Role of X-box Binding Protein 1 in Regulation of Retinal Müller Glia Metabolism

by

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Neuroscience Program
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Abstract

Müller glia play a critical role in maintaining retinal homeostasis through shuttling metabolic substrates to and from retinal neurons, releasing trophic factors, regulating neurotransmission, providing structural support, and regulating the extracellular environment. Thus, studying the regulation of Müller glia metabolism could provide helpful insights for understanding retinal dysfunction and degeneration. In this study, we investigate the role of X-box binding protein 1 (XBP1), a stress-inducible transcription factor, in regulation of retinal Müller glial metabolism. We hypothesize that deletion of XBP1 in Müller glia leads to increases in glycolytic capacity, which could in turn alter retinal metabolism. To test this hypothesis, we generated XBP1 conditional knockout (cKO) mice that lack XBP1 specifically in Müller glia using the Cre/LoxP system. Using a Seahorse extracellular flux metabolic analyzer, we measured mitochondrial respiration and glycolysis in the retina and in isolated Müller cells from XBP1 cKO mice. Our results demonstrate an increased rate of glycolysis in XBP1 cKO retinas and in XBP1-deficient Müller cells. These changes are associated with elevated glucose uptake and upregulation of glucose transporters GLUT1 and GLUT2. Additionally, two regulatory enzymes involved in glycolysis, pyruvate kinase and Hexokinase 1, are also upregulated in XBP1 cKO Müller cells. Taken together, our data suggest that XBP1 plays an important role in regulation of glucose metabolism in retinal Müller glia, which, although consisting of a relatively small population of retinal cells, could consequently influence retinal metabolic profile and function.
Chapter 1: Introduction

1.1 The importance of studying retinal Müller glia

As humans, vision is undoubtedly the sense we depend most upon. Disorders of the visual system are both costly to the healthcare system and devastating to individuals. Current treatment for most retinal degenerative disorders is extremely limited, and for the most part only preventative measures can be taken. Loss of retinal neurons in mammals is irreversible and characterizes most vision loss in retinal degenerative diseases such as diabetic retinopathy, macular degeneration, and retinitis pigmentosa. In the US, diabetic retinopathy is the leading cause of blindness in the working age population.

Müller glia have become a particularly interesting target for looking at the causes and prevention of retinal degenerative disease because of their supportive role in the retina and their ability to dedifferentiate and aid in repair of retinal damage in several species. Additionally, Müller glia dysfunction has been found to precede neuronal death in neurodegenerative disorders. Understanding how the loss of function in Müller glia affects pathology in the retina overall is a highly relevant area of research, because it can lead to treatments for early stage retinal disease prior to the onset of irreversible retinal neuron death and eventual blindness. Müller glia regulation of retinal metabolism is of particular interest in this field because neurotransmission in the retina is tightly coupled to metabolism, and metabolic stress resulting in neuronal injury occurs in most retinal degenerative diseases.
1.2 The role of Müller Glia in Retinal Metabolism

The neural retina is made up of cells that can be classified into two major types: neurons and glial cells and each of these cell types consists of multiple sub-populations. Retinal neurons include bipolar cells, amacrine cells, horizontal cells, ganglion cells, and photoreceptors. Rod and cone photoreceptors hyperpolarize in response to dim and bright light, respectively. Horizontal cells send inhibitory inputs to photoreceptors to help the cells respond to changes in brightness of visual signals. Bipolar cells along with amacrine cells help transmit the photoreceptor signal to retinal ganglion cells, which then exit the retina through the optic disc and form the optic nerve via their axons. Three different glial cell types are present in the retina: astrocytes, microglia, and Müller glia. Müller glia and astrocytes are characterized as macroglia cells because of their ability to support surrounding neurons and maintain the blood-retina barrier (BRB) (Kolb, 1995). Müller glia cell bodies are located in the inner nuclear layer, but they have long processes that extend in both directions and allow them to interact with nearly all the retinal cell populations.

The retina is one of the body’s most energy demanding tissues. Due to the need of neurons to create large ionic concentration gradients to carry out transmission of their signals, generated primarily via the Na+/K+ ATPase, an enormous amount of energy is required for neural tissue to function. In addition, the retina requires an even greater amount of energy relative to the brain, related to the need to maintain the depolarizing dark current. Thus, photoreceptors as well as other retinal neurons require a massive amount of energy to function. In general, retinal neurons rely on mitochondrial oxidative phosphorylation for energy, whereas glial cells rely on glycolysis and have the capacity for oxidative phosphorylation if needed (Winkler et al., 2000). Under metabolically stressful conditions such as low glucose or anoxia, Müller glia can increase their
rate of glycolysis in order to obtain ATP (Toft-Kehler et al., 2017a). Understanding the limit to which Müller glia are capable of adapting to metabolic stress will be important for understanding their clinical significance in preventing retinal degenerative disorders. Because retinal neurons in humans do not regenerate, understanding the mechanisms of metabolic support carried out by Müller glia that can protect against inner retina neuron death is of interest.

Retinal metabolism is highly compartmentalized due to the different activity and metabolic needs of retinal neurons and glial cells (Hurley et al., 2015). For example, retinal ganglion cells (RGCs), which are unmyelinated, require continuous blood and energy supply in order to obtain sufficient ATP for maintaining cell viability and function. This high energy demand is tightly monitored and controlled by Müller glia (Rueda et al., 2016; Toft-Kehler et al., 2017a; Toft-Kehler et al., 2017b). In addition, the anatomical features of the retina also contribute to the metabolic compartmentalization in that the inner and outer retina have very distinct access to blood supply. The inner two-thirds of the neurosensory retina is supplied by the retinal vascular network. The outer one-third of the retina, consisting of primarily retinal photoreceptor cells, is supplied by the choriocapillaries. The outer plexiform layer receives blood supply from both the retinal and choroidal vascular systems. The differences in the blood supply mechanism render the inner retina relatively more hypoxic than the outer retina (Yu and Cringle, 2001). As such, there is a significant need for Müller glia to provide additional metabolic support to the inner retinal neurons.

1.3 The glycolytic pathway in Müller glia and other pathways in retinal energy production

As mentioned above, glycolysis, which is favored by most glial cells, is one of the primary cellular energy pathways used by eukaryotes. The glycolytic pathway consists of ten enzyme catalyzed reactions, which convert glucose to two molecules pyruvate, and generates a net of 2
ATP molecules and one molecule of NADH. In general, glycolysis takes place in the cytoplasm of a cell, where each of the steps needs to be enzyme catalyzed. The glycolytic reactions can be categorized into two classes: reversible and irreversible. Pyruvate kinase (PK), phosphofructokinase (PFK), and hexokinase (HK) catalyze the irreversible reactions. Additionally, three of the enzyme steps are considered to be regulatory, which include step 1 catalyzed by HK, step 2 catalyzed by phosphoglucone isomerase (PGI), and step 7 catalyzed by phosphoglycerate kinase (PGK) (Figure 1-1).

