Retinal GABA Distribution of Hyperglycaemic Zebrafish

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Abstract

Diabetes is the leading cause of new cases of blindness in the US and causes blindness in 70% of patients with diabetes for more than fifteen years due to continued high blood sugar levels (hyperglycemia). To try to determine the effects of high blood sugar on the retina, hyperglycemia was induced in zebrafish by alternating their environment between 0% and 2% glucose solutions. Fish were sacrificed, and blood glucose levels measured every two weeks; blood glucose levels during this time were consistent with previous results. The retinas of weeks 0, 4, and 8 fish were cryostat sectioned and costained to identify cell nuclei (DAPI) and GABA distribution patterns. The staining in control fish showed GABA in horizontal processes and amacrine cell bodies. By week 4, the experimental group had less colocalization of GABA and DAPI in amacrine and horizontal cells and processes and more diffuse staining in the proximal inner nuclear layer. Horizontal cell process staining was absent in the week 8, experimental group. Amacrine cell labeling was not as continuous across the inner nuclear layer for the week 8, experimental group, and the GABA staining was diffuse and not confined to cell bodies. In some of the week 8, experimental samples, the inner nuclear and inner plexiform layers were thinner. These results indicate amacrine cell loss and possibly release of GABA following prolonged high blood sugar levels.

Background

High blood glucose levels, or hyperglycaemia, are a hallmark of diabetes. There are 23.6 million diabetic patients in the US, making up 8% of the population (American Diabetes Association, 2008). Type I diabetes, also called insulin dependent or juvenile onset diabetes, is an autoimmune disease which destroys the β-cells of the pancreas (Sherwood, 2005). β-cells

usually produce insulin which triggers blood sugar absorption. Without it, blood sugar levels remain high. Type II diabetes is triggered by reduced insulin production and/or target cells which are not as sensitive to insulin (Sherwood, 2005; Watkinson and Seewoodhary, 2008).

During diabetes, the body begins to metabolize proteins and fats leading to higher blood sugar levels and more acidic blood (Sherwood, 2005). Diabetes can lead to increased urine production and dehydration, damage to nerves and blood vessels, coma, or death (2005).

Damage to nerves and blood vessels can cause visual complications. Weakening of capillary walls can lead to aneurysms (Watkinson and Seewoodhary, 2008). Diabetes-induced metabolism changes can cause the formation of cataracts (2008). Central retinal vein occlusions and glaucoma can form due to blockages (2008). Other complications include hemorrhages, scar tissue formation, and retinal detachment (2008).

Diabetic retinopathy is the visual complication of focus in this research. 75% of patients with diabetes for more than fifteen years develop retinopathy, and it is the leading cause of new cases of blindness in the US (Gleeson, et al., 2007). Microvascular damage in the retina leads to diabetic retinopathy (Watkinson and Seewoodhary, 2008). The retinal capillaries close, causing tissue hypoxia and ischemia. This triggers an upregulation of VEGF and neurovascularization (Ambati, et al., 1997).

Diabetic retinopathy is characterized by ocular hemorrhages, lipid exudates, and growth of new blood vessels and connective tissue (Watkinson and Seewoodhary, 2008). Symptoms include floaters and deterioration of color and fine vision (2008). It is treated through blood glucose and blood pressure management, drug therapy to lower serum cholesterol levels to decrease exudates, laser photocoagulation, or vitrectomy (2008).

There are several models of research for diabetic retinopathy. Human vitreous samples are thought to be representative of retinal composition (Pulido, et al., 2007). ST-diabetic rats are injected with streptozotocin to kill β-cells (Gastinger, et al., 2006). Goto-Kakizaki rats are specially bred Wistor rats which have spontaneous, non-insulin dependent diabetes (Takeo-Goto, et al., 2002). The GK-rat model is interesting because it represents the early stages of diabetes as retinopathy is setting in.

Research has found that diabetic retinopathy includes a loss of retinal pericytes and an increase in the endothelial layer of blood vessels (Agardh, et al., 1997). There is a loss of amacrine and ganglion cells (Gastinger, et al., 2006) and a thinning of the inner nuclear and plexiform layers of the retina (Gleeson, et al., 2007). There is also a reduction in oscillatory potentials (Ambati, et al., 1997; Ishikawa, et al., 1996). When a flash of light excites a retina, the membrane potential creates multiple waves designated the a-wave, b-wave, and four oscillatory potentials (OPs). The OPs are thought to be created by amacrine cells, so their reduction indicates loss or malfunction of these cells.

