae. Pancreatic β-cells normally produce insulin, and when blood glucose levels rise following a meal, insulin is secreted to help get blood glucose into the cells to be used for energy. When pancreatic β-cells are lost (as in this model and Type I diabetes), insulin can no longer be made and secreted, and blood glucose levels rise, resulting in hyperglycemia.

Therefore, **I hypothesize that the onset of hyperglycemia by pancreatic β-cell ablation results in peripheral nerve changes.** The concept of changes to peripheral nerves is

derived from the notion that hyperglycemia causes diabetes, which in turn may lead to DPN. Previous data from the Clark lab shows that sensory neurons migrate away from the DRG in a glucose-induced hyperglycemic model (Fig. 1). A decrease in peripheral sensation may occur, possibly due to damage of the sensory neurons resulting from neuronal migration, leaving the neurons ineffective in responding to stimuli and sending proper electrical impulses to the CNS[9]. Based on this, and the knowledge that sensory nerve damage may occur prior to motor nerve damage, **I further hypothesize that sensory neuron dysfunction will occur in response to hyperglycemia by my β-cell ablation model.** Creating a DPN model with a characterized sensory neuronal phenotype will allow me to test established molecular drug libraries on thoughsands of zebrafish larvae at a time, something that could never be done in mammals.

Materials and Methods:

Zebrafish Model and Care:

In the Clark lab, we use a chemical-genetic cell ablation model to induce hyperglycemia in *Danio rerio*, or zebrafish, as they are more commonly known. Zebrafish are a valuable animal model because they are vertebrates and they share similar genetic traits with humans. Furthermore, zebrafish are easy to house and breed, and they yield a large amount of offspring at one time. The zebrafish are cared for by the student researchers in the lab, all of whom must complete an animal husbandry and ethics training through the CITI program to become certified to work with zebrafish. An animal care log is kept to track the feeding schedule, along with water conditions such as pH and temperature. Research conducted in the Clark lab is approved by Salisbury University's IACUC committee.

Transgenic Lines and Crossing:

I utilized a specific line of transgenic zebrafish, *Tg(ins;nfsB-mcherry)*. In these fish, a gene encoding nitroreductase (*nsfB*) from *Escherichia coli* is fused to a fluorescent probe, *mCherry*, and driven by the insulin (*ins)* gene in β-cells of the pancreas. The chemical-genetic ablation model relies upon these aspects of the transgene to work as this line allows for red fluorescence of the insulin producing β-cells for visualization prior to treatment, as well as ablation of these cells in treated fish. These fish were treated with a prodrug, MTZ, which acts against bacterial cells. In this model, it binds with the nitroreductase in β-cells, and this combination leads to a cytotoxic product that causes the cell ablation, or cell death, of these *nfsB*-expressing cells[10]. Ablation of insulin producing β-cells results in hyperglycemia and the loss of the red fluorescent cell marker, which I check for following treatment. Without β-cells to produce insulin, which initiates the intake and break down of glucose, blood glucose levels rise. To visualize the sensory neurons, I crossed *Tg(neuroD;GFP)* into the *Tg(ins;nfsB-mcherry)* line. The *neuroD* line causes green fluorescence of sensory neurons due to the green fluorescent protein (*GFP).*

Five days post fertilization (dpf), the treatment group was incubated in 10mM metronidazole (MTZ)/0.5% DMSO solution and the control group in 0.5% DMSO control solution for 48 hours. This solution was exchanged for fresh solution after the first 24 hours. Fish treated with MTZ were accessed after 48 hours (7dpf) for cell ablation using an epifluorescent microscope to visualize the pancreas. After ablation of insulin-producing cells, there is an absence of, or diminished *mCherry* fluorescence within the islet of the pancreas.

