DiaComp Progress report – Sandra Rieger, Ph.D.

A new *in vivo* model for imaging axon dynamics in injured diabetic zebrafish.

Diabetes mellitus affects over 26 million adults in the United States and 285 million people worldwide. These numbers are expected to nearly double in the next two decades. Neuropathy-mediated wound healing complications are a major problem in diabetes, with diabetic foot ulcerations (DFU) preceding approximately 84% of lower limb amputations. Long-term intensive care and consequences of amputation place immense burdens on individuals and their families as well as healthcare systems. While interdisciplinary clinical groups have made certain advances in DFU prevention and control of the disease, higher success could be achieved through a better understanding of the underlying causes. This lack of knowledge mostly derives from the complex etiology of DFU. Although the use of mammalian model organisms has proven to be highly important for the investigation of diabetes-associated complications, they are subject to technical limitations and high costs. New low-cost model systems are required allowing for *in vivo* analyses of the complexity of axon-wound interactions in live animals. With the power of *in vivo* imaging new concepts underlying the etiology of DFU could emerge. Here we propose to develop zebrafish larvae as a new *in vivo* model for elucidating basic mechanisms of DFU. Zebrafish larvae have several advantages over other model organisms in that they are optically clear and develop rapidly. This makes them suitable for genetic and pharmacological manipulations and for *in vivo* imaging. In addition, glucose metabolism in larval and adult zebrafish is highly similar to humans. Thus zebrafish larvae are ideal for investigations of diabetic complications. The goal of this proposal is to combine our previously established axon regeneration model in zebrafish larvae with genetic ablation of insulin-secreting pancreatic β-cells to analyze the behavior of acutely injured and neuropathic cutaneous axons under hyperglycemic conditions. This model will allow us to address basic questions that are key to understanding DFU etiology: 1) is there a temporal relationship between β-cell death and onset of neuropathy? 2) Do acutely injured cutaneous axons regenerate under hyperglycemic conditions? And 3) does acute injury initiate or aggravate neuropathy? Understanding these basic mechanisms will be fundamental for investigations on neuropathy-related chronic wound formation. This work has the potential for future pharmacological studies in zebrafish larvae with the goal to identify drugs that suppress the onset of neuropathy and prolong axon health.

We originally proposed two Specific aims that will provide important insight into the effects of hyperglycemia on zebrafish sensory neuron health:

Specific aim 1: Establish a larval zebrafish model for peripheral neuropathy induced by hyperglycemia.

In this aim our goal was to validate a previously established β-cell ablation model for inducing hyperglycemia in larval zebrafish and evaluate the effects of hyperglycemia on the peripheral axons of somatosensory neurons.

Specific aim 2: Elucidate dynamics of acutely injured axons in hyperglycemic larval zebrafish using *in vivo* time-lapse imaging.

Our goal here was to examine de- and regeneration behavior of peripheral sensory axons following acute injury under hyperglycemic conditions.

A progress report for Specific aim 1 followed by a description of studies to be carried out in the next year, as described in Specific aim 2, is presented.

Establish and characterize a larval zebrafish model for diabetic peripheral neuropathy.

The zebrafish has emerged as an invaluable genetic system for modeling human diseases, including diabetes. Peripheral neuropathy and chronic wound formation are major complications of diabetes. Although genetic tools have been established to induce β-cell death in zebrafish larvae to model hyperglycemia (*1, 2*), in-depth molecular characterizations of interference with glucose metabolism upon

β-cell ablation have not been performed. Furthermore, the effects of hyperglycemia on peripheral sensory axon health remain to be elucidated. In this proposal, we suggested to: 1) examine whether the genetic ablation of β-cells induces changes in glucose metabolism in larval zebrafish, 2) assess the effects of hyperglycemia on peripheral sensory axon morphology using *in vivo* confocal imaging, and 3) examine the effects of hyperglycemia on injury-induced axon de- and regeneration.

What have we achieved?