Research on neurotransmitter levels in the retina and changes due to diabetic retinopathy are varied. Glutamate has been found to increase (Ambati, et al., 1997; Lu, et al., 2007; Takeo-Goto, et al., 2002) or remain unchanged (Ishikawa, et al., 1996; Nishimura and Kuriyama, 1985). A decrease in glutamate to glutamine activity has also been found (Barber, et al., 2000; Lieth, et al., 1998). GLUT1, a transporter for glutamate was found to be monoubiquitinylated and degraded by lysosomes (Fernandes, et al., 2004). Dopamine decreases due to increased efflux from the cells (Nishimura and Kuriyama, 1985). Aspartate has been found to either increase in the inner segments of photoreceptors (Pulido, et al., 2007) or remain unchanged (Ambati, et al., 1997). Glycine has been reported to decrease (Takeo-Goto, et al., 2002) or remain unchanged

(Ambati, et al., 1997). Gamma aminobutyric acid, or GABA, which this research is focused on, has been found to increase overall (Ambati, et al., 1997), increase in amacrine cells and have a new presence in Müller cells (Ishikawa, et al., 1996; Ramsey, et al., 2006; Takeo-Goto, et al., 2002), and have no change (Nishimura and Kuriyama, 1985). A decrease in GABA-T activity, which breaks down GABA, has also been reported (Ishikawa, et al., 1996).

Diabetic retinopathy may be caused by an increase in glutamate and GABA which can be toxic. Normally, Müller cells uptake, convert, and transport glutamate and GABA keeping them at low extracellular levels (Takeo-Goto, et al., 2002). Ischemia can induce neuron damage, loss of detoxification methods (Ambati, et al., 1997), and changes in membrane potentials (Takeo-Goto, et al., 2002). These factors may lead to increases in glutamate and GABA levels. An increase in glutamate levels can lead to more ischemia (Ambati, et al., 1997), upregulation of VEGF (Ambati, et al., 1997), and an increased uptake by Müller cells (Takeo-Goto, et al., 2002). VEGF triggers vascularization (Ambati, et al., 1997). Increases in GABA concentration also trigger VEGF (1997) and increased uptake by Müller cells (Takeo-Goto, et al., 2002). It also may create competition, desensitization, or down-regulation of amacrine receptors leading to reduced OPs (Ishikawa, et al., 1996).

The model used in this research is the zebrafish. Zebrafish are inexpensive, develop quickly, and are easy to care for. Gleeson, et. al. induced hyperglycaemia in zebrafish by alternating their environment every 24 hours between normal water and a 2% glucose solution (2007). Zebrafish uptake water; when there is glucose in it, transporters begin to run backward, pumping the sugar into the blood. Alternation is necessary because the fish can osmoregulate and return to normal levels (2007). Zebrafish also have similar β-cells, insulin, receptors, and tyrosine-kinase substrates as humans (2007).

The non-diabetic retinal structure of zebrafish is similar to other vertebrates. Crooks and Kolb have performed immunocytochemistry on human retinas to detect glutamate, GABA, glycine and dopamine (1992). Similar to their findings, Connaughton, et. al. found GABA activity in horizontal and amacrine cells in the inner plexiform layer with projections into the inner nuclear layer (1999). It formed two bands. There were also active displaced amacrine cells in the ganglion cell layer (1999). Glutamate was found in the vertical pathway—cones, bipolar cells, and ganglion cells. There was also light labeling of amacrine cells adjacent to the inner plexiform layer and of horizontal cells in the inner nuclear layer (1999). Glycine was present in amacrine cells of the inner nuclear and plexiform layers and in interplexiform cells from the inner nuclear layer to the outer plexiform layer (1999). Dopamine was localized by staining for the dopamine synthesis enzyme, tyrosine hydroxylase (TOH), by Crooks and Kolb in humans and by Yazulla and Studholme in zebrafish. It was found in interplexiform cells and in a few cells in the inner nuclear layer with processes to the inner and outer plexiform layers (Crooks and Kolb, 1992; Yazulla and Studholme, 2001).

The zebrafish is valuable because it models early stages of diabetic retinopathy. Analysis of early stages may have more information on cause and effect relationships. The objectives of this research are to determine the effect of diabetic retinopathy on retinal GABA distribution in order to more fully understand how degradation occurs, to further test hyperglycaemic zebrafish as a model for diabetic retinopathy, and to support or refute possible mechanisms put forth by previous research.

Methods

Two groups of adult zebrafish were maintained for 8 weeks in 4L tanks on a 14 hour light, 10 hour dark photoperiod at 28°C. The experimental group was alternated between 0% and 2% glucose solutions every 24 hours. The water in the control group was also changed every 24 hours.

Every two weeks, 2-3 fish from each group were anesthetized in 0.02% tricaine solution and sacrificed, and blood glucose values were measured. The brainstem was hemisected, and the heads were kept in 4% paraformaldehyde at room temperature. Eyes were removed and placed back in 4% paraformaldehyde.

