Vulnerability of AII Amacrine Cells in Oxygen-induced Retinopathy

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Abstract

The retina is a patchwork of many different specialized neurons that all work together to pass visual stimuli to the brain for interpretation. These cells include the rod and cone photoreceptors and different classes of horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Of all these neurons, this study focuses largely on the rod pathway consisting of rods, cones, rod bipolar cells, and AII amacrine cells. The AII amacrine cell is an interneuron that is vital to the passing of visual signals from the rod photoreceptor along to the brain, contributing to vision in low-light conditions. However, it is unclear how AII amacrine cells and other retinal neurons in the rod pathway are altered in oxygen-induced retinopathy (OIR). OIR is a model used in animals that replicates the disease retinopathy of prematurity (ROP) that is seen in premature human infants. In this study we found that AII amacrine cells were substantially lost in OIR. However, the number of rod bipolar cells remained unchanged during OIR. These results suggest that the loss of AII amacrine cells in OIR could prove to be a crucial part of the cellular mechanism responsible for the loss of scotopic vision (night vision) seen in ROP patients.

Keywords: Retinopathy of prematurity (ROP), oxygen-induced retinopathy (OIR), retina, neovascularization, vaso-obliteration, hypoxia, rod, cone, rod bipolar cell, and AII amacrine cell

Introduction

The field of vision research is a vast and ever-growing field. The human eye and visual processes are so complex that there is much that is still to be discovered. One such complex structure within the eye, the retina houses many different intricate pathways that transmit visual stimuli to the brain, where it can be interpreted as images. Research on the retina has been conducted for several decades and yet there is still much more that has yet to be discovered, especially in regards to diseases of the eye.

Retinopathy of prematurity is an extremely debilitating disease of the eye that affects the retinas of newborn infants that are born pre-term (Good *et al*., 2005). Vasculature in the human retina begins to develop around the beginning of the second trimester and continues to develop all the way until a full-term birth. Without full time to develop, the vessels are not able to supply blood to the entire retina, resulting in areas of hypoxia. These conditions trigger an increase in vascular endothelial growth factor (VEGF) and insulin-like growth factor 1, which stimulate the formation of new blood vessels in these avascular areas. The new blood vessels are malformed, however, and leak blood into the retina. This is known as neovascularization, and can lead to formation of scar tissue and possible detachment of the retina (O'Connor *et al*., 2002). Over half of all premature babies born in the United States each year, approximately 15,000 total babies, develop some form of retinopathy of prematurity (National Eye Institute, 2014). The severity of this disease ranges from patient to patient but, in extreme cases, complete blindness can occur. This is a major reason why it is so important to study this disease and work to find ways to limit its damaging effects.

Research that is conducted to inspect the effects that retinopathy of prematurity (ROP) has on the retina often uses an animal model called oxygen-induced retinopathy (OIR). OIR is caused by placing young mice pups into a high-oxygen environment that, because their retinas are still developing, can cause blood vessels to regress, depriving the cells of oxygen once the animal is returned to typical oxygen levels (Scott & Fruttger, 2010). With most research to this point focusing on the problem of neovascularization, it is important to consider other retinal systems that are also affected by ROP. In milder cases of ROP where blindness does not occur, patients have been observed to have a decrease in visual acuity in instances of dim light, known as scotopic vision (Fulton *et al*., 2001). This signifies that there is an issue somewhere along the rod pathway (Figure 1), and so this experiment aims to discover the root of this problem. This study looks specifically at the AII amacrine cell and other major components of the rod pathway that are integral to scotopic vision. The main question to be answered by this project is if the hypoxic conditions caused by ROP are enough to inhibit the function of or even kill off any of these cells in the retina.

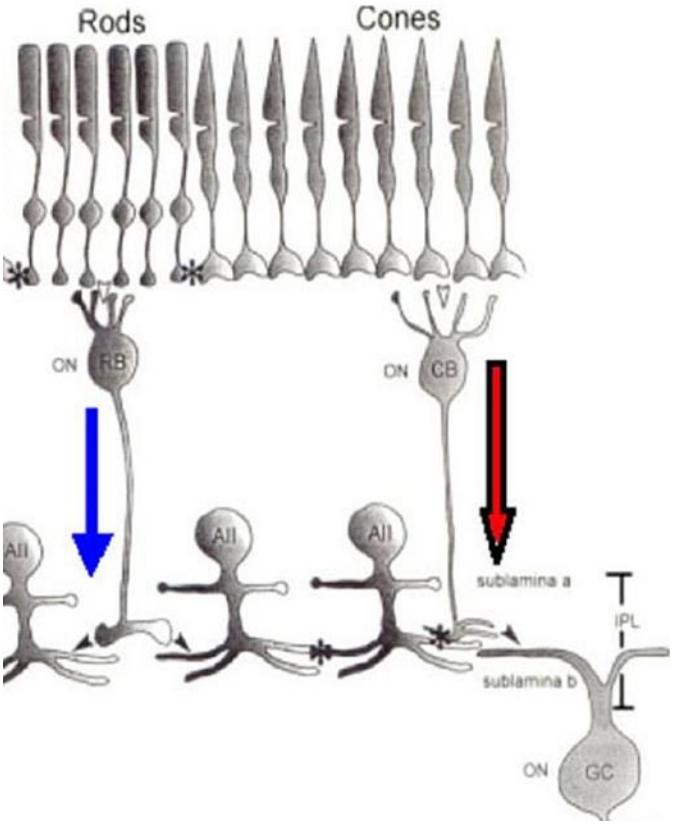

Figure 1: Rod pathway diagram. For vision to occur, photons of light are collected by either the rod or cone photoreceptors. If the rod photoreceptor is stimulated, it passes a signal along to the rod bipolar cell (RB near blue arrow). Unlike the cone bipolar cell (CB near red arrow), the rod bipolar cell does not make a direct connection with the ganglion cells to transmit its signals to the brain for interpretation. Rather, the AII amacrine cell acts as a bridge to allow the signal from the rod pathway to cross over to the cone pathway by making a synaptic connection with both the rod and cone bipolar cells. The signal can then propagate through the ganglion cell and to the brain via the cone bipolar cell. Figure from Mahnoosh & Connaughton.

