UCP2-DEPENDENT CHANGES IN MITOCHONDRIAL DYNAMICS
PROTECT THE RETINA FROM GLAUCOMA

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ABSTRACT

Glaucoma is a neurodegenerative disorder characterized by mitochondrial dysfunction and an increase in oxidative damage, leading to progressive retinal ganglion cell (RGC) degeneration. The oxidative status of RGCs is regulated intrinsically and also extrinsically by retinal glia. The mitochondrial uncoupling protein 2 (UCP2) relieves oxidative and neuronal damage in a variety of neurodegenerative disease models. However, the impact of Ucp2 on cell survival during sub-acute and chronic neurodegenerative conditions is not yet clear. Herein, we test the hypothesis that increased Ucp2 expression will improve retinal ganglion cell survival in a mouse model of glaucoma. We show that increasing retinal ganglion cell but not glial Ucp2 expression in transgenic animals decreases glaucomatous RGC death, but also that the PPAR-γ agonist rosiglitazone, an endogenous transcriptional activator of Ucp2, does not significantly alter RGC loss during glaucoma, suggesting differences in regulation of the protective transgene and the endogenous gene. We additionally hypothesized that deletion of Ucp2 in either RGCs or retinal glia would increase retinal damage and retinal ganglion cell death in a mouse model of glaucoma. Paradoxically, we found the reverse, and deletion of mitochondrial UCP2 decreased oxidative protein modification and reduced retinal ganglion cell death in male and female mice. This paradox was resolved after finding that Ucp2 deletion also increased levels of mitophagy in cell culture and retinal tissue. Together, our data show that both Ucp2 deletion and elevated Ucp2 expression facilitate increased mitochondrial function, either by reducing the generation of reactive oxygen species or by improving quality control, though transcriptional activation alone is insufficient to elicit this neuroprotective effect, motivating further research into the post-transcriptional regulation of Ucp2. These data support a model whereby certain forms of increased Ucp2 expression mediate neuroprotection during long-term oxidative stress, and may provide a therapeutic avenue for other chronic neurodegenerative conditions.
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Chapter 1. Introduction

I.I. Introduction to the Eye

The eye is divided into anterior and posterior chambers. The anterior chamber includes the cornea, iris, canal of schlemm, ciliary body, and lens (above the yellow line on Fig. 1). The cornea is a transparent mucus membrane that refracts light on the lens, which further refracts and focuses light on to the retina in the posterior segment. The iris is a pigmented tissue responsible for eye color, and can constrict or dilate according to light conditions so that the exquisitely light-sensitive retina in the posterior chamber is not flooded with excess light. The anterior chamber is bathed in aqueous humor, an optically clear fluid that provides nutritional support. Aqueous humor is produced by the ciliary body and drains from the eye through a spongy tissue called trabecular meshwork before entering the canal of

Figure 1.1. Anatomy of the Human Eye. The yellow dotted line indicates the boundary between the anterior and posterior chambers. Adapted Webvision - Gross anatomy of the human eye, by Helena Kolb (http://webvision.med.utah.edu/book/part-i-foundations/gross-anatomy-of-the-eye/)
schlemm, which returns aqueous humor to venous circulation. The trabecular meshwork and canal of schlemm are at the junction between the iris and the cornea. Just as the vascular system controls blood pressure by providing resistance against blood flow, trabecular meshwork provides resistance against aqueous humor drainage, and supports a homeostatic intra-ocular pressure (IOP) in the anterior segment. In humans this pressure is approximately 13-20 mmHg, but an imbalance between production and drainage of aqueous humor can elevate IOP and increases risk for glaucoma (Leske et al., 2003).

The posterior chamber (Fig. 1.1, below the yellow line) contains the vitreous body, the retina, pigment epithelium, choroid, sclera, and optic nerve. The retina senses light that passes through the cornea and lens and transmits this information to the brain through the optic nerve. The choroid lies posterior to the retina and provides vascular support from the ophthalmic artery, which ultimately supplies the inner retina. The sclera lies on the most lateral surface of the eye and covers roughly 80% of the eye’s surface. It provides structural support, and at its most anterior portion is continuous with the cornea. At its most posterior, the sclera surrounds the optic nerve. The optic nerve exits the eye and transmits visual signals to the rest of the central nervous system. It is composed of axons from retinal ganglion cells (RGCs), which at their anterior pole are surrounded by the lamina cribrosa, a connective tissue contiguous with the sclera that contains so called lamina cribrosa cells and optic nerve astrocytes. At the anterior pole of the optic nerve, these are referred to as optic nerve head astrocytes. In all other regions, optic nerve axons are ensheathed by oligodendrocytes, which mylinate most the optic nerve over most of its length. Changes in IOP within the anterior chamber have profound biomechanical effects on retinal ganglion cell axons and cells of the optic nerve head that may lead to the development of glaucoma.

I.I.1 Retinal Anatomy

The retina lies within the posterior chamber, and contains the neural machinery for phototransduction that is arranged in several well-defined layers, easily discernable in histological sections of both mice and humans (Fig. 1.2). The outer-most portion of neural retina tissue contains photoreceptors, the light-transducing cells that span most of the outer half of the neural retina. Photoreceptors constantly shed membranous disks for disposal by
retinal pigment epithelial (RPE) cells in the anatomically distinct area of the same name, which are non-neural cells stationed at the outer-most portion of the retina. From this region inwards, the retinal layers contain photoreceptor outer segments (OS), which contain the intact membranous disks used to sense light, inner segments (IS), which contain many of the photoreceptor mitochondria, and the outer nuclear layer (ONL), containing highly condensed photoreceptor nuclei. Photoreceptors constitutively release the neurotransmitter glutamate on to bipolar cell processes in the outer plexiform layer (OPL), a signal that is modulated by horizontal cells and amacrine cells. Nuclei of these cells lie in the inner nuclear layer (INL), and bipolar cells or amacrine cells interact with retinal ganglion cell (RGC) dendrites in the inner plexiform layer (IPL). RGC soma and nuclei are located in the ganglion cell layer (GCL), and axonal projections from the GCL that join up to form the optic nerve initially pass through the retinal nerve fiber layer (RNFL). The RPE to the OPL is considered the

Figure 1.2. Layers and Structure of the C57BL6/J Mouse Retina. The outer retina is the dorsal-most portion, and contains the non-neural retinal pigment epithelium (RPE), photoreceptor inner- and outer segments (IS and OS), the outer nuclear layer (ONL) and the outer plexiform layer (OPL). The inner retina contains the inner nuclear (INL), inner plexiform (IPL), ganglion cell (GCL), and retinal nerve fiber layers (RNFL).
outer retina, and the INL to the RNFL is considered inner retina.

Glial cells also span these layers, particularly Müller glia, which extend through the thickness of the retina (Reichenbach, 1989b). Their somata are localized within the inner nuclear layer, and possess major processes extending to the inner and outer layers of the retina, forming barriers known as the inner limiting membrane (bounded by the vitreal surface of the retina near the RNFL) and outer limiting membrane (bounded by the subretinal space). Astrocytes are also present in the retina in the RNFL/GCL, and are embryologically derived from optic nerve astrocyte populations (Watanabe and Raff, 1988). In humans they are a component of the lamina cribrosa, though in rodents, this area is mainly referred to as a ‘glial lamina’. Müller cells and astrocytes surround multiple retinal cell types and compartments in order to perform several supportive functions including but not limited to neurotransmitter uptake (Rauen et al., 1998), extracellular K\(^+\) buffering (Oakley et al., 1992), and the maintenance of neuronal energy supply (Poitry-Yamate et al., 1995; Tsacopoulos et al., 1998).

I.II. Introduction to Glaucoma

Glaucoma is a group of disorders, perhaps better categorized as a syndrome, which are marked by progressive visual decline due to retinal ganglion cell (RGC) dysfunction and death (Weinreb et al., 2014). There are several forms of the disorder that differ with respect to regional and ethnic prevalence (Miller, 1952; Leske et al., 2003; Tham et al., 2014). Combined across the world, there are approximately 64.3 million cases of glaucoma in individuals between 40-80 years of age, (3.54% of that age group), and by 2040 this number is expected to sharply increase, rising upwards of 111.8 million cases (Tham et al., 2014). The economic burden of glaucoma and disorders affecting the optic nerve is $5.8 billion per year in the United States (Wittenborn et al., 2013). Per individual with low vision or blindness, this amounts to a cost of $6,680 per year (Wittenborn et al., 2013), an estimate that doesn’t include a financial correlate for the loss in well being (approximated at $50,000/person/year and increasing with disease severity) (Wittenborn et al., 2013). With the growing population of glaucoma patients, these economic and social costs will also increase, making the proper diagnosis and treatment of glaucoma central for the future.
I.I.I. Risk Factors

A variety of risk factors are associated with glaucoma, but the most potent of these are age and intraocular pressure (IOP). IOP in the anterior chamber of the eye is normally (Mean ± SD) 13.3 ± 2.3 mmHg (De Moraes et al., 2013) but can easily reach ≥21 mmHg or greater without any symptomology. Patients with an IOP greater than 21 mmHg are considered to be at risk for glaucoma. Differences in IOP as small as 1 mmHg correlate with the chance of clinical progression (Leske et al., 2003), making the accurate estimation of IOP by clinical tools critical. Notably, accurate IOP estimation is difficult, as observer bias and differences between ‘gold-standard’ methods can account for inter-patient differences between 0.6-6 mmHg (Tonnu et al., 2005; Chihara, 2008). The risk of glaucoma also increases linearly with age (Leske et al., 2003; Friedman et al., 2004), though to an extent this may be a reflection of the duration of time a person has been exposed to other known or unknown risk factors (Boland and Quigley, 2007), including but not limited to IOP, and possibly reaching as far as microbiota composition (Astafurov et al., 2014; Chen et al., 2018). Ethnicity also plays an important role in glaucoma susceptibility and in the contraction of specific glaucoma subtypes. Individuals of African descent appear to be more susceptible to glaucoma than same-aged Caucasian or Hispanic populations (Friedman et al., 2004).

I.I.II Diagnostic Criteria

A diagnosis of glaucoma is the result of converging clinical evidence. These forms of evidence include changes in IOP or retinal morphology over multiple ophthalmic examinations (Prum et al., 2016). IOP is measured by tonometry, which uses puff of air or small probes to approximate IOP based on the resistance of the eye to deformation by an external force. Unfortunately, the extent and duration of IOP increases can differ greatly between glaucoma subtypes, within the same patients at different times of day (Asrani et al., 2000), and with different examiners (Tonnu et al., 2005), making diagnosis of a specific subtype highly difficult using IOP alone (De Moraes et al., 2013). In fact, IOP alone is not predictive of glaucoma. A significant proportion of asymptomatic patients may have an IOP of >21 mmHg, while others possess retinas or optic nerves damaged by glaucoma but present
with normal IOP levels (Anderson, 2011). Therefore the gold-standard detection methods have shifted away from IOP measurement and towards a multifactorial assessment of retinal and optic nerve structure and visual function over time (Prum et al., 2016). Macroscopic retinal structure can be seen during a fundoscopic examination, and spectral- and time-domain optical coherence tomography (SD-OCT/TD-OCT) can provide a more detailed and quantitative view of retinal and optic nerve structure, both used to diagnose glaucoma (Prum et al., 2016; Morejon et al., 2019). Using fundoscopic examination of the retina or SD-OCT/TD-OCT, one could qualitatively diagnose optic nerve damage from axon loss, which results in a deeper impression at the optic nerve head ("cupping"), or from direct measurement of optic nerve head and retinal nerve fiber layer thickness (Prum et al., 2016). Finally, visual field loss is the output of glaucoma and can be measured using perimetry (Prum et al., 2016). Well-correlated changes in these diagnostic criteria are the strongest indicators of glaucoma.

I.II.III. Glaucoma Subtypes and Genetic Associations

Glaucoma can be divided into roughly two types with different etiologies. The first and most common of these is primary open-angle glaucoma (POAG). Typical POAG is characterized by a chronic increase in IOP to >21 mmHg, which causes a progressive decrease in visual function and retinal thickness but is difficult to detect in its early stages (Morejon et al., 2019). As a consequence of the relatively slow and painless visual decline, estimated at -2.1 decibel/year, or a 7.3% loss in visual field per year (Nassiri et al., 2013; Sun et al., 2017b), POAG is often referred to as the "silent thief of sight", and is undiagnosed in an estimated 50-90% of affected individuals (Nayak et al., 2011). POAG is most frequently found in Africa, where it affects over 7.03 million individuals (4.2% of the population), but is also highly prevalent in South and Central America, with over 5.01 million cases (3.65% of the population), and in North America, with over 2.97 million cases (3.29% of the population). As of 2013, 44.1 million individuals were affected by POAG overall (Tham et al., 2014).

POAG patients can be subdivided based on unique features of the disease, such as juvenile open-angle glaucoma (JOAG), in which visual decline usually begins before 40
years of age and sometimes associated with polymorphisms in the *myocilin* gene (*MYOC*) (Wiggs and Pasquale, 2017). A different subgroup (33-50%) of patients experience progressive visual field loss despite having a ‘normal’ IOP (Anderson, 2011). This form is referred to as *normal tension glaucoma* (NTG), and it is unclear whether this form of glaucoma is mechanistically distinct from POAG. NTG is more prevalent in Asian countries such as Japan, South Korea, and China than in Europe and the Americas (Tham et al., 2014). The genetic basis for POAG and NTG are complex and partially overlapping. There are at least 29 genomic loci significantly associated with POAG (Wiggs and Pasquale, 2017; MacGregor et al., 2018). An Ingenuity analysis of biological pathways associated with these risk alleles does not inform a single model of disease pathogenesis, as the genes within risk loci have diverse biological functions ranging from lipid metabolism to cell division and migration (Wiggs and Pasquale, 2017).

Several genes associated with glaucoma play a critical role as modulators of mitochondrial function by regulating disposal of damaged mitochondria. For example, one of the risk factor alleles for glaucoma is on the thioredoxin reductase 2 gene (*TXNRD2*), which encodes a protein that regulates mitochondrial redox homeostasis (Bailey et al., 2016). A separate risk factor associated with POAG and NTG lies in the optineurin gene (*OPTN*) (Rezaie et al., 2002), which encodes a protein that localizes to damaged mitochondria and signals for their degradation (Wong and Holzbaur, 2014). Copy number variations in the *OPTN*-interacting tank-binding protein (*TBK*) gene also increase the risk of NTG (Davis et al., 2011; Fingert et al., 2011; Sirohi et al., 2015). POAG is also characterized by an increase in mitochondrial DNA (mtDNA) mutations in mitochondrial complex I genes (Sundaresan et al., 2015) and mtDNA transversions (Abu-Amero et al., 2006). Similar genetic abnormalities do not seem to occur in the nuclear DNA of POAG patients (Abu-Amero et al., 2009) and together these published data strongly suggest the presence of mitochondria-specific abnormalities in POAG, possibly related to a failure of cells to dispose of damaged mitochondria in some variants of the disease (Ito and Di Polo, 2017). The involvement of mitochondria in glaucomatous optic neuropathy is not particularly surprising, given that several forms of inherited mitochondrial disease manifest with degeneration of the optic nerve, including but not limited to dominant optic atrophy (DOA) and Leber’s hereditary
optic neuropathy (LHON), resulting from mutations in the mitochondrial fission-inducing GTPase *OPA1* (Yu-Wai-Man et al., 2011), or in several sites of mitochondrial DNA-encoded NADH:Ubiquinone Oxidoreductase (Complex I of the electron transport chain) genes (Yu-Wai-Man et al., 2002). Together these studies suggest an important connection between mitochondrial genetics as both affectors and effectors of optic nerve degeneration in both POAG and other degenerative disorders.