Eyes from weeks 0, 4, and 8 were equilibrated in 30% sucrose solution for at least overnight at 4°C. They were embedded in OCT media, crystat sectioned into 25μm thick sections, mounted onto silanated slides, and stored at -80°C.

Sections were warmed to room temperature, rinsed in PBS, and blocked with 5% goat serum, 0.3% Triton in PBS. Samples were probed for GABA with rabbit anti-GABA (1:200) overnight. One sample was used as a control of secondary antibody background staining by incubating it in PBS with no primary antibody. They were all rinsed in PBS and incubated with the secondary antibody TRITC-conjugated donkey anti-rabbit IgG (1:100) for two hours in the dark. Sections were washed with PBS, stained with DAPI (1:5,000), washed again with PBS, and covered with a glycerol/PBS solution before being coverslipped. Stacked, costained images were captured. The groups were compared for each time point.

Results

The blood glucose levels of zebrafish have been shown to increase after 24 hours in 2% glucose solution and return to baseline after 24 hours in normal water (Gleeson, et al., 2007). Figure 1 shows the fluctuations of blood glucose as determined by Gleeson, et. al. Inlayed are the levels measured in this experiment. The results from this experiment are consistent with previous findings.

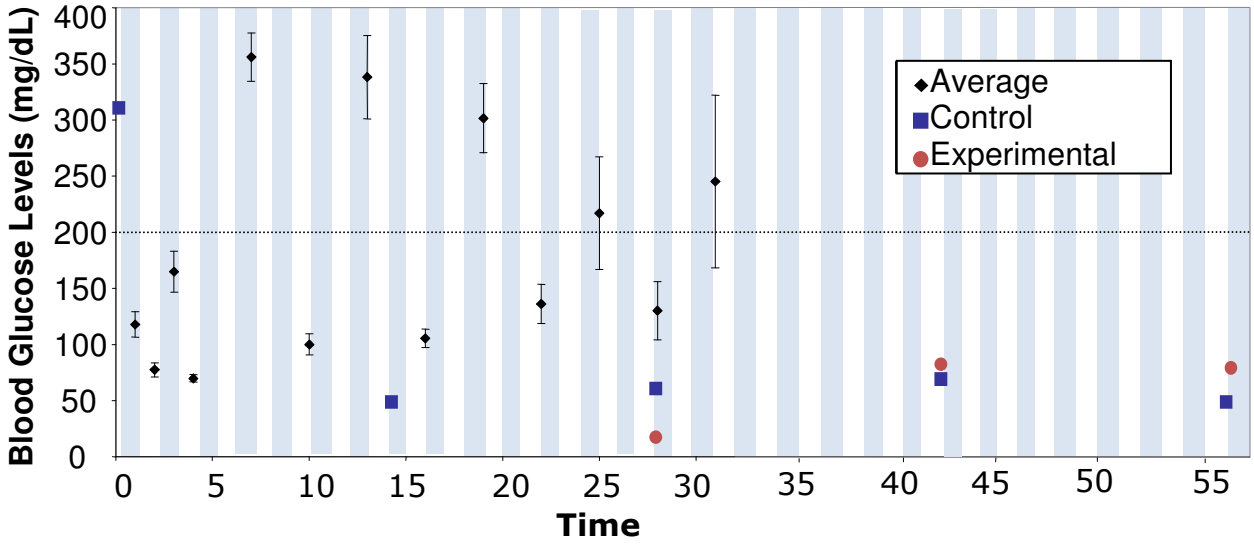

Figure 1: Blood glucose measurements were consistent with returns to control level after 24 hours in water - Shaded=2% glucose; unshaded=0%. Previous average values (n = 5) ± SE are indicated. The dashed line is at 200mg/dl. Any values above this line are considered to be hyperglycemic. The average values for control and experimental fish used in this study are superimposed. It is assumed that the fluctuations continue through two months.

Retinal sections were costained for DAPI and GABA. The GABA staining shown in Figures 2-7 had non-specific labeling in the outer segments of photoreceptors and in the inner plexiform layer; these areas were labeled even in sections with no primary antibody applied (figures not shown). The control, week 0 retinas (Figure 2) showed distinct amacrine cells in a continuous band along the inner nuclear layer with GABA staining surrounding the nuclei. The processes of the horizontal cells were also clearly stained for GABA.

