Exploring the Akt/PI3 Kinase Signaling Pathway in the Avian Retina

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

By

Zachary A. Goodman

The Ohio State University
April 2013

Project advisor: Professor Andy Fischer, Department of Neuroscience

Abstract

The Akt pathway is an important cell-signaling pathway that can affect many cellular processes including growth and proliferation. This study aims to explore the role of the Akt pathway in the formation of Muller glia-derived progenitors in the avian retina. The results show that pS6 (a readout of the Akt pathway) is expressed in embryonic retina but there are minimal levels in a mature, normal retina. Upon excitotoxic damage or FGF2 stimulation, pS6 is highly upregulated with a peak at 4h after damage. Furthermore, VO-OHpic trihydrate (VOTH), a PTEN inhibitor was able to increase the number of proliferating Muller glia four fold. We conclude that the Akt pathway is not active in a normal retina, the Akt/mTOR pathway is rapidly activated in damaged retinas, and this pathway stimulates the formation of Muller glia-derived progenitors.

Introduction

The retina is derived from the ventral diencephalon and contains the following neurons: rods, cones, bipolar, horizontal, bipolar, amacrine and bipolar cells. The primary photosensitive cells are the rod and cone cells located in the outer retina. Rods are responsible for vision in low light and cones enable color vision. Horizontal cells provide lateral inhibition and signal integration for the photoreceptor cells. The bipolar cells are responsible for transmitting signals from the photoreceptor cells to the ganglion cells and the signals are refined and processed by amacrine cells. Ultimately, the axons of the ganglion cells will come together to form the optic nerve to send signals to higher visual centers in the brain.

The aforementioned neurons that make up the retina are supported by the following glial cells: Müller glia, microglia and oligodendrocytes. Müller glia are the primary glial cell in the retina and have processes that span the entire length of the retina. Furthermore, Müller glia provide structural, metabolic, and synaptic support as well as ion homeostasis for the neurons in the retina. Oligodendrocytes are found in the inner retina and produce myelin for the axons of the ganglion cells. Lastly, microglia function as macrophages helping to rid the retina of any foreign organism or substance.

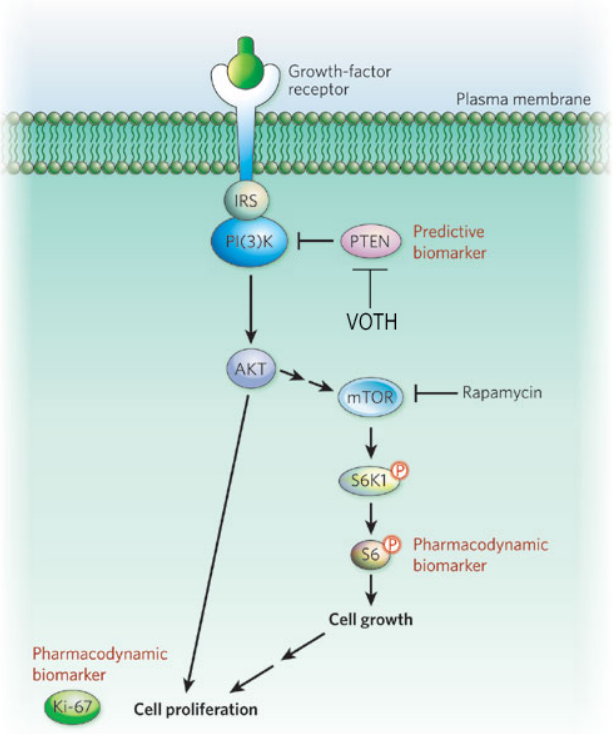
Akt, also known as Protein Kinase B (PKB), is the nucleus of a foundational signal transduction pathway with a pivotal role in many cellular processes. Cellular growth, migration, invasion, survival, proliferation, metabolism and angiogenesis all have links to Akt signaling. Furthermore, it has been shown that the Akt pathway communicates with other major signaling pathways such as NF-κB, Erk and JNK/p38, each of which having their own separate downstream effects on cellular processes (Manning and Cantley 2007). With far-reaching and well-studied effects, the Akt pathway can be selectively activated or inhibited with many different pharmacological agents. This paper will primarily focus on Akt signaling in the avian retina.

In previous works, it has been shown that excitotoxic damage (Fischer and Reh 2001) as well as FGF2/insulin in the absence of damage (Fischer et al. 2002) can drive the de-differentiation of Müller glia into progenitor cells in the avian retina and that these progenitor cells can then re-differentiate into amacrine and bipolar cells. Such findings can eventually be translated into potential cell-replacement therapies for degenerative retinal diseases. Therefore, I would like to further pursue the task of unraveling the complexities of the de-differentiation to re-differentiation pathways. The main goal of the