The second major form of glaucoma is *Primary angle-closure glaucoma* (PACG), which differs from POAG in both symptomology and progression. In PACG, the efflux of aqueous humor from the anterior chamber is impaired by a physical obstruction, normally the iris (Fig. 1.3), which occludes the irido-corneal angle, preventing aqueous humor outflow (Sun et al., 2017b). Due to the more dramatic nature of this obstruction in comparison to

![Diagram of the anterior chamber of the eye](https://nei.nih.gov/health/glaucoma/glaucoma_facts)

**Figure 1.3.** Schema of the anterior chamber of the eye, showing the flow of aqueous humor (green arrow), the dynamics of which are disrupted in many forms of glaucoma, either by occlusion of the irido-corneal angle or an increase in the resistance of trabecular meshwork to the outflow of aqueous humor. Image of from NEI facts about glaucoma page: https://nei.nih.gov/health/glaucoma/glaucoma_facts
POAG, IOP increases in PACG can reach >70 mmHg (Sun et al., 2017b). Despite the high magnitude of increase in IOP, the threshold value for PACG diagnosis is still >21 mmHg. PACG may also present with attacks of ocular or peri-ocular pain, nausea, and vomiting, making it more easily distinguishable from POAG (Sun et al., 2017b). This form is less prevalent in Africa and the Americas, but affects a greater proportion of the Asian population, with over 15.47 million cases in the 40-80 years old age group (1.09% of the population). In all other regions of the world, only between 0.26 and 0.85% of the population is affected, making it less prevalent than POAG, though still a major public health concern with >20 million affected individuals worldwide (Tham et al., 2014). As with POAG, some PACG cases appear to be familial, and significant genetic associations have been made between PACG and the *PLEKHA7*, *COL11A1*, *PCMTD1-ST18*, *EPDR1*, *CHAT*, *GLIS3*, *FERMT2*, *ABCC5*, and *DPM2* (Kong et al., 2011).

Similar to POAG, mitochondrial DNA variation is significantly elevated (>2 fold) in PACG patients, particularly in mtDNA encoding subunits of complex I and IV (Kumar et al., 2013). Other forms of glaucoma may also be enriched for mitochondrial abnormalities, as unsupervised gene clustering of RNA-seq data from glaucomatous RGCs from DBA2/J mice shows significant changes in mitochondrial gene expression. The accumulation in mitochondrial DNA abnormalities between different glaucoma varients suggests that increases in IOP are generally associated with mitochondrial mutations, and indeed this phenomenon can be found even in cultured RGCs exposed to elevated hydrostatic pressure (Zhang et al., 2016).

**I.II.IV. The Utility of Glaucoma Treatments**

Measurable RGC loss is apparent in 90% of eyes at diagnosis, and in 60% of eyes years before diagnostic visual field deficits (Sommer et al., 1991), implying the presence of an important time window during which treatment could prevent notable loss of visual function. The currently available FDA-approved treatments are exclusively drugs that decrease IOP, despite the fact that IOP is neither sufficient nor required for glaucoma (Jin and Noh, 2017). Unfortunately, the therapeutic benefit and main clinical goal of these treatments is limited to decreasing IOP (Prum et al., 2016). Though lowering of IOP is sometimes
protective against visual field deterioration (AGIS, 2000), this effect is incomplete and the disease will still progress in 25-50% of patients (Kass et al., 2002; Jin and Noh, 2017). These data provide a strong motivation for the development of adjuvant glaucoma therapeutics with separate mechanisms from those that exclusively reduce IOP (Howell et al., 2014; Yang et al., 2016; Kimura et al., 2017).

I.II.V. Models of Glaucoma

Glaucoma treatments are generated from data gathered first in model systems. The glaucomatous changes in eye, retinal, and optic nerve physiology are recapitulated to different extents using several distinct organisms and systems, including but not limited to hydrostatic pressure elevation in patient-derived optic nerve head astrocytes and IOP elevation in monkeys, rabbits, pigs, mice, rats, and zebrafish (Bouhenni et al., 2012). These model systems each have unique advantages and disadvantages relating to ease of manipulation and how closely they recapitulate the human disease. While experiments on human postmortem retinal or optic nerve tissue will represent glaucoma with a high degree of accuracy, one cannot perform experimental manipulations that are central to a mechanistic understanding of the disease. On the end of this spectrum, zebrafish breed and mature very quickly and are genetically mutable, allowing for many different manipulations, though it is unclear whether the molecular composition of zebrafish ganglion cells are similar to the molecular signature of human retinal ganglion cells, which unlike zebrafish cannot regenerate. Mice and rats lie in the middle of this spectrum, as they are genetically mutable, cost-effective, and possess retinas that are anatomically similar to those of humans, with a few caveats. The principal differences between rodents and humans are slight differences in baseline IOPs, differences in the proportion of rod and cone photoreceptors, their lack of a fovea (a concentration of cone photoreceptors in the central retina) or a lamina cribrosa per se, and overall poor vision relative to humans. These species differences are important to note, but do not at all preclude the use of mice or rats as a model system. Of particular relevance to glaucoma is their lack of a lamina cribrosa, but as other have noted, the area corresponding to a human lamina cribrosa in the mouse optic nerve shares many similar structural and cellular elements (May and Lütjen-Drecoll, 2002).
Within all of these organisms, glaucoma-dependent RGC loss is often modeled by applying physical damage to the optic nerve (crush or transection), chemical damage to the optic nerve (administration of superphysiological concentrations of RGC glutamate receptor agonists such as N-methyl-D-aspartate) or by surgically elevating IOP (retinal ischemia/reperfusion, photocoagulation of retinal veins, occlusion of the trabecular meshwork with microbeads). Among these models, those which cause RGC death via IOP elevation are the most mechanistically similar to normal glaucoma physiology, but it is important to note that IOP elevation exerts a physiological effect on more regions of the eye than the retina/optic nerve alone, and more cells within the retina/optic nerve than just RGCs. This potential limitation can be overcome using immunofluorescent labeling, or by more sophisticated techniques such as fluorescence activated cell sorting (Chintalapudi et al., 2017) or laser capture microdissection (Sutherland et al., 2018), but these are nonstandard and may cause artifacts in RGC gene/protein or function expression. Therefore, the relevance of in situ techniques such as ON crush, transection, or RGC excitotoxicity is their specificity towards RGCs.

Aside from surgical models of glaucoma, there also exist transgenic mouse strains with increased susceptibility for spontaneous increases in IOP-dependent or -independent RGC degeneration. In most genetic models, the mutations that cause IOP-dependent RGC degeneration are due to partial occlusion of aqueous humor flow. The DBA2/J strain is widely used for this purpose and has several genetic differences from C57BL6/J mice. The most important of these differences are mutations resulting in two amino acid substitutions in Tyrp1 and a premature stop codon in Gpnmb. Both of these genes are expressed in the iris, and deficiency leads to a combination of iris stromal atrophy and deposition of pigment in the trabecular meshwork (Chang et al., 1999), both impeding the flow of aqueous humor and thus increasing IOP (Anderson et al., 2001). Reintroduction of a wild-type Gpnmb variant on an otherwise normal DBA2/J mouse rescues them from RGC degeneration and these mice are used as a (mostly) genetically identical control for DBA2/J mice (Howell et al., 2007). The popularity of these mice and their age-dependent development of glaucoma makes these mice attractive as glaucoma models, but unfortunately the vast majority of commercially available transgenic mouse strains are generated on a C57BL6/J background. This limits the
ability of labs to easily generate new transgenic DBA2/J for the study of glaucomatous neurodegeneration or neuroprotection.

IOP can also spontaneously increase in mice with point mutations in *myocilin* (Tyr437His), the collagen type-I α1 subunit (multiple mutations), or overexpression of the calcitonin receptor-like receptor. Several other mouse lines exist in which RGCs are spontaneously dysfunctional or die without an increase in IOP, and although less commonly used, are available on a C57BL6/J genetic background. These lines are categorized in Table 1 of (Johnson and Tomarev, 2010). The most accurate of these genetic and surgical models will display phenotypes similar to what occurs in human glaucoma, including an elevation in IOP, progressive retinal ganglion cell death, and visual dysfunction. Notably, IOP is known to affect gene and protein expression in more than just RGCs (Hernandez et al., 2008), suggesting that a combinatorial use of glaucoma models (genetic vs. surgical or IOP-increasing vs. IOP-neutral) may provide strong confirmatory or mechanistic evidence to support that the treatment in given study is targeted to RGCs.

*I.II.VI. Locus of Damage in Glaucoma*

Glaucoma, either in humans or when modeled in animals is characterized by damage to anatomically distinct regions of the posterior segment, primarily the retina and the optic nerve (ON). Of the many different retinal cell types that exist, only retinal ganglion cells appear to die in mouse models of glaucoma (Kendell et al., 1995; Jakobs et al., 2005), and in glaucomatous human retinal tissue (Wax et al., 1998). This RGC death is topological and tends to most greatly affect inferior or superior portions of the peripheral retina (Mabuchi et al., 2004; Kwong et al., 2013). RGC death from these or any other regions results in a wedge shaped pattern of optic nerve fiber loss that can be found in immunolabeled retinal whole-mounts or upon fundoscopic examination (Hoyt et al., 1973; Soto et al., 2011). Notably, the regional distribution of RGC loss has not been replicated in all studies of glaucoma, and there are differences in the loci of RGC degeneration with different model systems of glaucoma, different mouse strains, and different levels of severity (Schaub et al., 2017). Some of the species- or strain-dependent differences in RGC loss and distribution may result from differences in the biomechanical properties of the human and mouse optic nerve heads, the
structure of which have historically been posited to be critical for RGC degeneration (Yan et al., 1994; Quigley, 1995; Schaub et al., 2017). The theoretical mechanism relating mechanical strain of RGC axons to apoptotic RGC death (Quigley et al., 1995; Kerrigan et al., 1997) is dysfunction in RGC axonal transport (Quigley, 1995; Takihara et al., 2015), which leads to a primary pathological insult in retinal ganglion cells as well as changes in the activity or function of optic nerve head and retinal glia (Dai et al., 2012). These glaucoma-associated phenomena are categorized below as “Ganglion Cell Intrinsic” or “Ganglion Cell Extrinsic” mechanisms of degeneration, though there are several hypothetical routes by which these distinct mechanisms can be unified.

I.II.VI.I. Ganglion Cell Intrinsic

Following IOP elevation in animals or humans, RGCs undergo multiple pathological changes, including dysfunction in the axonal transport of brain-derived neurotrophic factor (BDNF) (Quigley et al., 2000) and mitochondria (Takihara et al., 2015), as well as an increase in hypoxia (Chidlow et al., 2017), mitochondrial dysfunction (Coughlin et al., 2015), and oxidative damage (Tezel et al., 2005; Lin and Kuang, 2014; Jassim and Inman, 2019). These phenotypes may be linked, as glaucoma associated mutations in optineurin are sufficient for BDNF deficiency, which in turn is sufficient for mitochondrial dysfunction (Shim et al., 2018). Glaucoma causes an accumulation of mitochondria at the optic nerve head (Minckler et al., 1978), and in animal models disrupts the number of mitochondria transported along axons and decreases the size of transported mitochondria (Coughlin et al., 2015; Takihara et al., 2015). Decreased overall transport suggests that fewer mitochondria are present to provide bioenergetic support to glaucomatous RGC axons, and the smaller size of the mitochondria that are present is associated with the accumulation of damage and decreased bioenergetic function (Coughlin et al., 2015). In addition to supplying ATP, mitochondria are also a frequent source of oxidative damage (Angelova and Abramov, 2016). Oxidative damage results in mtDNA mutation, protein modification, and lipid peroxidation. Since oxidative damage is clearly a factor both in animal models (Tezel et al., 2005; Kimura et al., 2017) and human glaucoma (Feilchenfeld et al., 2008; Tezel et al., 2010), there is a strong likelihood that it results from mitochondrial dysfunction. Interestingly, the changes
observed in glaucoma are generally thought to localize to a small area of the retina and optic nerve, but recent studies have also found more systemic changes in mitochondrial function and redox state. More specifically, human glaucoma is associated with a decrease in systemic lymphocyte mitochondrial complex I function (Van Bergen et al., 2015), a decrease in circulating reduced glutathione (Gherghel et al., 2005), and an increase in systemic oxidative stress, measured by levels of the oxidative DNA modification 8-hydroxyguanosine (Himori et al., 2016). These changes would not be expected if glaucomatous mitochondrial dysfunction is solely a consequence of increased IOP, and may represent underlying differences between otherwise healthy people and those that are susceptible to glaucoma.

I.II.VI.II. Ganglion Cell Extrinsic

In addition to the changes which occur in retinal ganglion cells, IOP-induced biomechanical changes to the retina and optic nerve are sensed by glia (Dai et al., 2012), which in the retina and optic nerve are astrocytes, oligodendrocytes, microglia, and müller glia. Although they are necessary for fast neurotransmission, the overall evidence suggesting that oligodendrocytes contribute to glaucoma pathophysiology is weak, and will not be covered in this thesis. The other glial cell types are each sensitive to the retinal and optic nerve microenvironment, and following a damaging stimulus respond by effecting a program of changes in cell morphology (Lye-Barthel et al., 2013; Bosco et al., 2016), gene expression (Qu and Jakobs, 2013), secretion of pro- and anti-inflammatory signals (Huang et al., 2009), and metabolic/anti-oxidative support to neurons (Malone and Hernandez, 2007; Chidlow et al., 2017), in a process that can be categorized as glial “activation”, “reactivity”, or “gliosis”. Gliosis can take different forms, with either neuroprotective (Sun et al., 2017a) or neurodestructive effects (Bringmann et al., 2009). These effects are central for the biology of glaucoma, and are briefly summarized below.

Müller glia are radial cells unique to and penetrating the entire thickness of the retina. They possess many functions essential for the maintenance of RGC and retinal physiology. One of the most central of these functions is to provide metabolic support, performed through calcium-dependent regulation of vessel dilation (Newman, 2015; Biesecker et al., 2016), control of lactate levels (Poitry-Yamate et al., 1995; Lindsay et al., 2014), or through the
hydrolysis of glycogen stores (Poitry-Yamate and Tsacopoulos, 1992). The metabolic activity of mülle glia is important for maintenance of neural survival, as fluorocitrate-mediated inhibition of glial citric acid cycle activity potentiates retinal damage following global inhibition of glycolysis by iodoacetate (Zeevark and Nicklas, 1997). Together, the data strongly suggests that mülle glia provide metabolic support to retinal neurons, which is particularly important in light of the multiple mitochondrial abnormalities that occur in glaucoma.

Müller glia also provide powerful anti-oxidative support to RGCs through the release of glutathione. In the rat and monkey retina, the majority of glutathione is localized to mülle glia (Schütte and Werner, 1998; Carter-Dawson et al., 2004). Upon the induction of hypoxia, total (oxidized and reduced) rat retinal glutathione re-localizes to RGCs (Schütte and Werner, 1998), implying active glutathione export from mülle glia, a phenomenon observed more directly in cultured astroglial cells (Kannan et al., 2000).

The most well-known and consistent sign that mülle glia are affected by glaucoma is an up-regulation of the intermediate filament protein GFAP (Tanihara et al., 1997; Lam et al., 2003). If there are any glaucoma-related changes in anti-oxidative or metabolic support to retinal neurons, they have yet to be thoroughly characterized. It is known that the mülle cell glutamine synthetase activity is impaired during glaucoma (Moreno et al., 2005), but the evidence for toxic glutamate accumulation on the glaucomatous retina has largely been discredited (Lotery, 2005). Elevated IOP in the monkey retina does significantly increase mülle glial glutathione levels (Carter-Dawson et al., 2004), though more recent data in mice show the opposite effect of raised IOP (Jassim and Inman, 2019), suggesting either model- or species-dependent reactions of glutathione to stress. Regardless, we have yet to find a study that has clearly outlined the effects of glaucoma on the oxidative/reductive state of mülle cell glutathione levels, so overall the contribution of mülle glia to metabolic changes in glaucoma is still an area worthy of further research.
Generally, astrocytes are functionally similar to müller glia, though this similarity is more clear between astrocytes in other regions of the central nervous and müller glia in the retina, as both regulate blood flow, buffer extracellular potassium concentrations, recycle synaptic glutamate for intracellular conversion to glutamine, and up-regulate GFAP in response to neural damage (Bringmann et al., 2009). Astrocytes of the retina appear to be primarily associated with blood vessels (Fig. 1.4A), and are not even present in the avascular retina (Stone and Dreher, 1987). Unlike müller glia, astrocyte GFAP expression is

Figure 1.4. Localization and Structure of Retinal Astrocytes. GFAP-labeled astrocytes are present throughout the retina, but the association between their processes and retinal blood vessels (enclosed by yellow lines) is particularly visible in retinal whole-mounts (A). (B) In 10 um sections of the retina, the localize strictly to the ganglion cell layer/retinal nerve fiber layer (yellow lines), where they contact retinal ganglion cells and their axons. (C) Many of these astrocytes are localized to the optic nerve, where RGC axons converge and eventually exit the eye.
constitutive, and clearly labels cell bodies and processes in the ganglion cell and nerve fiber layers of the retina (Fig. 1.4B), regardless of changes in IOP (Lam et al., 2003). They appear to ensheath axons at the optic nerve head (Fig. 1.4C) and mediate trans-cellular degradation of RGC mitochondria (Davis et al., 2014). They also cover blood vessels, and are proposed to stimulate angiogenesis (West et al., 2005). Similarly, blood vessel growth negatively stimulates astrocyte proliferation and down-regulates VEGF expression (West et al., 2005). Their spatial proximity to blood vessels exposes them to increased oxygen levels, which increases their sensitivity to alterations in oxygen levels associated with IOP (Yu and Cringle, 2001) such as hypoxia (Chidlow et al., 2017; Jassim and Inman, 2019), but increases the risk that they will contract oxidative damage. In glaucoma, oxidative damage to both retinal glia and the blood vessels they ensheath is particularly pronounced (Feilchenfeld et al., 2008). The reason for this damage is unclear, as spectroscopic examination of retinal and optic nerve head oxygen saturation shows only minor changes in glaucoma (Olafsdottir et al., 2014; Türksever et al., 2015; Li et al., 2016), though molecular markers of tissue hypoxia such as heme oxygenase 1, the hypoxia inducible factor 1-α, and tissue pimonidazole labeling are each increased in glaucomatous retinas of animal models with elevated IOP (Chidlow et al., 2017; Jassim and Inman, 2019). The generation of oxidants during tissue hypoxia is not an uncommon observation (Clanton, 2007), and regardless of the mechanistic basis, this oxidative injury may represent an insufficient ability of retinal astrocytes to detoxify ROS in glaucoma. A gene expression analysis of human glaucomatous optic nerve head astrocytes reveals upregulation of pro-inflammatory, metabolic, and compliment activation pathways. These changes suggest that the dominant reaction of retinal astrocytes to damage is to signal to and activate resident immune cells at the site(s) of glaucomatous damage, possibly to beneficial (Harder et al., 2017) or destructive effect (Williams et al., 2016).