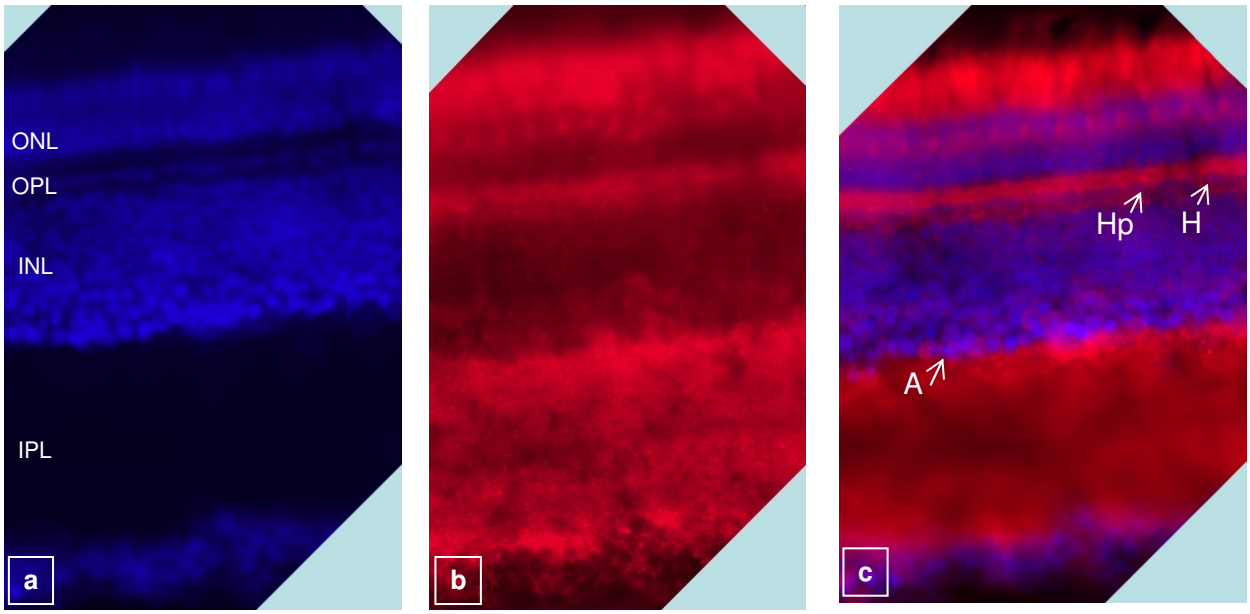

Figure 2: Control, week 0 retinas show GABA staining in amacrine and horizontal cells and clear horizontal processes - a-DAPI staining, b-GABA staining, c-costaining of GABA and DAPI; A-amacrine cells body, Hp-horizontal process.

The control, week 4 retinas (Figure 3) were similar to week 0. Amacrine nuclei were stained for DAPI and were surrounded by a GABA containing region. Horizontal cell processes were stained for GABA, and the cell bodies were outlined by GABA staining.