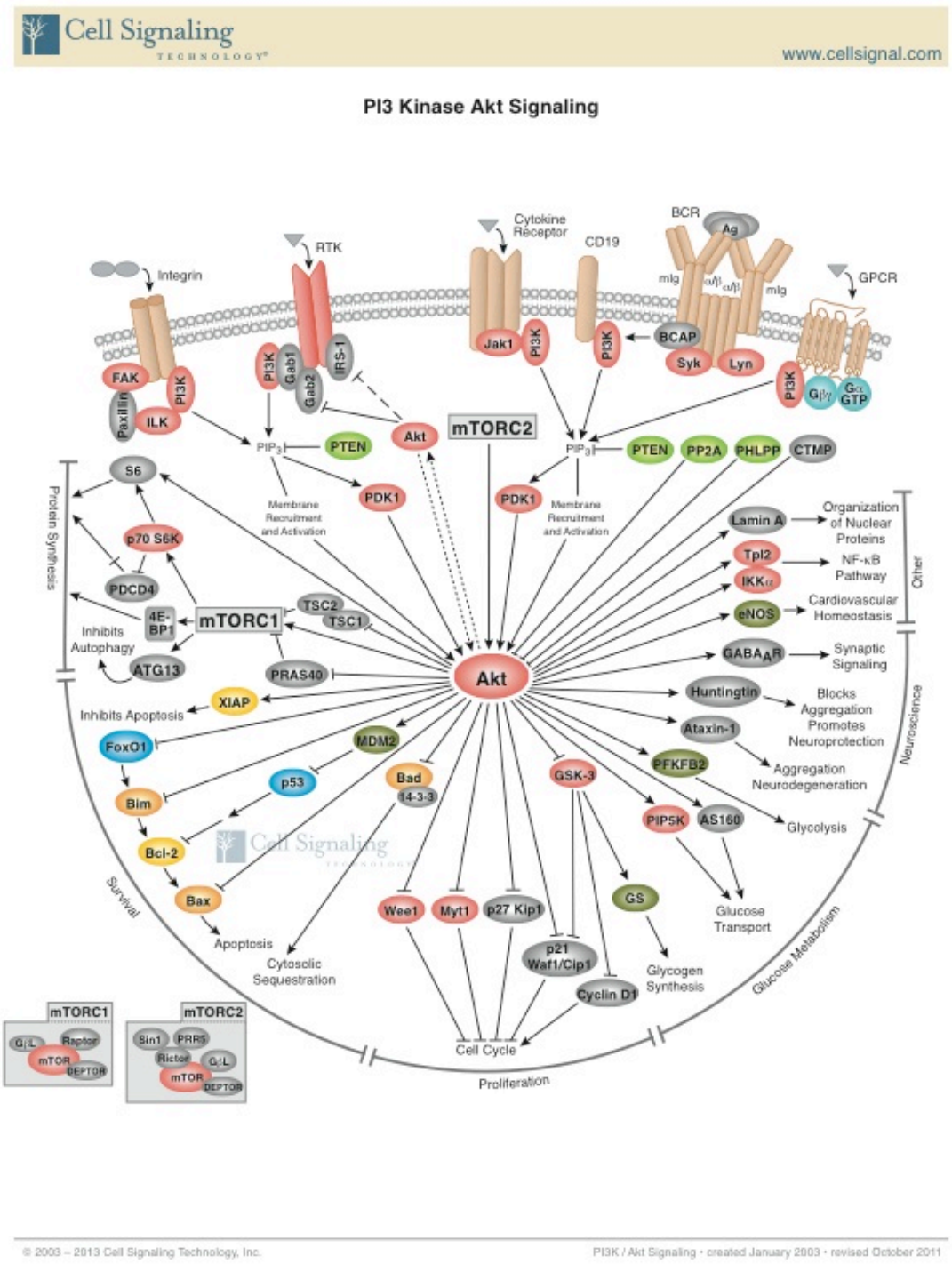
current study was to examine Akt signaling in a normal and stimulated avian retina as well as the pathway's role in the formation of Müller glia derived progenitors.

Phosphorylated S6 protein (pS6), a component of the mammalian 40S ribosomal subunit, was chosen as a downstream readout of the Akt pathway. S6 Kinase (S6K) is responsible for the translational control of 5'TOP mRNAs that code for translational machinery and elongation factors necessary for cell proliferation and growth. S6 is regulated by S6K through multiple phosphorylations (Dufner and Thomas 1999).

VO-OHpic trihydrate (VOTH), a small molecule PTEN inhibitor was chosen to manipulate the Akt pathway. Inhibition of PTEN would allow for the uncontrolled phosphorylation of PIP_2 to PIP_3 by PI3 Kinase, leading to the successive activation of Akt, mTORC1, p70S6 kinase [the cytoplasmic isoform of S6K (Dufner and Thomas 1999)] and ultimately the phosphorylation of S6 as shown in the diagram below (Sawyers 2008).



The phosphatidylinositol-3-OH kinase (PI(3)K)–PTEN–mTOR signalling pathway is aberrantly activated in many tumors, leading to dysregulation of cell growth and proliferation. Activation of the pathway can be assessed by biomarkers such as loss of PTEN mRNA or protein production in tumor tissue. Biochemical inhibition of mTOR by rapamycin can be assessed by biomarkers such as the abundance of the phosphorylated form of the ribosomal protein S6, and its therapeutic effects on tumor cells can be assessed by the proliferation marker Ki-67. IRS, insulin-receptor substrate; S6K1, ribosomal protein S6 kinase, 70-kDa, polypeptide 1 (Sawyers 2008).



Different branches of the Akt pathway. Taken from Cell Signaling.

Materials and Methods

Animals

The use of animals was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. Newly hatched leghorn chickens (Gallus gallus domesticus) were obtained from the Department of Animal Sciences at the Ohio State University and kept on a cycle of 12-h light, 12-h dark (lights on at 7:00 a.m.). Chicks were housed in a stainless steel brooder at about 28°C and received water and Purina^{TM} chick starter ad libitum.

Intraocular Injections

Intraocular injections were performed as described previously (Fischer et al., 2009). In all experiments, 20 μL of vehicle containing the test compound was injected into the experimental (right) eye, and 20 μL of vehicle alone was injected into the control (left) eye. The vehicle was DMSO (Dimethyl sulfoxide; 30% DMSO in saline). Test compounds included NMDA (N-methyl-D-aspartate; 20 μmol (full) and 5 μmol (quarter) per dose; Sigma-Aldrich), recombinant human FGF2 (250 ng/dose; R&D Systems), VOTH (VO-OHpic trihydrate; 1μg/dose; Sigma-Aldrich).

We used six different injection paradigms: (1) Paradigm A—on post-hatch day 8 (P8) the left eye received a single injection of saline and the right eye received VOTH. Retinas were harvested 1, 2, 4, and 24h later. (2) Paradigm B—on P7 the left eye received an injection of saline and the right eye received VOTH. Retinas were harvested 4h and 48h later. (3) Paradigm C—on P7 both the left and right eyes received a quarter dose of NMDA. Then, 4h later, the right eye received a dose of VOTH. 24h after the

injection of VOTH, the right retina was given a second dose of VOTH. 48h after the first VOTH injection, the right retina received a dose of VOTH+BrdU. The retinas were harvested 72h after the first dose of VOTH. (4) Paradigm D – on P7 the left eye received injections of saline and the right eye received two consecutive daily doses of FGF2. The retinas were harvested 2 days after the first injection of FGF2. (5) Paradigm E – on P7 the left eye received saline and the right eye was treated with a full dose of NMDA. Retinas were then harvested 1, 2, and 3 days after the first injection of NMDA. (6) Paradigm F – on P8 the left eye received saline and the right eye received a quarter dose of NMDA. The retinas were harvested 4h later.