Microglia are the only immunological cell type in the undamaged retina (Chen et al., 2018). Microglial reactivity is tied in part to astroglial reactivity and the same signals that reduce the pro-inflammatory phenotype of one will do so for the other (Balasingam and Yong, 1996; Wang et al., 2014). Reactive microglia are visible following IOP elevation, but are preceded by RGC axon and müller cell gliosis, implying that these cells react to RGC
damage, and may even catalyze a form of ongoing damage, but are likely not responsible for any causative insult (Son et al., 2010).

In summary, glaucoma is a highly prevalent and costly disease that frequently manifests with elevated intraocular pressure, mitochondrial abnormalities, oxidative stress, and gliosis of multiple retinal/optic nerve glial cell types. In the past, much of the focus has been on decreasing raised IOP, but alternative modalities of treatment are now in development. The modality of treatment with which we are most interested is the modulation of mitochondrial function, which we believe is key to protect both neural energy supply and oxidative status.

I.III. Introduction to Mitochondrial Uncoupling Proteins

I.III.I. Overview of Mitochondrial Function

Mitochondria are organelles present in most eukaryotic cells. They come in a variety of shapes and have a 2-layered membrane structure that forms a distinct environment within cells. This environment is host to several multi-protein complexes that oxidize high-energy molecules such as pyruvate, acyl-carnitine, and amino acids in to reduced nicotinamide adenine dinucleotide (NAD$^+$→NADH), flavin adenine dinucleotide (FAD$^+→FADH_2$), and utilize the reduction potential of these two high-energy molecules and the Krebs cycle intermediate succinate as substrates of the electron transport chain (ETC, Fig. 1.5). The ETC translocates protons across the inner mitochondrial membrane, concentrating them within the intermembrane space. The electrochemical gradient formed by these protons is known as the proton-motive force ($\rho$), which drives the synthesis of adenosine triphosphate (ATP) by adenosine diphosphate and inorganic phosphate in the F$_1$F$_0$-ATP synthase. ATP is the main cellular energetic currency and in most eukaryotic cells, mitochondria are the principal source of ATP. Mitochondrial function and consequent production of ATP are highly regulated.

I.III.II. Mitochondrial Membrane Potential and Reactive Oxygen Species

One important consequence of ATP production is the simultaneous generation of free radicals, which result from the interaction of electrons in the ETC with molecular oxygen to
form superoxide radicals ($O_2^-$). Superoxide is highly reactive, and belongs to a group of molecules termed ‘reactive oxygen species’ (ROS; $O_2^-$, HO’, $H_2O_2$), that when elevated can disrupt cellular function by oxidizing proteins, lipids and carbohydrates. A high proportion of

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Figure 1.5. UCP2 Transports Protons down their Electrochemical Gradient in the Mitochondrial Electron Transport Chain. Cartoon of the mitochondrial inner membrane depicting the proton translocation activity of the NADH:Ubiquinone oxidoreductase (complex I), the Ubiquinone:Cytochrome C oxidoreductase (Complex III), and cytochrome C oxidase as well as their electron transfer reactions to ubiquinone. These reactions establish the mitochondrial transmembrane potential, which powers ATP synthase activity. UCP2 (right) is also present in the inner mitochondrial membrane and catalyzes leak of protons from the intermembrane space to the mitochondrial matrix, diminishing this transmembrane electrochemical potential. Ucp2 is activated and inhibited by various factors, noted in the figure and referred to in text.
cellular ROS are the result of electron leakage from the mitochondrial electron transport chain, and there are at least 11 such sites of electron leak (Brand, 2016).

While most cells are partially protected from basal levels of ROS by endogenous intramitochondrial and extramitochondrial antioxidant defense systems (including superoxide dismutase, catalase, thioredoxins, peroxiredoxins, glutathione), a non-zero rate of ROS production combined with the rapid diffusion of these molecules will eventually result in damage to mitochondrial components, particularly in pathogenic states dominated by mitochondrial dysfunction and elevated rates of ROS production. The rate of ROS production is positively correlated with $\rho$. $\rho$ is normally -170 to -200 mV, and is composed of an electrical potential (the mitochondrial membrane potential; $\Psi_m$) and a chemical gradient (mitochondrial pH). In polarized mitochondria (a more negative $\Psi_m$), there is a greater driving force for ATP synthesis, but also an elevated rate of electron leakage and consequent ROS production. Small depolarizations in $\Psi_m$ (which is roughly 85% of $\rho$) can decrease the rate of ROS generation (Korshunov et al., 1997; Miwa et al., 2003). An important consequence of a lowered $\Psi_m$ is a decrease in the driving force for ATP production, and drastically depolarized mitochondria are considered nonfunctional due to vanishing rates of ADP phosphorylation. However, the relationship between ROS and $\Psi_m$ is nonlinear, and small depolarizations in $\Psi_m$ below maximum hyperpolarization drastically decrease the generation of ROS such as $\text{H}_2\text{O}_2$ without drastically altering ATP levels. This point is central to the hypothesis that “mild uncoupling” of mitochondria will decrease the production of ROS, particularly in pathogenic states where mitochondria may not properly function (Korshunov et al., 1997; Miwa et al., 2003).

Both $\Psi_m$ and $\rho$ are determined by basal and inducible proton leaks. Basal proton leak is a consequence of partial mitochondrial membrane permeability to protons, though quantitative estimates of this permeability may be exaggerated due to damage of mitochondrial membranes acquired over the isolation process. Inducible proton leak has been found to varying degrees in mitochondria from many different tissues and is sensitive to various factors such as thyroid and steroid hormones as well as long chain fatty acids (Starkov, 1997). Much of the cellular proton leak was attributable to the adenine nucleotide translocase (ANT), which uses the $\Psi_m$ to power ATP/ADP exchange across the inner
mitochondrial membrane. ANT is one of the most highly expressed mitochondrial proteins, so electrogenic molecule exchange results in proton leak. There are several other electrogenic mitochondrial transporters, though the majority of these are expressed at low levels and at normal levels of activity do not induce a notable magnitude of proton leak.

I.III.III. The Mitochondrial Uncoupling Proteins

In addition to ANT proteins, mitochondrial uncoupling proteins (UCPs) are another principal source of endogenous proton leak. The UCP family consists of 5 members, UCP1, UCP2, UCP3, UCP4 and UCP5/BMCP1, each of which falls within the SLC25 family of mitochondrial solute carriers. Characterization of the UCP family began with UCP1, which was found in brown adipose tissue to be expressed at levels comparable to the ANT (8% of total mitochondrial protein). In this tissue, UCP1 catalyzes a proton leak that generated sufficient heat to induce non-shivering thermogenesis and thus maintain body temperature (Matthias et al., 2000). Ucp1 knockout mice are incapable of sustaining non-shivering thermogenesis (Matthias et al., 2000; Nedergaard et al., 2001), suggesting that combinatorial activity of the other UCPs (UCP2-5) were insufficient to generate an increase in body temperature, likely a result of their relatively low protein expression in mitochondria (0.1-1% of Ucp1) (Pecqueur et al., 2001). Despite this finding, others have hypothesized low-levels of thermogenesis from different UCPs, such as UCP2, the levels of which in the brain correlate with small but biologically relevant increases in tissue temperature (~1°C). These increases in temperature were proposed to elevate the rate of synaptic vesicle exocytosis and neurotransmission (Horvath et al., 1999). There was insufficient follow-up on this finding however, and the overall consensus is that UCP2-UCP5 do not mediate thermogenesis (Matthias et al., 2000), which is perhaps unsurprising given that a protein BLAST search of the amino acid identity between human UCP1 and UCP2 proteins is only 59%, a number which decreases for other UCP family members. Additionally, the tissue distribution of UCP1 and other UCPs are very dissimilar (Nicholls and Locke, 1984; Boss et al., 1997; Mao et al., 1999; Yu et al., 2000; Pecqueur et al., 2001; Richard et al., 2001; Rupprecht et al., 2012), and when combined, these data suggest that the UCPs other than UCP1 mediate physiological functions other than thermogenesis.
I.III.IV. Ucp2 Regulates Proton Leak and the Production of ROS

Given the role of proton leak in the regulation of ROS, it was suggested that UCPs regulate endogenous reactive oxygen species (ROS) production by decreasing $\Delta \rho$ (Nègre-Salvayre et al., 1997). This hypothesis is supported by several forms of evidence, beginning with characterizations of Ucp2 knockout mice, in which the generation of ROS increased by 80% in isolated macrophages, which was of a sufficient magnitude to completely protect mice from Toxoplasma Gondii. infection (Arsenijevic et al., 2000). Additionally, increasing UCP2 levels, or adding factors that increase or sustain UCP2 activity (for example: fatty acids and coenzyme Q$_{10}$), decreases ROS generation, while Ucp2-knockout or factors that inhibit UCP2 (genepin and purine nucleotides) increase ROS. It is important to note that some of these factors independently show anti-oxidative properties (Noh et al., 2013), but the evidence for Ucp2-dependent mitochondrial uncoupling is strong overall. UCP2 is a 309 amino acid (33 kDa) protein situated in the inner mitochondrial membrane (Figure 1.5). It has three transmembrane domains and catalyzes proton leak by flipping proton bound fatty acids across the mitochondrial membrane (Berardi and Chou, 2014).

I.III.V. Regulation of Ucp2 mRNA and Protein

Ucp2 is expressed in most types of tissue, and can be transcriptionally activated by several different factors, that bind upstream of the Ucp2 gene, including Stat3 (Lapp et al., 2014), Pgc-1$\alpha$ (Oberkofler et al., 2006), and several PPARs (Villarroya et al., 2007). Foxa1 and Smad4 are also able to bind to the Ucp2 promoter region and act as transcriptional repressors (Vatamaniuk et al., 2006; Sayeed et al., 2010).

Despite strong evidence for Ucp2-mediated proton leak, there have however been several discrepancies in the literature showing that some tissues have moderate-to-high levels of Ucp2 RNA or UCP2 protein, but no appreciable proton leak. This may result from differences in tissue and condition-specific regulation of Ucp2. To be more specific, there is substantial Ucp2 transcription in several tissues, but the translation of Ucp2 is inhibited by multiple mechanisms, including several microRNAs that can bind its 3’ untranslated region (Chen et al., 2009; Sun et al., 2011; Jiang et al., 2013). These microRNAs can either target Ucp2 for destruction by the RNA-induced silencing complex, or just prevent translation of
the Ucp2 transcript (Carrington and Ambros, 2003). Ucp2 translation is also regulated by the RNA binding protein, hnRNP-K, which allows for rapid induction of UCP2 protein synthesis upon stimulation by angiopoietin-I (Tahir et al., 2014) and possibly insulin (Ostrowski et al., 2004). Finally, Ucp2 translation can be inhibited by an upstream 111 bp open reading frame (uORF) (Hurtaud et al., 2006), and this regulation is abolished by physiological levels of glutamine, though the relevance of this uORF to Ucp2 physiology is unclear (Hurtaud et al., 2007). However, since glutamine may increase citric acid cycle activity by increasing levels of glutamate and α-ketoglutarate, glutamine-stimulated UCP2 translation may be a means of compensating for increased ROS derived from greater electron transport chain activity. As with the other mechanisms of posttranscriptional regulation, there has yet to be a complete profile of which physiological circumstances dictate whether translation will be inhibited. Notably, our own investigation in to whether glutamine increases uncoupling did indeed show that glutamine can stimulate leak respiration (Appendix, Fig. A1), though we determined this respiration to be independent of Ucp2, and more likely a consequence of increased drive from citric acid cycle-derived NADH, as pyruvate was sufficient to stimulate the same effect, also independent of Ucp2 deletion (Figure A2).

Together, these regulators of translation can create conflicting views of UCP2 protein levels based on RNA data alone, which is particularly important, as high concentrations of mitochondria are necessary for western blot analysis (Derdak et al., 2009) and many of the commercially available antibodies used to detect UCP2 protein are unreliable (Pecqueur et al., 2001). Even with a helpful system for the detection of Ucp2, the protein is constitutively degraded, with a half-life of ~30 minutes (Rousset et al., 2007), and if precautions are not taken to prevent this degradation, it will likely hamper the detection of any Ucp2-dependent signal.

In addition, UCP2 protein activity is tightly regulated and can be induced by superoxide (Echtay et al., 2002) or by modification with glutathione (Mailloux et al., 2011). Superoxide and oxidized glutathione are products or mediators of oxidative stress, and that UCP2 activity results from these oxidative stress mediators suggests that UCP2 activation is a form of feedback regulation to prevent greater levels of ROS (Echtay et al., 2002).
I.I. Overall Hypothesis and Aims of the Thesis

ROS levels increase during glaucoma, and glaucomatous damage to the retina is limited by anti-oxidative agents (Yang et al., 2016). However, anti-oxidative therapies typically detoxify ROS only after they are generated, providing a chance for any given oxidant molecule to cause damage prior to detoxification. UCP2 decreases the generation of ROS, which may have a more potent anti-oxidative effect. Increased \textit{Ucp2} expression is protective in animal models of ischemia-reperfusion (Mattiasson et al., 2003), Parkinsonism (Andrews et al., 2005), Epilepsy (Diano et al., 2003), and even within the excitotoxic retina (Barnstable et al., 2016). These studies each imply that the function of UCP2 is protective, and together show the constancy of this result across time, in different labs, and in different disease models. These data lead us to test the \textit{overall hypothesis that Mitochondrial Uncoupling Protein 2 Protects Retinal Ganglion Cells from Death in Glaucoma}.

A shared feature of previous studies is that models of neurodegeneration were performed acutely, with animals sacrificed within a week of exposure to a single damaging stimulus. Neurodegeneration however is a source of persistent damage over time, and the question of whether UCP2 is protective during chronic neurodegeneration is still unanswered. Glaucoma is a chronic neurodegenerative condition, and when modeled in mice, closely approximates the evolution of neurodegeneration over time. This leads us to \textit{test the hypothesis that elevated \textit{Ucp2} expression is neuroprotective against retinal ganglion cell loss during chronic neurodegeneration}.

We have also discussed how glaucoma involves pathogenic changes to both neurons and glia, and how oxidative stress increases in glia of the retina and optic nerve. The effect of modulating glial oxidative stress on neurons is unclear as well. A decrease in oxidative damage to glia could increase glial antioxidant supply to, or improve metabolic support of RGCs. This leads us to \textit{test the hypothesis that increasing glial \textit{Ucp2} expression will protect RGCs in a non-cell autonomous manner}.