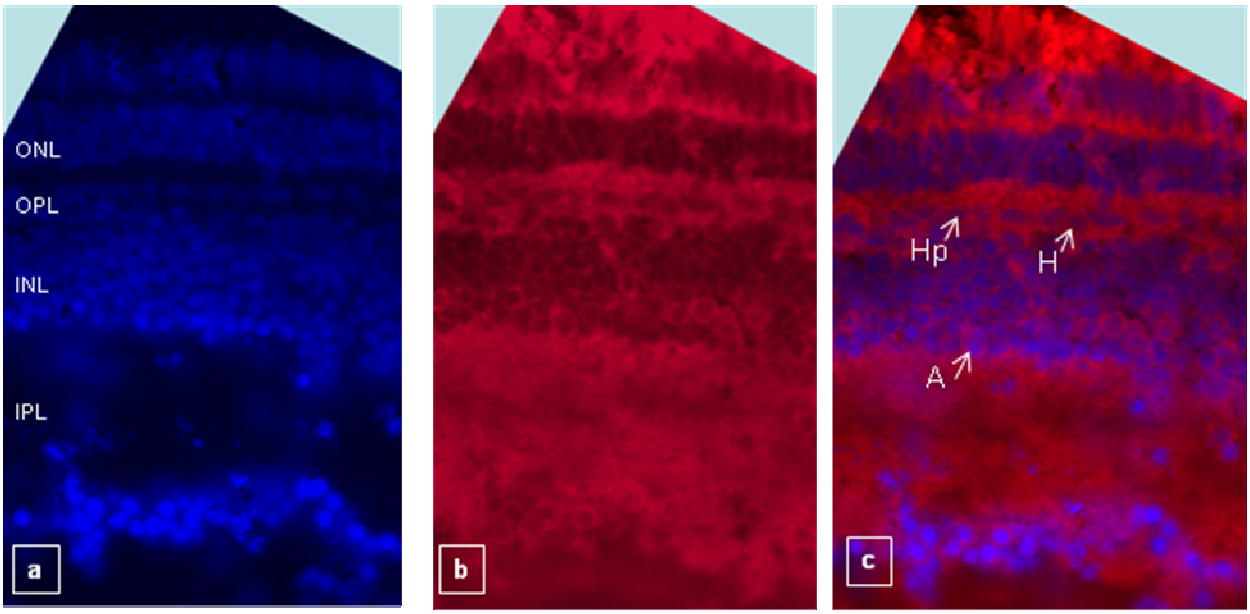

Figure 3: Control, week 4 retinas show GABA staining in amacrine and horizontal cells and clear horizontal processes – a-DAPI staining, b-GABA staining, c-costaining of GABA and DAPI; A-amacrine cells body, H-horizontal cell body, Hp-horizontal process.

The experimental, week 4 retinas (Figure 4) GABA staining of amacrine and horizontal cell bodies was observed. The proximal inner nuclear layer had more diffuse GABA staining than in the control retinas from the same week (Figure 3b vs. 4b). The horizontal processes were also stained for GABA as in the control, week 0 retinas (Figure 3).

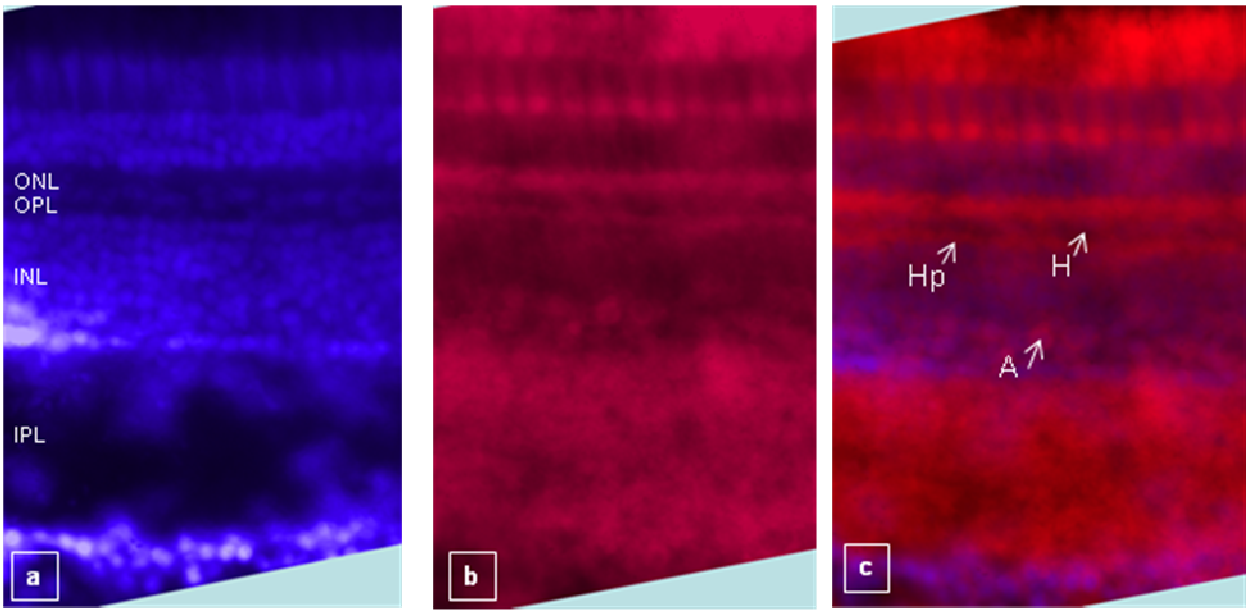

Figure 4: Experimental, week 4 retinas show some GABA staining in and around amacrine and horizontal cells and clear horizontal processes - a-DAPI staining, b-GABA staining, c-costaining of GABA and DAPI; A-amacrine cells body, H-horizontal cell body, Hp-horizontal process.

The control, week 8 (Figure 5) had distinct amacrine cells along the inner nuclear layer with GABA staining surrounding the nuclei. The processes of the horizontal cells were also clearly stained for GABA.