Fixation, Sectioning and Immunocytochemistry

Tissue dissection, fixation, cryosectioning, and immunolabeling were performed as described previously (Fischer et al., 1998, 2009).

Working dilutions and sources of antibodies used in this study included; rabbit anti-pS6 was used at 1:750 (2215S; Cell Signaling Technologies); rabbit anti-pERK1/2 was used at 1:200 (137F5; Cell Signaling Technologies); rabbit anti-pCREB was used at 1:600 (87G3; Cell Signaling Technologies); goat anti-Sox2 was used at 1:1,000 (Y-17; Santa Cruz Immunochemicals); mouse anti-Brn3a was used at 1:50 (Chemicon; MAB1585); rabbit anti-TFBP was used at 1:400 (Dr. J.J. Lucas; SUNY); rat anti-BrdU was used at 1:200 (OBT0030S; Serrotec). Secondary antibodies included donkey-anti-goat- Alexa488, goat-anti-rabbit-Alexa488, goat-anti-mouse- Alexa488/568, rabbit anti-goat Alexa488 and diluted to 1:1,000 in PBS plus 0.2% Triton X-100.

Cell-distinguishing markers in the retina

pS6 is marker of the activated Akt pathway. Sox2 is expressed by Müller glia and cholinergic amacrine cells. Brn3a is expressed ganglion cells. TFBP is expressed by oligodendrocytes. pCREB and pERK are readouts of the activated MAPK pathway. HuC/D is expressed amacrine and ganglion cells. Bromodeoxyuridine (BrdU) is a thymidine analog that gets incorporated into DNA during DNA replication.

Photography, Measurements, Cell Counts, and Statistical Analyses

Photomicrographs were taken by using a Leica DM5000B microscope equipped with epifluorescence and a 12 megapixel Leica DC500 digital camera. Identical illumination, microscope and camera settings were used to obtain images for quantification. Images were optimized for color, brightness and contrast, and double-labeled images overlaid by using Adobe Photoshop^{TM}6.0. Areas were sampled from 5.4 MP digital images. BrdU cell counts were made from four different animals. Due to inter-animal variability, all counts were standardized by (treated/control) *100. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the same region of retina (21,2600μ^2) for each data set.

Results

Since pS6 was chosen as a readout of the Akt pathway, it was important to establish a baseline for pS6 expression in an untreated retina. pS6-immunoreactivity is present in the axon terminals of the photoreceptors (bright green band in fig 1a). The labeling in the axon terminals of photoreceptors is likely to be a non-specific cross-reaction, given that this pattern of labeling is not perturbed by experimental paradigms that are expected to influence levels of pS6. In addition, pS6-immunoreactivity was observed in a few ganglion cells [positive for the ganglion cell marker Brn3a (fig 1f,i)] and oligodendrocytes [positive for TFBP (fig 1l)]. pS6 was not observed in Sox2-expressing mature Müller glia in the inner nuclear layer (INL) (fig 1b,c).

In addition to mature retinas, patterns of pS6 expression were determined in embryonic retinas. Eggs were incubated and harvested at embryonic day 4 (E4), E6, E8, E12 and E15. At E4 and E6, during early stages of retinal development, pS6 is primarily expressed in maturing ganglion cells, as evidenced by pS6 positive cells which are also positive for HuD/C, a cytoplasmic neuronal marker (fig 2f,i). By E8, there is continued pS6 staining in maturing neurons, but also in presumptive progenitors in the developing INL (fig 2c). At E12, pS6 is present in relatively few maturing neurons and appears in a few scattered Müller glia (fig 2j). Furthermore, a few cell in the cilliary marginal zone (CMZ; contains retinal progenitors) display pS6 expression (fig 2k). Interestingly, at E15 pS6 appears to be highly expressed in maturing Müller glia (Fig. 2l).