Finally, if UCP2 is neuroprotective and present within the retina, it is likely active during the course of glaucoma. In that case, lower levels of \textit{Ucp2} should exacerbate a glaucomatous insult, leading us to \textit{test the hypothesis that decreasing \textit{Ucp2} expression in a chronic model of neurodegeneration will increase RGC loss}. The data we have gathered
to address these questions both supports pre-existing hypotheses of UCP2 function and mechanism, and also discovers a new avenue through which lower levels of *Ucp2* can also protect neural cells of the retina.
Chapter 2. Materials and Methods

II.I. Ethical approval

These studies were carried out in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (8th edition). The protocol (CATS IACUC ID: PRAMS201546432) was approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

II.II. Animals and Breeding

The following studies used a combination of C57BL6J (WT) and transgenic mice, all housed at an ambient temperature of 25°C, 30-70% humidity, a 12-hr light-dark cycle (light from 7:00 AM – 7:00 PM), and ad libitum access to normal rodent chow. All transgenic mouse strains were bred to a C57BL6J background and expressed components of a Lox-CreER\textsuperscript{T2} system, described in further detail below.

II.III. Cre mice

B6.Cg-Tg(GFAP-cre/ER\textsuperscript{T2})505Fmv/J (GFAP-creER\textsuperscript{T2}, JAX Stock#: 012849) were generated using the GFAP promotor, –2163 to +47 bp relative to the RNA start site (+1). This promotor is active in Gfap-expressing cells of mice, particularly in radial glial cells early in development, and in a smaller population of astrocytes, Müller cells, as well as subventricular zone stem cells after early development (Tanihara et al., 1997; Ganat et al., 2006). In all creER\textsuperscript{T2} variants, the recombinase is a fusion product of the bacteriophage P1 cre recombinase and an estrogen receptor regulatory subunit. Stimulation of the estrogen receptor regulatory subunit with the estrogen receptor modulator and tamoxifen metabolite 4-hydroxytamoxifen stimulates creER\textsuperscript{T2} translocation to the nucleus and subsequent LoxP excision (Zhang et al., 1996), allowing for temporal control of gene expression in addition to the spatial control resulting from promoter use (Zhang et al., 1996). For GFAP, the temporal control is particularly important, as many neural precursor cells express GFAP, and without temporal control LoxP may be excised in both neural and glial populations.
Tg(Thy1-creER<sup>T2</sup>,-EYFP)HGfng/PyngJ (Thy1-creER<sup>T2</sup>, JAX Stock#: 012708) were designed so that both creER<sup>T2</sup> and the fluorescent label YFP would be expressed selectively in projection neurons (Young et al., 2008). Most mouse lines using different variant of the Thy1 promoter show sparse creER<sup>T2</sup> expression (Young et al., 2008), and the particular line of mice we use overcomes inefficient creER<sup>T2</sup> expression. Our own comparison of nuclear labeling with Hoechst 33258, the retinal ganglion cell marker RBPMS, and YFP expression shows the presence of YFP in 81% of RBPMS+ cells and 32% of all ganglion cell layer nuclei.

We stimulated cre–mediated recombination promoted in 1-4 month old mice by daily intra-peritoneal injections of 100 mg/kg tamoxifen (Sigma, T5648) dissolved in sunflower seed oil (Sigma, S5007) for 8 days. Ucp2<sup>fl/fl</sup> or Ucp2<sup>KI</sup> mice were injected with tamoxifen at the same time points as experimental subjects, to control for the effects of tamoxifen exposure.

II. II. II. LoxP mice

We used B6;129S-Ucp2<sup>tm2.1Lowl</sup>/J (Ucp2<sup>fl/fl</sup>, JAX Stock#: 022394) and Ucp2K<sup>flox/flox</sup> (Toda et al., 2016) transgenic mouse strains, in which cre recombinase-mediated LoxP excision causes either Ucp2 deletion or overexpression, respectively. Ucp2<sup>fl/fl</sup> mice contain LoxP sites flanking exons 3 and 4 of the endogenous Ucp2 gene. Cell type-specific creERT<sup>2</sup> activity results in cell type-specific Ucp2 deletion. We additionally used a Ucp2KOK<sup>flox/flox</sup> mice, bred from whole body Ucp2-knockout mice (B6.129S4-Ucp2<sup>tm1Lowl</sup>/J, JAX Stock#: 005934) (Zhang et al., 2001) and Ucp2K<sup>flox/flox</sup>, which were generated by introducing the mouse Ucp2 cDNA in to an AscI site of a cassette targeted to the ROSA26 locus (Toda et al., 2016). In this cassette, the inserted cDNA was preceded by a LoxP site-flanked STOP codon and followed by an IRES-eGFP sequence, so that normally the cDNA would not be expressed, but following cre recombinase mediated LoxP excision, the stop codon would be removed, allowing for expression of the Ucp2 cDNA and eGFP under the endogenous mouse ROSA promoter (Sasaki et al., 2006). Notably, the expression of eGFP was weak and photobleached easily, so visualization of this fluorophore required either (A) minimal manipulation of the tissue before microscopic analysis, or (B) antibody labeling with anti-
GFP followed by a AlexaFluor488 antibody, which completely overlapped with the GFP fluorescence spectrum. We re-derived Ucp2KI\(^{\text{flox/flox}}\) mice by breeding Ucp2KOKI\(^{\text{flox/flox}}\) mice (provided by Dr. Sabrina Diano, Yale University), with wild-type C57BL6J mice colony to produce mice heterozygous for both alleles. These heterozygotes were then crossed to produce Ucp2KI\(^{\text{flox/flox}}\) (Ucp2\(^{KI}\)) lacking a Ucp2-KO allele at the endogenous Ucp2 locus.

To produce mice in which Ucp2 would be selectively deleted in Gfap- or Thy1-expressing cells following treatment with tamoxifen, Ucp2\(^{\text{fl/fl}}\) and Ucp2\(^{\text{KI}}\) mice were crossed with GFAP-creER\(^T2\) or Thy1-creER\(^T2\) mice, and offspring heterozygous for LoxP sites were respectively crossed with Ucp2\(^{\text{fl/fl}}\) or Ucp2\(^{\text{KI}}\) mice to generate offspring used in our studies. To ensure complete Ucp2 deletion, only mice with both LoxP alleles were used.

II.III. Rosiglitazone Treatment

Rosiglitazone (RSG) was fed to WT mice by grinding 4 mg pills (Avandia, GSK) with a mortar and pestle and mixing them in to ground normal mouse chow. We measured daily food consumption based on changes in the weight of specialized feeding containers, and adjusted the amount of RSG used based on food consumption for a maximally stable drug dosage. RSG was fed to mice beginning 2 days prior to microbead injection and does not alter intra-ocular pressure. During this study, we estimate an average RSG consumption of 28.2 mg RSG/kg mouse/day.

II.III. Genotyping

Tissue from ear punches was boiled for 10 minutes in a 10 mM Tris, 0.1% SDS, 1 mM EDTA lysis buffer, followed by an overnight incubation at 56°C in lysis buffer supplemented by 50-250 ug/mL proteinase K. Enzyme activity was halted by boiling samples for 5 minutes prior to use in PCR. Genotyping PCR reactions used the following generic format (next page):
This recipe was used in combination with several PCR protocols and primer sets to detect specific sequence features. The primer/protocol set and precise sequence features are listed below in Table 1.

Table 1. Specific applications of genotyping PCR primers and protocols

<table>
<thead>
<tr>
<th>Primer Set/Protocol</th>
<th>Locus Amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>CreER&lt;sup&gt;T2&lt;/sup&gt;</td>
<td>The presence of either Thy1-CreER&lt;sup&gt;T2&lt;/sup&gt; or GFAP-CreER&lt;sup&gt;T2&lt;/sup&gt;.</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;lox&lt;/sup&gt;</td>
<td>The presence of LoxP-flanked exons 3-4 on endogenous Ucp2</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;x&lt;/sup&gt;</td>
<td>The CreER&lt;sup&gt;T2&lt;/sup&gt;-mediated excision of Ucp2 exons 3-4</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;KI&lt;/sup&gt;</td>
<td>The insertion of a Ucp2 cDNA at the Rosa26 locus</td>
</tr>
<tr>
<td>Rosa26&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>The wild-type Rosa26 locus</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>The presence of endogenous wild-type Ucp2</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;KO&lt;/sup&gt;</td>
<td>The absence of endogenous wild-type Ucp2</td>
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</tbody>
</table>

Table 2. Primers used for genotyping

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
<th>Product Size (bp)</th>
<th>Citation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ucp2&lt;sup&gt;lox&lt;/sup&gt;</td>
<td>ACCAGGGCTGTCTCCAAGCAGG</td>
<td>AGAGCTGTTCGAA CACCAGGCCA</td>
<td>~268 (WT) ~301 (LoxP)</td>
<td>(Robson-Doucette et al., 2011)</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;x&lt;/sup&gt;</td>
<td>AGAGCTGTTCGAA CACCAGGCCA</td>
<td>TAGAGGAGGTGG TGTTCCAGCTC</td>
<td>409</td>
<td>(Robson-Doucette et al., 2011)</td>
</tr>
<tr>
<td>Ucp2&lt;sup&gt;KI&lt;/sup&gt;</td>
<td>AAAATCGCTCTGA GTTGTTATCACTA</td>
<td>CGTTCATATGGGA ACATACGTCATTA</td>
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<td></td>
</tr>
<tr>
<td>Rosa26&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>AAAATCGCTCTGA GTTGTTATCACTA</td>
<td>GCAAAGGATCAAC AAAAGTGTACTA</td>
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<tr>
<td>Ucp2&lt;sup&gt;x&lt;/sup&gt;</td>
<td>GACTGCTCTGGA AAAAGGCGCCT</td>
<td>CTCAAAAGGTGC CTCCCCGA</td>
<td>400</td>
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</tr>
<tr>
<td>Ucp2&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>CTCAGAAAGGTGC CTCCCGA</td>
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<td></td>
</tr>
<tr>
<td>CreER&lt;sup&gt;T2&lt;/sup&gt;</td>
<td>GCAACGAGGTGATG AGGTTCCGAAG</td>
<td>TCCCGCCGATAAC CAGTGAAACAG</td>
<td>307</td>
<td>(Ganat et al., 2006)</td>
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</table>
### Table 3. CreERT2 genotyping protocol

<table>
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<th>Temp (°C)</th>
<th>Time (mm:ss)</th>
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<tr>
<td>2</td>
<td>95</td>
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<tr>
<td>4</td>
<td>74</td>
<td>1:00</td>
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<tr>
<td>5</td>
<td>Go To #2</td>
<td>29x</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>5:00</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
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</tbody>
</table>

### Table 4. Ucp2\(^{KO}\) and Ucp2\(^{WT}\) genotyping protocol

<table>
<thead>
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<th>Step</th>
<th>Temp (°C)</th>
<th>Time (mm:ss)</th>
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<tbody>
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</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1:00</td>
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<tr>
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<td>29x</td>
</tr>
<tr>
<td>6</td>
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<td>10:00</td>
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<tr>
<td>7</td>
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</tbody>
</table>

### Table 5. Rosa26WT and Ucp2KI genotyping protocol

<table>
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<th>Step</th>
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<th>Time (mm:ss)</th>
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</thead>
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<tr>
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<td>0:30</td>
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<tr>
<td>3</td>
<td>61.1</td>
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<tr>
<td>4</td>
<td>72</td>
<td>0:45</td>
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</table>

### Table 6. Ucp2Flox and Ucp2\(^{Δ}\) genotyping protocol

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<th>Time (mm:ss)</th>
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<tbody>
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<td>95</td>
<td>1:00</td>
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<tr>
<td>3</td>
<td>73 (-1°/cyc)</td>
<td>1:00</td>
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<tr>
<td>4</td>
<td>72</td>
<td>0:30</td>
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<tr>
<td>5</td>
<td>Go To #2</td>
<td>15x</td>
</tr>
<tr>
<td>6</td>
<td>95</td>
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<td>72</td>
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</tr>
<tr>
<td>11</td>
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</tr>
</tbody>
</table>

**II.IV. Primary Cortical Astrocyte Culture**

Primary mouse cortical astrocytes were isolated from postnatal day 1-5 mice as previously described (Sarafian et al., 2010; Lapp et al., 2014). Briefly, mice were decapitated and brains were removed from the skull. In tissue culture medium, a small portion of superior cerebral cortex was pinched off of the brain using curved forceps. Meninges were removed from this region, and the tissue was subsequently triturated with a sterile flame-polished glass Pasteur pipette until it formed a single cell suspension (approximately 20 passes through the
pipette). The suspension was filtered through a 70 µm cell strainer (Corning, Cat#: 352350) to remove larger debris, centrifuged at 500 x g and 4°C for 5 min, resuspended in growth medium (Dulbecco’s Modified Eagle’s Medium/Ham’s F12 supplemented with 2 mM L-glutamine, 15 mM HEPES, 10% fetal bovine serum, and 10 ng/mL gentamicin), and plated in a T-25 tissue culture flask. Cells were grown at 37°C in a 5% CO2/balance air atmosphere, with medium replacement every 2-3 days. After the cells reached confluency, between 7-14 days in vitro (DIV), contaminating cells were shaken off by rotating at 250 RPM overnight. Astrocyte-enriched cultures were plated at 15-30,000 cells/well on black tissue-culture-treated 96-well plates (Corning, Cat# 3603), 96-well seahorse plates (Agilent, Cat# 101085-004), or 12- or 18-mm diameter circular glass coverslips and used at passage #2 or 3, allowing at least 48 hours following medium replacement before experimentation. We treated all primary astrocytes from Ucp2<sup>fl/fl</sup>, Ucp2<sup>Kl</sup>, Ucp2<sup>fl/fl</sup>; GFAP<sup>-creER</sup><sup>T2</sup>, Ucp2<sup>Kl</sup>; GFAP-creERT<sup>T2</sup> mice with 1 µM 4-hydroxytamoxifen (Sigma, Cat#: H6278) for 24 hours, and washed it out for 24 more hours prior to any experimentation.

**II.V. In vitro Measurement of Mitochondrial Membrane Potential**

We determined mitochondrial membrane potential (Ψ<sub>m</sub>) in primary cortical astrocytes using the potentiometric fluorescent dye TMRE (50 nM in pre-warmed culture medium, ImmunoChemistry, Cat#: 9103, Ex/Em:530/590), or the ratiometric potential-sensitive dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetracyethyl-benzimidazolylcarbocyanine iodide (JC-1; 2 µg/mL in pre-warmed culture medium; Thermo-Fischer, Cat#: T3168). Either probe was loaded for 30 minutes at 37°C. Following probe loading, cells were washed 3 times in pre-warmed 1x PBS supplemented with 1 mM glucose and 2 mM GlutaMax (Thermo-Fischer, Cat#: 35050-061). Assays contained controls where cells were co-treated with the probe and a membrane permeant protonophore carbonyl cyanide-4-(trifluoromethoxy)phenyl-hydrazone (FCCP, 1-10 µM, Caymen Chemical, Cat#: 15218). FCCP depolarizes Ψ<sub>m</sub> and confirmed that uncoupling induced the correct direction of change in probe fluorescence (i.e. that the assay was operating in non-quenching mode). Probe fluorescence was determined at Ex/Em: 530/590nm for TMRE and at 485/530nm and 530/590nm for JC-1 on a microplate reader (BioTek Synergy II), and was measured once/min for at least 10 minutes. These
measurements were averaged for each well and normalized to control cells lacking cre recombinase.

**II.VI. In vitro Measurement of Oxidative Status**

We determined the effect of UCP2 on oxidant production using both the general redox-sensitive probe chloromethyl-2′,7′-di-chlorofluorescein diacetate (CM-H$_2$-DCFDA, 40 µM; Thermo-Fischer, Cat#: C6827) and the mitochondrial superoxide probe MitoSox (5 µM, Thermo-Fischer, Cat#: M36008).Cells were washed with 1x PBS and loaded with either probe added to pre-warmed 1x PBS supplemented with 1 mM glucose and 2 mM GlutaMax (Thermo-Fischer, Cat#: 35050-061) for 30 minutes at 37°C. Excess probe was washed out, and we measured probe oxidation in the plate reader’s kinetic mode, which took serial measurements of CM-H$_2$-DCFDA or MitoSox fluorescence over time at 485/530nm or 530/590nm, respectively. The increase in probe fluorescence (ΔF) over 10-30 minutes was divided by initial fluorescent intensity (F$_0$). This rate of increase in fluorescence was normalized to the mean ΔF/F$_0$ of control cells lacking cre recombinase. As a positive control, we used the mitochondrial complex III inhibitor Antimycin A (5 µM) to stimulate ROS production and confirm the utility of these redox-sensitive probes.