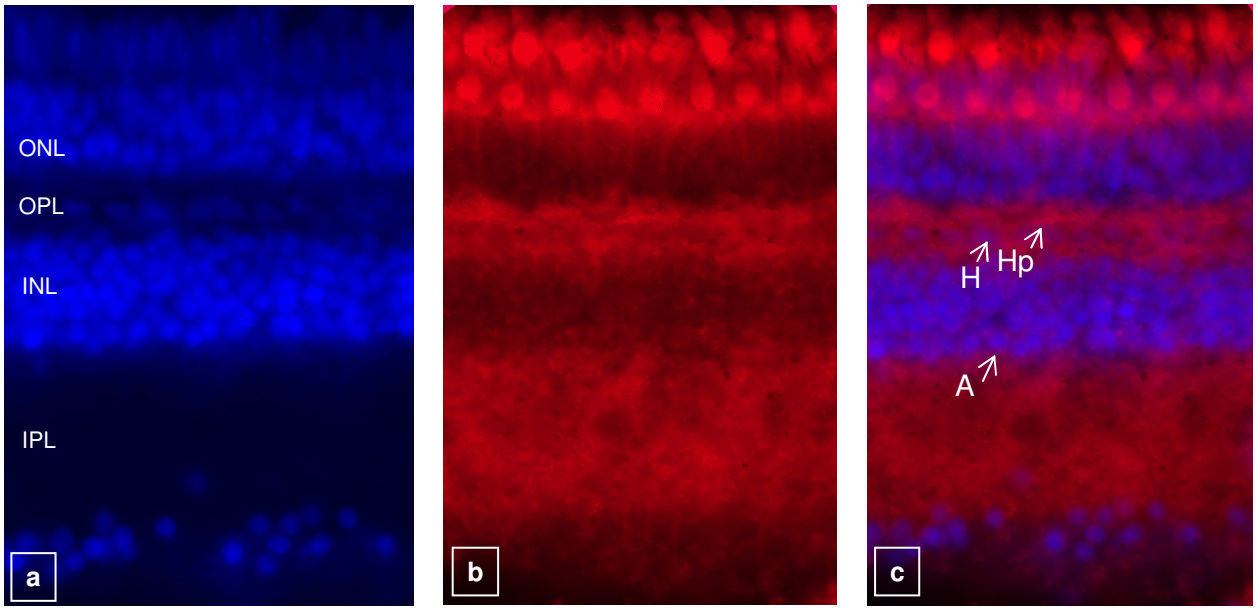

Figure 5: Control, week 8 retinas show GABA staining around the nuclei staining in amacrine cells and clear horizontal processes – a-DAPI staining, b-GABA staining, c-costaining of GABA and DAPI; A-amacrine cells body, Hp-horizontal process.

In the experimental, week 8 retinas (Figure 6), the amacrine cells did not form a continuous band along the inner nuclear layer. There was less colocalization and more diffuse staining in the proximal inner nuclear layer of GABA (Figure 5bvs Figure 6b). There was no clear horizontal cell process labeling.

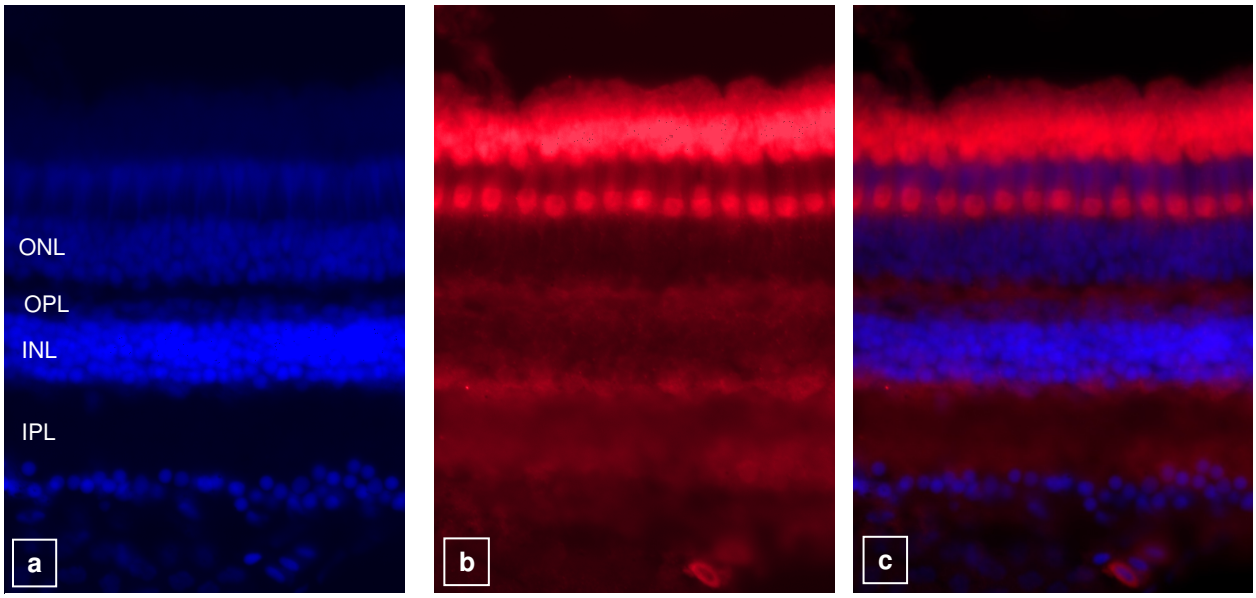

Figure 6: Experimental, week 8 retinas do not have clear amacrine cell body staining, and the staining is not as prominent. The processes of horizontal cells are not stained for GABA - a-DAPI staining, b-GABA staining, c-costaining of GABA and DAPI