The AII amacrine cell is a well-known and often studied type of amacrine cell. Its primary function is providing a bridge that carries the sensory signals from the rod photoreceptor and rod bipolar cell over to the cone bipolar cell (Figure 1). The signal is passed from the cone bipolar cell to the ganglion cell where it then leaves the eye and enters the brain for signal interpretation and processing (Wassle *et al.,* 2004). Without this cell, the electrical impulses would end suddenly at the rod bipolar cell and no message would be sent to the brain. Although much is known already about this particular cell, no research has been done to explore how these cells react to the unfavorable conditions of certain diseases like retinopathy of prematurity. The original thought for the reduction in scotopic vision in ROP patients was that rod photoreceptors were themselves damaged and thus causing this visual deficit, but now the preliminary data from this experiment has shown that AII amacrine cells may also have an impact on this. In the future, these and any further findings will hopefully help to better understand, treat, and possibly eliminate the effects of ROP.

Aims and Objectives

As mentioned previously, retinopathy of prematurity is a disease that affects about 15,000 prematurely-born babies each year in the U.S. About one third of these infants will go legally blind due to the disease. These babies develop the disease as a result of the retinal vasculature not being given the time to fully form. This used to be compounded by the high levels of oxygen administered to premature babies immediately after birth by nurses trying to save their lives, but with better understanding of the disease in recent years this has dramatically decreased. The

retina then tries to rapidly grow blood vessels in these areas which can result in weak and malformed blood vessels that leak blood, leading to scarring and possible retinal detachment.

Aims

1. To investigate if oxygen-induced retinopathy (OIR) has an impact on the viability of AII amacrine cells and other rod pathway neurons in the mouse retina.
2. To examine if the oxygen treatment influences the activity of the surviving cells.
3. To study if the possible loss of and/or damage to the AII amacrine cells is a contributing factor to the degradation and loss of scotopic vision (night vision) seen in retinopathy of prematurity (ROP) patients.
4. To test possible drugs or compounds to see if they provide protection to neurons in the retina or reverse damage done by the conditions of oxygen-induced retinopathy.

Objectives

1. It has been already shown that dopaminergic amacrine cells in the retina die due to the low-oxygen environment caused by OIR, so knowing if other cells die as well could lead to a break-through in the treatment of patients with ROP.
2. If the cells do not completely die there remains the possibility that they are damaged enough to lose some of their functioning capabilities, which would also be important to know in the possible treatment of ROP.
3. Although it is hypothesized that a loss of AII amacrine cells would reduce rod-stimulated vision since they are a vital link in the rod pathway, no studies have yet focused on this and so it must first be confirmed and better understood.

4. Finding a neuroprotective agent that could reverse the damage done to these cells or protect them from future damage could lead to treatment and ultimately a better life for those struggling with the effects of ROP.

Methods

Animals:

The mice used in this experiment were housed in their own separate room in Oakland University's Biomedical Research Support Facility and taken care of each day by the staff of the BRSF. This experiment requires the use of wild-type mice (mice without genetic mutation) that are divided into two groups, the oxygen treated experimental group and age-matched controls which are not subjected to any form of treatment. To create the oxygen-induced retinopathy model, cages containing seven-day old (P7) mice pups and their mothers were placed into a sealed Plexiglas chamber where the oxygen level was constantly maintained at 75%. The air in this chamber is more than three times more saturated with oxygen than the approximately 21% oxygen saturation found in normal atmospheric air. Since the pups' retinas are still developing during this time, the severe increase in oxygen will cause blood vessel regression as a smaller number of blood vessels are needed to be able to supply the tissue with the same amount of oxygen. After five days of treatment at the age of twelve days (P12), the litters were returned to normal housing conditions and atmospheric oxygen levels. When the mice are removed from the chamber and placed into normal oxygen levels, the retina is then deprived of oxygen and must grow new blood vessels to meet the demand for oxygen, thus mimicking the process of ROP. Four time points were used in this experiment; P12 when the mice pups are removed from the

chamber, P17 because it is the time of greatest blood vessel reformation (neovascularization), P25 because neovascularization has ended and the retina is fully vascularized once again, and P42+ because anywhere past this point the mouse is an adult.