Müller glia rely primarily on aerobic glycolysis to generate ATP and only derive a small amount of ATP from oxidative phosphorylation despite the much higher ATP yield from this process. This is also known as the Warburg effect, or the predominance of glycolysis over oxidative phosphorylation, which allows Müller glia to spare oxygen for surrounding neurons in hypoxic conditions (Winkler et al., 2000; Roesch et al., 2008; Hurley et al., 2015). Compared to other retinal cells, Müller glia are more viable and can survive in acute conditions of low glucose, oxygen deprivation, or mitochondria inhibition by utilizing different energy pathways. In low glucose conditions they rely on oxidative phosphorylation more than glycolysis, while under hypoxic conditions or mitochondrial inhibition they would utilize glycolysis as the principal metabolic pathway (Tien et al., 2017). However, in conditions with combined glucose deprivation and mitochondrial inhibition, Müller glia will be unable to survive due to the lack of an alternative pathway to produce ATP. The potent glycolytic capacity renders Müller glia more resistant to retinal ischemia than neurons; yet sustained and severe ischemia will deplete their stored glycogen, which is required for glycolysis, leading to cell death.

There has been debate on whether Müller glia rely solely on the glycolytic pathway consisting
of the traditional 10 enzyme catalyzed reactions, or also utilize the pentose phosphate pathway (PPP)/ hexose monophosphate shunt (HMPS) pathway, which runs in parallel to glycolysis (Buse et al., 2002; Buse, 2006; Semba et al., 2014). The PPP/HMPS is an alternative glucose oxidation pathway, which converts glucose 6 phosphate ‘shunted’ from the first reaction of glycolysis to ribose-5-phosphate, NADPH, and erythrose-4-phosphate. It is believed that both pathways likely take place in Müller glia, because the cells express genes and enzymes required for these pathways. Furthermore, the PPP generates NADPH through consumption of organic carbon and NADPH is vital for preventing oxidative stress in highly metabolically active retina tissue. Thus, the partial use of the PPP is plausible.

Figure 1-1. Glycolysis, de novo serine synthesis, and hexosamine biosynthesis in Müller glia. The glycolytic pathway is the primary energy pathway utilized by Müller glia. The pathway is characterized by ten enzyme catalyzed reactions. Enzymes are listed in the table corresponding to the numbers above each arrow. In addition to glycolysis, glycolytic intermediates are also shuttled to alternative pathways including the hexosamine biosynthetic pathway and the de novo serine synthesis pathway. Condensed versions of the de novo serine synthesis pathway (DNSSP) and the hexosamine biosynthetic pathway (HBSP) are shown highlighting relevant reactions, products, and enzymes to the UPR and metabolism. The HSP converts fructose-6 phosphate and glutamine to glucosamine-6 phosphate and glutamate via the non-glycolytic enzyme GFAT. The HSP
produces O-GlcNAc, which is used to carry out post translational modifications on cellular proteins. The de novo serine synthesis pathway consists of 3 enzyme-catalyzed steps, through which Müller glia convert 3- phosphoglycerate to serine, which is then provided to retinal neurons. The net reaction of glycolysis yields 2 molecules of ATP and produces pyruvate, which is subsequently used by the Müller glia for the Kreb’s cycle, or shuttled to surrounding neurons. (GFAT=glutamine:fructose-6-phosphate aminotransferase, OGT= O-GlcNAc transferase).

Another important function of Müller glia is to carry out glutamate-glutamine cycling, which is critical for preventing glutamate excitotoxicity in the retina (Bringmann et al., 2009; Vohra et al., 2017). Glutamate released from retinal ganglion cells (RGCs) during neurotransmission is taken up by Müller glia through excitatory amino acid transporters (EAATs) and converted to glutamine through glutamine synthase (GS). Glutamine is then released back to retinal neurons, where it is converted back to glutamate for neuronal signal transduction. In conditions of low glucose, glutamate can be metabolized by glutamate dehydrogenase and enter the tricarboxylic acid (TCA) cycle in Müller glia, which couples neurotransmission activity to metabolism. Similarly, Müller glia can utilize lactate released from retinal neurons to synthesize glutamine; thus the levels of extracellular lactate can be used as a marker of retinal energy homeostasis.

1.4 The implication of the unfolded protein response in retinal metabolism

One of the cellular responses, which can be triggered by metabolic stress and regulates the cellular metabolic pathways is the unfolded protein response (UPR). The UPR is an adaptive response activated by endoplasmic reticulum (ER) stress, a condition of misfolded and unfolded proteins accumulating in the ER. Factors such as environmental stress, increased need for protein synthesis, hypoxia, and inflammation, lead to ER stress. Under homeostatic conditions, ER membrane proteins protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), which function as the ER stress
sensors, are bound to glucose-regulated protein 78 (GRP78) and kept inactive. Upon ER stress, GRP78 dissociates from these ER membrane proteins and binds to unfolded/misfolded proteins. The dissociation activates the ER stress sensors and initiates the UPR.

The UPR branch of interest for this study is the IRE1 branch. When IRE1 is unbound from GRP78 at the ER membrane, it is activated through dimerization and auto-phosphorylation. The activated form of IRE1 is capable of splicing a 26 nucleotide exon from XBP1 mRNA. This process creates a more stabilized form of XBP1, which translocates to the nucleus and acts as an extremely potent transcription factor. Spliced-XBP1 (XBP1s) can then promote expression of several downstream pro-survival targets, including lipogenic genes, gluconeogenic genes, and additional UPR chaperones (Zhang et al., 2015; Pirelli et al., 2016). In addition to its role in the unfolded protein response, XBP1 has also been shown to regulate several metabolic genes, including forkhead box protein O1 (FOXO1), angiopoietin-related protein 3 (ANGPTL3), liver carboxylesterase 1 (CES1), and hexokinase 2 (HK2) (Yoshida et al., 2001; Acosta-Alvear et al., 2007; Zhou et al., 2011). Additionally, XBP1 is linked to regulating two pathways that couple the UPR to metabolism in the retina, the hexosamine biosynthetic pathway (HBSP), and the de novo serine synthesis pathway (DNSSP). The HBSP uses a glycolytic intermediate, fructose-6-phosphate (F6P) and glutamine to generate glucosamine-6-phosphate (Gln-6-P) and glutamate from the enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT). XBP1 has been shown to regulate GFAT expression. The end result of the HBSP is production of UDP-GlcNAc, which is converted by O-GlcNAc transferase (OGT) to β-linked N-acetylglucosamine (O-GlcNAc), which can then carry out post translational O-glycosylation of proteins (Marshall et al., 1991; Semba et al., 2014; Wang et al., 2014). The DNSSP converts 3-phosphoglycerate (3PG), a glycolytic intermediate to serine via three enzyme catalyzed reactions to biosynthetically provide
serine to cells. This pathway is important for Müller glia, which synthesize serine and shuttle it to neurons, which lack expression of the DNSSP enzymes. Disturbances to the DNSSP caused by metabolic or oxidative stress lead to decreased mitochondrial ATP production and upregulation of ER chaperone protein heat shock protein 72 (HSP72) (Zhang et al., 2018).