The thicknesses of the inner nuclear and plexiform layers of all control retinas (Figure 7a, b, d) were similar. The experimental, week 4 retinas (Figure 7c) showed little change from any of the control retinas. Some of the experimental, week 8 retinas, such as the sample shown in Figure 7b, had inner nuclear and plexiform layers which were thinner than the controls.

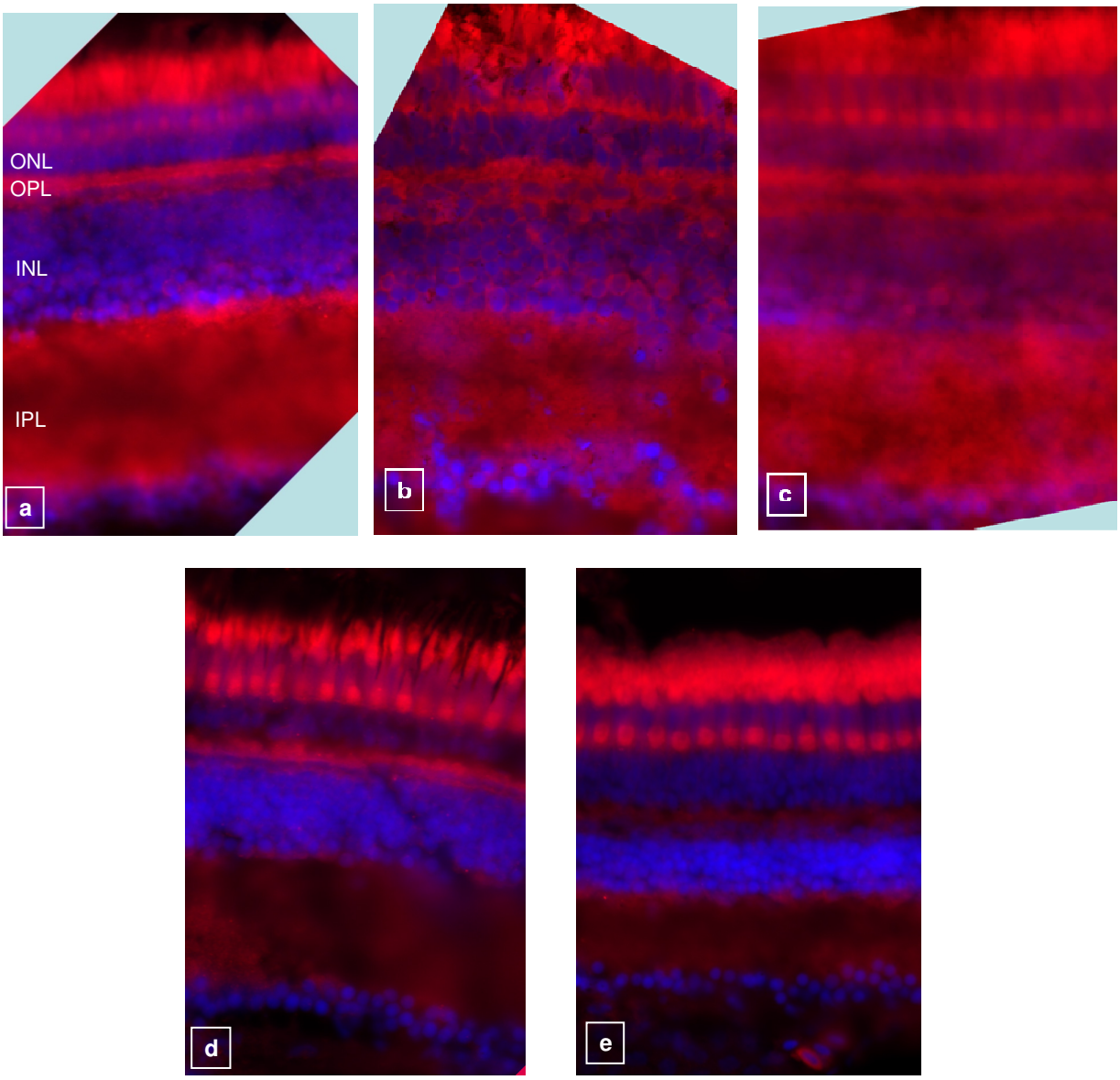

Figure 7: The inner nuclear and inner plexiform layers of some experimental, week 8 retinas are thinner - Retina samples costained for GABA (red) and DAPI (blue): a-control, week 0, b-control, week 4, c-experimental, week 4, d-control, week 8, e-experimental, week 8. ONL=outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer; IPL=inner plexiform layer.