**II.VII. Seahorse Extracellular Flux Analysis**

Oxygen consumption rate (OCR) was used as a proxy of oxidative metabolism. We performed a ‘mitochondrial stress test’ on cultured astrocytes (seeded at 30,000 cells/well) using inhibitors of electron transport added in the following sequence at the indicated final concentrations: oligomycin (25 µg/mL; Caymen Chemical), FCCP (1 µM), antimycin A (1 µM) + rotenone (1 µM). OCR with each reagent was measured 4-5 times and total assay time was roughly 2-3 hours. Following the assay, we measured DNA concentration in each well for normalization of OCR traces, and analyzed basal respiration (untreated), maximal respiration (respiration with FCCP), and uncoupling (respiration with oligomycin/basal). For these measurements, mean non-mitochondrial respiration (OCR following treatment with antimycin A and rotenone) was subtracted from each sample.
II.VIII. Anesthesia and Euthanasia

For IOP measurement, anesthesia was initially induced using 5% isoflurane vaporized in air (v/v), with a flow rate of 1 L air/minute. Once mice lost their righting reflex, isoflurane levels were maintained at 1.5% for the duration of the experiment.

For electroretinography, optical coherence tomography, and microbead injection, mice were anesthetized with an intraperitoneal injection of 100-110 mg ketamine/kg mouse supplemented with 10-11 mg xylaine/kg mouse. If anesthesia had not reached sufficient depth 5 minutes after injection, we administered ¼ volume doses until it was. Following any procedures, mice were placed in their home cage, which was left to warm on a heating pad maintained at 37°C. Positive recovery from anesthesia was determined based on the mouse locomotion and responsiveness, and once this was confirmed, mice were placed back in their home area.

The microbead or PBS injection protocol additionally involved penetration of the highly pain-sensitive cornea, which required additional topical anesthesia (0.5% proparacaine hydrochloride eyedrops), applied 5 minutes before the procedure.

Mice were euthanized using the Euthanex SmartBox system, which is connected to a CO₂ supply tank and automatically regulates CO₂ dispersion to minimize anesthesia-associated stress or pain. We followed CO₂-mediated asphyxiation with cervical dislocation to confirm complete euthanasia of all mice.

II.IX.I. Microbead Injection-Preparation

Microbead suspensions were prepared the day of injection. 1 µm (Polysciences, Cat#: 07310-15) and 6 µm diameter microbeads (Polysciences, Cat#: 07312-5) were first sterilized in 100% ethanol for 5 minutes and pelleted at 10,000 x g for 1 minute prior to re-suspension at 1.5x10⁷ beads/µL (1 µm beads) and 3x10⁶ beads/µL (6 µm beads). A day or less prior to bead injections, we pulled glass micropipettes with an upright pipette puller (Narishige Group, Japan), and broke the tip to an outer diameter of approximately 50-100 µm. The tips of these pipettes were beveled at two orthogonal 30° angles with fine-grade sandpaper on a micropipette beveler (Sutter Instruments, Novato, CA, USA). Pipettes were filled with dH₂O and connected to a Hamilton syringe (Hamilton Company Reno, NV, USA) mounted on a
micromanipulator (to increase ease of access) through polyethylene tubing (also filled with dH2O).

II.IX.II. Microbead Injection-Surgery

We modeled glaucoma in mice by elevating IOP in 2-4 month old mice of both genders similarly to the Cone et al. ‘4+1’ protocol (Sappington et al., 2010; Cone et al., 2012), but without the use of a viscoelastic substance. At least 24 hours prior to bead injection, we took a baseline IOP measurement. Immediately prior to bead injection we anesthetized mice with 110 mg/kg ketamine and 11 mg/kg xylazine. We treated mice with topical proparacaine hydrochloride (Akorn) to further anesthetize the cornea and either phenylephrine hydrochloride or tropicimaide to dilate the pupil and decrease the chance of damage to the iris during the surgery. Following systemic and topical anesthesia, we injected 2 µL each of 1- and 6-µm polystyrene microbeads at concentrations of 1.5x10⁷ beads/µL and 3x10⁶ beads/µL, respectively, through a 50-100 µm cannula penetrating the cornea at an angle roughly parallel to the axis formed by the iris. With the mouse eye facing upwards during the surgery, this axis is roughly parallel to the benchtop. Bead injection occurred over 10-30 seconds, with slower injection times more desirable for bead migration to the aqueous humor outflow pathway. As an internal control, 4 µL of sterile 1x phosphate buffered saline (PBS) was injected in to the contralateral eye. Injections of beads or PBS were followed by injecting a 0.5-1 µL air bubble, which prevented the outflow of beads following pipette removal. Proper injection and placement of this air bubble is critical to ensure bead-induced IOP elevation, and bead outflow was potentially a major source of variability during this procedure. Following bead injection, we measured postoperative IOP every 3 days for up to 30 days. Following terminal IOP measurements, mice were euthanized. For measurements of gene expression, protein levels, and mitochondrial function, mice were euthanized 3 days following microbead injection, as IOP is generally maximal or near maximal at this post-operative time point. In this model, bead induced increases in IOP persist for at least 30 days, but in our hands fall
II.X. Measurement of Intra-ocular pressure (IOP)

Intra-ocular pressure (IOP) was measured in mice anesthetized by 1.5% isoflurane in air (v/v) using an Icare® TonoLab (Icare Finland Oy, Espoo, Finland) rebound tonometer, both before and after injection with polystyrene microbeads. Each reported measurement is the average of 18 technical replicates/mouse/eye. Though the amount of time under anesthesia has been shown to affect IOP, mice were anesthetized for <10 minutes and yielded stable mean IOP measurements, particularly in control eyes. However, the work of Ding et al., 2011 leads us to believe that our method of measurement underestimates IOP values by at least 3 mmHg, and possibly more in bead-injected eyes (Ding et al., 2011). Mice were included in this study if their individual IOP was elevated by ≥3 mmHg or if a paired t-test of IOP over time between microbead and PBS-injected eyes was statistically significant (p<0.05).

II.XI. Electroretinography (ERG)

The electrophysiological response of the retina to a series of flashes of light was recorded from anesthetized mice. All ERG recording used a Ganzfield ColorDome™ connected to an Espion e2 system (Diagnosys Inc., Boston), which was housed in a dark room and performed under dim red light. Prior to any recordings, mice were dark-adapted overnight. Whiskers were trimmed and pupils dilated with 1% tropicamide (Bausch and Lomb) or 1% atropine (Bausch and Lomb). The corneas of both eyes were covered with GenTeal eye lubricant (Novartis) or an artificial tear solution to prevent them from drying out. Animals were placed on a warmed (37°C) table with a plastic headrest, and a gold loop electrode with an external-facing protective plastic lens was placed over the hydrated cornea. A platinum needle reference electrode was inserted subcutaneously and a ground electrode was attached to the right hind foot. Following electrode placement, the dome generated flashes of white light from 1X10^6 to 100 cd*s/m², and the electrical responses from the retina were recorded.

II.XII. Optomotor reflex testing

To determine the optomotor reflexes of mice, and individual mouse eyes, we utilized
methods developed by Glen Prusky and colleagues (Prusky et al., 2004). Mice were placed on a circular platform enclosed on all sides by computer monitors. These monitors projected a virtual reality environment consisting of rotating black vertical bars of varying frequency and contrast. To avoid bias, the frequency and contrast of these bars is controlled by a program (Optometry; Cerebral Mechanics, Inc., Lethbridge, AB, Canada) and cannot be seen by the experimenter. The animal’s head position was tracked by the experimenter through an overhead digital camera, and was used to adjust the apparent distance of vertical bars. A lateral head and neck movement following the direction of the bars indicated a positive optokinetic response. Experiments to determine spatial frequency threshold and contrast sensitivity were conducted in technical triplicates, both before and after microbead-injection.

**II. XIII. Cytochrome-C oxidase Histochemistry**

In situ cytochrome-C oxidase (COX) activity was assayed using a modified procedure described by (Wong-Riley, 1979; Murphy et al., 2012). Briefly, following euthanasia, unfixed mouse eyes were harvested, immediately frozen in OCT on dry ice (~78°C), and cut in to 10 µm sections. Control and experimental samples were frozen and sectioned on the same slide to minimize potential slide-to-slide variability. Slides were incubated in an assay solution containing 4 mM freshly dissolved 3’,3’-diamidobenzadine, 100 µM cytochrome C (Sigma, Cat#: 2037), and 5 U/mL catalase (Sigma, Cat#: E3289) for 1h at 37°C. Assay control slides were additionally exposed to 5 mM NaN₃, which inhibits COX activity. Slides were imaged in brightfield at 20x magnification. Analysis of staining intensity was performed using the H-DAB setting of the ImageJ color deconvolution tool and subtracting the result from background image intensity.

**II. XIV. Histology and Immunocytochemistry**

Immunolabeling of sectioned retinal tissue was performed as previously described (Pinzon-Guzman et al., 2011). Briefly, whole eyes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 1x PBS overnight at 4°C. The next day, eyes were washed 3 times in 1x PBS and divided in half with a scalpel blade.
One half was frozen and sectioned, while the other was labeled as a whole-mount. Frozen tissue were embedded in a 2:1 mixture of 20% sucrose in PBS and OCT (Electron Microscopy Sciences), cooled to -20°C, and cut at a 10 µm thickness. Samples for each experiment were located on the same slide to control for assay variability. Prior to immunohistochemical labeling, we unmasked antigens in a boiling sodium citrate buffer (10 mM, pH 6.0) for 30 minutes. We subsequently labeled oxidative protein carbonyls on eye sections using an OxyIHC kit (EMD-Millipore, Cat#: S7450), in accordance with the manufacturer’s instructions. For all other labels, we permeabilized sections in 0.5% Triton-X-100 in PBS (PBS-T) and blocked them with 5% nonfat milk in PBS-T. Following blocking, we incubated sections in primary antibodies (Table 7) diluted in the blocking buffer overnight at 4 °C. The following day, sections were washed 3x with PBS-T and incubated in secondary antibody with 1 µg/mL Hocheest-33258 for 2-3 hours. After three washes, slides were mounted with 0.5% n-propyl gallate in 1:1 glycerol: PBS. For particularly low-abundance proteins, we labeled sections with a VectaStain Elite ABC kit (Vector Laboratories), which uses a biotin-streptavidin-Horseradish peroxidase (HRP) complex to increase signal sensitivity. Retinal whole mounts used a similar protocol as normal fluorescent labeling of tissue sections, but with the following modifications: antigens were not unmasked, tissue was incubated in primary antibody for 6 days at 4°C and in secondary overnight at 4°C.

Table 7. Antibodies used in this study

<table>
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<tr>
<th>Protein</th>
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<td>DI-1549</td>
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**II.XV. Cell Immunocytochemistry**

Immunocytochemistry of glass coverslipped cells was performed using a protocol similar to the one described for immunohistochemical labeling of retinal sections. Medium was replaced with pre-warmed 1x PBS for 5 min and cells were subsequently fixed at RT in 4% paraformaldehyde for 30 minutes. Cells were washed 3 times in 1x PBS prior to permeablization for 15 minutes with 0.2% Triton-X-100 in PBS (PBS-T). All subsequent labeling steps and antibody dilutions were identical to those used in immunohistochemical labeling of retinal sections.

**II.XVI. Microscopy**

Labeled tissue was imaged on a Fluoview FV1000 confocal microscope (Olympus) at a resolution of 512x512 to 1024x1024 pixels/image and 10-20 µs/pixel. For any given experimental comparison, fluorescence acquisition parameters were held constant, and later measured using the ImageJ measurement tool. Unless otherwise specified, measurements are the mean intensity of the area bounded by the ganglion cell and outer nuclear layers.

3’,3’-diamidobenzadine (DAB)-labeled tissue was imaged in brightfield using an Olympus BX50 microscope, also using identical acquisition parameters for any given label. Staining intensity was derived using the H-DAB vector of the ImageJ Color Deconvolution tool background was subtracted from each image, resulting in a numerical semiquantitative measure of oxidative tissue stress.
II.XVII. Analysis of Mitochondrial Morphology and Mitophagy

We monitored mitochondrial morphology, size, and number in TOMM20-immunolabeled cells using the MiNa V1 plugin (Valente et al., 2017). This plugin uses the CLAHE local contrast enhancement algorithm and tophat filtering (Iannetti et al., 2016) to detect individual spherical or tubular mitochondria as well as mitochondrial networks formed by multiple tubular mitochondria. Morphology was detected in individually selected cells, and mitochondrial morphology was analyzed in >30 cells per condition.

We measured colocalization between LC3B and TOMM20 using the ImageJ plugin “Coloc 2” and reporting the Manders’ overlap coefficient corresponding to the TOMM20 image channel. In this program, each channel is automatically thresholded, and the reported coefficient represents the number of colocalized pixels (in this case between thresholded LC3B and TOMM20 channels) divided by the number of total thresholded pixels for the TOMM20 channel. The number is therefore a representation of what % of mitochondria are undergoing mitophagy.

Tissue LC3B puncta were detected using the ImageJ “Find Maxima...” tool with a noise tolerance of 1000, which of all the methods tested best recapitulates a visual search for LC3B puncta. The number of puncta detected were divided by the total area imaged for a metric of puncta density.

II.XVIII. Retinal Ganglion Cell Counting

RGC density was estimated in the ganglion cell layer by counting RNA-binding protein with multiple splicing (RBPMS) immuno-labeled cells in retinal whole-mounts (Rodriguez et al., 2014). We used RBPMS due to the high concordance between RGC soma labeling with this marker and with other well-characterized markers including β-III-tubulin and retrograde immunogold labeling, normally (Rodriguez et al., 2014) and after optic nerve damage (Kwong et al., 2011). This concordance suggests that loss of RBPMS labeling was due to loss of RGCs and not to loss of RBPMS expression. For each retina, RBPMS+ cells were counted across 3-4 well-separated fields, with each field measuring 317.95 µm x 317.95 µm and centered 1000 µm from the optic nerve head. Cell counts were obtained from contrast-enhanced images using the ImageJ cell counter plugin. The observer counting RGC
numbers in these images was blinded to the identity of each sample. Mean±SEM and median RGC densities were 4283±83 and 4169 cells/mm², respectively in PBS-injected Ucp2^{fl/fl} retinas, and 4758±113 and 4738 cells/mm² in data pooled from wild-type C57BL6/J and Ucp2^{KI} controls, roughly comparable to values found in the literature for this marker (Rodriguez et al., 2014; Wang et al., 2017). RGC densities did not significantly vary with retinal quadrant following IOP elevation, so our images were therefore averaged across all retinal quadrants.

II.XIX. RNA Isolation and Quantitative Real-Time PCR

Flash frozen cells or tissue were lysed in TRIzol (Thermo-Fischer, Cat#: 15596018) and RNA precipitated using the manufacturer’s recommended procedure. Final RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer prior to reverse transcription. We reverse transcribed 300-1000 µg RNA using SuperScript III (Thermo-Fischer, Cat#: 18080093) with random hexamers. cDNA was amplified with iQ SYBR Green Supermix (Bio-Rad, Cat#: 1708882) and quantitated on a Bio-Rad iCycler. qPCR primers were designed to span intron-exon junctions or bridge an exon-exon junction, and reactions were followed by a melt curve analysis to confirm the amplification of a single PCR product. All results were normalized against the TATA-box binding protein (Tbp) cDNA using the ΔΔC_t method. Tbp is stably expressed during both mouse retinal development (Adachi et al., 2015) and in retinal endothelial cells treated with innate immune system stimulants (Wei et al., 2013).

Table 8. Primers used to analyze gene expression

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### II.XX. Western blot analysis

Freshly dissected retinal tissue was lysed in RIPA buffer supplemented with Halt protease inhibitor cocktail (Thermo-Fischer, Cat#: 78430) and a phosphatase inhibitor cocktail set II (EMD-Millipore, Cat#: 524625). Following 10 seconds of sonication, protein concentration was estimated with a DC protein assay (BioRad, Cat#: 5000111). Protein was denatured with a combination of Laemmli buffer (at a 1x final concentration, e.g. a 2% final concentration of sodium dodecyl sulfate) and by boiling samples for 5 minutes. 20-30 µg denatured retinal homogenate was loaded on a 4-12% Criterion TGX polyacrylamide gel (Bio-Rad, Cat#: 5678124) and run at 240 V for 35 minutes prior to semi-dry transfer to a nitrocellulose membrane (pre size: 0.45 µm, Bio-Rad, Cat#: 1620115) at 16 V for 40 minutes. Leftover protein was visualized with Coomassie Blue staining, and the sum of Coomassie–labeled histone bands were used for protein normalization.