As it stands, the role of XBP1 in Müller glia metabolism, and on Müller glia regulation of retinal metabolism remains unclear. This goal of this study is to identify how XBP1 deletion alters Müller glia and whole retina metabolism.
Chapter 2: Materials and Methods

2.1 Animals

Müller-specific XBP1 knockout mice were generated by crossing XBP1 flox/flox mice, which contain two loxP sites flanking exon 2 of the XBP1 gene (Kaser et al., 2008), with Müller-specific Cre transgenic mice, which express Cre recombinase in Müller cells (Hetz et al., 2008; Ueki et al., 2009; Wang et al., 2010). XBP1 f/f, Müller Cre+ mice that lack XBP1 in only Müller cells are referred to as Müller-XBP1 cKO. XBP1 f/f, Müller cre- littermates are used as control and referred to as wild type (WT). The care, use, and treatment of all animals were in strict agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology and with the IACUC guidelines set forth by the University at Buffalo.

2.2 Müller cell isolation and cell culture

Müller cells were isolated from retinas of postnatal day 7 WT and Müller-XBP1 cKO mice following a two-day protocol. On day 1, eyes were enucleated and kept overnight in DMEM with 1% penicillin and streptomycin and 2 mM glutamine. On day 2, the eyes were trypsinized for 1 hour at 37º C and retinas were dissected out carefully to avoid removing retinal pigmented epithelium (RPE). Retinas were mechanically dissociated with scissors as much as possible and centrifuged for 5 min at 800 RPM. Supernatant was removed and the pellet was resuspended and pipetted vigorously in culture medium (DMEM/F12 50/50, 10% FBS, 1% P/S and 1% glutamine), and seeded into a 60 mm tissue culture dish. Cells appeared after 4-5 days and were passaged after becoming confluent approximately every 2 days.
2.3 Western blotting

Mouse retinas or cultured cells were lysed using radioimmunoprecipitation (RIPA) buffer with 1% sodium orthovanadate, protease inhibitors, and phenylmethane sulfonyle fluoride (PMSF). The bicinchoninic acid (BCA) assay was used to determine sample protein concentration per manufacturer’s instructions. Samples were diluted with 2X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA) and heated to 65º for 5 minutes. Protein lysates were loaded onto 10% SDS-page gel. Gels were wet transferred onto Biotrace membrane for 3.25 hours at .34 amps. Membranes were then blocked with 3% skim milk prior to primary antibody incubation. Primary antibodies were incubated overnight at 4º C, and secondary antibodies were incubated the following day for 2 hours at room temperature in dilutions of 1:10,000. Specific primary antibody information is included in Table 2-1. Blots were developed using chemiluminescence kit (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific, USA) and visualized under Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA). All results were normalized to β-actin expression on the corresponding membrane.

Table 2-1. Western blot antibody information.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>Rabbit, polyclonal</td>
<td>1:500</td>
<td>Novus</td>
</tr>
<tr>
<td>KDEL (for GRP78)</td>
<td>Mouse, monoclonal</td>
<td>1:1000</td>
<td>Abcam</td>
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<td>ERP29</td>
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<tr>
<td>AMPK</td>
<td>Rabbit, polyclonal</td>
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<td>Cell signaling</td>
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<tr>
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<td>Cell Signalining</td>
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<td>Sigma</td>
</tr>
<tr>
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<td>1:500</td>
<td>Thermo fisher</td>
</tr>
<tr>
<td>HK1</td>
<td>Rabbit, monoclonal</td>
<td>1:1000</td>
<td>Cell signaling</td>
</tr>
</tbody>
</table>
2.4 Immunohistochemistry

Retinal sections: Eyes were enucleated, washed, and fixed in solution of 4% paraformaldehyde in phosphate-buffered saline (PBS) for 45 minutes. Eyes were rinsed in PBS on a shaker plate 3 times and then sunk in 30% sucrose in PBS overnight. The next day, fixed eyes were frozen in optimum cutting temperature compound (OCT) blocks in ethanol cooled on dry ice. Blocks were sectioned into 8 um sections and mounted onto super-frost plus slides. Slides were dried overnight and rehydrated the next day in PBS. Sections were rinsed 3 times with PBS, and ready to be used for primary and secondary incubation.

Cultured Müller glia: Cells were seeded onto coverslips coated with poly-d-lysine (PDL) in 12- well culture plates and grew overnight. Cells were fixed in 4% paraformaldehyde for 20 minutes and rinsed 3 times with PBS. Fixed cells were blocked with solution of 1% triton and 1% N,O-bis(trimethylsilyl)acetamide (BSA) in PBS for 1 hour at room temperature prior to primary incubation. After rinsing, cells were incubated with primary antibodies (Table 2-2) overnight at 4º. Cells were incubated with secondary antibodies at a dilution of 1:800 for 1 hour at room temperature. Images were taken using the Olympus BX53 microscope (Olympus, Tokyo, Japan).

Table 2-2. Immunohistochemistry antibody information.

<table>
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<tr>
<th>Antibody</th>
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<tr>
<td>GS</td>
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<td>Sigma</td>
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<td>Vimentin</td>
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<td>1:800</td>
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<tr>
<td>GFAP</td>
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<td>Dako</td>
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<td>AMPK</td>
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<tr>
<td>PAMPK</td>
<td>Rabbit, polyclonal</td>
<td>1:800</td>
<td>Cell signaling</td>
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</table>
2.5 Lactate and pyruvate concentration assay

L-lactate and pyruvate concentrations in retinal tissue lysates or cell culture medium were measured using L-Lactate Assay Kit (ab65331) and Pyruvate Assay Kit (ab65342) per manufacturer’s instructions. Levels of lactate and pyruvate were normalized to protein concentrations in each sample measured by BCA assay.

2.6 Glycolysis stress test and mitochondria stress test

The seahorse XFe24 extracellular flux analyzer is a relatively new assay that measures changes in metabolism in real time. The machine inserts highly sensitive probes into an assay plate containing wells with sample and culture medium. The probes are capable of detecting small changes in concentration of oxygen and flux of protons into the medium which are used to quantify changes in rates of respiration, without damaging the cell or tissue samples. Specifically, oxygen consumption is used to quantify changes in mitochondrial respiration, and extracellular acidification rate (or proton flux) is used to quantify changes in the rate of glycolysis.

Glycolysis and mitochondria stress tests were carried out on cell and tissue samples using the Seahorse XFe24 Extracellular Flux Analyzer. Retinal tissue explants of 1 mm² were obtained from 6-8 month old mice and plated onto 24 well Seahorse assay plates with ganglion cell layer (GCL) side down. Müller glia were seeded at a density of 20,000 cells per well in a 24 well seahorse assay plate coated with poly-d lysine (PDL) and grew for 2 days prior to the experiment.

Drug injections resulting in final well concentration of 10 mM glucose, 1 μM oligomycin, and 50 mM 2-D-glucose were performed per manufacturer’s instructions. The glycolysis stress test consists of 3 basal measurements, followed by an injection of glucose and three more
measurements, followed by an injection of oligomycin and three measurements, and finally an injection of 2-DG followed by 3 final measurements. An example of the time-course and injection protocol is shown in Figure 2-1. The same procedure was used for the mitochondria stress test, using injections of Oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone/antimycin A respectively, instead.

Figure 2-1. Representative images of Seahorse XFe 24 glycolysis stress test and mitochondria stress test. The image on the left shows an example of the time course and injection strategy used in the glycolysis stress test. The image on the right shows the timing of injections and number of measurements taken in a mitochondrial stress test (www.agilent.com).