Glucose Assay:

A glucose assay (Glucose Colorimetric/Fluorometric Assay Kit, BioVision) was performed to determine if hyperglycemia was achieved in the treated group. For this assay, 20 fish from both treatment and control groups were flash frozen at -80°C. These samples were thawed in assay buffer, homogenized, and centrifuged at 13,000 rpm for 1 minute. After centrifugation, the supernatant was collected and the pellet was discarded. Diluted glucose standard of 1 nmol/µl and 1, 2, 3, 4, 6, 8, 10 µl is added to wells on a plate, and the final volume brought to 50 µl with Glucose Assay Buffer. 16 µl of supernatant sample is added to 34 µl assay buffer. Finally, a mixture of 46 µl Glucose Assay Buffer, 2 µl Glucose Probe, and 2 µl Glucose Enzyme Mix per sample was added to each well. The Glucose Enzyme Mix oxidizes glucose, creating a product that reacts with dye to express a pink color. The plate was incubated in the dark at 37°C for 30 minutes. After this period, the absorbance is measured at 570 nm in a microplate reader, and analyzed with a Spectromax pro (SpectraMax i3, Molecular Devices). The Spectromax sends light through the samples and, depending on the concentration of the material within those samples, a certain amount of light is absorbed. This absorbance is measured by the Spectromax and is directly proportional to the glucose concentration within the samples.

Fixing and Mounting:

Both treated and control groups were fixed and mounted, separately, at 7 dpf. The fish were fixed in a solution of 4% paraformaldehyde (PFA) overnight. They were then set into agar gel molds in a vertical orientation. After the molds hardened, the agar was cut into blocks containing the fish, and set into 30% sucrose solution overnight. This removes excess water from

the tissues of the fish to prevent the formation of ice crystals while making sections with the cryostat.

Cryostat:

A cryostat (Leica, USA) was used for taking cross sections of zebrafish in agar blocks. The blocks were set onto circular disks with the heads of the fish facing up, and frozen to the disks with histoprep at approximately -40°C. Once frozen, the disk was attached to the stage, and 10μm sections from the blocks were cut. These sections were set onto slides, and coverslips were attached using aquapoly/mount (Poly Science, catalog #18606) and 1x phosphate buffer saline (PBS).

Epifluorescent Microscope:

I acquired images for both control and treated zebrafish using an inverted fluorescent microscope (EVOS FL Auto Imaging System, Thermo Fisher Scientific). All images were imported into ImageJ (NIH) for visualization purposes (not for analyses) where adjustments were limited to levels, contrast, color matching settings, and cropping. Due to the transgenes within the fish, the β-cells and sensory neurons could be visualized. The β-cells, before ablation, fluoresce red from the *mCherry* probe and sensory neurons are indicated by the green fluorescent protein (*GFP*). Thus, the condition of the pancreas pre- and post-treatment as well as changes to sensory neurons can be visualized.

Data Quantification:

The data are represented as ± Standard Error of the Mean (SEM). A two-tailed independent samples t-test was used to analyze the differences between two groups for the glucose assay and neuron migration count data. Graph Pad Prism 7.0 (Graph Pad Software, Inc.,

San Diego, CA) was used for statistical analyses and graph preparation. Differences between groups were considered significant when $p < 0.05$.

Counts were taken for migrating sensory neurons defined as those outside of the DRG. This study was completed in triplicates for both control (DMSO) and treated (MTZ + DMSO) groups. The neuron counts and glucose assay results were generated into visuals to show the significant difference between control and treated fish for the number of migrating neurons and blood glucose levels.

Specific Aim:

It is the aim of the Clark lab to characterize a zebrafish larvae model of DPN. The primary goal of my study was to determine how hyperglycemia presents itself in the PNS, specifically in regards to sensory neurons in zebrafish larvae. This study was comprised of two aims: (1) induce hyperglycemia with an established ablation model and (2) analyze treated fish for changes in sensory nerves. Experiments were conducted following the methods outlined in the paradigm below (Fig. 2).