The membrane was washed in 1x Tris buffered saline with 0.1% Tween-20 (TBS-T) and blocked in TBS-T with 2% (w/v) blotting grade blocker (blocking buffer, Bio-Rad, Cat#: 1706404). Membranes were incubated in primary antibodies (Table 9) diluted in blocking buffer overnight at 4°C. Membranes were washed in TBS-T and incubated in secondary antibody for 2 hours at room temperature prior to generation of chemiluminescent bands with Pierce ECL Western Blotting Substrate (Thermo-Fischer, Cat#: 32106) and visualization on photosensitive films (Thermo-Fischer, Cat#: 34091). Band density was determined from scanned developed films using ImageJ.
Table 9. Antibodies used for Western Blotting

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II.XXI. Statistical analysis

Quantified data are represented by that group’s mean±sem unless otherwise indicated. We performed all statistical analyses in GraphPad Prism. Linear trends were analyzed with a linear regression. We determined the statistical effect of one independent variable on 2 groups using a Student’s t-test or paired sample t-test in cases where samples were matched (e.g. the control was the contralateral eye of the same animal). We analyzed the effect of one variable on >2 groups using a one-way ANOVA with a Bonferroni’s (Chapter 3) or Holm-Sidak (Chapter 4) post-hoc analysis. We analyzed the effect of 2 variables using a 2-way ANOVA with a Bonferroni’s (Chapter 3) or Holm-Sidak (Chapter 4) post-hoc analysis. The statistical significance threshold was p<0.05 for all tests.
Chapter 3. Overexpression of Ucp2 in Retinal Ganglion Cells is Neuroprotective*

*This chapter contains data from the submitted manuscripts “Cell Autonomous Neuroprotection by the Mitochondrial Uncoupling Protein 2 in a Mouse Model of Glaucoma” and “Mitochondrial Uncoupling Protein 2 Knockout Promotes Mitophagy to Decrease Retinal Ganglion Cell Death in Glaucoma”.

III.I. Baseline IOP Values for C57BL6/J and Transgenic Mice

Glaucoma is a disorder characterized by an elevation in IOP. Therefore, it was critical that the baseline IOP of all mice was similar to that of non-glaucoma controls. We determined the IOP of all genotypes tested (Fig. 3.1A) and of male and female C57BL6/J

![Figure 3.1. Baseline Aqueous Humor Dynamics. (A) Genetic background does not significantly alter IOP between C57BL6/J control and transgenic strains prior to bead injection, and neither does (B) gender. (C) IOP measurement standard error also increased as a function of mean IOP.](image-url)
mice, finding no notable difference (Fig. 3.1B). This suggested that aqueous humor dynamics of these mice were unperturbed by Ucp2 deletion or overexpression. We were also concerned about the reliability of tonometry to measure IOP, and compared mean IOP with the standard error measurement (SEM) of the technical replicate used to derive mean IOP (Fig. 3.1C). We found a drastic increase in IOP standard error with greater IOP levels, particularly with an IOP >20 mmHg, making it difficult to be certain of exact IOP value when elevated.

Figure 3.2. The Microbead Occlusion Model of Glaucoma. (A) Timeline for the microbead model used in our study, with relevant experimental endpoints highlighted. (B) Photograph of an anesthetized mouse immediately following microbead-injection. (C) brightfield image of a retinal section 30 days following bead injection, with important anatomical structures labeled and showing the localization of beads to the irido-corneal angle (between the dotted red lines). (D) In a group of C57BL6/J mice, bead injection causes a sustained increase in IOP, (n=10).
III.II. Microbead Injection Impedes the Outflow of Aqueous Humor

Figure 3.2A illustrates the paradigm used to model glaucoma in the transgenic animals of this study. These mice are pre-treated with 10 mg/kg tamoxifen for 8 days (~6 days prior to injection), baseline IOP measured (3 days prior to injection), and beads injected (day 0). Three days following bead injection, retinal tissue was flash frozen for analysis of COX activity or isolation of protein or RNA. Thirty days following bead injection, retinal tissue was isolated for cell counting or analysis of oxidative stress or GFAP. Beads (white) initially occlude the eye (Fig. 3.2B), but eventually migrate to the irido-corneal angle (Fig. 3.2C), where they raise IOP over a sustained period of time (Fig. 3.2D). Wild-type C57BL6/J and transgenic mice were treated identically, except that C57BL6/J mice were never pre-treated with tamoxifen. In a group of pilot mice injected with microbeads, and not sacrificed 30 days after injection, IOP elevation persisted for almost 2 months (~57 days, n=2), though notably in some animals IOP elevation is not persistent following bead injection. Other users of the microbead model found that bead injection in 6-week old C57BL6/J mice can support an increase in IOP that is sustained over at least 24 weeks (Khan et al., 2015).

III.III. Microbead Injection-Induced IOP Elevation Determines RGC Loss Regardless of Region.

Following the microbead injection paradigm in Ucp2^{fl/fl} mice, we found a significant (p=0.0175, df=12, n=14) but overall weak relationship (R^2=0.39) between the bead-induced increase in IOP and a decrease in RGC survival, measured by a decrease in the density of retinal ganglion cell soma labeled with the protein marker RNA-binding protein with multiple splicing (RBPMs) in retinal whole-mounts between PBS- and microbead-injected eyes of the same mice, 1000 um from the optic nerve head (Fig. 3.3A). In the subset of Ucp2^{fl/fl} mice where we marked retinal region during dissection, there was no clear difference in RGC density between the superior and inferior portions of the same retina, either in PBS- or bead-injected eyes (Fig. 3.3B). Based on these data, our measurements of RGC density and loss throughout this thesis are averaged across multiple retinal regions.
III. Microbead Injection-Induced IOP Elevation Impairs Retinal and Visual Function

We determined the effect of microbead injection on two different measures of retinal function in C57BL6/J mice. Following 30 days of bead-induced IOP elevation (Fig. 3.4A), we measured the optomotor reflex in response to different frequencies of black bars, with better seeing eyes better able to distinguish higher frequencies of bars. The spatial frequency “threshold” is the point beyond which mice can no longer detect the presence of more moving bars, as sufficiently small and frequent black bars against a white background can appear as grey to an observer with poor vision. These tests are performed in awake, freely moving C57BL6/J mice, and the un-injected eyes of these mice have a spatial frequency threshold, or visual acuity, of 0.39±0.03 cycles/° (n=6). This trended towards increase to 0.46±0.03 in PBS-injected control eyes (n=5), and was significantly decreased to 0.17±0.03 with bead injection (p<0.05, Fig. 3.4B). We obtained similar findings of decreased vision with bead injection upon measuring contrast sensitivity threshold in the same animals, but did not include these data due to the high inter-mouse variability in measurements of this
parameter. We measured retinal function with electroretinography (ERG), which measures electrical responses of retinas to calibrated flashes of white light. Figure 3.4C shows example ERG traces and points out the component waved measured in Fig. 3.4D and Fig. 3.4E. The A-wave (Fig. 3.4D), a negative deflection in voltage <100 ms post-stimulus which reflects

![Figure 3.4](image)

Figure 3.4. Retinal and Visual Responses to Bead Injection. (A) IOP increases following bead injection. (B) Visual function, measured by spatial frequency threshold, is impaired in bead-injected C57BL6/J eyes (n=5-6). (C) Example ERG traces and (D-E) analysis of component waveform intensity with increasing light intensity. The A-wave (D) and B-wave (E) respectively represent photoreceptor and inner retinal responses, both of which are impaired by bead injection (n=2-3). ****p<0.001
photoreceptor activity, is reduced in amplitude by bead injection, as is the B-wave (~100-200 ms after a stimulus), which reflects the activity of multiple inner retinal cell types. Photoreceptors and the A-wave are normally unaffected in the DBA2J mouse model of glaucoma which also experiences elevated IOP (Bayer et al., 2001), so the decrease in visual or retinal function may correspond to a decrease in penetration of light through the eye due the physical presence of beads, which may block a portion of this light. Notably, the B-wave is active despite no obvious photoreceptor response, suggesting that retinal activity is reduced, but not absent from these retinas (Fig. 3.4E).

Figure 3.5. Impaired mitochondrial function in bead-injected Ucp2<sup>fl/fl</sup> retinal tissue. (A) Representative histochemical labeling of retinal tissue sections 3 days following bead injections, and (B) measurement of DAB intensity (n=5). (C) mtDNA/nDNA was also measured in this tissue, and this measure of mitochondrial mass was unchanged 3 days after bead injection (n=3), but was significantly decreased by 30 days (n=8). *p<0.05
III.V. Microbead Injection Decreases Mitochondrial Function in the Retina

We determined the effect of microbead injection on mitochondrial function by (3.5A-B) measuring cytochrome C oxidase (COX)-dependent oxidation of 3',3'-diamidobenzadine (DAB), which results in the development of brown color on tissue sections. Relative COX activity was determined in ImageJ, following spectral de-convolution so that oxidized DAB was specifically measured. We also approximated mitochondrial mass by measuring mtDNA/nDNA in retinal tissue sections, and determined mtDNA/nDNA of bead-injected retinas over PBS-injected controls three (n=3) and thirty days (n=8) after bead injection (Fig. 3.5C). This ratio was close to 1 three days following bead injection, indicating no significant change in mitochondrial mass soon after bead injection. This ratio was significantly reduced thirty days after bead injection (p=0.037, df=9, Fig. 3.5C). Together these data suggest that bead injection decreases mitochondrial function and eventually mass.

III.VI. Microbead Injection Increases Oxidative Stress in the Inner Retina

One stable mark of oxidative damage is accumulation of lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) (Malone and Hernandez, 2007). HNE can modify histidine residues on proteins to form a stable adduct that we used as a proxy for oxidative stress in tissue. To determine whether retinas are stressed in the microbead glaucoma model, we compared HNE labeling in the retinal layers of microbead- and PBS-injected eyes. HNE reactivity was significantly increased to 159±29% of contralateral PBS-injected eyes within the inner retina 30 days following bead injection (p=0.0035, df=36, n=5), but not the outer retina (98±23% of contralateral control). Analysis of individual inner retinal layers showed increases in HNE but none of these reach significance following adjustment for multiple comparisons, suggesting that although the effects of IOP on oxidative stress may occur in multiple cell types, its main effect is within the retinal region that degenerates in glaucoma (Fig 3.6A-B).
Increases in bead-induced oxidative damage to the retina are supported by increases in the expression of multiple antioxidant genes. We monitored levels of retinal *Hmox1*, *Nfe2l2*, *Ucp2*, *Sod2*, and *C1Qa* expression in a group of untreated (naïve) mice, and from PBS-injected, and microbead-injected mice, 3 days following bead injection (n=3, df=6). Bead-injection increased the expression of *Hmox1* (856±294%, p=0.0217), *Nfe2l2* (149±14%, p=0.033), and *C1Qa* (728±295%, p=0.048) relative to contralateral bead-injected retinas, and caused a smaller trend towards increase in *Sod2* (117±35%, p=0.73) and notably in *Ucp2* as well (366±196%, p=0.29, Fig. 3.7). In all cases where we measured gene expression, there was no notable or statistically significant difference between retinas of naive and PBS-injected mice of the same genotype. In all subsequent experiments we used
contralateral PBS-injected retinas as an internal control for microbead-induced IOP elevation, and consider it useful as a proxy for the normal retina. Together these data strongly suggest that the bead-injected retina is oxidatively stressed, and that the increase in bead-injection-induced changes in gene expression are insufficient to prevent retinal ganglion cell lipid peroxidation, measured by 4-HNE intensity in the inner retina.

**III. VII. Exogenous Uncoupling Agents Decrease Mitochondrial ROS Production**

The positive association between mitochondrial membrane potential ($\Psi_m$) and the production of reactive oxygen species (ROS) has been well characterized in isolated mitochondria, and we tested the hypothesis that mild mitochondrial uncoupling stimulated by an exogenous protonophore will decrease mitochondrial ROS in intact cells. We treated primary cortical astrocytes with FCCP at a low concentration to uncouple mitochondria.
without completely dissipating the $\Psi_m$, and found that 10 nM FCCP depolarized the mitochondrial membrane potential ($\Psi_m$) to $88\pm4\%$ of control levels, whereas $\Psi_m$ was $34\pm2\%$ of control in astrocytes treated with 10 µM FCCP, a concentration routinely used to maximally depolarize mitochondria (10 µM; Fig. 3.8A).

We tested the hypothesis that uncoupling will reduce ROS production generated as a consequence of mitochondrial dysfunction. To test this hypothesis we loaded cells with the mitochondrion-targeted superoxide probe MitoSox and treated them with the mitochondrial complex III inhibitor Antimycin A (AA; 5 µM). AA significantly increased the rate of MitoSox oxidation ($p<0.001$, Fig. 3.8B), but this increase was partially attenuated in cells simultaneously treated with AA and 10 nM FCCP ($p<0.05$, Fig. 3.8B). These data show that
uncoupling decreases the generation of ROS by cultured astrocytes with dysfunctional mitochondria.

**III.IX. Microbead Injection increases Uncoupling Protein 2 Expression in an IOP dependent Manner**

If uncoupling is protective, *Ucp2* levels should increase to prevent or decrease the accumulation of oxidative damage following bead injection. To determine whether *Ucp2* expression is positively or negatively related to bead-injection and IOP elevation, we measured retinal *Ucp2* expression as a function of IOP in *Ucp2*0/0 mice. Bead injection significantly increased IOP (p<0.05, Fig. 3.9A), and we found that the elevated IOP was correlated with retinal *Ucp2* expression \( (r^2=0.8, \ p=0.0001 \ \text{Fig. 3.9B}) \). When we immunolabeled the retinas of PBS- and bead-injected C57BL6/J mice for UCP2 however, we did not notice any striking increase in staining. Retinas were diffusely labeled, except for the ganglion cell layer, where UCP2 labeling appeared more intense \( (n=4, \ \text{Fig. 3.9C}) \). Retinas from full body *Ucp2* knockout mice were used as a control to determine antibody specificity. Notably when the same antibody is used to determine UCP2 protein levels in a western blot, many bands are labeled outside the molecular weight of UCP2 at 33 kDa. Most of these bands are unchanged even in mitochondria-dense lysate form full-body UCP2-knockout mice, which has hampered out ability to determine UCP2 protein levels in tissue from transgenic animals (Popova et al., *personal communication*).
III.X. Uncoupling Protein 2 Decreases Ψ<sub>m</sub> and ROS Production

Because UCP2 protein levels did not appear to increase significantly during glaucoma, we artificially increased *Ucp2* using transgenic mice that can overexpress it selectively in *Thyl*-expressing projection neurons (RGCs in the retina) and in *Gfap*-expressing astrocytes and müller glia. These mice were bred to contain the genetic constructs
illustrated in Fig. 3.10A, except for control mice, which did not possess a copy of cre recombinase.

Figure 3.10. *Ucp2* Overexpression and Construct Localization in the Mouse Retina. (A) Genetic constructs used to stimulate increased *Ucp2* gene expression, and (B) consequent retinal *Ucp2* expression following LoxP excision in *Ucp2*\(^{KI}\); *GFAP-creER\(^{T2}\) and *Ucp2*\(^{KI}\); *Thy1-creER\(^{T2}\) mice. We also imaged the localization of fluorescent proteins co-expressed with *Thy1-creER\(^{T2}\) (C-D, bottom), or expressed following stop codon excision in *Ucp2*\(^{KI}\); *GFAP-creER\(^{T2}\) mice (C-D, top). This was imaged in retinal sections (C) and whole-mounts (D) also labeled with the RGC marker RBPMS and/or the nuclear label Hoechst-33258. The white arrows indicate regions of each cre variant that are structurally distinct, including müller glia fibers (C, top), astrocytes (D, top), and RGC soma (C-D, bottom).
Following eight daily 100 mg/kg/day tamoxifen injections, we found that GFAP-creER$^{T2}$ expression increased Ucp2 transcript levels to 165±14% of control (p<0.01, n=6), and Thy1-creER$^{T2}$ increased Ucp2 to 229±77% of Ucp2$^{KI}$ controls (p<0.05, n=3, Fig. 3.10B). Following exposure to tamoxifen, cells express eGFP, pictured in Fig. 4C, though notably, YFP, which spectrally overlaps with eGFP, is expressed both before and after tamoxifen injection in Thy1-creER$^{T2}$ retinas (Fig. 3.10C shows typical patterns of these fluorescent labels in retinal sections), and in our hands is much easier to detect.