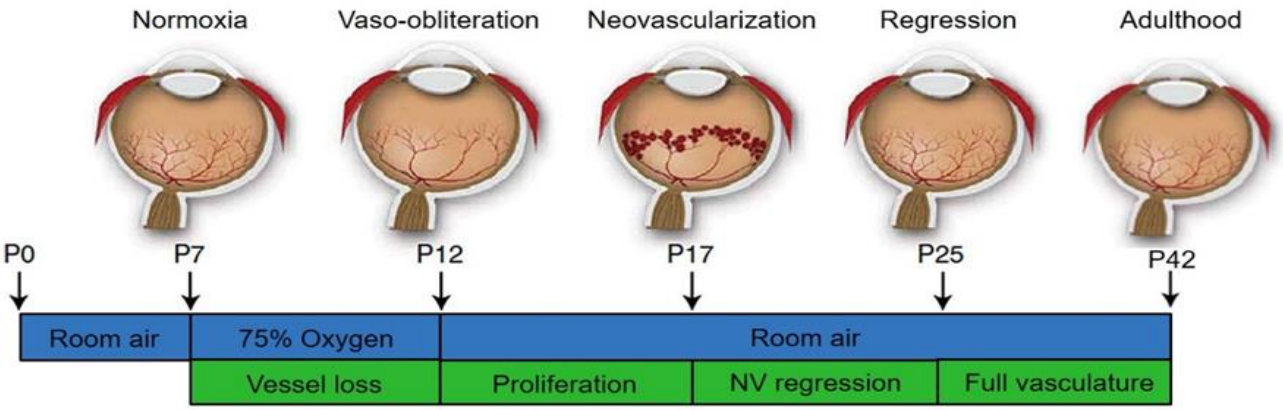

Figure 2: Oxygen-induced retinopathy model. Mice are born at postnatal day zero (P0) with incomplete retinal vasculature, similar to the underdeveloped vasculature seen in premature infants. One week after birth (P7), mice are exposed to a 75% oxygen-containing atmosphere. During this time, a period of hyperoxia (surplus of oxygen) occurs in the retina. To compensate for this overabundance of oxygen, blood vessels in the central retina regress (vaso-obliteration). After five days of treatment, the mice are returned to a normal atmosphere (about 20% oxygen). The reduced vasculature is incapable of delivering adequate oxygen to the cells of the retina, causing hypoxia (lack of oxygen) to ensue. The hypoxic condition of the central retina triggers neovascularization to begin at P12 and reach its peak at P17, after which time the abnormal blood vessels begin to regress and regular vasculature takes its place. By P25, the retinal vasculature has completed its re-formation and finally at P42 the mouse is considered to be an adult. Figure from Connor, et al. with modification.

Retinal Dissection for Wholemounting:

At either age P17 or P42, the mice were humanely euthanized and their eyes removed. Using the proper dissecting tools and a Bausch & Lomb microscope, the cornea and lens were removed. The eyes were then fixed in 4% paraformaldehyde for thirty minutes to allow for easier removal of the retina. The sclera and choroid layers were then carefully peeled back, allowing

for the retina to be separated from the rest of the eye cup. At this point the retina should retain its cup-like shape and was put back into the 4% paraformaldehyde for an additional thirty minutes to finish the fixation process. The procedure for immunohistochemically staining the retinas could then proceed.

Retinal Dissection for Slicing:

At one of the four ages specified earlier, the mice were humanely euthanized and their eyes removed. Using the proper dissecting tools and a Bausch & Lomb microscope, the cornea and lens were removed. The eyes were then fixed in 4% paraformaldehyde for one hour. The whole eye cup was then washed in 0.1M phosphate buffered saline (PBS) for 15 minutes and then transferred into a vile containing a 10% sucrose mixture for one hour. The eyes were then transferred into a vile containing a 20% sucrose mixture for another hour. Next, the eyes were placed into a solution of 30% sucrose where they remained overnight. The following day the eyes were transferred into a 2:1 mixture of 30% sucrose and OCT embedding compound for 45 minutes. The 2:1 mixture was then poured into plastic freezing blocks and partially frozen by placing the block into a bath of methanol and dry ice. When the mixture was about half frozen, the block was removed from the methanol and the eye cup was carefully placed into the sucrose/OCT mixture, taking care to note which direction the eye was facing. Only one eye was to be frozen in each block. The freezing block was then placed back into the methanol and dry ice to finish the freezing process. The frozen blocks were then wrapped in cellophane and aluminum foil, labeled, and placed into the -80°C freezer for storage until slicing.

Retinal Slicing:

When ready, the frozen blocks were pulled out of the -80°C freezer and placed into the chamber of the Leica CM3050 S Cryomicrotome in preparation for slicing. The objective and chamber temperatures of the cryomicrotome were both always set to -20°C. The frozen sucrose/OCT mixture was removed from the freezing block and mounted onto the objective using more OCT. Using a new microtome blade, 14 micrometer (μm) thick slices were taken and placed onto a slide, each slide capable of holding twelve slices. These freshly prepared slides were then placed back into the -80°C freezer until needed for the staining procedure.

Immunohistochemistry for Wholemounting (AII Amacrine Cells):

After fixation in 4% paraformaldehyde, the retinas were washed in 0.1M PBS for 15 minutes and then blocked for 2 hours in a solution of 1% bovine serum albumin, 0.3% Triton-X 100, and 0.1M PBS. A rabbit polyclonal primary antibody against Dab-1 (concentration - 1:500) was added to a new mixture of blocking solution. After 1 day of incubation at room temperature, lectin (1:100) was added to the primary antibody solution in order to stain for blood vessels. The retinas were incubated in this solution for an additional day for a total of two days spent in the primary antibody. The retinas were washed in PBS 4 times for 5 minutes each and then 4 times for 10 minutes each (for a total of one hour). The retinas were then transferred to a mixture of blocking solution and Alexa Fluor 594 donkey anti-rabbit IgG secondary antibody (1:500). After 2 hours of incubation, the retinas were rinsed in PBS by the same 4x5/4x10 procedure and mounted on slides. A scalpel was used to make four cuts in the retina at right angles to each other in order for the retina to lay flat. A drop of Vectashield mounting solution was used for the

preservation of the fluorescence, and the slide was finally coverslipped and sealed with nail polish.