2.7 RNA isolation and quantitative real-time PCR

RNA was harvested from cultured Müller cells or retinas of adult WT and cKO mice. Primers specific for target genes were used and are shown in Table 2-3. PCR was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Average threshold cycle (CT) value of fluorescence units was used to analyze mRNA levels. CT values were normalized to 18S and analyzed using the formula: $2^{(\Delta\text{CT of WT} - \Delta\text{CT of cKO})}$, where $(\Delta\text{CT of WT}) = (\text{CT of target gene} - \text{CT of 18S for WT})$, and $(\Delta\text{CT of cKO}) = (\text{CT of target gene} - \text{CT of 18S for cKO})$. 
Table 2-3. qRT-PCR primer information.

<table>
<thead>
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<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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<td>GTAACCCGTTGAACCCCATT</td>
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<td>ENO1</td>
<td>GATGGACGGGCACAGAGAATAAA</td>
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<td>HK1</td>
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<tr>
<td>XBP1</td>
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<td>AGCTGAGTGCAAACGACATA</td>
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</table>

2.8 Glucose uptake assay

Glucose uptake was measured using a fluorescent glucose uptake cell-based assay kit (Cayman Chemical, Ann Arbor, MI, USA), which measures the glucose uptake via glucose transporter 1 (GLUT1), per manufacturer’s instructions. Müller glia were seeded onto PDL coated 96 well plates and grew overnight. Cells were gently rinsed with glucose free medium, and culture medium was replaced with glucose free medium containing 2-NBDG, a fluorescent deoxyglucose analog provided from the assay kit. For control wells, apigenin, a glucose transport blocker was
added to the reaction mixture. Glucose uptake was measured after 4 hours of incubation using a microplate reader capable of detecting fluorescence at excitation and emission wavelengths of 485 nm and 535 nm. Results were normalized to control wells and cell density in each well, which was recorded using fluorescence imaging.

2.9 Statistical analysis

Data analysis was completed in excel. Statistical values are expressed as mean ± SD. Statistical analyses were performed using unpaired Student’s t test for two-group comparison. Statistical significance was accepted as p < 0.05.
Chapter 3: Results

3.1 Confirmation of XBP1 knockout efficiency in retinal Müller Glia-specific conditional XBP1 knockout (XBP1 cKO) mice

Müller glia-specific XBP1 cKO mice were generated by crossing XBP1 flox/flox mice and VMD2-cre mice (Hetz et al., 2008; Ueki et al., 2009). A diagram of XBP1 cKO mouse generation is shown in Figure 3-1. The specificity of cre expression to Müller glia in the VMD2-cre mice was initially demonstrated by Ueki et al. (Ueki et al., 2009) and was further validated by Sri Rao (Fliesler lab, SUNY Buffalo) (Figure 3-2). To confirm XBP1 deletion in Müller glia, expression of cre and XBP1 was measured in isolated Müller glia. As shown in Figure 3-3, cre protein was only expressed in Müller cells derived from Müller-XBP1 cKO mice but not in WT mice. In addition, XBP1 mRNA expression, measured using qRT-PCR, was reduced in XBP1 cKO Müller

![Figure 3-1. Generation of Müller-XBP1 cKO mice.](image)

The floxed XBP1 allele was generated by insertion of lox p sites to flank exon 2 of the XBP1 gene. The floxed XBP1 mice were bred with VMD2-Cre expressing mice resulting in the generation of a Müller cell specific XBP1 cKO mouse (XBP1 f/f, Müller cre+). XBP1 f/f, Müller Cre- mice were used as controls for all experiments.
glia by over 60% (Figure 3-4), suggesting a successful deletion of XBP1 deletion in Müller glia of XBP1 cKO mice.

Figure 3-2. Cre expression is localized to Müller glia. Representative images of retinal sections from a postnatal day 60 (P60) WT mouse or a VMD2 Cre - GFP mouse. Cre expression is visualized by GFP (green) in retinal layers, which confirms robust Cre expression localized to Müller glia (Figure courtesy of Sriganesh Ramachandra Rao, obtained prior to publication with permission from Dr. Steven J. Fliesler).

Figure 3-3: Western blot of Cre expression in isolated Müller cells from XBP1 cKO mice. Western blotting was used to examine Cre-recombinase protein expression in cultured Müller cells derived from XBP1 cKO and WT mice. Cre expression was detected in XBP1 cKO cells but not in WT cells.
3.2 Conditional XBP1 deletion in Müller glia does not affect mitochondrial respiration

Previous experiments have established that metabolic changes in Müller glia impact whole retina metabolism (Skytt et al., 2016; Tchernookova et al., 2018; Zhang et al., 2018). Several models have demonstrated that metabolic stress induced via high glucose conditions, disruption to the de novo serine synthesis pathway, and hypoxia lead to mitochondrial dysfunction in Müller glia and eventual apoptosis, which in turn contributes to loss of other retinal cells (Xin et al., 2013; Tien et al., 2017; Zhang et al., 2018). While XBP1 is known to have a role in regulating metabolism via gluconeogenesis, carbohydrate metabolism, glycolysis, and lipid metabolism, its specific effect on mitochondrial respiration in Müller glia was unclear. Furthermore, many of the downstream targets of XBP1 appear to be conditionally and cell type specifically expressed (Acosta-Alvear et al., 2007). Therefore, it would be important to establish the effect of XBP1 deletion in Müller glia on retinal metabolism.

Using the Seahorse XFe24 Analyzer, we measured both mitochondrial respiration and
glycolysis in real time in retinal explants. Although previous studies support that Müller glia rely minimally on oxidative phosphorylation, the retina overall is very reliant on oxidative phosphorylation. Thus, it would be necessary to evaluate the changes in both metabolic pathways that can be possibly induced by XBP1 knockdown in Müller glia. Using Seahorse XFe24 mitochondrial stress test, we first measured mitochondrial respiration in retinal explants from Müller-XBP1 cKO and WT mice. We found that there is no significant difference in oxygen consumption rate (OCR) of XBP1 cKO and WT retinas, suggesting that the mitochondrial respiration is not affected by XBP1 down-regulation in Müller glia (Figure 3-5). This is consistent with previous findings in which decreases in OCR are only seen when using a mouse model of dysfunctional photoreceptors, due to the fact that they are the primary users of oxidative phosphorylation in the retina, and they greatly outnumber other retinal cells (Kooragayala et al., 2015).

To further confirm that XBP1 deletion in retinal Müller glia does not affect oxidative phosphorylation of the retina, we examined AMPK and p-AMPK protein expression in the retina by western blot analysis and immunohistochemistry. AMPK is an important sensor of cellular energy, regulated by changes in the ratio of cellular AMP/ATP (Gowans and Hardie, 2014). Because most cellular ATP is derived from oxidative phosphorylation in the mitochondria, AMPK is a relatively specific indicator of changes in this energy pathway. Western blotting showed no significant difference in protein expression of AMPK or p-AMPK in retinas from Müller-XBP1 cKO and WT retinas (Figure 3-6). Additionally, immunostaining showed no difference in AMPK expression in each retinal layer between Müller-XBP1 cKO and WT retinas (Figure 3-7).
In retinal degenerative diseases, increased UPR signaling has been linked to mitochondrial dysfunction, and continued mitochondrial dysfunction leads to apoptosis in both neurons and Müller glia (Toft-Kehler et al., 2017b). Furthermore, it suggested that any changes to Müller cell/whole retina metabolism induced by XBP1 deletion were not directly affecting mitochondrial function of retinal cells because the rate of oxidative phosphorylation and the ratio of ATP/AMP remained consistent between the WT and XBP1-cKO retinas.