Specific aim 1

1. Depletion of insulin in a genetic β-cell ablation model

To deplete insulin-secreting pancreatic β-cells and monitor loss of β-cell mass through decreased fluorescent protein expression in the endocrine pancreas, we utilized a genetic ablation technology. In this system, the *nfsB* gene, encoding the bacterial enzyme Nitroreductase (NTR), is fused to the gene encoding the red fluorescent protein mCherry and expressed in β-cells under the insulin promoter. If the prodrug metronidazole (MTZ) is added to dechorionated zebrafish embryos, NTR converts MTZ into a toxin, whereby β-cells undergo apoptosis without affecting neighboring cells. This system has been successfully utilized for ablation of β-cells (*1, 2*), macrophages (*3*), pineal gland cells (*4*) (our lab) and peripheral sensory neurons (our lab). For β-cell and insulin depletion, we obtained the T*g(insulin:NTR-mCherry)* transgenic zebrafish strain from the lab of Dr. Michael Parsons (Johns Hopkins University) and raised these fish to breeding age. Their offspring were obtained and at 24 hours post fertilization (hpf) screened for fluorescent transgene expression. Only homozygous embryos with transgene expression in β-cells were selected. For ablation, we dechorionated zebrafish embryos at 24 hpf using Pronase (see **Protocol 1** for methodology of dechorionation) and treated the embryos either with 0.5 % DMSO as control or 10 mM MTZ/ 0.5 % DMSO (see **Protocol 2** for methodology of β-cell ablation using the NTR/MTZ system) for 48 hours (until 3 days post fertilization, dpf), similar to previous reports (*1*). Although we found variation in the ablation efficiency between individual zebrafish treated with MTZ (Figure 1), we observed a strong reduction in fluorescent transgene expression in 29 % of the larvae after 48 hours of treatment (Figure 1C), whereas 71 % of the larvae showed a partial ablation (Figure 1B). MTZ treatment for 1 day sufficiently ablated β-cells only in 15 % of the analyzed larvae. We never observed ablation in DMSO-treated control larvae after 24 and 48 hours (Figure 1A). Because β-cells regenerate in zebrafish (*2*), we also monitored the re-appearance of

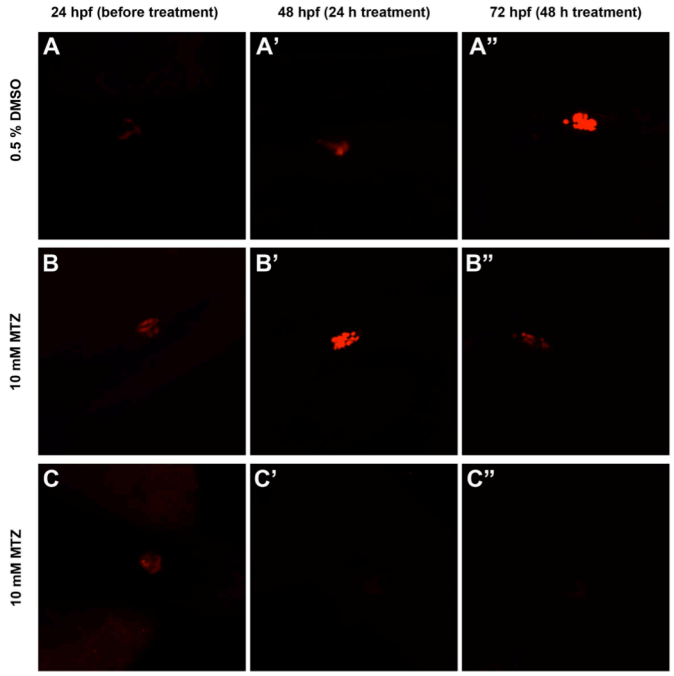

Figure 1. Ablation of β-cells in Tg(*ins:NTR-mCherry*) transgenic zebrafish larvae. (**A**) NTR-mCherry expression in pancreatic β-cells of control embryos at 24 hours post fertilization (hpf), before (A) and after 24 h (A') or 48 h (A") of DMSO treatment. The DMSO-treated larva shows an increase in fluorescence over time, suggesting that the pancreas is still developing between 24 and 72 hpf. (**B, C**) Comparison between partial (B) and full (C) β-cell ablated larva. Shown are β-cells before treatment at 24 hpf (B, C), which appear to have equal fluorescence intensity. Larva 1 (B') shows increased fluorescence after 24 hours, whereas larva 2 (C') shows complete ablation of β-cells at this time. At 48 hours of MTZ treatment, the fluorescence is strongly reduced in both larvae (B", C").

mCherry fluorescence over time. We however did not observe a recovery in fluorescence until at least 8 dpf (not shown).