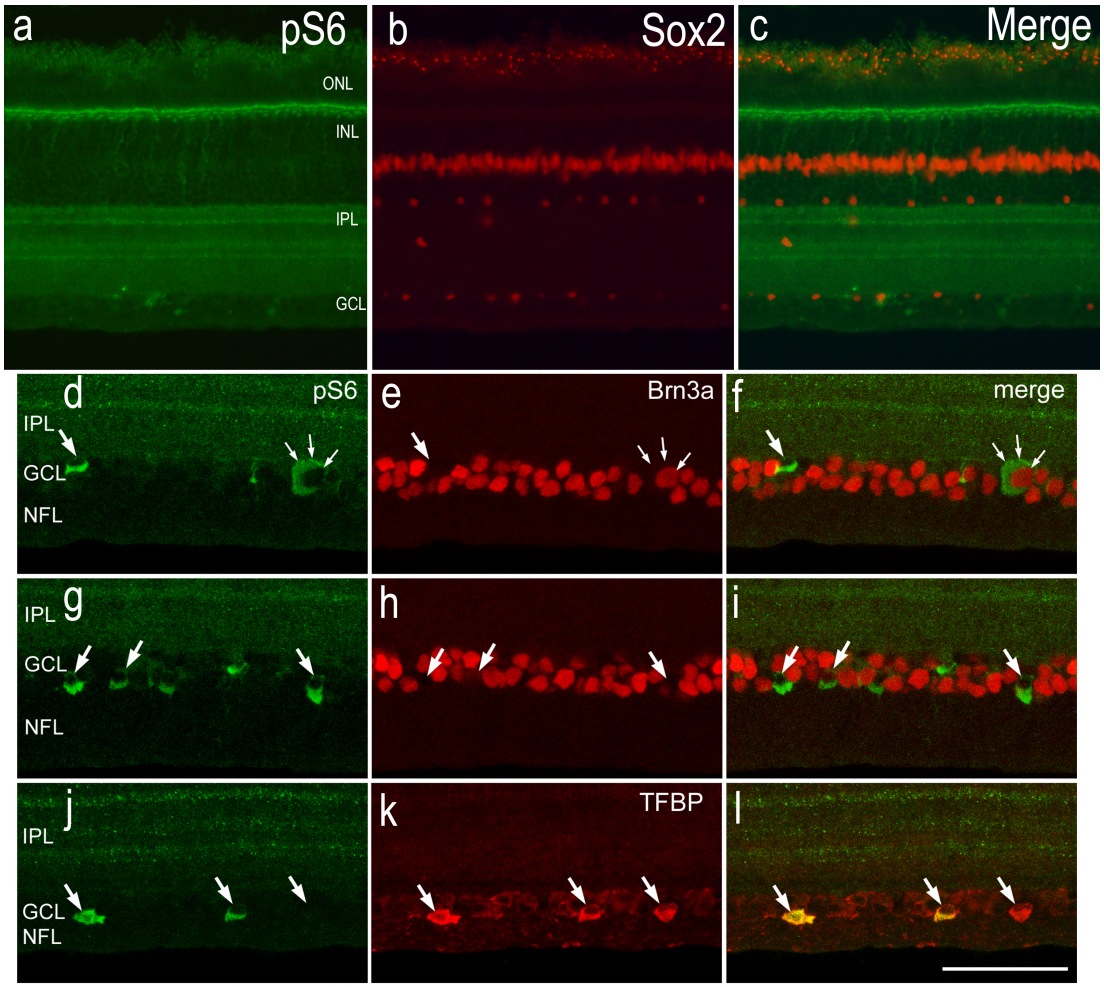


Figure. 1. pS6 expression in a normal retina. Retinas were harvested 4 hours after injection with sterile saline. **(a-c)** pS6, Sox2 expression and colocalization in a normal retina. **(d-i)** pS6, Brn3a and colocalization in a normal retina. **(j-l)** pS6, TFBP expression and colocalization in a normal retina.

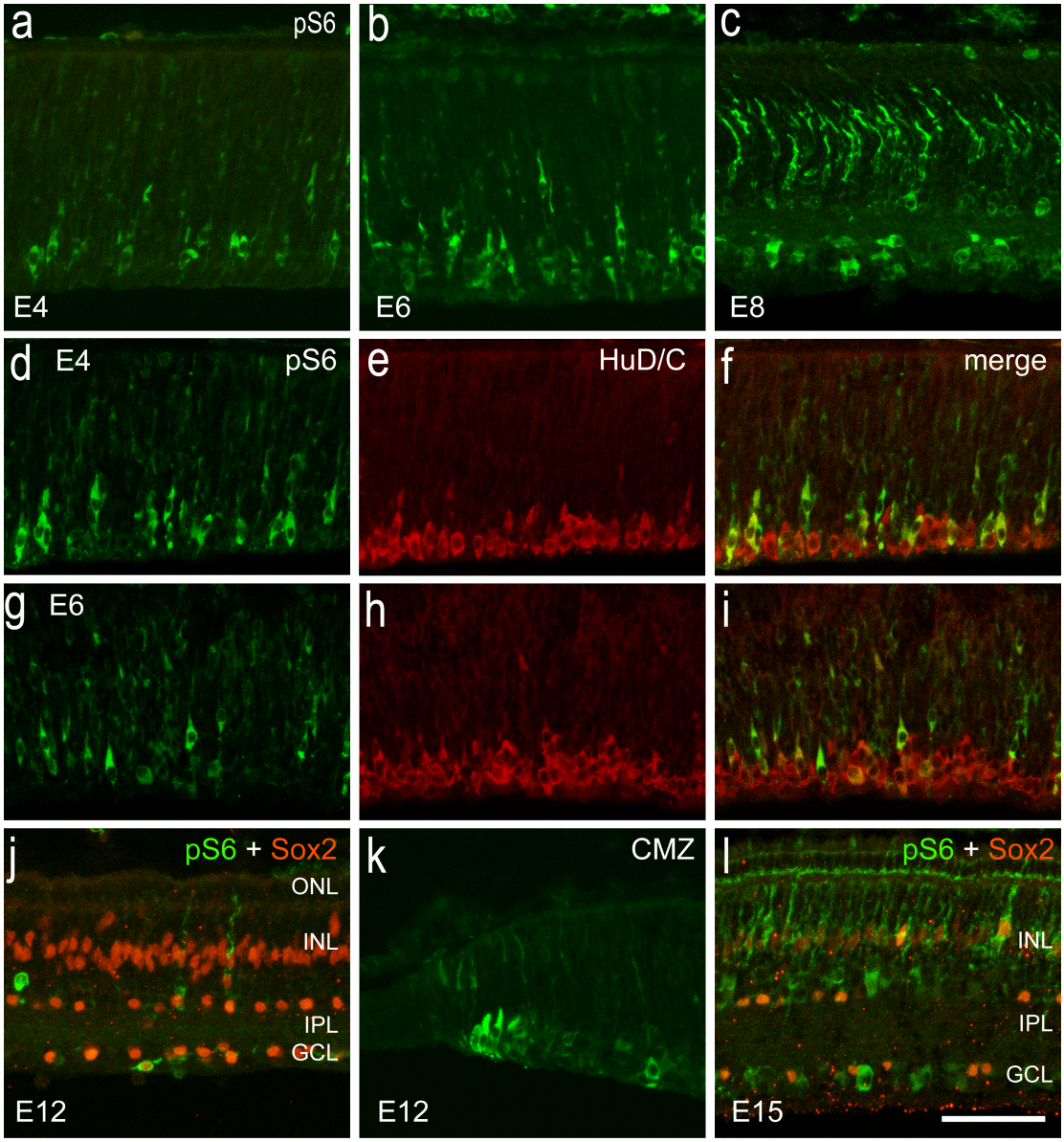


Figure. 2. pS6, Sox2 and HuD/C expression in the embryonic retina. **a-c)** pS6 expression at E4, E6, E8. **d-i)** Expression of pS6, HuD/C and colocalization at E4 (d-f) and E6 (g-i). **j)** Colocalization of pS6 and Sox2 and E12. **k)** Expression of pS6 in the CMZ at E12. **l)** colocalization of pS6 and Sox2 at E15.