Immunohistochemistry for Slicing:

In preparation of the staining procedure, slides to be used were removed from the -80°C freezer and thawed out at room temperature on the lab bench. Once thawed, a hydrophobic marker was used to trace around the slices to ensure that any liquids dropped onto the slices would remain there and not spill off the slide. The slices were then rinsed with PBS for 15 minutes prior to putting blocking solution on them for 2 hours. After being drained of the blocking solution, the slide was immersed in the primary antibody solution overnight at room temperature. When staining for cone photoreceptors, a rabbit polyclonal anti-cone arrestin primary antibody (1:10,000) was used. When the slides were instead stained for the rod bipolar cells, a rabbit polyclonal anti-PKCα primary antibody (1:500) was used. The following day, the slides were rinsed with PBS per the same 4x5/4x10 procedure used for wholemounting. The slides were then immersed in secondary antibody for 2 hours. Alexa Fluor 594 donkey anti-rabbit IgG secondary antibody (1:500) was used to visualize both the cones and rod bipolar cells, a major reason as to why they were not stained for on the same slides. Prior to washing with PBS, the slides that were stained for cones were double stained to also visualize rod photoreceptors. To do this, DAPI from Calbiochem (1µL DAPI: 50mL PBS) was placed onto the slides for 5 minutes. After that five minutes, all slides were washed with PBS using the previously stated procedure. Finally, the slides were drained of excess PBS, a drop of Vectashield mounting solution placed onto the slices, and the slide coverslipped and sealed.

Imaging and Analysis for Wholemounts:

Tile scans of whole retinas for the images of the blood vessels (channel 2) were taken using a 10x objective using the MosaiX function on a Zeiss Axio Imager Z.2 microscope. Similar tile scans were made at 20x to visualize the AII amacrine cells (channel 7). These images were then stitched into one image large image using the NIS Elements software program. The single, unstitched pictures of the cells were taken at 40x and carefully noted from what part of the retina each photo was taken at.

Imaging and Analysis for Slicing:

Single images of all three cell types were taken of both central and peripheral portions of each retina using the 20x and 40x objectives. The channel 7 filter was used to visualize the cones and rod bipolar cells. The channel 1 filter was used for the DAPI-stained rod photoreceptors. For the retinas that were double stained for both cones and rods, images were taken using each filter at the same focus position prior to moving on to the next location. Care was taken to ensure that images were taken from both the central and peripheral retina and were labeled as such when saving the images.

Results and Discussion

Oxygen-induced Retinopathy Model:

Due to the overabundance of oxygen present when the mice pups are living in the oxygen chamber, a signaling cascade occurs in the inner retina that tells the blood vessels to begin to regress. This is the only major difference present between retinopathy of prematurity present in

humans and the OIR model that researchers use to mimic the disease's effects. In the human form of the disease, the area with the lack of blood vessels is found in the periphery of the retina rather than the central retina as is the case in OIR. This is due to the baby being born prior to the vasculature fully forming, which progresses outwards from the inner retina. Although this dissimilarity between the two models of the disease has no impact on the data gathered from this experiment, it does point out a major reason why the cells of the rod pathway were specifically targeted in this study. Due to the high acuity of the cone photoreceptor, they are best served to be centrally located within the retina where the lens can best focus the incoming light from our surroundings. Thus, the ratio of photoreceptors located in the outer retina of a human is exclusively made up of rods. As a result, the rods and its accompanying cells have the greatest risk of being affected in humans dealing with retinopathy of prematurity, therefore having the utmost importance in a study such as this.

To ensure that the OIR model was successfully working in this experiment, wholemount retinas were stained with lectin to visualize the blood vasculature. Images were taken of both control and OIR retinas at the ages of P17 and P42. At each of these ages, the control retinas should exhibit fully formed and complete vasculature throughout the entire retina. In contrast, the P17 OIR retina should show signs of avascularization and neovascularization in the central retina. By P42, however, the OIR retinas should appear as if the vasculature had returned to normal. As Figure 3 reveals, the retinas behaved as they should per the OIR model, meaning that the rest of experiment could move forward with confidence (image on following page).