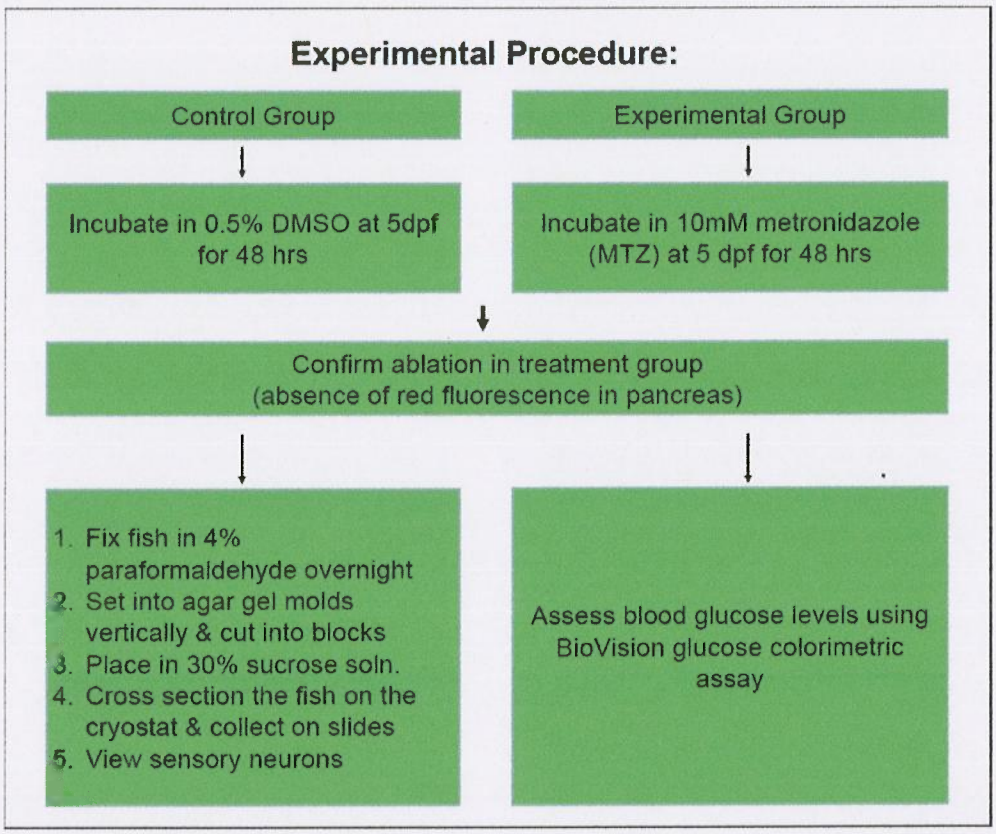

Figure 2. Experimental Paradigm. This procedure was followed for every experiment which consisted of both a control group and treatment group. In addition to incubation in their respective solutions, fish either underwent processing for visualizing neurons, or were used in a glucose assay to determine if the treatment in MTZ resulted in hyperglycemia for the treated fish.

Aim 1: Induce hyperglycemia in zebrafish larvae

The treatment group from a line of *Tg(ins;nfsB-mcherry); Tg(neuroD;GFP)* zebrafish underwent β-cell ablation as described in the methods. The pancreatic β-cells are tagged with *mCherry*, and fluoresce red when viewed beneath an epifluorescent microscope; however, following ablation, this fluorescence should not appear due to loss of cells that express *mCherry*. I determined β-cell ablation by the lack of *mCherry* expression, which suggests potential hyperglycemia of the treated fish. In addition, I performed the glucose assay outlined above to assure hyperglycemia was induced in the treatment group after β-cell ablation.

Aim 2: Observe and analyze changes to the sensory neurons

I hypothesized that, following induced hyperglycemia, sensory neurons would migrate from the DRG, contributing to the effects of peripheral neuropathy that are associated with DPN. At 5dpf, larvae from the *Tg(ins;nfsB-mcherry); Tg(neuroD;GFP)* line were split into control and treatment groups. The treatment group was induced with hyperglycemia by incubation in 25 ml of 10mM MTZ solution. After 48 hours, I confirmed ablation of pancreatic β-cells, which caused hyperglycemia and, potentially, peripheral neuropathy. To analyze the sensory neurons for migration, the *GFP* transgene is tagged to sensory neurons in the zebrafish, allowing for visualization by green fluorescence seen with an epifluorescent microscope. I compared the placement of sensory neurons of both the control and treated groups to search for potential migration of the sensory neurons in the treated zebrafish.

Results:

Induced Hyperglycemia

A glucose assay, as described above, was performed on both control and treated groups to determine blood glucose levels. The absorbances collected from the Spectromax pro were quantified into pmol glucose per larvae. This data was used to determine that the zebrafish that underwent treatment and ablation had higher blood glucose levels than the control ($*p<0.05$), providing support that the treated fish were induced with hyperglycemia (Fig. 3).