Having found that we can successfully ablate β-cells in larval zebrafish, we wanted to confirm successful insulin depletion by assessing insulin expression with quantitative PCR. Comparing DMSO and MTZ treated larvae at 3 dpf and 8 dpf (when larvae were allowed to recover for 5 days after treatment), we found a strong reduction in the insulin expression at both days in β-cell depleted animals (Figure 2). These data demonstrate that 1) similar to humans, insulin in zebrafish larvae is also produced in β-cells and 2) β-cell depletion with the genetic ablation system sufficiently decreases insulin expression levels, thus allowing us to model type I diabetes.

2. Defining hyperglycemia in zebrafish larvae

Having shown that β-cell ablation reduces insulin gene expression, we next wanted to determine whether β-cell ablation is sufficient for induction of a hyperglycemic phenotype in larval zebrafish. Because we cannot not utilize blood glucose measurements due to the low amounts of blood present in zebrafish larvae (e.g. terminal bleeding of one adult zebrafish is just sufficient for a single blood glucose measurement) we needed to define hyperglycemia using alternative readouts. We analyzed therefore the differential gene expression of metabolic factors implicated in glucose metabolism using quantitative PCR (Figure 2, see also **Protocol 3**). For the gene expression analysis, we compared DMSO-treated and MTZ-treated Tg(*ins:NTR-mCherry*) larvae at 3 and 8 dpf (**Table 1** shows the treatment scheme and **Table 2** shows the primer sequences for the analyzed genes).

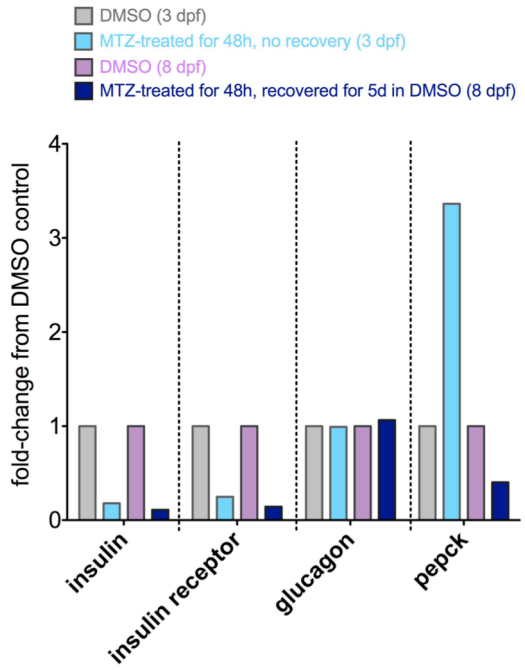

Figure 2. Gene expression analyses of *insulin*, *insulin receptor*, *glucagon*, and *pepck* with quantitative PCR. We compared gene expression in larvae treated with DMSO alone or 10 mM MTZ/ 0.5 % DMSO at 3 and 8 dpf. *insulin* expression was reduced on both days, suggesting that 48 h MTZ treatment is sufficient to deplete Insulin levels up to at least 8 dpf. Also, *insulin receptor* expression was downregulated, suggesting a feedback regulation between Insulin and Insulin receptor. *glucagon* expression did not differ from controls at both days, whereas *pepck* expression was strongly upregulated in β-cell ablated larvae at 3 dpf and downregulated at 8dpf, demonstrating a dynamic regulation, implying that these differences depend on acute and chronic insulin depletion.