Discussion

Because the fish were always sacrificed at the end of a 24 hour period in 0% glucose water, their blood glucose levels were low. This was consistent, however, with previous research by Gleeson, et. al., with the assumption that the trend of increased blood glucose following immersion in 2% glucose and then a return to normal levels in 0% glucose would continue for two months (2007). The control, week 0 fish had very high blood glucose levels (Figure 1). This was most likely due to being fed the morning of sacrifice. In order to avoid interference of food intake with glucose levels, the fish were not fed for the remainder of the experiment. This research also must rely on previous demonstration of blood glucose fluctuation because very few measurements of blood glucose levels were recorded successfully due to not enough blood being available to make measurements.

The control retinas (Figures 2, 3, and 5) showed similar staining patterns to one another and to previous research by Connaughton, et. al. (1999). This indicated that any stress from lack of food and transport did not affect the retinas. These retinas showed horizontal cell nuclei surrounded by GABA staining with GABA staining in the processes as well. The amacrine cells also had GABA staining confined to the cell bodies. The amacrine cells formed a continuous band along the proximal inner nuclear layer. Due to the non-specific staining of the inner plexiform layer, the processes of amacrine cells could not be analyzed.

Week 4, experimental retinas (Figure 4) still showed amacrine and horizontal colocalization and staining of horizontal cell processes. They also showed, however, more diffuse staining along the proximal inner nuclear layer instead of clearly defined cell body staining. This may indicate that by 4 weeks, some of the amacrine cells have begun to release GABA. By week 8, the experimental retinas (Figure 6) had amacrine cells which were not as

prominently stained for GABA. The GABA was not kept in the cell bodies and instead stained the proximal inner nuclear layer diffusely. The week 8 experimental retinas showed more diffuse GABA staining than the week 4, experimental retinas. Additionally, the amacrine cells were not continuous along the proximal inner nuclear layer. By week 8, there had been a detectable loss of labeled amacrine cells suggesting they released GABA. The horizontal cell processes were also not clearly defined in the week 8, experimental retinas (Figure 6). This may be due to a loss of GABAergic processes or retention of GABA in the horizontal cell bodies.

The week 4 and week 8 experimental retinas showed a decrease in amacrine cell body GABA and an increase in diffuse GABA over time. It cannot be determined from this data whether this indicates external GABA or less sequestered internal GABA. Normal cells have very little extracellular GABA levels due to detoxification mechanisms and uptake by Müller cells (Ambati, et al., 1997; Takeo-Goto, et al., 2002). Research by Ambati, et. al. using vitreous samples were able to detect an increase in extracellular GABA (1997). An increase in extracellular GABA may be due to neuron damage from ischemia. An increase in extracellular GABA levels can upregulate VEGF, triggering vascularization (1997). The results presented here may support this mechanism which could be an early means of the VEGF upregulation and neurovascularization characteristic of diabetic retinopathy.

The release of GABA causing VEGF upregulation would be further exasperated by a loss of amacrine cells. Other models of diabetic retinopathy have reported a loss of amacrine cells (Ambati, et al., 1997; Gastinger, et al., 2006; Gleeson, et al., 2007; Ishikawa, et al., 1996). This was further supported by the week 8, experimental retinas which showed a discontinuous band of amacrine cells along the proximal inner nuclear layer. In order to further study this mechanism, the thicknesses of the inner nuclear and plexiform layers were analyzed. The control retinas and

week 4, experimental retinas were all similar (Figure 7a-d). The thickness of these layers in the week 8, experimental retinas varied. Some, as shown in Figure 7e, were thinner. These layers are where amacrine cell bodies and their processes lie. A loss of amacrine cells during prolonged hyperglycemia is therefore supported. The variation in layer thickness of week 8, experimental retinas may be due to genetic differences as to how well the cells deal with stressors such as hyperglycaemia before dying.

The data collected here could be supported by the further analysis of week 4 and 8 retinas to increase the sample size and the analysis of week 2 and 6 retinas which have already been isolated. The contribution of glutamate and dopamine should also be explored using similar methods as those used here.

The hyperglycaemic zebrafish is an early stages model of diabetic retinopathy. Similar to some previous findings, this research has found progressive loss of GABA-positive amacrine cells, a possible release of GABA, and decrease in inner nuclear and plexiform layer thicknesses. It has also found the retention or loss of horizontal GABAergic cell processes which has not been previously reported.

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