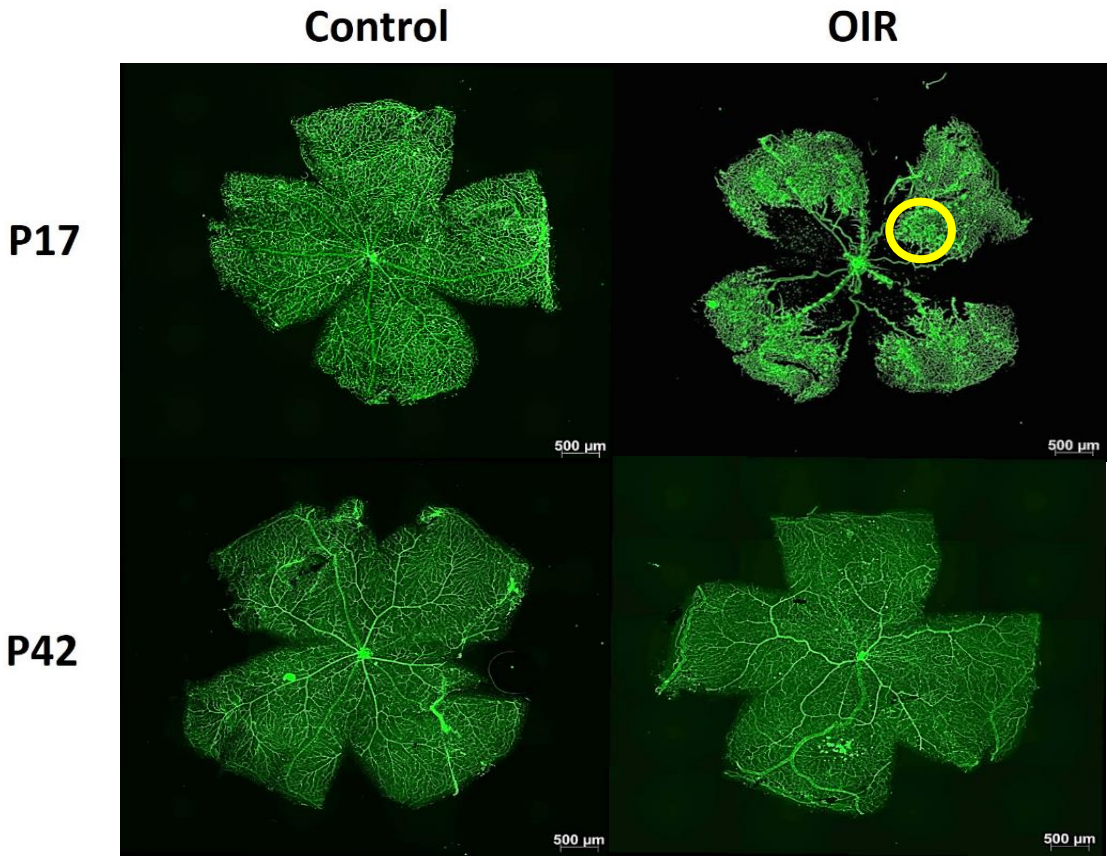

Figure 3: Vasculature progression in OIR. The above pictures are used to confirm that the oxygen treatment was successful in producing the OIR model in the mice. The P17 control retina (top left) displays full and complete vasculature throughout the entire retina. In the P17 OIR retina (top right), however, vaso-obliteration is clearly identifiable in the central retina while neovascularization (circled in yellow) is seen just peripheral to the area of avascularization. The remaining two images on the bottom are the P42 control and OIR retinas (left and right respectively). They appear to be very similar as the P42 OIR retina has regained full vasculature in its central region and neovascularization has completely disappeared.

AII Amacrine Cells:

As mentioned previously, the AII amacrine is a critical intermediate in transmitting the electrical impulse that begins in the rod photoreceptor. These amacrine cells can be found all throughout the retina, but they are most abundant in the central retina. This coincides with the

fact that they make connections with the cone bipolar cells that are mostly localized to the inner retina. With the knowledge that the oxygen-induced retinopathy model eliminates some of the vasculature from the central retina, the next step was to determine what impact this would have on the cells of the retina. An assumption could be made that, with no blood vessels present, a lack of oxygen would lead to the eventual death of any cells in the immediate area. This turned out to be true in the case of the dopaminergic amacrine cell when a similar study done by our lab revealed that they were susceptible to these ischemic conditions. In contrast, cholinergic amacrine cells were found by the same study to be immune to OIR's hypoxic conditions, exhibiting no cell loss in the inner or peripheral retina (Spix *et al.,* 2016). Mice of the age of P17 and P42 were used for this experiment to see if AII amacrine cells are lost in OIR. At the age of P17, the cells of the inner retina would have been exposed to a low oxygen environment for five days. At P42, the mouse is considered to be fully grown and any possible regrowth of cells should have occurred by this point, if it were to happen at all.

When looking first at the P17 control retina, it was evident that there was a uniform covering of the retina in AII amacrine cells (Figure 4). There were no sections in either the central or peripheral retina of the control that showed any reduction of cells. The P17 OIR retina, however, showed an extreme deficit of cells in both the central, avascular area and the area in the mid-periphery where neovascularization occurs. The central retina showed an absolute lack of cells where not even one cell can be seen on the 40x image (Figure 4). The only areas in the central retina where some cells did remain viable were those areas directly immediate to the major blood vessels that survived the oxygen treatment since they were responsible for supplying blood to the vessels of the peripheral retina that were not affected by the treatment. It is likely that these vessels were able to supply enough oxygen and nutrients to the surrounding cells and

thus able to keep them alive. When moving outwards to where neovascularization was the most prevalent, we begin to see more cells than what was present in the inner retina, but still less than half of the cells found in the same location in the control retina. When moving further outward to the edges of the retina, there is no longer a disparity between the control and OIR retinas and the cell densities are equal (data not shown).