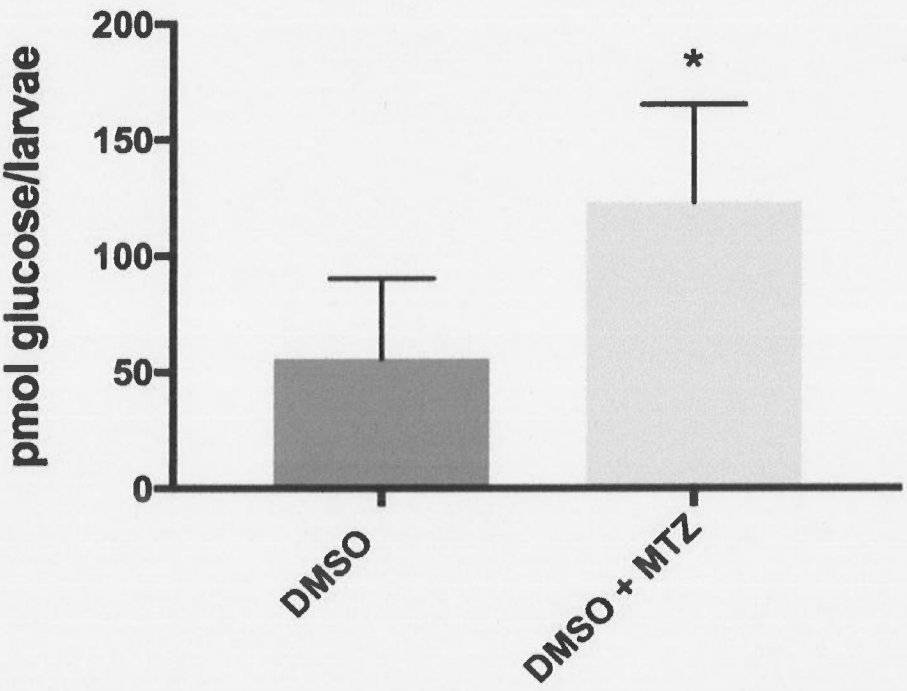

Figure 3. MTZ treated fish were hyperglycemic There is a significantly higher average blood glucose concentration in the MTZ treated fish (DMSO + MTZ) compared to the control fish (DMSO) *$p<0.05$.

Sensory Neuron Changes

An EVOS FL Auto Imaging System (Thermofisher Scientific) was used to view migrating sensory neurons and obtain Z-stacked images of sections from prepared slides. Neuron counts for both control and MTZ treated fish were completed in triplicates, utilizing three experiments consisting of both a control group and treatment group. Based upon the visualization (Fig. 4) and counts (Fig. 5) of sensory neurons outside of the DRG, we conclude that sensory neurons migrated away from the DRG in hyperglycemic fish. Although neurons migrated in control fish as well, there were significantly more neurons migrating in the MTZ treated fish (****$p<0.0001$).

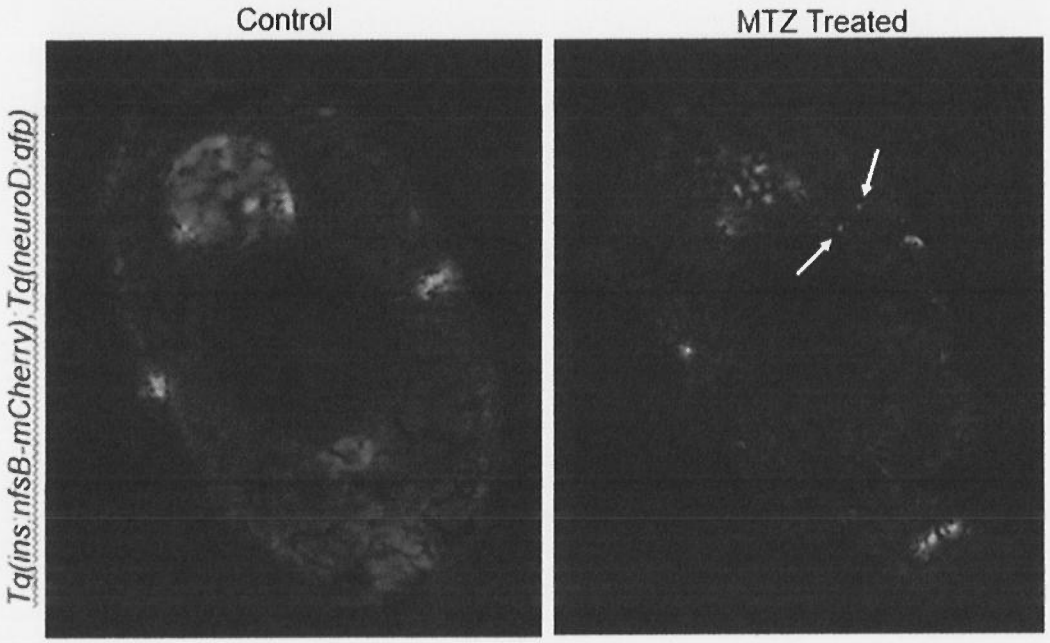

Figure 4. Neurons migrate in MTZ treated fish Sensory neurons (white arrows), tagged with *Tg(neuroD;GFP)* transgene, migrate from the DRG in zebrafish treated with MTZ solution at 5 and 6dpf, while they remain clustered in the DRG in the control fish.