![Figure 3-5. Mitochondrial respiration in Müller-XBP1 cKO and WT retinal explants. The Seahorse mitochondrial stress test showed no difference in mitochondrial respiration between WT and Müller-XBP1 cKO retina explants. Mitochondrial respiration is measured as oxygen consumption rate (n=3).](image)

![Figure 3-6. Western blot analysis of AMPK and p-AMPK in XBP1 cKO and WT retinas. Western blot for AMPK and pAMPK expression showed no significant difference between groups. Protein expression was normalized to B-actin. Left panel shows a representative image of western blot results. Right panel shows quantification of all results. (n=4).](image)
3.3 XBP1 Deletion in Müller glia increases metabolic capacity in Müller-XBP1 cKO retinas

Decreases in glycolysis in Müller glia has been used as an indicator of cellular or metabolic stress in the retina, such as oxidative stress, mitochondrial dysfunction, or vascular leakage. Next, we examined glycolytic respiration by glycolysis stress test in Müller-XBP1 cKO retina explants in real-time. This test measures glycolysis over the course of two hours during which three injections are used to isolate glycolytic respiration. A basal measurement of glycolysis is taken at the start of the test. Then oligomycin, an inhibitor of mitochondrial respiration (specifically ATP synthase) is injected. Following this, more glucose than would be biologically available is provided to the samples. Finally, 2-DG, a nonmetabolizable form of glucose is injected. Treatment of retinal explants with oligomycin inhibits mitochondrial respiration and causes the tissue to rely solely on glycolysis. Glycolytic rate was measured by highly sensitive probes which can measure proton...
efflux by the tissue into the medium, which increases as the rate of glycolysis increases. The glycolysis stress test showed a subtle increase in glycolytic rate in Müller-XBP1 cKO retinal explants, and a significant increase in metabolic capacity, which compares basal glycolytic respiration to stressed glycolytic respiration (Figure 3-8).

**Figure 3-8. Glycolysis Stress Test on Müller-XBP1 cKO and WT retina explants.**

The glycolysis stress test showed an increase in glycolytic respiration, measured by ECAR in Müller-XBP1 cKO explants compared to controls. This increase in glycolytic respiration was non-significant, and the graph above shows the overall trend seen using results from one glycolysis stress test. The increase in metabolic potential from stressed and baseline ECAR was significant (n=5, p<0.05). The increase is of interest because it suggests that changes to the Müller cell population do impact the whole retina.
In order to better understand the mechanism behind the increase in glycolysis in the retina, qRT-PCR was used to observe expression of glycolytic enzymes in whole retina samples. Based on findings from a paper which found increases in expression of glycolytic genes following selective laser ablation of Müller glia, the initial glycolytic target genes examined were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase A (LDHa), LDHb, enolase 1 (ENO1), and ENO2 (Chung et al., 2013). GAPDH catalyzes the sixth step of glycolysis where glyceraldehyde 3-phosphate is converted to 1,3-bisphosphoglycerate. The enzyme is linked to ER to Golgi vesicle shuttling, making it an interesting target when looking at XBP1 which links ER stress to metabolism. Additionally, in models of diabetic retinopathy GAPDH accumulates in the nuclei of Müller glia (Lind et al., 2013). LDHa and LDHb are two subunits of the LDH enzyme which catalyzes the interconversion of the end products of glycolysis- lactate and NAD+ to pyruvic acid and NADH. Theoretically, an increase in LDH would correspond to an increased rate of glycolytic activity (Winkler et al., 2000). ENO1 and ENO2 catalyze the ninth step of glycolysis where 2-phosphoglycerate (2PG) is converted to Phosphoenolpyruvate (PEP). ENO1 has been studied in depth in cancer research because it is regulated by hypoxia inducible factor 1α (HIF1α), which is a critical regulator of cancer cell metabolism (Zheng et al., 2016). Cancer cell metabolism is somewhat informative for Müller glia metabolism because both cell types exhibit the Warburg effect, the preference to carry out glycolytic respiration in both the presence and absence of oxygen, and both are capable of regulating surrounding blood vessels. XBP1 has been shown to regulate, HIF1α in both normal and hypoxic conditions in breast cancer and 293 cell, with XBP1 knockdown causing HIF1α downregulation (Chen et al., 2014b). qRT-PCR results showed no significant difference in expression of the glycolytic target genes in RNA isolated from whole retina samples (Figure 3-9). Again, because the deletion of XBP1 is specific
to Müller glia, whole retina qRT-PCR results could be difficult to interpret. Because of this, qRT-PCR and seahorse experiments were also carried out on isolated XBP1 cKO and WT Müller glia.

**Figure 3-9. Expression of glycolytic genes in Müller-XBP1 cKO and WT retinas.** Target genes were selected based on results from a study showing that micro-dissection of individual Müller glia resulted in upregulation of GAPDH, ENO1 and ENO2, and LDHa and LDHb. No significant difference was detected between whole retina samples from Müller-XBP1 cKO and WT retinas. (N=4).

**Figure 3-10. Expression of glycolytic regulatory genes in Müller-XBP1 cKO and WT retinas.** PK, PFK, and HK1 catalyze irreversible reactions in glycolysis and have an important role relative to other enzymes in regulating the forward rate of reaction. In Müller-XBP1 cKO retinas PK was significantly upregulated, (n=3, p<0.05).
3.4 XBP1 deletion in Müller glia increases glycolytic capacity of isolated Müller glia

As mentioned, the conditional deletion of XBP1 only occurs in a small population of retinal cells, which has the limitation of producing results that may be hard to detect in samples using the whole retina. Therefore, it would be important to study metabolic changes in isolated Müller glia cells.

First, we confirmed the purity of Müller cell cultures using Müller cell markers glutamine synthase (GS), retinaldehyde-binding protein (CRALBP), glial fibrillary acidic protein (GFAP) and vimentin using IHC staining (Figures 3-11 a-c). Results show greater than 90% of cultured cells were Müller glia. GS, CRALBP, and vimentin are established markers of Müller glia (Tackenberg et al., 2009; Otteson and Phillips, 2010). GFAP is only expressed in vivo in Müller glia in conditions of cellular stress such as retinal diseases (Lupien et al., 2004). However, in cultured conditions Müller glia can express GFAP, possibly due to the lack of interactions with neurons and RPE that occur in vivo (Li et al., 1993; Lewis and Fisher, 2003).