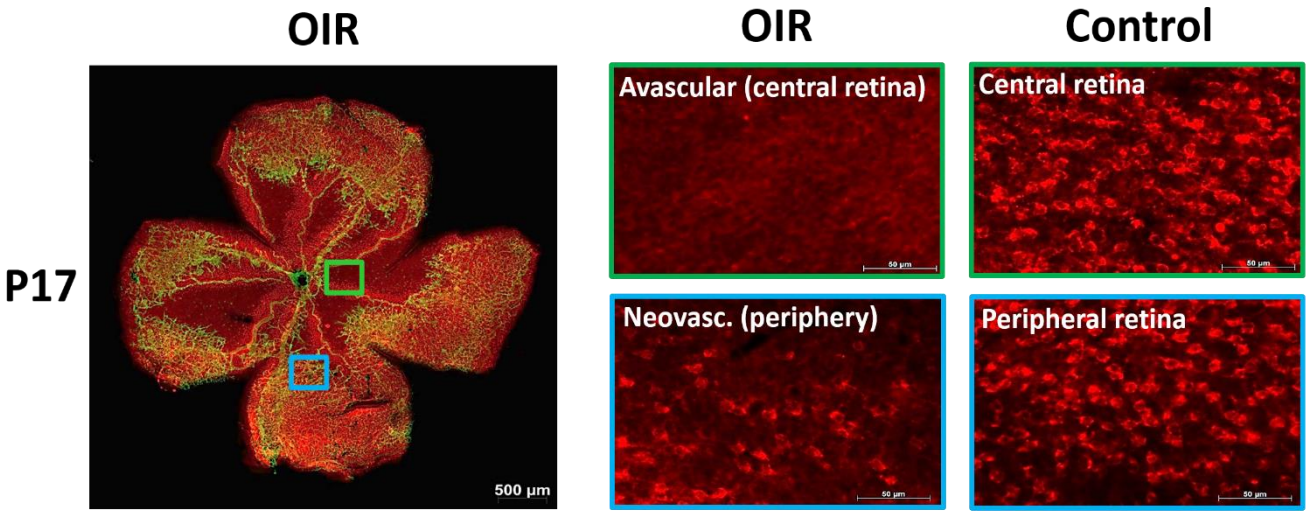

Figure 4: P17 AII amacrine cell death. The large image on the left displays a wholemount picture of the P17 OIR retina showing AII amacrine cell density (red) and blood vessels (light green). The wholemount nicely shows the correlation between the vascular regression in the central retina and the loss of AII amacrine cells (dark red in avascular region). The smaller images on the right compare the cell density of the OIR (left column) and the control (right column) retinas. Across the rows, the two images compare increased zoom images taken from similar areas on the different retinas, more specifically the sites of avascularization (green boxes) and neovascularization (blue boxes) in the OIR model. In the avascular region of the OIR retina there is complete loss of cell density while the neovascular region exhibits a considerable reduction in cell density when compared to the controls.

The P42 control retina, like the P17 control, exhibits uniform cell viability across the entire retina. Since cell loss was evident in the P17 OIR retina, it would be reasonably expected that its P42 counterpart would show similar loss. If there appeared to be a greater amount of cell

viability, however, it would implicate that cell regrowth would have had to have taken place in the 3+ weeks since P17. When looking at the images of the P42 retina, there is, in fact, cell loss similar to that of the P17 retina. Although the vasculature has regrown in the central retina and neovascularization has ceased, large patches of cell loss persist and are evident in the inner retina (Figure 5). The return of oxygen and nutrients to this area after the regrowth of the blood vessels was not enough to reverse the devastating effects of the OIR model. Like in the case of the P17 retinas, there was not a difference in cell numbers in the outer edges of the retina when comparing the P42 OIR retina to the control (data not shown). Also like the P17 OIR retina, cell viability near the larger vessels in the central retina remains.