First, we assessed *insulin receptor* expression, as we hypothesized that loss of insulin may regulate the expression of the *insulin receptor*. Because receptor downregulation is a hallmark of type II diabetes, which is however dependent on chronically elevated insulin levels, we expected that loss of insulin might lead to an upregulation of *insulin receptor*. In contrast to our expectations, we observed that *insulin receptor* expression was downregulated at both days in β-cell ablated larvae, suggesting that *insulin receptor* expression in larval zebrafish may be regulated by insulin. Given these results, we have identified the *insulin receptor* as a marker for assessing changes in glucose metabolism in insulin-depleted larvae.

As originally proposed, we also analyzed the expression of other genes implicated in glucose metabolism, such as *phosphoenolpyruvate carboxylase* (*pepck)*. PEPCK has been demonstrated to be transcriptionally modified by several glucoregulatory hormones, including insulin and glucagon. It, therefore, represented an excellent marker for changes in glucose metabolism in β-cell ablated larvae. *pepck* expression has been furthermore shown to be differentially regulated in larval zebrafish treated with 40 mM glucose (*5*). In our ablation model, we observed that

pepck expression was elevated at 3 dpf and low at 8 dpf when compared to DMSO controls, suggesting a dynamic regulation that differs under acute and chronic conditions of insulin depletion. Because downregulation of *pepck* mRNA expression has also been shown in the glucose treated larvae (*5*), it suggests that acute insulin depletion may not be sufficient for hyperglycemia to develop and that only chronic insulin depletion raises blood glucose levels.

Because PEPCK has been demonstrated to be stimulated by glucagon (*6*), we also analyzed *glucagon* expression and expected a similar regulation. Surprisingly, *glucagon* expression did not differ between control and β-cell ablated larvae at 3 dpf and 8 dpf, implying that the expression of this gene does not depend on insulin depletion. Alternatively, another *glucagon* paralog, *glucagon b*, may be insulin-responsive instead, for which we have not yet examined the expression. Taken together, these results suggest that genetic ablation of β-cells using the NTR/ MTZ system is sufficient to induce changes in glucose metabolism in zebrafish larvae.

Table 1. Gene expression analysis with quantitative PCR to define a hyperglycemic phenotype in Tg(*ins:NTR-mCherry*) larval zebrafish

Treatment begin	Agent	Treatment length	Recovery (days)	Collection of mRNA	Gene expression analysis
24 hpf	0.1% DMSO	48 h	0	3 dpf	*insulin, pepck, glucagon a, insulin receptor a*
24 hpf	10 mM MTZ	48 h	0	3 dpf	*insulin, pepck, glucagon a, insulin receptor a*
24 hpf	0.1% DMSO	48 h	5	8 dpf	*insulin, pepck, glucagon a, insulin receptor a*
24 hpf	10 mM MTZ	48 h	5	8 dpf	*insulin, pepck, glucagon a, insulin receptor a*

Table 2. Primer sequences for the analysis of gene expression in Tg(*ins:NTR-mCherry*) larvae via qPCR

Gene name	Gene symbol	5'-3' forward primer	5'-3' reverse primer
insulin	ins	ccc ttt atc tgg tct gtg gc	ttc ctt atc agc tcg gca tg
phosphoenolpyruvate carboxylase	*pepck*	gag aac agc acc atc ctc ag	tca ccg ttt tac tct cca cac
glucagon a	*gcga*	aag act tcg ttc agt ggc tc	tgg gtt gtc cgg att tta gc
insulin receptor a	*insra*	ata aga atg atc ggg agt gtg g	tgc ttg cat gag gat gga c
Elongation factor alpha (control gene)	*ef1a*	tct aca aat gcg gtg gaa tcg	gag caa tgt caa tgg tga tac c

3. Determining the effects of β-cell ablation and insulin depletion on the development of peripheral neuropathy in larval zebrafish