We isolated cortical astrocytes from Ucp2$^{KI}$ and Ucp2$^{KI}$; GFAP-creER$^{T2}$ mouse pups and tested the hypothesis that Ucp2 overexpression will decrease $\Psi_m$ and the generation of

![Figure 3.11](image_url)

Figure 3.11. Increased Ucp2 Decreases $\Psi_m$ and ROS (A) TMRE fluorescence ($\Psi_m$, n=5-7) or (B) JC-1 fluorescence ratio (n=4) in normal or Ucp2 overexpressing primary cortical astrocytes, with FCCP-treated controls. (C) The relative rate of increase in MitoSox fluorescence (n=3-4) in primary cortical astrocytes from Ucp2$^{KI}$ and Ucp2$^{KI}$; Gfap-creER$^{T2}$ pups. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.
ROS. We measured $\Psi_m$ with the vital dye TMRE, which accumulates in mitochondria proportionally with their potential $Ucp2$ knock-in depolarizes $\Psi_m$ to 72±7% of control levels (p=0.0095, Fig. 3.11A), with 10 µM FCCP decreasing TMRE fluorescence to 54±5% of controls (p=0.0002). This finding was confirmed using the ratiometric probe JC-1, which equilibrates in mitochondria as red fluorescent aggregates and outside as green fluorescent monomers. The ratio of aggregate/monomers is proportional to $\Psi_m$, which we found to be lower in 4-hydroxytamoxifen (4-OHT) pretreated $Ucp2^{KI}; GFAP-creER^{T2}$ astrocytes, which should express $Ucp2$ at greater levels, compared to untreated controls (p<0.05, n=4, Fig. 3.11B). Ucp2 overexpression also decreased the production of ROS, monitored by the change in MitoSox fluorescence over time and normalized to the mean fluorescent intensity of $Ucp2^{KI}$ control samples (p=0.043; Fig. 3.11C). Together, these data show that increased $Ucp2$ expression decreases $\Psi_m$ and mitochondrial ROS, which may be similar in mechanism to the protective effects promoted by 10 nM FCCP.

III.XII. Ucp2 Overexpression In Thy1+ Neurons, But Not Gfap+ Glia Protects RGCs From Death Following Microbead Injection

We injected microbeads or PBS into the anterior chambers of these mice, elevating IOP by an average of 5.3 mmHg in $Ucp2^{KI}$ control mice, 2.4 mmHg in $Ucp2^{KI}; GFAP-creER^{T2}$ mice, and 7.5 mmHg in $Ucp2^{KI}; Thy1-creER^{T2}$ mice (Fig. 3.12A). Bead injection in control mice caused a significant loss in RGCs (1018±88 cells/mm$^2$ for a 19±3% reduction in RGCs; n=11) that was attenuated in mice overexpressing $Ucp2$ in RGCs ($Ucp2^{KI}; Thy1-creER^{T2}$, 492±175 cells/mm$^2$ for a 10±4% reduction; n=9), but not in $Ucp2^{KI}; GFAP-creER^{T2}$ mice (824±225 cells/mm$^2$ for a 15±4% reduction, n=6, Fig. 3.12B-C). These data demonstrate that $Ucp2$ decreases RGC loss due to elevated IOP over a sub-acute timeframe, and also that the beneficial effects of $Ucp2$ are cell autonomous, as $Ucp2$-overexpression in GFAP-positive glia is insufficient to decrease glaucoma-related RGC loss (Fig. 3.12B-C).
Figure 3.12. Increased Ucp2 Expression in RGCs but not Astrocytes and Müller Glia Decreases Retinal Ganglion Cell Loss. (A) Difference IOP between bead- and PBS-injected eyes before and following intraocular surgery. Beads injection elevated IOP over a 30 day period in Ucp2<sup>KI</sup> (n=11), Ucp2<sup>KI</sup>; Gfap-creER<sup>T2</sup> (n=6), Ucp2<sup>KI</sup>; Thy1-creER<sup>T2</sup> (n=9) eyes. (B) RGC loss in retinas in retinal whole-mounts from Bead- and PBS-injected eyes of the indicated genotypes. (C) Representative images of retinal whole-mounts labeled for RBPMS, quantified in (B). *p<0.05.
III.XIII. Ucp2 Overexpression Selectively Decreases Oxidative Damage In Thy1+ Neurons, But Not Gfap+ Glia

The protective effects of Ucp2 expression coincided with decreases in oxidative protein carbonylation, measured by OxyIHC labeling. Bead-injected retinas from Ucp2\textsuperscript{KI}; Thy1-cre\textsubscript{ER}\textsuperscript{T2} mice (n=4) were labeled 27±6% less strongly than corresponding Ucp2\textsuperscript{KI} controls (p<0.05, n=7). In contrast, labeling of Ucp2\textsuperscript{KI}; GFAP-cre\textsubscript{ER}\textsuperscript{T2} retinas was nonsignificantly reduced by 11±11% relative to controls (n=3, Fig. 3.13A-B).

Figure 3.13. Increased Ucp2 Expression in RGCs but not Astrocytes and Müller Glia Decreases Oxidative Stress. (A) Representative labeling of fixed retinas from bead-injected Ucp2\textsuperscript{KI} (n=7), Ucp2\textsuperscript{KI}; Gfap-cre\textsubscript{ER}\textsuperscript{T2} (n=3), and Ucp2\textsuperscript{KI}; Thy1-cre\textsubscript{ER}\textsuperscript{T2} (n=4) eyes for oxidative stress-derived protein carbonylation. The right panels contain close-ups of somata in the GCL, likely RGCs. A single section was labeled without using the 2’,4’-dinitrophenylhydrazine derivitization reagent to confirm the specificity of the primary antibody to towards dinitrophenylhydrazones. The intensity of DAB labeling is quantified in (B). *p<0.05
III.XIV. Rosiglitazone Treatment Increases Ucp2 Transcription But Does Not Prevent RGC Loss Following Bead Injection

Ucp2 transcription is in part regulated by a PGC1-α/PPAR-γ axis (Chen et al., 2006; Donadelli et al., 2014). Rosiglitazone (RSG) is an FDA-approved PPAR-γ agonist. We confirmed that retinal Ucp2 expression increases 24 hours following an IP injection of 10 mg/kg rosiglitazone (p<0.05, Fig. 3.14A), and hypothesized that due to transcriptional

Figure 3.14. Rosiglitazone Increases Ucp2 transcription but does not Alter Glaucomatous RGC loss. (A) Microbead injection increases IOP to a similar extent in Control-(n=3) and rosiglitazone (RSG)-fed mice (n=4). (B) Intraperitoneal RSG increases Ucp2 transcript levels in the mouse retina (n=3) (C) increased IOP leads to RGC loss, though dietary RSG does not appear to alter RGC loss. (D) Representative RBPMS labeling in whole-mount retinas from PBS- and Bead-injected WT mice. *p<0.05
activation of Ucp2, dietary rosiglitazone would confer the same resistance to damage in glaucoma as transgenic Ucp2 overexpression. To test this hypothesis, we increased IOP in control- and RSG-fed WT mice (Fig. 3.14B) and measured RGC loss 30 days following bead injection. The difference in RGC density between the PBS- and bead-injected eyes in WT control mice was 569±170 cells/mm² (n=3), compared to 961±210 cells/mm² in RSG-fed mice (n=4; 3.14C-D). The degree of cell loss was generally lower than in Ucp2<sup>KI</sup> controls, which can be explained by the more advanced age of these mice (3.8 months), which has been demonstrated to reduce the effectiveness of RGC loss following bead injection (Cone et al., 2010). Regardless, the result of this pilot study on the effects of RSG ran contrary to our expectations and did not decrease retinal ganglion cell death, and in these pilot data trended towards increasing RGC loss (Fig. 3.14C-D).

Conclusions

Similar to what was found in isolated mitochondria by (Korshunov et al., 1997; Miwa et al., 2003), decreases in Ψ<sub>m</sub> also decrease ROS production following mitochondrial dysfunction in cells (Fig. 3.8). Since we show that glaucomatous tissue is oxidatively stressed (Fig. 3.6-7), we reached the hypothesis that partial dissipation of mitochondrial Ψ<sub>m</sub> may decrease oxidative stress by decreasing the generation of mitochondrial ROS in glaucoma. This anti-oxidative role for UCP2 is backed by several studies (Diano et al., 2003; Lapp et al., 2014), and in those data as well as our own, increasing Ucp2 expression is negatively correlated with Ψ<sub>m</sub> and mitochondrial ROS (Fig. 3.11). Ucp2 expression appears to increase with increasing IOP (Fig. 3.9), which suggested to us the possibility that microbead-induced IOP elevations cause compensatory increases in Ucp2, as they do with other antioxidant systems (Chidlow et al., 2017). We do however note our re-analysis of publically available data generated by (Howell et al., 2011), who performed a microarray to analyze gene expression in the DBA2/J mouse retina, and found an initial increase and gradual decrease in Ucp2 expression that correlated with glaucoma severity (Appendix, Fig. B1). We do suggest however that decreases in Ucp2 with increasing glaucoma severity may be a reflection of the reduction in ganglion cell number. Regardless, it is clear that if elevated Ucp2 levels are neuroprotective, than normally during glaucoma, that elevation is either insufficient in
magnitude, too transient, or occurs at the wrong time. IOP is different at different times of day (Appendix, Fig. C1), and perhaps the increase in Ucp2 is sufficient to protect against the damaging effects of elevated IOP during the daytime, but not at night when mouse IOP peaks.

We tested the hypothesis that Ucp2 is protective, and unlike in previous studies, our model of neurodegeneration was not a transient stressor (Diano et al., 2003; Mattiasson et al., 2003; Andrews et al., 2005; Barnstable et al., 2016), but a sustained increase in IOP (Huang et al., 2018). The data show that when overexpressed in Thy1+ retinal ganglion cells, Ucp2 is protective against death and associated with decreasing levels of oxidative damage (Fig. 3.12). Our data also show that Ucp2-mediated neuroprotection is dependent on cell type, as increased Ucp2 levels in Gfap-expressing glia does not significantly alter retinal ganglion cell loss or oxidative stress-derive protein carbonyls compared to controls (Fig. 3.12-13). A much larger sample size may be sufficient to demonstrate a neuroprotective effect, supported by a trend towards decreased oxidative stress and RGC loss in Ucp2KI, GFAP-creERT2 mice, but overall the data argue for much weaker if any Ucp2-mediated neuroprotection from glial cells compared to RGCs. This seems to suggest that changes in mitochondrial dynamics within Gfap-expressing glia of the retina may not be central for the progression of glaucoma or the amelioration of that progression, an unexpected result given the many changes they undergo over the course of the disease (Woldemussie et al., 2004) and the protection they give to RGCs (Kawasaki et al., 2000). An alternative possibility to explain why Müller and astroglial Ucp2 overexpression is not protective may be that the increase in Ucp2 occurs to a lesser extent than Thy1-creERT2-dependent Ucp2 overexpression in RGCs. In each case however, the cell-type specific promotor determines the localization of creERT2 and not the genetic elements upstream and downstream of the transgenic Ucp2 cDNA, which are identical for all transgenic mice used in this chapter. The true test of this would be to determine Ucp2 expression in each of these cell types isolated directly from the retina using a cell sorting technique that minimally damages RGC axons and Müller cell radial processes.

Rosiglitazone is a PPAR-γ-dependent transcriptional activator of Ucp2 (Medvedev et al., 2001; Chen et al., 2006) in retinal tissue (Fig. 3.14A), though in vivo, the dose of RSG we used does not seem to promote Ucp2 mediated neuroprotection in the microbead model of
glaucoma. PPAR-γ appears to be expressed with high specificity in Müller glia cells of the rodent retina (Zhu et al., 2013), and while the failure of rosiglitazone to protect RGCs was initially surprising, it likely increases the transcription of glial \textit{Ucp2}. \textit{Ucp2} overexpression in \textit{Gfap}-expressing glia failed to protect RGCs, so our experiments using RSG-fed and \textit{Ucp2} \textit{KI}; \textit{GFAP-creER}\textsuperscript{T2} mice largely support each other. PPAR-γ agonism with pioglitazone is sufficient to decrease RGC loss following optic nerve crush in rats (Zhu et al., 2013) or retinal ischemia/reperfusion injury in mice (Zhang et al., 2017), suggesting that glial \textit{Ucp2} expression may be protective against more acute retinal insults. Alternatively, our study only uses a single dose of RSG and does not show a dose response relationship between either RSG concentration and any known RSG-dependent genes or between RSG concentration and RGC death, which may be critical in order to determine whether the dose of RSG we use is ineffective because it is too low and thus stimulating an increase in \textit{Ucp2} to an insufficient extent.

Notably, increases in neural PPAR-γ are possible following optic nerve crush (Zhu et al., 2013), which would allow for RSG-stimulated \textit{Ucp2} mRNA expression. Even so, endogenous \textit{Ucp2} is likely subject to multiple post-transcriptional regulatory mechanisms (Donadelli et al., 2014), unlike the \textit{Ucp2} derived from our transgenic mice, which lacks these regulatory regions (Toda et al., 2016). A larger study of \textit{Ucp2} and PPAR-γ in retinal disease that uses multiple models and agonists/antagonists may yield a clearer picture that captures the cell type specific dynamics of these factors, and changes during different paradigms of retinal damage.

Overall, these data suggests that stimulating an increase in RGC \textit{Ucp2} expression decreases RGC loss. To elicit activity of this neuroprotective molecule in vivo, the field should focus efforts on determining which factors regulate \textit{Ucp2} transcription as well as translation and functional activity in neurons.
Chapter 4. *Ucp2* Deletion in Either Retinal Ganglion Cells or Astrocytes and Müller Glia is Neuroprotective

*This chapter contains data from the submitted manuscript: “Mitochondrial Uncoupling Protein 2 Knockout Promotes Mitophagy to Decrease Retinal Ganglion Cell Death in Glaucoma”.*

IV.I. *Ucp2* deletion Increases Mitochondrial Membrane Potential ($\Psi_m$) and Oxidative Stress

When artificially increased, *Ucp2* decreases $\Psi_m$ and oxidative stress, so we predicted the opposite, that *Ucp2* deletion would increase $\Psi_m$ and oxidative stress. We deleted *Ucp2* selectively in *Thy1*-expressing RGCs or *Gfap*-expressing astrocytes and müller glia using the same creER$^{T2}$ expressing transgenic mice illustrated in Fig. 3.10. However, these cre mice were bred with *Ucp2*$^{\beta\beta}$ mice with LoxP sites flanking exons 3 and 4 of the endogenous *Ucp2* gene (pictured in Fig. 4.1A). As before, *Ucp2*$^{\alpha\beta}$ lacking any cre transgene were treated as controls. In 4-OHT pretreated primary cortical astrocytes from *Ucp2*$^{\alpha\beta}$ and *Ucp2*$^{\beta\beta}$; GFAP-creER$^{T2}$ mouse pups, we amplified a PCR product specific to *Ucp2*-deleted cells (*Ucp2*Δ), shown in Fig. 4.1B. Following tamoxifen injections in vivo, we confirmed cre-mediated *Ucp2* deletion by measuring retinal *Ucp2* transcript, which in *Ucp2*$^{\alpha\beta}$; GFAP-creER$^{T2}$ and *Ucp2*$^{\beta\beta}$; *Thy1*-creER$^{T2}$ mice decreased to 54±14% and 58±13% of levels found in *Ucp2*$^{\beta\beta}$ controls (Fig. 4.1C). This extent of decrease in *Ucp2* was close to the expected value, as it appears to be mainly expressed within cells of the inner retina (Fig. 3.9C).