Next, we determined if conditional XBP1 deletion affects cell death and/or proliferation, which could significantly influence cellular metabolism. Cell proliferation was measured using BrdU (figure 3-12) staining and cell death was measured using TUNEL staining (figure 3-13). Results showed no difference in cell death or cell proliferation between Müller-XBP1 cKO and WT Müller glia.
Figure 3-11. Characterization of Müller glia using CRALBP. Cultured Müller glia were stained with IHC antibodies Vimentin, GFAP, GS, and CRALBP (figures 3-11a-c) to ensure culture wasn’t contaminated with other retinal cells. Vimentin, GS, and CRALBP are established markers of Müller glia. GFAP expression in Müller glia should not be present in Müller glia in vivo, but does occur in vitro.
Figure 3-12. Analysis of cell proliferation in XBP1 cKO and WT Müller cells. No difference in cell proliferation was detected between WT and XBP1-cKO Müller cells.

Figure 3-13. Analysis of apoptosis in XBP1 cKO and WT Müller cells by TUNEL assay. The TUNEL assay showed no differences in number of cells undergoing apoptosis. Cells were tested in standard culture conditions (no treatment induced). Results showed <50 cells per 1000 cells undergoing apoptosis. This suggests that XBP1 deletion doesn’t impact viability of Müller glia.

The glycolysis stress test was carried out to determine if XBP1 deletion increases glycolysis in isolated Müller glia. Results showed a significant increase in overall glycolytic
capacity, glycolytic reserve, stressed glycolytic capacity, and metabolic capacity (figure 3-14). Glycolytic capacity is the cells maximal rate of glycolytic respiration, carried out in the presence of more glucose than would ever be biologically available. Glycolytic reserve represents the difference in glycolytic respiration, measured by ECAR after mitochondrial respiration is inhibited via oligomycin until 2-DG is injected which competitively inhibits PGI. This has implications for the cells ability to provide energy in low oxygen conditions or under mitochondrial stress, specifically that XBP1 has an inhibitory role on the cells ability to carry out glycolysis. It’s possible that this result was taking place in the glycolysis stress test on XBP1-cKO retinal explants, but the result was masked by the presence of retinal photoreceptors.

As was done in retinal samples, we examined the expression of GAPDH, LDHa, LDHb, ENO1, and ENO2 in Müller glia isolated from XBP1 cKO and WT mice. Similar to the results in whole retinas, no significant difference in gene expression of GAPDH, LDHa, LDHb, ENO1, and ENO2 was detected. This could be due to the fact that these enzymes catalyze reversible reactions. Following this result, we examined other major glycolytic enzymes, including HK1, PK, and PFK, which catalyze the irreversible reactions in glycolysis (Rueda et al., 2016). These reactions are critical for directing glucose metabolism toward the glycolytic pathway. In addition, PFK catalyzes the rate-limiting step of glycolysis. Notably, these enzymes all take place before the sixth step, which converts glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate and generates NADH and a proton. The release of the proton into the culture medium is what the ECAR specifically quantifies in the seahorse XFe24 analyzer. No significant changes in expression PFK was found between groups. Upregulation of PK and HK1 were seen in Müller-XBP1 cKO cells (figure 3-15). HK1 upregulation was examined further using western blot to see if the change was also reflected in protein expression. The change in HK1 specifically was pursued because several studies have
demonstrated the regulation of HK, and not PK by XBPI (Kaser et al., 2008; van Schadewijk et al., 2012; Liu et al., 2016). Western blot results showed no significant change in expression of HK1 in XBPI cKO and WT Müller glia (Figure 3-16).

**Figure 3-14. Increased glycolytic capacity and metabolic potential in XBPI-deficient Müller cells.** XBPI cKO Müller glia exhibit an increased glycolytic capacity throughout the glycolytic stress test (n=4, p<0.05). Metabolic potential compares stressed glycolytic respiration to basal glycolytic respiration (Stressed ECAR). XBPI deletion increases metabolic potential (n=4, p<0.05). Increased glycolytic respiration increases the proton flux in the cell plate (ECAR). Stressed ECAR is measured after the injection of oligomycin which inhibits mitochondrial respiration and forces the cells to rely on glycolysis (n=4, p<0.05). XBPI-cKO Müller glia exhibit increased stressed ECAR (n=4, p<0.05).
Figure 3-15. Expression of glycolytic regulatory enzymes in XBP1 cKO and WT Müller cells. qRT-PCR analysis of glycolytic regulatory enzymes showed small increases in expression of PK, PFK, and HK1. Only upregulation of HK1 in Müller-XBP1 cKO mRNA was found to be statistically significant (n=4, p<0.05).

3.5 XBP1 Deletion in Müller glia leads to increased expression of GLUT1 and GLUT2

Given the lack of changes in glycolytic enzyme gene expression, we speculate that other mechanisms involving glucose uptake may contribute to the increase in glycolysis. Glucose is the carbon substrate of glycolysis, and because of its larger size and polar uncharged nature, it’s unable to cross the lipid bilayer without a transporter. Two glucose transporters have been previously established to exist in Müller glia, GLUT1 and GLUT2, which are regulated in response to glucose availability and cellular energy demand (Kumagai et al., 1994). Increase in these transporters would likely correspond to an overall increase in glucose in the cell, which would provide more substrate for the glycolytic reactions and increase the production of glycolytic products. Both western blotting and qRT-PCR confirmed that GLUT1 and GLUT2 are significantly up-regulated in Müller-XBP1 cKO Müller glia compared to WT (Figure 3-16) (n=5, p<0.05).

To understand the consequences of increased expression of glucose transporters in Müller glia, glucose uptake was also measured in the cells. Glucose uptake was measured using an assay...
that measures glucose uptake via GLUT1. The assay provides fluorescent data, which can be quantified via a micro plate reader and confirmed via fluorescent microscopy to check cell morphology during the assay. The glucose uptake assay showed a significant increase in glucose uptake in Müller-XBP1 cKO Müller glia compared to WT (Figure 3-17) (n=3, p<0.05).

One limitation with measuring glucose uptake via GLUT1 is that it is difficult to confirm that the increased glucose is actually being shuttled to the glycolytic pathway. However, one of the possible ways to support this theory is by looking at the levels of the glycolytic products in the samples. Oxidation of glucose in glycolysis yields two molecules of pyruvate which is also interconverted to lactate. To examine changes in the amount of lactate and pyruvate being produced by the cells, an assay measuring lactate and pyruvate in the cell media was used.

Lactate and pyruvate concentrations were of interest because Müller glia regularly shuttle these metabolites to surrounding neurons, and because pyruvate is the resultant product of aerobic glycolysis, and lactate of anaerobic glycolysis. Additionally, increases of pyruvate in the retina can have a protective effect against ROS by inhibiting toxic oxidative modifications to proteins and sulphydryls. Oxidative modifications, particularly to enzymatic proteins can damage the catalytic site and cause inactivity (Hegde et al., 2010). No change was observed in the concentration of lactate in culture medium, which could be in part because the cells are primarily relying on aerobic glycolysis under standard culture conditions. The concentration of pyruvate in the conditional knockout culture medium was significantly decreased relative to the control culture medium. Because lactate levels were relatively similar, but pyruvate concentration was significantly decreased in the XBPI deficient Müller glia, the ratio of lactate/pyruvate was therefore increased in the cKO cells (Figure 3-18).
Figure 3-16. Expression of GLUT1, GLUT2, and HK1 in XBP1 cKO and WT Müller cells. qRT-PCR results of GLUT1 and GLUT2 mRNA expression in XBP1-cKO Müller glia relative to control, n=6, p<0.05). Western blot of GLUT1, GLUT2, and HK1 with corresponding quantification of protein expression relative to β-actin (n=5, p<0.05 for GLUT1 and GLUT2).