To assess whether changes in glucose metabolism affect somatosensory neuron health, we labeled somatosensory neurons in Tg(*ins:NTR-mCherry*) transgenic zebrafish larvae with fluorescent transgenes. We either transiently injected a CREST3:GFP transgene, which we previously characterized (*7*) to mosaically label sensory neurons, or by crossing the Tg(*ins:NTR-mCherry*) fish with Tg(*isl2b:GFP*) transgenic fish, in which all sensory neurons are labeled with green fluorescent protein (GFP) (*8*). We treated zebrafish larvae according to the above-described scheme and imaged the peripheral axons of sensory neurons at 8 dpf (see **Protocol 4** for confocal imaging methodology). We chose to analyze 8 dpf larvae because of the changes in the expression of genes implicated in glucose metabolism at this stage (see section 2). Control larvae were incubated either in embryo medium (0.03% Instant Ocean Aquarium Salt) or in 0.5% DMSO (Figure 3). Sensory axons in embryo medium-incubated larvae (Figure 3A, A')

appeared morphologically similar to DMSO-incubated larvae (Figure 3B, B') at all days analyzed, suggesting that RB axons are healthy in the presence of DMSO. In contrast, when larvae were incubated in MTZ, we observed massive axon degeneration in distal terminals of the axons (Figure 4A, A', arrowhead).

Because chronic glucose treatment has been shown to modulate expression of genes implicated in glucose metabolism in larval zebrafish (*9*), we analyzed the direct effects of glucose on axon health and whether glucose treatment could be used as an alternative method to induce peripheral neuropathy. In this assay, we incubated embryos from 24 hpf until 8 dpf with 40 mM glucose, according to previous studies. When examined with confocal imaging we observed a loss of axon density (Figure 4B, B', arrow), suggesting that sensory neurons degenerate in the presence of glucose. Taken together, these data demonstrate that peripheral axons of somatosensory neurons in zebrafish larvae are affected by disturbed glucose metabolism, similarly to humans. This model provides the basis for further investigations of the mechanisms underlying diabetic peripheral neuropathy in the larval zebrafish.