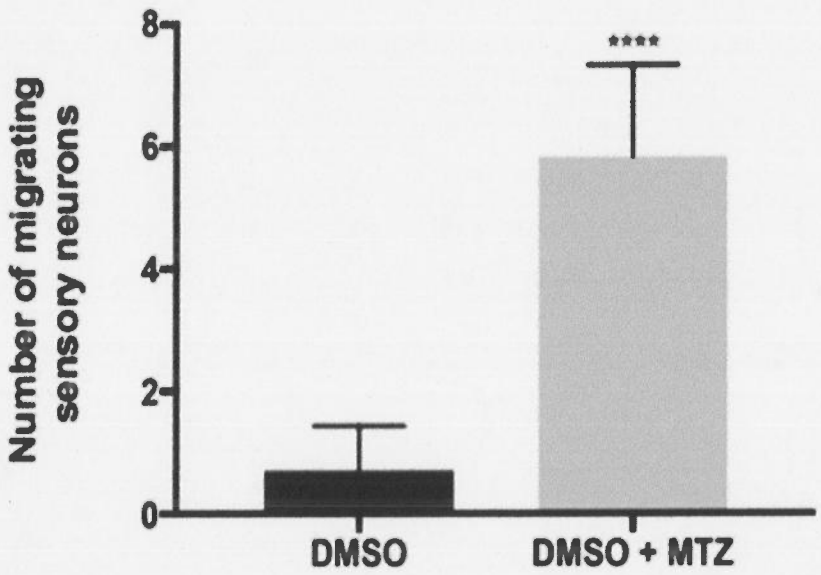

Figure 5. Sensory neurons migrate away from the DRG following hyperglycemic induction. There is a statistically significant difference in the number of migrating neurons between MTZ treated (DMSO + MTZ) and control (DMSO) fish ****$p<0.0001$.

Discussion:

Although DPN affects a large portion of the population and contributes tremendously to the cost of health care, treatment is limited to pain management. Diabetes affects about 8.3% of adults worldwide, 50% of those afflicted presenting with DPN, and the prevalence of diabetes is expected to increase by 69% by 2030. Currently, the United States spends about $245 billion in health care costs for DPN and other complications of diabetes. With the increased presence of diabetes and DPN around the world, the physical, financial, and societal burdens will also continue to increase. There are no current FDA approved therapies or approved curative medications in the US or UK for DPN most likely due to the complexity and unpredictability of the condition. Thus far, we depend upon the management of pain symptoms for neuropathic treatments; however, even these provide minimal and limited pain relief[11].

Until an animal model exists that can determine and manipulate the underlying molecular mechanisms causing the onset of DPN, the issues and complications associated with this condition will continue. Here, I proposed the use of zebrafish to model the acute changes in peripheral nerves following the onset of hyperglycemia. I found that zebrafish larvae are induced with hyperglycemia by chemical-genetic β-cell ablation and this ablation results in peripheral nerve changes. Specifically, I observed the migration of sensory neurons away from the DRG which may provide a reason for loss of sensation experienced by humans with DPN. These results suggest that this may serve as a robust model for DPN. By utilizing an animal model that can compare to the condition in humans, scientists will gain a better understanding of DPN and its effects on the nervous system. Once the phenotype is characterized, testing for potential treatments to reverse the effects of the condition can be carried out.

Wright, *et. al.* found that migrating neurons originating from the DRG may become incorporated into the sympathetic ganglia, a component of the sympathetic nervous system important for involuntary events within the body, following a decrease in voltage-gated sodium channel levels[12]. Interestingly, there has been some evidence that voltage-gated sodium channel expression decreases in diabetic sensory neurons as well[13]. With this knowledge, future research will involve analyzing sodium channel levels following induced hyperglycemia and exploring whether or not these migrating neurons have a specific trajectory towards the sympathetic ganglia. Furthermore, I am curious as to the role these neurons may play once they reach their new environment.