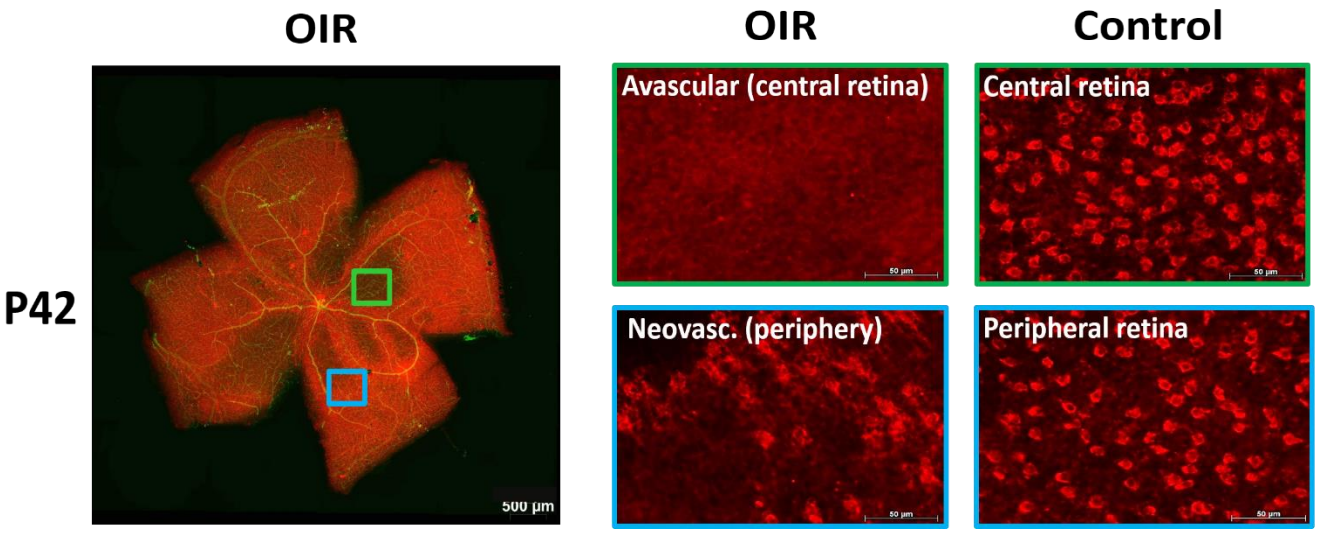

Figure 5: P42 AII amacrine cell death. The large image on the left shows a wholemount picture of the P42 OIR retina showing AII amacrine cell density (red) and blood vessels (light green). The wholemount reveals that, despite the regrowth of the vasculature present in the central retina, there are vast areas of cell loss that remain in the area (dark red in central region beneath the vasculature). The smaller images on the right compare the cell density of the OIR (left column) and the control (right column) retinas. Across the rows, the two images compare increased zoom images taken from similar areas on the different retinas, more specifically the sites of avascularization (green boxes) and neovascularization (blue boxes) in the OIR model. In the region where avascularization would have occurred, there is complete loss of cell density in the OIR retina. In the comparable region where neovascularization would have occurred, there is a considerable reduction in cell density.

Rod Bipolar Cells:

Once the signal is originated at the rod photoreceptor, it is then passed along to the rod bipolar cell. Like the AII amacrine cell, the rod pathway does not function if the rod bipolar cell in nonfunctional or nonexistent. The rod signal would simply terminate at the axonal terminals of the rod if the rod bipolar cell and its dendrites were not there to receive it. Similar to the AII amacrine cell study, mice were either subdivided into an oxygen treated group or a control group. After staining, images were taken once again at locations in the central and peripheral retinas. Retina slices were used to visualize the rod bipolar cells rather than wholemounting because we wanted to be able to view the entire length of the cell from its cell body all the way down the axon to the axonal terminals. If using a wholemount, none of this would be visible as the image would be taken while looking down upon the cell and not from the side.

To better monitor the progression of cell loss, if any, images were taken at the four different ages mentioned previously. Images taken at P12 showed no difference between the control and OIR retinas. Furthermore, there was no variance between the images taken from the central retina and those taken from the periphery. Since the oxygen treated mice at this age were sacrificed immediately after being removed from the chamber, it comes as no surprise that there was not any cell loss at this point as the rod bipolar cells had not yet been exposed to an oxygen deficit after vaso-obliteration. At P17, however, there was once again no change between the OIR model and the control (Figure 6). At a time when AII amacrine cell loss was already complete, the rod bipolar cells have remained intact in all the regions of the retina. These results continued to be true when looking at the P25 and the P42+ images. There was no cell loss evident in the central retina where it was believed to have had the highest likelihood of happening. Whether it was between the central and peripheral regions of the control retinas,

central and peripheral regions of the oxygen treated mice, or a comparison across the two treatment groups, no discernable difference can be made between any of the images. In this way, the rod bipolar cell is like the cholinergic amacrine cell in that it is able to resist cell death during the hypoxic conditions of oxygen-induced retinopathy.