Figure 3-17. GLUT1-mediated glucose uptake is increased in XBP1 cKO and WT Müller cells. Glucose uptake activity of the GLUT1 transporter was measured via a probe specific to the transporter. A significant increase in glucose uptake was found in Müller-XBP1 cKO cells after 4 hours. Results were analyzed relative to control wells which had glucose uptake blocked via apigenin. (n=6, P=0.02).
Figure 3-18. Analysis of lactate and pyruvate production from XBP1 cKO and WT Müller cells. No change was detected in the amount of l-lactate in cell culture medium between groups. There was a significant decrease in the amount of pyruvate in cell culture medium for Müller-XBP1 cKO Müller glia (n=6, p<0.05).
Discussion

In the present study, we examined the impact of XBP1 deletion in Müller glia on retinal metabolism using Müller glia-specific XBP1 cKO mice. Our findings suggest that conditional deletion of XBP1 in Müller glia leads to a significant increase in glycolysis in isolated Müller cells and an increase in retinal glycolysis, without altering mitochondrial respiration. The increase in glycolytic respiration is concomitant with alterations in the expression of genes involved in regulating the glycolytic pathway. Furthermore, XBP1 deletion increases the expression of GLUT1 and GLUT2 and enhances glucose uptake in Müller glia. The fact that the changes in Müller glia, which only constitute about 16% of mouse retinal cells, impact the metabolism of the whole retina emphasizes the importance of Müller glia in maintaining retinal homeostasis, as well as the potency of XBP1 as a transcription factor in regulation of metabolic signaling cascade (Jeon et al., 1998).

One major finding from our study is that ablation of XBP1 results in an increase in glycolytic respiration and metabolic capacity in retina Müller glia. While XBP1 is known as a main UPR effector that regulates the transcription of genes involved in the ER protein folding and degradation, studies over the past decade have identified a new function of XBP1 in regulation of glucose and lipid metabolism and have implicated XBP1 in metabolic disorders (Sha et al., 2011). An early study by Lee and associates reports that deletion of XBP1 in mouse liver results in profound hypocholesterolemia and hypotriglyceridemia, suggesting a critical role of XBP1 in lipid production (Lee et al., 2008). Interestingly, the regulation of lipid metabolism by XBP1 appears to be independent of ER stress and does not require the UPR. In addition to lipogenesis, XBP1 has been shown to regulate glucose production and release, glucose metabolism, and insulin resistance.
For example, XBP1 regulates FOXO1 in hepatocytes, adipocytes, and pancreatic cells. FOXO1 upregulates gluconeogenesis in the liver by increasing the transcription of two gluconeogenic enzymes, glucose-6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK). In liver cells, XBP1 binds to FOXO1, and targets this protein for proteasome degradation, consequently suppressing glucose production (Zhou et al., 2011; Piperi et al., 2016).

The role of XBP1 in glucose metabolism appears to be controversial. In glioma cells, XBP1 deletion inhibits glycolysis through downregulation of HK2, an isoform of hexokinase, which is the enzyme that phosphorylates glucose to glucose-6 phosphate in the first step of glycolysis (Liu et al., 2016). In contrast, a recent study shows that the UPR activation induced via tunicamycin (TM) on human neuroblastoma cell line SK-N-SH significantly reduced mitochondrial respiration and glycolysis, which was measured using the Seahorse XF96 analyzer. Furthermore, they found that specific inhibition of the IRE1 branch of the UPR via 4u8C during ER stress restored mitochondrial respiration and glycolysis to non-stressed levels. This study provides evidence for the role of IRE1 activation in reducing glucose metabolism both through mitochondrial respiration and glycolysis (van der Harg et al., 2017). Because XBP1 is one of the most potent downstream targets of activated IRE1, it is logical to speculate that inhibitory effect of IRE activation could be at least in part through XBP1, which was unfortunately unexplored in their study. In line with these findings, we demonstrate that XBP1 deletion increases glycolytic respiration and glycolytic capacity in retinal Müller glia, suggesting that XBP1 may play an inhibitory role in regulation of glycolysis. Notably, we performed the glycolytic measurements under homeostatic conditions. It is well established that hypoxia, oxidative stress, and high glucose conditions impair glucose metabolism in Müller glia, which could drastically influence glycolysis and oxidative phosphorylation (Xi et al., 2005; Krugel et al., 2011; Tien et al., 2017). How XBP1 deletion
impacts glycolysis under these disease relevant stress conditions is yet to be investigated.

Our study also suggests that XBP1 deletion does not affect mitochondrial respiration in resting retinal Müller glia. Relative to glucose metabolism, there are few reports published to date that investigate the role of XBP1 in regulation of mitochondrial function. Previously, we demonstrated that deletion of XBP1 in retinal pigment epithelium results in increased mitochondrial reactive oxygen species (ROS) production and apoptosis (Zhong et al., 2012; Chen et al., 2014a). However, how XBP1 deletion or overexpression affects mitochondrial function and energy production remains elusive. While in the present study, we did not observe any difference in mitochondrial respiration between the XBP1-deficient Müller cells and WT controls, we can’t exclude the possibility that XBP1 plays a more prominent role in maintaining mitochondrial function under stress conditions. It is also possible that the effect of XBP1 on regulation of mitochondrial function is cell type specific. Therefore, it would be worthwhile to determine how XBP1 deletion effects mitochondrial respiration in Müller glia under conditions of high glucose and oxidative stress, which are high relevant to retinal disorders including diabetic retinopathy and retinal degeneration.

Another main finding from our study is that XBP1 deletion increases glucose uptake in Müller glia via GLUT1. To our knowledge, there is no published study demonstrating a role of XBP1 in regulation of glucose transporters or glucose uptake. GLUT1 is a glucose transporter highly expressed in cells involved in barrier functions such as RPE and Müller glia, which maintain the blood retina barrier (Kumagai et al., 1994). GLUT1 is the major glucose transporter expressed in Müller glia. GLUT2 monitors glucose uptake and metabolism in cell types, including Müller glia by acting as a glucose sensor, and helps Müller glia maintain glucose homeostasis (Watanabe et al., 1994). In the present study, we found that deletion of XBP1 significantly increases the
transcription of GLUT1 and GLUT2 genes in Müller glia. The changes were further confirmed at the protein level. The exact mechanisms by which XBP1 regulates GLUT1 and GLUT2 remain unclear. We speculate that XBP1 could upregulate GLUT1 and GLUT2 expression through activation of hypoxia inducible factor 1α (HIF1α), a transcription factor induced by hypoxia and many other stress conditions. The regulation of GLUT1 and GLUT2 by HIF1α is well established across cell types and species, wherein increased HIF1α expression typically results in increases in GLUT1 and GLUT2 expression (Chen et al., 2001; Hayashi et al., 2004; Wan et al., 2017). In breast cancer cell lines, XBP1 deletion decreases HIF1α expression (Chen et al., 2014b), suggesting a critical role of XBP1 in HIF1α activation. Additionally, previous research has shown that HIF1α regulates genes that promote vascular permeability in hypoxic Müller glia (Xin et al., 2013). Therefore, potential pathways mediated by HIF1α, through which XBP1 regulates GLUT1 expression and glucose uptake in Müller glia, warrant further investigation in Müller cells.