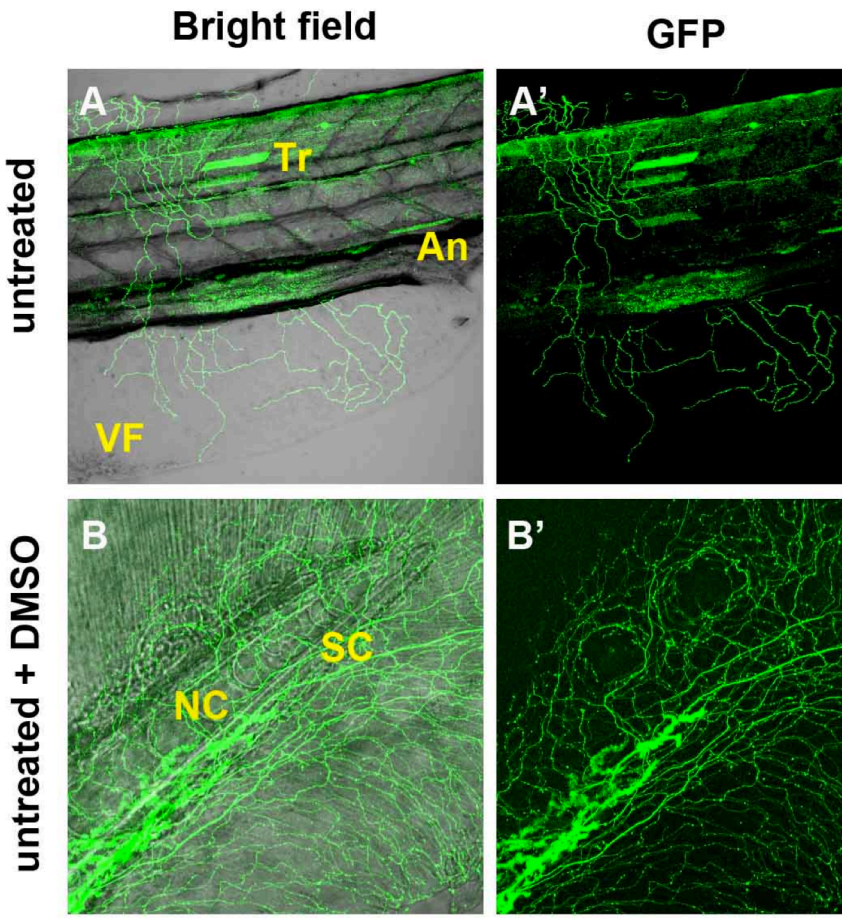

Figure 3. Confocal imaging of peripheral sensory axons in transient and stable transgenic larvae. Anterior is left, posterior is right. (**A**) Morphology of a peripheral axon of a single GFP-labeled sensory neuron, captured in an untreated zebrafish larva at 8 dpf. The peripheral axon arborizes in the dorsal (upper) and ventral (lower) sides of the medial trunk. (**B**) Morphology of peripheral axon arbors in a Tg(*isl2b:GFP*) transgenic larva, in which all sensory neurons express GFP. The larva incubated in 0.5 % DMSO shows a wildtype-like morphology of sensory axons, similar to the untreated larva (A). *Abbreviations:* (A) *Tr, trunk; An, anus; VF, ventral fin.* (B) *NC, notochord; SC, spinal cord.*

Specific aim 2

In this section, we will present studies planned to achieve Specific aim 2 during the extended project period.

The goal of the second aim is to characterize in more detail the peripheral neuropathy phenotype: 1) we will quantify the extent of axon degeneration in hyperglycemic larvae; 2) we will determine whether injury aggravates the onset of peripheral neuropathy, and 3) we will examine whether we observe a loss of regenerative capacity in acutely injured axons.

1) We will assess the temporal relationship between β-cell ablation and axon degeneration in β-cell ablated and glucose-treated larvae and monitor sensory axon degeneration with confocal imaging daily between 2 and 8 dpf. To be quantitative, we will trace axon debris in control and hyperglycemic larvae and further count the number of axons per μm^2. For quantitative analyses, we have obtained the Imaris Imaging software and a Filament Tracer module (Bitplane, Switzerland). In addition to these assessments of axon health, we will examine the extent of hyperglycemia in the imaged larvae by using an assay in which we can indirectly measure overall glucose levels present in the larvae. This assay is

based on the production of hydrogen peroxide (H_2O_2) during oxidation of glucose to D-glucono-1,5-lactone by glucose oxidase. H_2O_2 levels can be visualized with the chemical fluorescent probe Amplex Red (Life Technologies, USA). This method has been shown to precisely determine glucose concentrations in zebrafish embryos (*9*). To assess glucose-dependent oxidation, we will collect control and hyperglycemic larvae after imaging, homogenize them and incubate with glucose oxidase, horseradish peroxidase, and Amplex Red probe. We will detect glucose-mediated oxidation by assessing fluorescence at excitation and emission spectra of 535 and 590 nm, respectively, in a BioTek Powerwave XS plate reader (BioTek Instruments, USA). We hypothesize that ROS levels are highest in those larvae in which we observe severe degeneration of sensory axons.