Next, retinas were damaged with *N*-methyl-D-aspartate (NMDA; 0.5 µmol NMDA; fig 3a-d, or 2 µmol NMDA; fig 3e-p) which is an excitotoxin that primarily kills amacrine cells (Fischer and Reh 2001). The retinas were harvested at 4h (fig 3a-d), 1 day (fig 3e-h), 2 days (fig 3i-l) and 3 days (fig 3m-p) after injection with NMDA. Expression of pS6 in the control central retina is relatively low (fig 3a,e,i,m) with only a slight increase in peripheral regions of control retinas from eyes injected with saline (fig 3c,g,k,o). Upon damage, there is a large upregulation of pS6 in the INL in both central and peripheral retina at 4h (fig 3b, d) and 1 day (fig 3f, h) with no noticeable difference between central and peripheral. Expression levels of pS6 taper off in the damaged central retina by day 2 (fig 3j), with a decreased level of pS6 in the treated periphery (fig 3l) as compared with 4h and 1 day after NMDA injection. By day 3, there is only slight pS6 expression in the central and peripheral treated retinas (fig 3n,p) and wild type pS6 levels in both the central and peripheral control retinas (compare fig 3m,o to fig 1a,c).

It was previously shown that a combined injection of FGF2 and insulin causes the Müller glia to become proliferating progenitor cells in the same manner as in NMDA-damaged retinas (Fischer et al. 2002). Injections of FGF2 alone are known to enhance the ability of Müller glia to become progenitor cells (Fischer et al. 2009a); (Ghai et al. 2010). Thus, we tested whether FGF2 influenced the expression of pS6. Retinas were injected with two consecutive daily doses of FGF2 (250 ng) and harvested 2 days after the first injection of FGF2. This caused an increase in pS6 expression in the INL similar to the levels seen in the central retina after 1 day of NMDA damage (compare fig 4d to fig 3f). The cells expressing pS6 are Sox2-positive Muller glia (fig 4f).

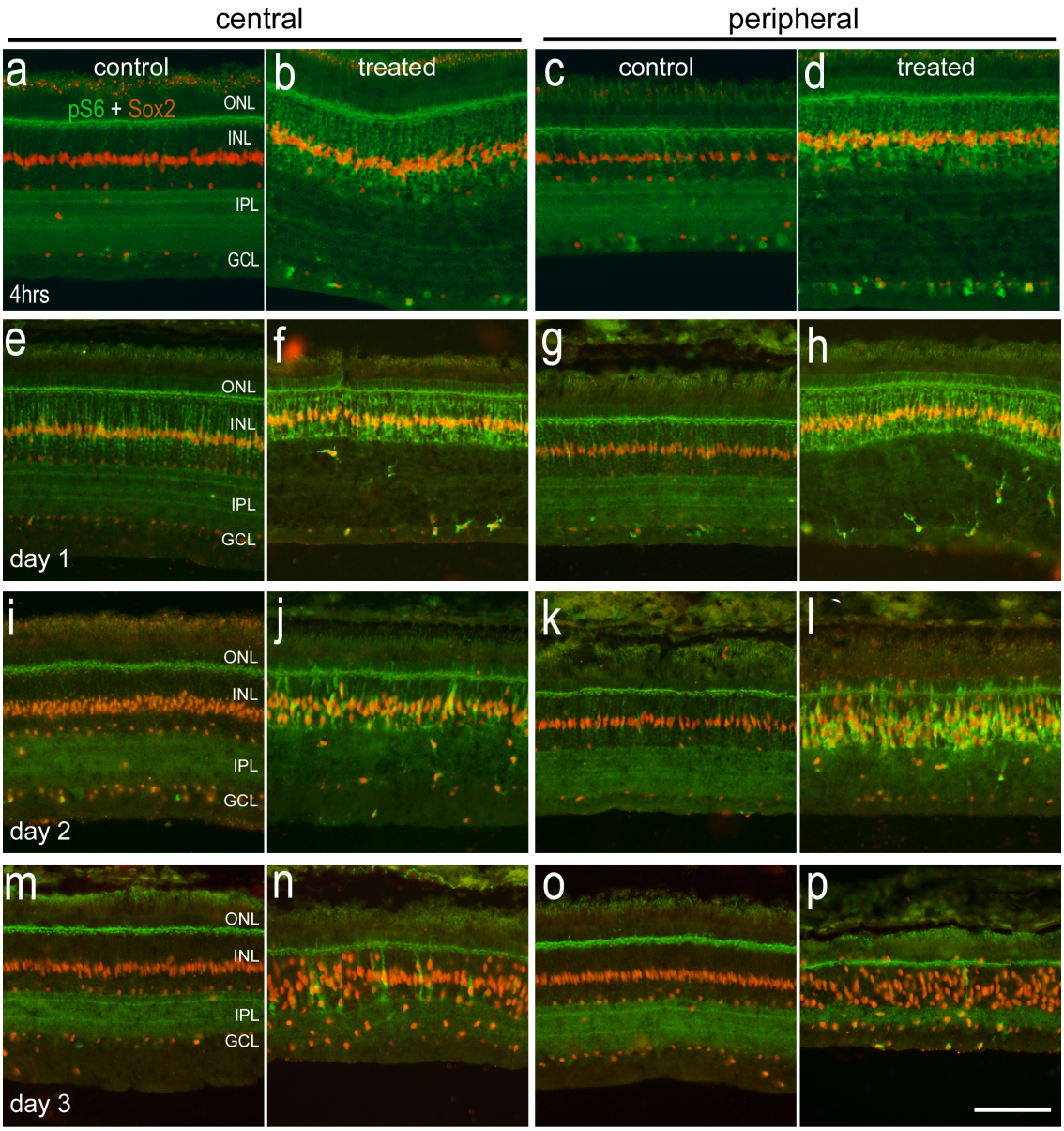


Figure. 3. Expression of pS6 and Sox2 after NMDA damage. For a-d, retinas were given a quarter dose (0.5 μmol) of NMDA at P8. For e-p, retinas were given a full dose (2.0 μmol) of NMDA at P7. The control retinas were given sterile saline. The retinas were harvested at 4h (a-d), 1 day (e-h), 2 days (i-l) and 3 days (m-p) after injection with NMDA. **(a-p)** Colocalization of pS6 and Sox2 in the central and peripheral retina at 4h (a-d), 1 day (e-h), 2 days (i-l), 3 days (m-p).