The increase in glycolytic respiration, without changes in cell proliferation and viability in XBP1 cKO Müller glia in this study, is interesting and is somewhat different from the results seen in other models investigating the effect of XBP1 knockdown on metabolism. Most studies suggest that XBP1 deletion decreases metabolic capacity and consequently reduces cell viability and cell proliferation. In glioma cells, XBP1 knockdown caused a decrease in glycolysis and a reduction in expression of HK2 (Liu et al., 2016). In breast cancer tumor cells, XBP1 knockdown decreases the expression of HIF1α, which in turn causes downregulation of GLUT1, PDK1, and VEGFA, leading to reduced cell viability. Results of our study show that XBP1 knockdown in non-stimulated Müller cells increases glycolytic respiration along with enhanced GLUT1 and GLUT2 gene expression, protein expression, and activity. However, cell viability and proliferation appear to be unaffected. Several possibilities could account for this discrepancy. First, cell survival and
proliferation are controlled at multiple levels and a moderate increase in glucose uptake and glycolysis may not be sufficient to impact cell viability and growth. Second, XBP1 has a large number of downstream target genes, many of which are involved in regulation of cell survival, cell proliferation and differentiation. Therefore, the specific regulation of gene expression by XBP1 in Müller glia should be investigated by transcriptome analysis in future studies.

Our data from the qRT-PCR experiments show that there is no significant difference in the expression of glycolytic enzymes, with the exception of hexokinase, in XBP1 cKO Müller glia. The upregulation of hexokinase was pursued with western blot and results showed similar protein expression of HK between WT and XBP1-cKO Müller glia. These results are somewhat surprising, because we have observed an increase in glycolysis in both isolated Müller cells and in the retina of XBP1 cKO mice. While the exact mechanism remains elusive, we suspect that this contradictory finding may be associated with the nature of the reactions catalyzed by these enzymes being mostly reversible and the role of the cytosolic glycolytic enzymes in other reaction pathways. For example, many glycolytic enzymes are also used in the pentose phosphate pathway and gluconeogenesis. Although the enzymes are expressed at similar levels in normal Müller-XBP1 cKO and WT retinas, they may be utilized to different extent for breakdown of glucose in glycolysis or in other energy pathways. Future studies are needed to test the functionalities of these enzymes in each specific metabolic pathway in Müller glia.

The use of Seahorse XFe24 analyzer in our study was very advantageous because it allowed us to examine Müller cell metabolism in vivo and in vitro. The seahorse instrument is ideal for looking at metabolism in tissue and cells because the conditions in the assay are gentle enough as to not impair cell and tissue function, and highly sensitive to any changes in assay medium, making
reported changes in ECAR and OCR very reliable. A previous study examined retinal photoreceptor metabolism using Seahorse analyzer (Perron et al., 2013). They found that cone photoreceptor cells (661W) exposed to oxidative stress suffer loss of mitochondrial reserve capacity and shift from oxidative phosphorylation to aerobic glycolysis. Moreover, the cells lose the ability to manage mitochondrial produced reactive oxygen species (ROS) due to mitochondrial membrane fragmentation and heterogeneity, and eventually undergo apoptosis (Perron et al.). Using a mouse model of peroxisome proliferator-activated receptor alpha (PPARα) knockdown, another study measured retinal metabolism using the Seahorse analyzer and demonstrated that lipids are the primary substrate for oxidative phosphorylation in the retina, as opposed to glucose which is used for aerobic glycolysis, and that loss of PPARα impairs retinal lipid metabolism and mitochondrial function (Perron et al., 2013; Pearsall et al., 2017). In another study, the authors looked at retinal Müller cell function in high glucose conditions using the Seahorse XF 24 analyzer and found that high glucose causes mitochondrial fragmentation and mitochondrial membrane damage in Müller cells, which leads to release of cytochrome C and eventual apoptosis (Tien et al., 2017). These results indicate that the mitochondria in Müller glia are sensitive to high glucose conditions. Future studies are planned to investigate how XBP1 deletion affects mitochondrial function in Müller glia with exposure to high glucose.

**Study limitations and future directions**

The metabolic pathways in Müller glia are extremely complex. This is one of the major challenges of this study. Müller glia have a well-known function in shuttling energy substrates to surrounding neurons. It is also difficult to determine the specific energy substrate or product such as glucose or pyruvate to be used directly by a metabolic pathway. For example, we observed an
increase in glucose availability in XBP1-cKO Müller cells. However, in addition to glycolysis, glucose is also a reactant in oxidative phosphorylation and a precursor to the reactants in hexosamine biosynthesis and serine synthesis. Thus, it would be worthwhile to examine the levels of glycolytic intermediates in the XBP1 cKO Müller glia in future studies.

Another limitation lies in the measurement of pyruvate and lactate levels. The decrease in lactate and pyruvate in XBP1 cKO Müller glia is surprising due to the increased rate of glycolysis, which would theoretically produce the products of lactate and pyruvate. However, Müller glia are known to shuttle lactate and pyruvate to the surrounding neurons. It is possible that the substrate shuttling is reduced in cultured cells, since the cells lack the glia-neuron interactions that signal the need to shuttle energy substrates out of a cell.

Our current research focuses largely on the direct relationship between XBP1 deletion and metabolic consequences in Müller glia and how the metabolic changes in Müller glia would affect the metabolism of the whole retina. In the future it would be worthwhile to examine if increased glycolytic capacity had a positive effect on visual function and retinal neuron viability, in particular during pathologic conditions related to human diseases. Moving forward it would be interesting to investigate the effects of XBP1 deletion in Müller glia on retinal neurodegeneration and function over an extended time period or in a diseased model, such as diabetic retinopathy. In addition, the critical role of Müller glia in supporting surrounding neurons would also be an important future target. Co-culturing Müller glia with retinal ganglion cells and photoreceptors would be useful to investigate how XBP1 deletion in Müller glia directly affects neuronal cell survival and function 

*in vitro*. In particular, it would be interesting to re-measure lactate and pyruvate medium concentration from these cultures, and potentially look at lactate and pyruvate transporter
expression in Müller glia and neurons and Müller glia and RPE. It would additionally be interesting to see how XBP1 deletion in Müller glia impacts the shuttling of biosynthesized serine to retinal neurons, because serine biosynthesis runs in tandem with glycolysis.

Finally, it would be interesting to further explore how XBP1 specifically regulates GLUT1 and GLUT2. Future experiments would be worthwhile to overexpress XBP1 in Müller glia and examine changes in GLUT1 and GLUT2 expression and glycolytic respiration. It would be also be worthwhile to look at how XBP1 deletion and overexpression in Müller glia impacts HIF1α expression and whether HIF1α mediates the effects of XBP1 on regulation of Müller glia glucose uptake, glucose metabolism, cell survival and function under stress conditions.
References