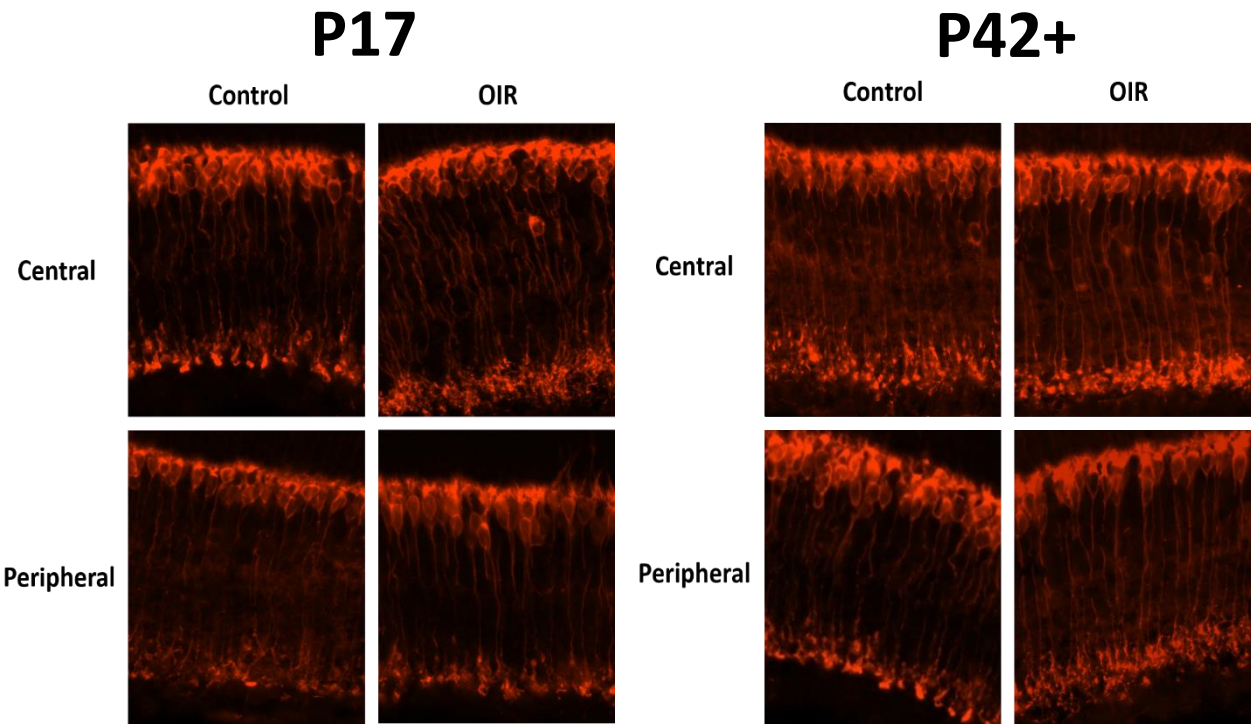

Figure 6: Rod bipolar cells. Staining for rod bipolar cells with PKCα was done on P12, P17, P25, and P42+ retinas. In this figure, pictures taken from P17 and P42+ retinas were used. Images of both the central and peripheral regions were taken for the OIR treated mice as well as their age-matched controls. The images show no discernable difference in cell morphology or number between the two treatment groups, nor a difference between the avascular central region of the retina and the normal peripheral retina of the oxygen-treated mice. The images from the other ages not shown displayed the same results.

On-going Experiments

Rods and cones are the basic functional unit of sight as they are capable of taking variations in light and transform that into signals that can be interpreted by the brain as sight. For this study, it was also important that we look at these two critical cells in addition to the AII amacrine and rod bipolar cells. Preliminary testing was done on the rods/cones and images were taken (Figure 7), but not enough data was collected to make a definitive statement on their survival in OIR. Another crucial finding that would further this study would be to look at the functionality of the surviving cells. This could be done using a western blot to measure the amount of protein present in the retina that is specific to a particular cell. According to Spix et al. (2016), although approximately 50% of dopaminergic amacrine cells were lost in the OIR model, there was almost a 75% reduction in the production of tyrosine hydroxylase in the OIR retinas. Tyrosine hydroxylase is involved in the production of dopamine and as such is only found in the retina in dopaminergic amacrine cells. This disparity in the percentages means that even though half of the cells survived, they did not retain their full capacity for producing dopamine. If this were the case, then there would be a 50% decrease in tyrosine hydroxylase to go along with the 50% reduction in cells. It will be interesting to find out if the remaining AII amacrine cells can function normally or if they were also negatively affected by OIR, just not to the point of dying. Likewise, the rod bipolar cells were able to survive the hypoxic conditions of OIR, but there is no guarantee that these cells retain complete functionality. Finally, an eye test called an electroretinogram (ERG) can be administered to the mice to gauge the impact that AII amacrine cell loss has on vision. This process works by measuring the action potential waves of the cells within the retina. If the oxygen treated mice show a significant decrease in the wave action

created by the AII amacrine cell, this means that the rod signal is being interrupted at that cell, possibly leading to a deficit in scotopic vision.

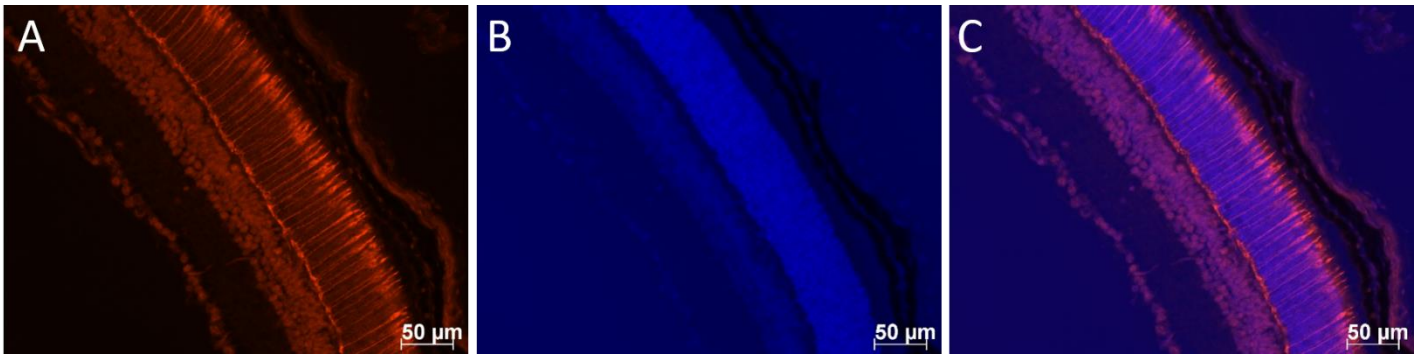

Figure 7: Rod and cone overlay. Image A and B are photos taken from the same location on the retina visualizing the cones and rods respectively. Image C merges the two images together, overlaying the rods and cones on top of one another.

Conclusion

In conclusion, the conditions present in oxygen-induced retinopathy are detrimental to the AII amacrine cells in the central, avascular retina. Cell death in OIR was observed primarily at P17, meaning that the cells died between P12 and P17 when the retina was in a hypoxic state. No major additional cell loss was identified at P42 compared to P17, but neither was a reversal of cell death detected at this time point either, likely signifying that the cell loss is permanent. Surviving AII amacrine cells were concentrated in the areas of the retina that remained vascular throughout the course of the disease, most notably in the most peripheral areas of the retina and the areas directly surrounding large blood vessels that did not regress during the hypoxic conditions. In contrast, the rod bipolar cells were able to resist the hypoxic conditions and survive throughout the OIR retina. Taken together, the results suggest that the loss of AII amacrine cells play a larger role in the reduction of scotopic vision in ROP patients than was previously expected.

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