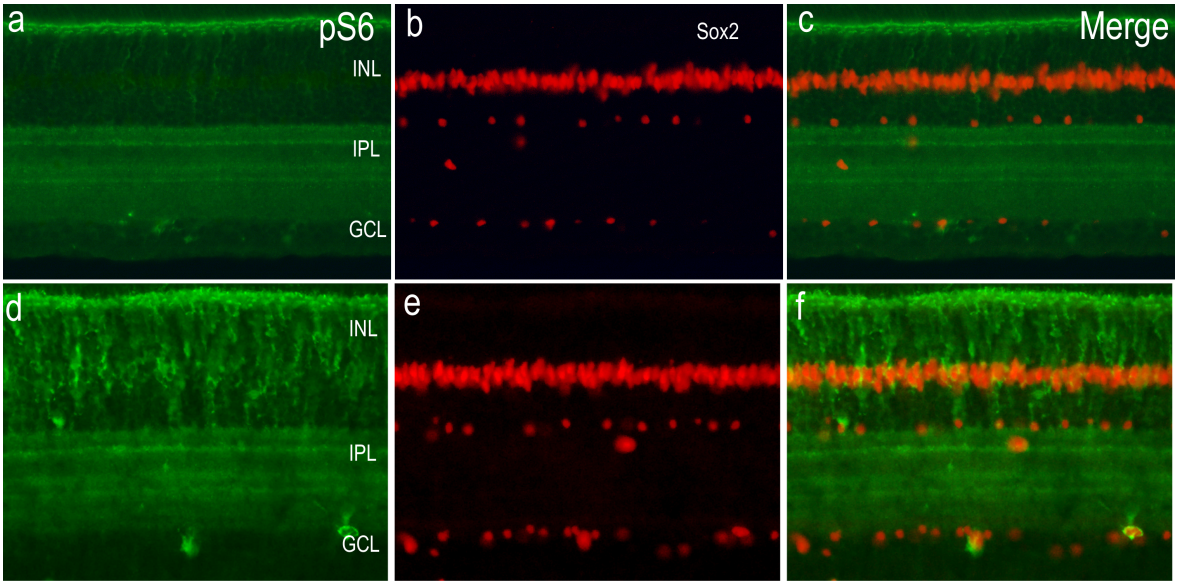


Figure. 4. FGF2 induced expression of pS6. The control retinas were given sterile saline and the treated retinas received 2 doses of FGF2 (250 ng). **(a-c)** Expression of pS6, Sox2 and colocalization in the control retina. **(d-f)** Expression of pS6, Sox2 and colocalization in the treated retina.

The next step in these experiments was to assay the effects of PTEN-inhibitor (VOTH) in the retina. VOTH was injected and retinas were harvested 1h, 2h, 4h, 1 day and 2 days after injection with VOTH. To assess the specificity of the PTEN inhibitor, we probed for off-target effects and determined whether MAPK-signaling was affected in the Müller glia; a read-out of glial reactivity (Fischer et al. 2009a/b). Both pCREB (fig 5a-d) and pERK (fig 5e-h) were expressed in the central and peripheral retina. There were no consistent expression patterns of pCREB (fig 5a-d) across all time points (1hr, 2h, 4h, 1 day and 2 days). Furthermore, there were no differences in pERK expression in the central and peripheral retina (fig 5e-h). Overall expression levels of pCREB and pERK were low as compared to a previous work (Fischer et al. 2009a). There does not appear to be a difference in pS6 levels between retinas treated with VOTH (fig 5j,l) and the control (fig 5i,k), suggesting that PI3K-signaling may be very low in normal, healthy retinas.