Additional research involves looking into how sensory neurons respond when β-cells are allowed to regenerate after chemical-genetic ablation. This is possible as zebrafish are known for having the ability to regrow and restore damaged tissues[14]. To accomplish this, the larvae will be

removed from the MTZ solution, which will allow them to regenerate their previously ablated pancreatic cells and reverse the neuronal damage. The larvae will be examined for possible proliferation of the neurons, and whether or not they remain at their origin within the DRG following regeneration. This may lead to a small molecule drug screen to determine possible molecular mechanisms and therapeutics.

Conclusion:

Research on peripheral neuropathy is important due to the vital role peripheral nerves play in forming the bridge between the brain and environment. This condition, increasingly prevalent throughout the world, is debilitating to the lives of many people, diminishing their ability to interact with their environment. With continued research to find and test animal models for DPN, people can better understand peripheral neuropathy with the goal of developing effective treatments and solutions for the condition.

References

1. Abraira, Victoria E. and David D. Ginty. "The Sensory Neurons of Touch." *Neuron* 79.4 (2013): 618-639.

2. Kaplan, S., E. Odaci, B. Unal, and M. Fornaro. "Chapter 2 Development of the Peripheral Nerve." *International Review of Neurobiology* 87 (2009): 9-26.

3. Corsino, Leonor, K. Dhatariya, and D. Umpierrez . "Management of Diabetes and Hyperglycemia in Hospitalized Patients." *Endotext [Internet].* South Dartmouth (MA): MDText.com, Inc.; 2000.

4. Tesfaye, Solomon and Dinesh Selvarajah. "Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy." *Diabetes Metab Res Rev* 28 (2012): 8-14.

5. Lodish, H., et al. 2000. Overview of Neuron Structure and Function. In W. H. Freeman (Ed.), *Molecular Cell Biology 4th edition* (Section 21.1). Retreived from https://www.ncbi.nlm.nih.gov/books/NBK21535/

6. Freburg, Laura A. 2016. Chapter 2: Functional Neuroanatomy and the Evolution of the Nervous System. In Nina Taylor (Ed.), *Discovering Behavioral Neuroscience: An Introduction to Biological Psychology.* Cengage Learning, Boston. Pages 28-30 & 65.

7. Petrlik, Joan C. "Diabetic Peripheral Neuropathy." *The American Journal of Nursing* 76.11 (1976): 1794-1797.

8. Howe, Kerstin, Matthew D. Clark, Carlos F. Torroja, James Torrance, Camille Berthelot, 408 Matthieu Muffato, John E. Collins, et al. "The Zebrafish Reference Genome 409

Sequence and Its Relationship to the Human Genome." *Nature* 496.7446 (2013):498–503.

9. Kline, Kristina M., D. G. Carroll, and K. F. Malnar. "Painful Diabetic Peripheral Neuropathy Relieved with Use of Oral Topiramate." *Southern Medical Journal* 96.6 (2003): 602-605.

10. Curado, S., D. Y. R. Stainier, and R. M. Anderson. "Nitroreductase-Mediated Cell/tissue Ablation in Zebrafish: A Spatially and Temporally Controlled Ablation Method with Applications in Developmental and Regeneration Studies." *Nature protocols* 3.6 (2008): 948–954.

11. Javed, S., U. Alam, and R. A. Malik." Treating Diabetic Neuropathy: Present Strategies and Emerging Solutions." *Rev Diabet Stud* 12.1-2 (2015): 63-83.

12. Wright Melissa A., W. Mo, T. Nicolson, and A. B. Ribera. "In vivo evidence for transdifferentiation of peripheral neurons." *Development* 137.18 (2010): 3047–3056.

13. Hong, S., T. J. Morrow, P. E. Paulson, L. L. Isom, and J. W. Wiley. "Early painful diabetic neuropathy is associated with differential changes in tetrodotoxin-sensitive and -resistant sodium channels in dorsal root ganglion neurons in the rat." *J Biol Chem* 279.28 (2004): 29341-29350.

14. Gemberling, M., T. J. Bailey, D. R. Hyde, and K. D. Poss. "The zebrafish as a model for complex tissue regeneration." *Trends in Genetics* 29.11 (2013): 611-620.