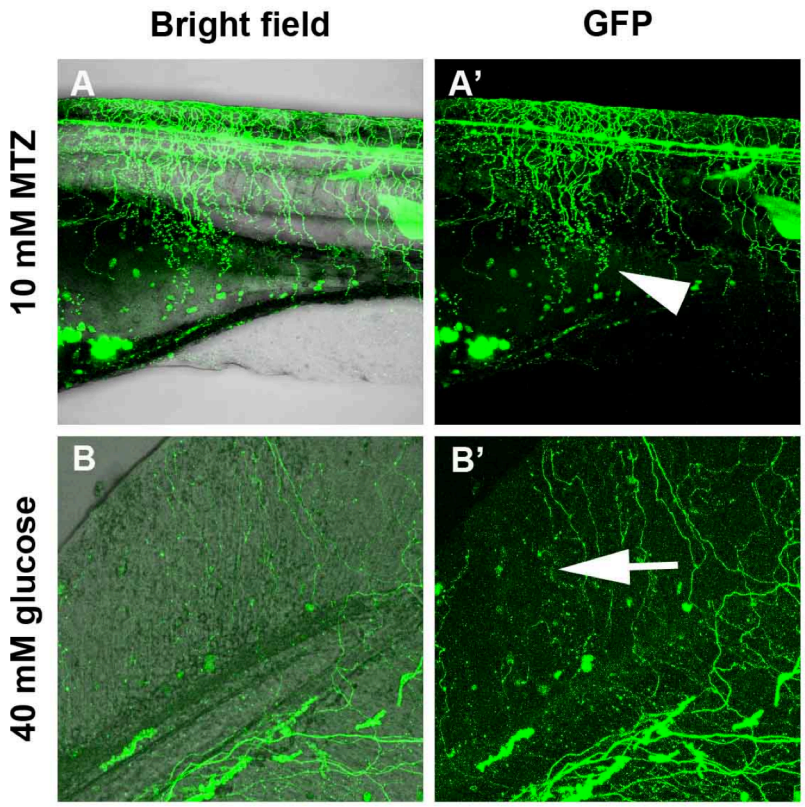

Figure 4. Peripheral axons of sensory neurons at 8 dpf incubated in 10 mM MTZ (A) and 40 mM glucose (B). (**A, A'**) Massive degeneration (arrowhead) of a peripheral sensory axon in a larva treated with 10 mM MTZ. In this larva, multiple neurons are transiently labeled following *CREST3:Gal4UAS-GFP* transgene injection into 1-cell stage embryos. (**B, B'**) Loss of axon arbors in a Tg(*isl2b:GFP*) transgenic larva treated for 7 days with 40 mM glucose. Excessive glucose induces sensory neuron death, as revealed by decrease axon density (arrow in B').

2) To determine whether injury aggravates the onset of peripheral neuropathy, we will assess axon health in 3 dpf control and hyperglycemic larvae following tail fin amputation. I previously showed that injury of peripheral axons initially induces axon degeneration in axon terminals, which can be up to 150 µm of the axon terminals depending on the extent of the injury. Within 2 hours however, the debris is typically cleared away and healthy axons begin to regenerate (*7*). We expect that under hyperglycemic conditions, axon debris is either not cleared away and/ or axon degeneration is aggravated and progresses. This would suggest that injury could stimulate the onset of peripheral neuropathy under hyperglycemic conditions.

3) To examine the regenerative capacity of axons in β-cell ablated/ glucose-treated larvae, we will acutely injure axons by amputating the tail fin at 3 dpf. We will utilize time-lapse confocal imaging to monitor axon regeneration for 12 hours following amputation, and we will subsequently quantify axon growth using NIH Image J software (*10*).

Summary & Future Perspectives

The strength of this proposal is that we can utilize *in vivo* imaging in larval zebrafish to characterize hyperglycemia-induced peripheral neuropathy. In the past year, we have been able to characterize the genetic changes associated with hyperglycemia in larval zebrafish using a genetic β-cell ablation model. We further discovered that hyperglycemic zebrafish larvae, either due to β-cell ablation or glucose treatment, develop peripheral neuropathy similarly to humans. In the next year, we will focus primarily on performing imaging analyses and using quantitative analyses to characterize in more detail the

degeneration phenotype. We will also assess whether injury initiates or aggravates degeneration and whether the regenerative capacity of axons is compromised prior to the onset of peripheral neuropathy, as this could be a first indicator of compromised axon health.

With completion of the proposed studies, we will have a better understanding of the dynamics of peripheral sensory neuron de- and regeneration under hyperglycemic conditions. These characterizations will set the framework for further studies on the underlying molecular mechanisms leading to peripheral neuropathy in hyperglycemic zebrafish. Once these basic analyses are completed, we can utilize larval zebrafish as a pharmacological screening tool for drugs that slow down, prevent, or reverse these phenotypic changes in sensory neurons. We can furthermore begin to assess the role of hyperglycemia in wound healing complications using wound-healing assays in zebrafish.

We expect to publish this work following the completion of the second year.

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