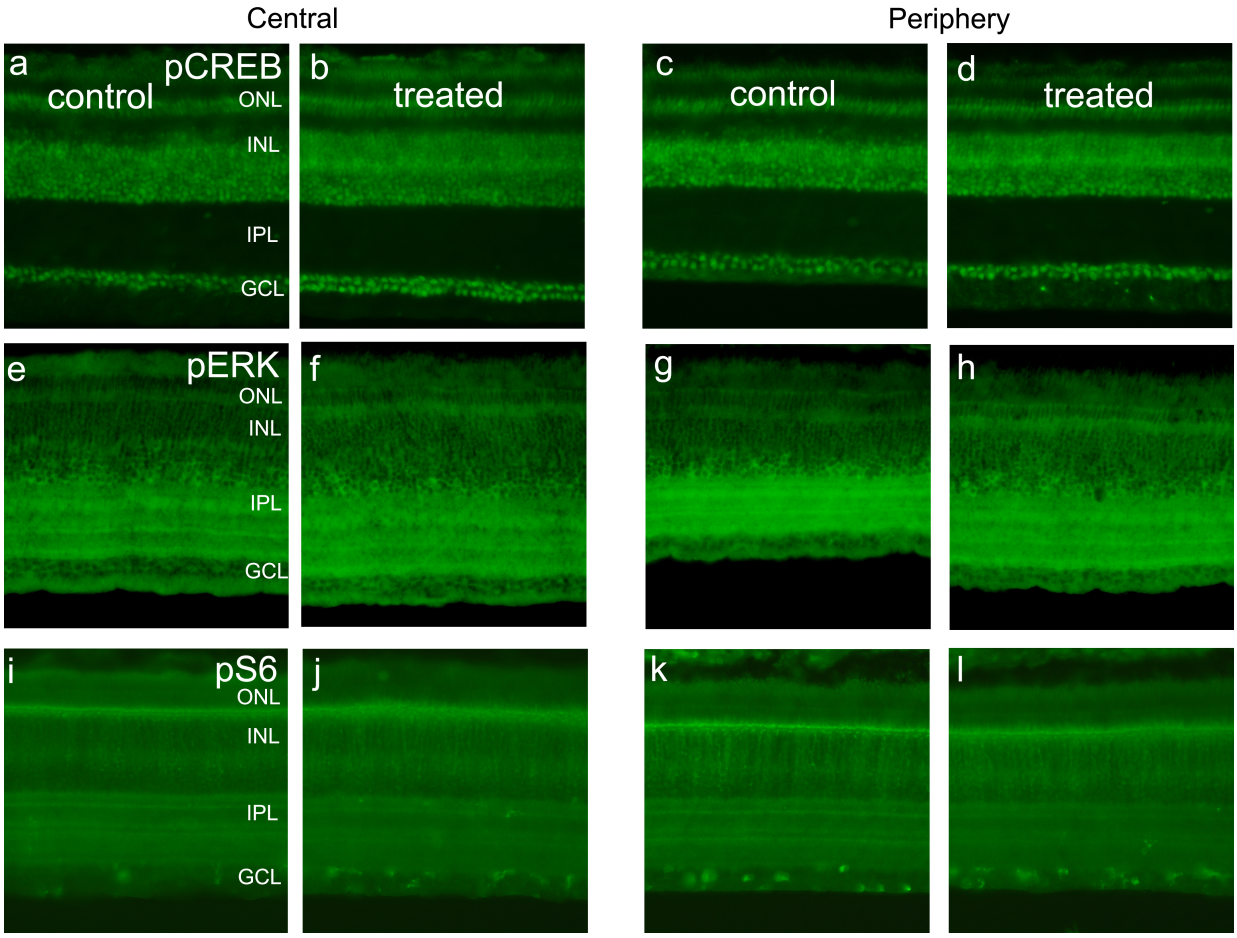


Figure. **5.** VOTH treatement. The pERK, pS6, pCREB retinas were harvested 1h, 4h and 4h respectively after 1 dose of VOTH (1.0 μg). The control retinas were given sterile saline. **(a-d)** pCREB expression in the central and peripheral retina. **(e-h)** pERK expression in the central and peripheral retina. **(i-l)** pS6 expression in the central and peripheral retina.

pS6 is highly up regulated in Müller glia in NMDA-damaged retina, suggesting that PI3K/Akt/mTOR-signaling is activated. Accordingly, we tested whether further activation of this pathway with VOTH influenced the formation of proliferating Müller glia-derived progenitors. Retinas were injected with 0.5 μmol of NMDA followed by a dose of VOTH 4h later. 24h later, another dose of VOTH was administered. Then, 48h after the first injection with VOTH, a dose of VOTH+BrdU was given and the retinas were harvested 72h after the first VOTH injection. The VOTH appeared to slightly increase levels of pS6 in peripheral regions of the NMDA-damaged retinas (Fig. 6b,d). Injection of BrdU allowed for the marking of proliferating cells. Retinal areas of 21,2600μ^2 were surveyed and cells positive for both BrdU and Sox2 in the INL (proliferating Muller glia) were counted. NMDA-damaged retinas treated with VOTH had an average of 427% and 139% increase in number of Sox2/BrdU-positive Müller glia in central (n=4) and peripheral retina (n=3) respectively (fig 6f,h; fig 6i).

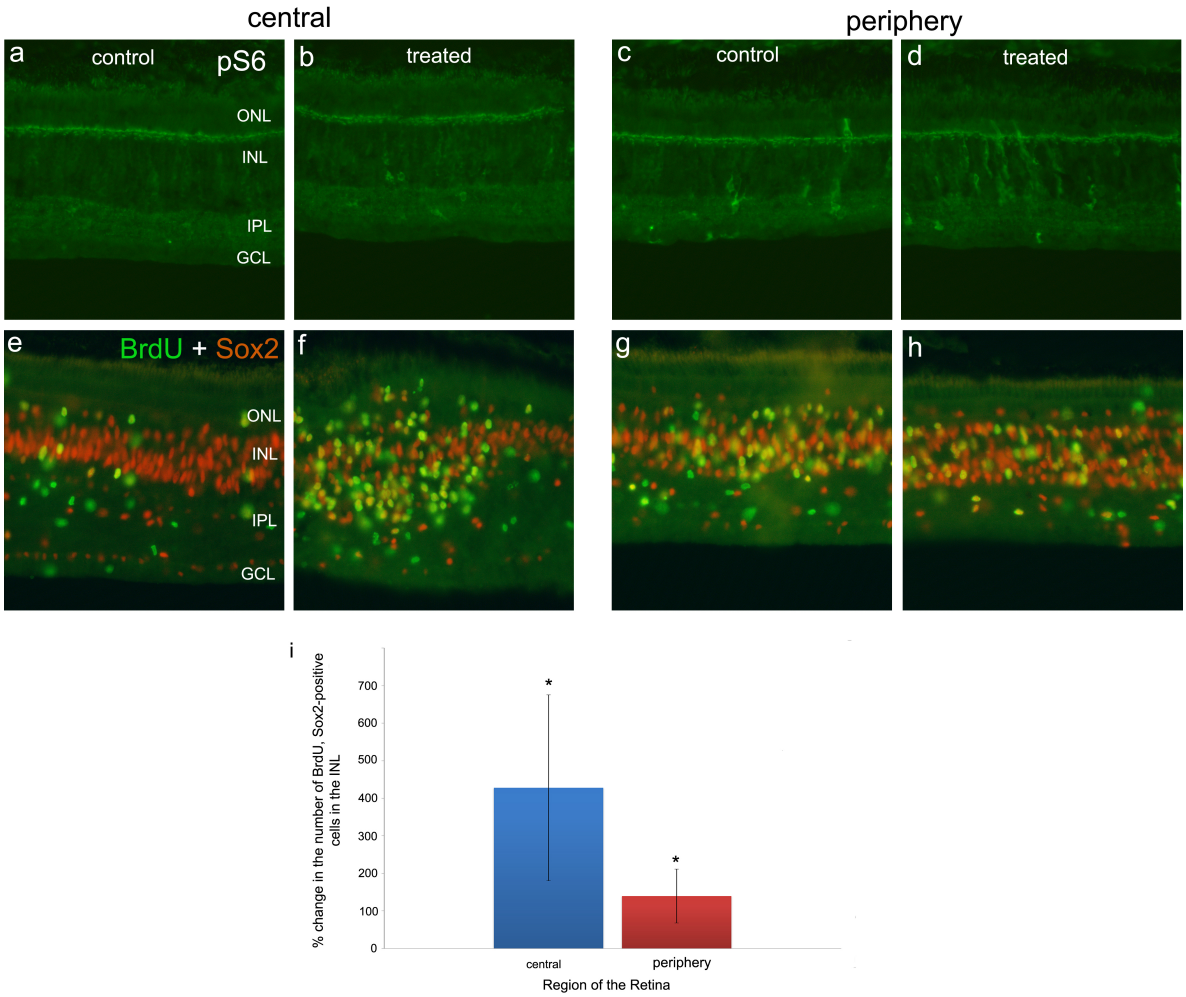


Figure. **6**. VOTH after NMDA damage. Both control and treated retinas were given a quarter dose (0.5 μmol) of NMDA. 4h later the treated retinas received a single dose of VOTH (1.0 μg). 24h after the first dose of VOTH, the treated retinas received another dose of VOTH. 48h after the first injection of VOTH, the retinas received a dose of VOTH+BrdU. The retinas were harvested 72h after the first injection of VOTH. An area of 21,2600μ^2 pixels was used for the BrdU cell counts. **(a-d)** pS6 expression in the central and peripheral retina. **(e-h)** BrdU and Sox2 colocalization in the central and peripheral retina. **(i)** A histogram illustrating the means and standard deviations for the % change in BrdU, Sox2-positive cells in the INL (proliferating Müller glia) in the central and peripheral retina. Significance (*P < 0.05) of difference was calculated using a 1-tailed students t-test.

Discussion

The results show that pS6 is only expressed in a small subset of ganglion cells and oligodendrocytes in normal chick retina. The data from embryonic retinas suggests that expression of pS6 is symptomatic of immature neurons and glia because it is expressed in immature ganglion cells, E8 progenitors and maturing Muller glia. There is a significant increase in pS6 expression upon damage with NMDA, and FGF2 stimulation indicating that pS6 expression is not solely a response to acute retinal damage. Furthermore, following NMDA-treatment, pS6 expression peaks at 4h suggesting that the PI3K/Akt/mTOR pathway is rapidly activated in Müller glia after damage to retinal neurons.

VOTH by itself does not induce a response in the retina as evidenced by the inconsistent expression of pCREB, and normal levels pERK and pS6 in Müller glia. This finding suggests that there is normally little or no PI3K-signaling in normal, healthy Müller glia and that VOTH specifically acts on the Akt pathway. However, in NMDA-damaged retinas, VOTH caused an increase in proliferating Muller glia-derived progenitors. It is expected that the VOTH should increase retinal levels of pS6 in damaged retinas. However we failed to detect large, widespread increases in pS6 at 3 days after NMDA-treatment. Further, experiments are required to test whether levels of pS6 in Müller glia are affected by VOTH shortly after NMDA-treatment when levels are normally elevated following damage.

In conclusion, the results indicate that Akt signaling is not active in a normal retina and that pS6 is a reliable readout of the pathway. Akt signaling is selectively activated in Müller glia in response to NMDA damage as well as FGF2 stimulation.

Further, enhanced activation of the PI3K pathway by inhibiting PTEN in Müller glia increased the number of proliferating glia-derived progenitors.

The results of these experiments provide a foundation for understanding Akt/mTOR signaling during retinal development and following retinal damage. In addition, these studies suggest that PTEN can be targeted pharmacologically to enhance the formation of Müller glia-derived progenitors and, possibly, neuronal regeneration. The next step, with regards to the increased levels of Müller glia proliferation (BrdU, Sox2-positive cells in the INL), would be a TUNEL stain in order to determine if VOTH has neuro-protective qualities. Furthermore, there is evidence to support that Oct4, Akt and Hmgb2 form a feedback loop to maintain pluripotency in mouse embryonic stem cells (Campbell and Rudnicki 2013). Therefore, there should be staining or RT-PCR for Oct4, Klf4 and Nanog (all pluripotency factors) in order to determine whether the dedifferentiating Müller glia are expressing pluripotency factors.

Additionally, new injection paradigms should be used. The retinas should be damaged with NMDA and then injected with rapamycin, an inhibitor of mTORC1, theoretically leading to the down regulation of pS6 expression. This paradigm can be modified by administering VOTH before the rapamycin in order to determine whether the inhibition of mTORC1 can negate the activating effect of VOTH. If pS6 levels are found to be high, that result would indicate that pS6 activation is occurring by a secondary signal transduction pathway. Lastly, VOTH can be injected after FGF2 + insulin, in order to determine whether the formation of Müller glia-derived progenitors can be enhanced by further activating the Akt/mTOR pathway in the absence of retinal damage.

Acknowledgements

I would like to thank Dr. Fischer for all of his guidance and support throughout this project. I would also like to thank Chris, Donika, Levi, Lillia, and Bill for all of the help that they have given me with my lab work.

Works Cited

Campbell PA, Rudnicki MA. 2013. Oct4 Interaction with Hmgb2 Regulates Akt Signaling and Pluripotency. Stem Cells.

Dufner A, Thomas G. 1999. Ribosomal S6 kinase signaling and the control of translation. Exp Cell Res 253(1):100-9.

Fischer AJ, McGuire CR, Dierks BD, Reh TA. 2002. Insulin and fibroblast growth factor 2 activate a neurogenic program in Muller glia of the chicken retina. J Neurosci 22(21):9387-98.

Fischer AJ, Reh TA. 2001. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci 4(3):247-52.

Fischer AJ, Scott MA, Ritchey ER, Sherwood P. 2009a. Mitogen-activated protein kinase-signaling regulates the ability of Müller glia to proliferate and protect retinal neurons against excitotoxicity. Glia 57(14):1538-1552.

Fischer AJ, Scott MA, Tuten W. 2009b. Mitogen-activated protein kinase-signaling stimulates Muller glia to proliferate in acutely damaged chicken retina. Glia 57(2):166-81.

Ghai K, Zelinka C, Fischer AJ. 2010. Notch signaling influences neuroprotective and proliferative properties of mature Muller glia. J Neurosci 30(8):3101-12.

Manning BD, Cantley LC. 2007. AKT/PKB signaling: navigating downstream. Cell 129(7):1261-74.

Sawyers CL. 2008. The cancer biomarker problem. Nature 452(7187):548-52.