To investigate the effects of *Ucp2* deletion on cellular function, we used primary cortical astrocytes cells from *Ucp2*$^{\beta\beta}$ and *Ucp2*$^{\alpha\beta}$; GFAP-creER$^{T2}$ mice. We first determined the effect of *Ucp2* on mitochondrial transmembrane potential ($\Psi_m$), oxidative stress, and cell respiration using these astrocytes. We measured $\Psi_m$ with the vital dye JC-1, which equilibrates in mitochondria as red fluorescent aggregates and outside as green fluorescent monomers. The ratio of aggregate/monomers is proportional to $\Psi_m$, and we found that *Ucp2* deletion (n=7) increased $\Psi_m$ to 120±7% of *Ucp2*$^{\alpha\beta}$ (n=7) cells (p=0.013, df=15). As a control, we applied the membrane-permeant protonophore FCCP (1 µM, n=3), which reduced $\Psi_m$ to 72±4% of controls (p=0.013, df=15, Fig. 4.2A).
Figure 4.1. Cell type-specific *Ucp2* Deletion in Mice. (A) Schematic diagram of transgenic mouse genotypes for mice used in this study, the primer locations for genotyping these animals, the region of DNA excised by Cre recombinase activity (B) Example genotyping PCR of 4-hydroxytamoxifen pretreated astrocytes from *Ucp2<sup>fl/fl</sup>* (lanes 1-2) and *Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> (lanes 3-4) mice. The primers in this reaction only amplify when the >1000bp LoxP-flanked region of the *Ucp2* gene is removed. (C) *Ucp2* expression is reduced to roughly 50% of controls in the retinas of *creER<sup>T2</sup>* expressing animals, suggesting that *Ucp2* is mainly expressed in RGCs and in *Gfap*-expressing glia, at roughly the same levels.
We independently confirmed this Ucp2-deletion-dependent increase in \( \Psi_m \) using another membrane permeant potentiometric dye, TMRE. Similarly to JC-1, TMRE showed a significant increase in \( \Psi_m \) with glial Ucp2 deletion (n=4-5, Fig. 4.2B), and a decrease with Ucp2 deletion increases \( \Psi_m \), ROS, and mitochondrial respiration. \( \Psi_m \) was determined using (A) the ratiometric and potentiometric vital dye JC-1 (n=7-8), or (B) the potentiometric dye TMRE (n=4-5) in primary cortical astrocytes from biologically distinct Ucp2\(^{fl/fl}\) or Ucp2\(^{fl/fl}\); GFAP-creER\(^{T2}\) mice. Treatment with 1 \( \mu M \) FCCP (n=3) was used as an assay control. (C) In the same cell types, the rate of CM-H\(_2\)-DCFDA oxidation is also increased by Ucp2 deletion (n=7) or with 4 \( \mu M \) Antimycin A (n=5). (D) Seahorse XF96 measurement of cellular oxygen consumption demonstrating that Ucp2 deletion increases basal and maximal mitochondrial oxygen consumption rate (OCR) in primary astrocytes, notably Ucp2 deletion did not alter uncoupled respiration (n=3). *p<0.05, **p<0.005.

Figure 4.2. Ucp2 Deletion Increases \( \Psi_m \), ROS, and Mitochondrial Respiration.
exposure to 1 µM FCCP (n=2). Oxidative stress was measured using CM-H$_2$DCFDA, a cell permeant probe that becomes fluorescent upon oxidation, and we measured the rate ($\Delta F/F_0$) of probe oxidation over 30 minutes. Relative to $Ucp2^{fl/fl}$ controls, $Ucp2$ deletion increased oxidative stress to 133±12% (p=0.0452, df=16, n=7). As a control to verify assay function, $Ucp2^{fl/fl}$ cells were treated with 4 µM Antimycin A, which non-significantly increased oxidative stress to 122±10% of controls (p=0.13, df=16, n=5, Fig 4.2C). We measured oxygen consumption rate (OCR) using a seahorse extracellular flux assay in $Ucp2^{fl/fl}$ and $Ucp2^{fl/fl}; GFAP-creER^{T2}$ astrocytes. Following each assay we normalized respiratory activity to DNA concentration. Basal respiration (untreated) over time was greater in $Ucp2^{fl/fl}; GFAP-creER^{T2}$ cells (p<0.0001, df=1, n=3), as was maximal respiration (p=0.0004, df=1, n=3). Basal respiration is a measure of normal mitochondrial function, and maximal respiration corresponds reflects the ability of mitochondria to meet a high energy demand by oxidizing citric acid cycle metabolites and consuming oxygen at a greater rate, not limited by the transmembrane proton gradient. Together the data suggested that $Ucp2$ deletion unexpectedly enhanced mitochondrial function in cells (Fig. 4.2D).

IV.II. Ucp2 Deletion Prevents RGC Loss Following Microbead Injection

We injected beads in to mice with $Ucp2$ selectively deleted in Gfap- or Thy1-expressing cells. On average, bead injection increased mean IOP by 9.5±1.5 mmHg in $Ucp2^{fl/fl}$ mice, 3.1±0.7 mmHg in $Ucp2^{fl/fl}; GFAP-creER^{T2}$ mice, and 8.3±1.7 mmHg in $Ucp2^{fl/fl}; Thy1-creER^{T2}$ mice (Fig. 4.3A). In addition to our own data (Fig. 3.3A) others have demonstrated that the extent of IOP increase in the microbead model does not always correlate well with the extent of RGC loss (Cone et al., 2010), so differences in the extent of IOP increase between $Ucp2^{fl/fl}$, $Ucp2^{fl/fl}; Thy1-creER^{T2}$, and $Ucp2^{fl/fl}; GFAP-creER^{T2}$ mice should not significantly alter the extent of retinal ganglion cell death.
To determine the effect of \( \text{Ucp2} \) deficiency on glaucomatous RGC death, we counted the density of RBPMS+ RGCs on whole-mount retinas from \( \text{Ucp2}^{\text{fl/fl}}, \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{\text{T2}} \) and \( \text{Ucp2}^{\text{fl/fl}}, \text{Thy1-creER}^{\text{T2}} \) retinas (Fig. 4.3B-C). We determined the effect of glaucoma on RGC loss, and found a significant decrease in bead-induced RGC loss between

![Image](image_url)

**Figure 4.3. Ucp2 Deletion Protects Against Glaucoma** (A) IOP difference between microbead and PBS-injected \( \text{Ucp2}^{\text{fl/fl}}, \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{\text{T2}} \), and \( \text{Ucp2}^{\text{fl/fl}}, \text{Thy1-creER}^{\text{T2}} \) eyes over 30 days following a single microbead injection. (B) Example RBPMS-labeled RGCs in PBS- and Bead-injected eyes from \( \text{Ucp2}^{\text{fl/fl}} \) (n=29), \( \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{\text{T2}} \) (n=10), and \( \text{Ucp2}^{\text{fl/fl}}; \text{Thy1-creER}^{\text{T2}} \) (n=13). Scale bar (red) = 100 µm. Differences in RGC density between PBS- and Bead-injected eyes are quantified in (C). A difference of “0” indicated no retinal ganglion cell death. **p<0.01.
Ucp2<sup>fl/fl</sup> control retinas (607±148 cells/mm<sup>2</sup> in, df=49, n=29), Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> (79±195 cells/mm<sup>2</sup>, p=0.04, n=11), and Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> retinas (-169±151 cells/mm<sup>2</sup>, p=0.0039, n=13, Fig. 4.3B). These data suggest that surprisingly, reduced retinal Ucp2 levels decreased RGC loss following IOP elevation.

To mitigate concerns that the lesser extent of IOP elevation in Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> mice is the reason that fewer RGCs are lost following bead injection, we analyzed RGC loss in a subset of bead-injected mice chosen such that mean IOP increases were close to those of Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> mice and <0.1 mmHg different between all groups. In this subset, Ucp2-dependent differences in RGC loss persisted, with a mean RGC loss was 695 cells/mm<sup>2</sup> in Ucp2<sup>fl/fl</sup> controls (n=9), -79 cells/mm<sup>2</sup> in Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> mice (p=0.04, n=11), and -56 cells/mm<sup>2</sup> in Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> mice (p=0.04, n=7). RGC loss in these subgroups was similar to the larger sample of mice, and these data suggest that the protective effect of Ucp2 governs RGC loss to a greater extent than does the degree of IOP elevation.

**IV.III. Reduced buildup of oxidative stress and retinal gliosis in the retinas of Ucp2-deficient mice**

To determine the effect of Ucp2 on retinal oxidative stress, we measured HNE immunoreactivity in retinas of bead-injected eyes from Ucp2<sup>fl/fl</sup>, Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup>, and Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> mice (Fig. 4.4A-B). Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> (n=8) and Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> (n=7) accumulated significantly less HNE (p=0.02 and 0.03 respectively, df=20) than Ucp2<sup>fl/fl</sup> controls (n=8), providing a quantitative proxy for the qualitative finding that the inner retina was less oxidatively damaged (Fig. 4.4B).
Increases in retinal GFAP are a sign of müller cell reactivity, and in Ucp2fl/fl mice, bead injection increased GFAP intensity to 239% of control (p=0.0085, df=61, n=13-23). In Ucp2fl/fl; GFAP-creER<sup>T2</sup> (n=7), and Ucp2fl/fl; Thy1-creER<sup>T2</sup> eyes (n=8). Scale bar=30 µm. *p<0.05.

Increases in retinal GFAP are a sign of müller cell reactivity, and in Ucp2fl/fl mice, bead injection increased GFAP intensity to 239% of control (p=0.0085, df=61, n=13-23). In Ucp2fl/fl; GFAP-creER<sup>T2</sup> or Ucp2fl/fl; Thy1-creER<sup>T2</sup>, bead injection did not significantly increase our semi-quantitative measure of GFAP labeling intensity, though there was a trend toward increase for each genotype (Fig. 4.5A-B). This is visualized in representative retinal sections of bead-injected transgenic mice, within which Gfap+ müller cell fibers are detectable but reduced in overall intensity relative to controls (Fig 4.5A). Neuroprotection of RGCs in transgenic mice was associated with a decrease in HNE labeling intensity, and we

Figure 4.4. Ucp2 deletion in glia or retinal ganglion cells decreases retinal oxidative stress. (A) Representative 4-hydroxynonenal (green) and Hoechst33258 (blue)-labeled images and (B) quantification of 4-hydroxynonenal labeling in the inner retinas of bead-injected Ucp2fl/fl (n=8), Ucp2fl/fl; GFAP-creER<sup>T2</sup> (n=7), and Ucp2fl/fl; Thy1-creER<sup>T2</sup> eyes (n=8). Scale bar=30 µm. *p<0.05.
hypothesized that Ucp2 deletion altered mitochondrial dynamics or physiology in such a way as to reduce mitochondrial oxidant production.

**IV. IV. Ucp2 Deletion Alters Mitochondrial Dynamics In Cortical Astrocytes**

We labeled untreated (n=5) and 4-hydroxytamoxifen (1 µM)-pretreated (n=4) primary astrocytes from Ucp2<sup>fl/fl</sup>; GFAP-cre<sup>T2</sup> mice for the translocase of the outer mitochondrial membrane (TOMM20) to visualize mitochondrial dynamics (Fig. 4.6A). We quantified mean mitochondrial size, number, mass, and network size using the ImageJ plugin MiNA V1 (Valente et al., 2017). There was a non-significant trend towards decrease in overall mitochondrial mass per cell (p=0.31, df=7, Fig. 4.6B), a significant decrease in the number of mitochondria/cell (p=0.035, df=7, Fig. 4.6C), and an increase in mean area (p=0.0061, df=7, Fig. 4.6D). The overall number of mitochondrial networks/cell was unaltered. Together the images of these mitochondria and the quantified data suggest a slight increase in the number
Figure 4.6. *Ucp2* Deletion Does Not Alter Mitochondrial Biogenesis and Increases Astrocyte Mitophagy. (A) Contrast-enhanced and auto-segmented images of Hoechst-33258 (blue)- and TOMM20 (white)-labeled mitochondria in control and 4-hydroxytamoxifen pretreated *Ucp2<sup>fl/fl</sup>; GFAP-creERT<sup>2</sup> primary cortical astrocytes (B-E) Quantification of mitochondrial morphological characteristics through measurement of (B) Overall area per cell (mitochondrial mass, µm<sup>2</sup>), (C) the number of mitochondria per cell, and (D) mean area per mitochondrion (µm<sup>2</sup>) (n=5 biological replicates, derived from different mice with ≥6 cells per biological replicate). Scale bar = 32 µm. (E) Expression of genes associated with mitochondrial biogenesis and function in cultured astrocytes, showing that *Ucp2* deletion does not increase factors that regulate biogenesis, but does decrease the expression of *Sod2* and *cytochrome B* (n=4). *p<0.05*
of more fused mitochondria under normal cell culture conditions (Fig. 4.6A).

A healthy pool of mitochondria is maintained through a balance of mitochondrial autophagy (mitophagy) and biogenesis (Piantadosi et al., 2008; Uittenbogaard and Chiaramello, 2014; Ito and Di Polo, 2017). The decrease in mitochondrial number could result from a decline in mitochondrial biogenesis, or an increase in the disposal of damaged mitochondria. We tested the first of these hypotheses by determining the expression of three genes involved in mitochondrial biogenesis, PolgA, Tfam, and PGC1-α. These genes were not significantly altered by Ucp2 deletion in primary Ucp2<sup>fl/fl</sup>; GFAP-creER<sup>T2</sup> astrocytes, though the expression of nuclear and mitochondrial genes associated with mitochondrial function (CytB, Sod2) were significantly decreased (p=0.037 and 0.024 respectively, df=27, n=3-4, Fig. 4.6E), which is indicative of a decrease in the transcription of a portion of mitochondrial genes.

We next tested whether the decrease in mitochondrial number and increase in size resulted from activation of mitophagy. We measured the subcellular distribution of the autophagosome adapter protein LC3B and the mitochondrial outer membrane marker TOMM20 in primary cultures of cortical astrocytes (Fig. 4.7A). Using the Coloc2 plugin on ImageJ, we determined the Manders’ overlap coefficient corresponding to the proportion of co-localized (Lc3b+ TOMM20+) pixels over TOMM20+ pixels (tM2). Ucp2 deletion significantly increased tM2 from 0.044±0.019 to 0.128±0.024 (p=0.033, df=6, n=3-4, Fig. 4.7B). tM2 also increased to a greater extent in Ucp2<sup>β/β</sup> astrocytes treated with 10 µM FCCP for 3h, a positive control treatment known to reproducibly induce mitochondrial fragmentation and mitophagy (0.246±0.074).
Figure 4.7. Increased Cellular Mitophagy with \(Ucp2\) Deletion. Representative images (A) and quantification of colocalization (B) between LC3B (green) and TOMM20 (red) in \(Ucp2^{0/0}\) (n=5) and \(Ucp2^{0/0}; \text{Gfap-creER}^{T2}\) astrocytes (n=3), showing a significant increase in colocalization with \(Ucp2\) deletion. Cell identity was confirmed using the astrocyte marker GFAP (white) and the DNA label Hoechst-3328 (blue). *\(p<0.05\)
To recapitulate these findings in tissue, we reevaluated mitochondrial biogenesis in retinal tissue from \( \text{Ucp}^2 \text{fl/fl}, \ \text{GFAP-creER}^{T2}, \ \text{Thy1-creER}^{T2} \) mice, by measuring the gene expression of \( \text{PolgA}, \ \text{Tfam}, \ \text{PGC1-\alpha} \). Similar to our findings in primary cortical astrocytes, we found no differences in the expression of these factors (Fig. 4.8A). We also measured the expression of several genes related to mitophagy in the retinas.
of bead- and PBS-injected transgenic mice (Fig. 4.8B, n=3/group). We observed genotype- and condition-specific increases in the expression of Pink1, Park2, Bnip3L, and Lc3b. Specifically, there was an overall significant increase in Pink1 expression in Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub> mice regardless of bead injection (p=0.038), a general increase in Park2 expression in Ucp2<sup>β/β</sup>; GFAP-creERT<sub>2</sub> mice, which was only significant following bead injection (p=0.016). Bnip3l was non-significantly elevated in Ucp2<sup>β/β</sup>; GFAP-creERT<sub>2</sub> retinas, but only with PBS-injection (p=0.087). Conversely, Bnip3l expression was significantly elevated in the retinas from Bead-injected Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub> mice but not in PBS-injected controls (p=0.039). Lc3b expression significantly increased in bead-injected Ucp2<sup>β/β</sup>; GFAP-creERT<sub>2</sub> retinas (p=0.02) and reached a near significant increase in Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub> retinas (p=0.058). The expression results suggest that there is an increase in retinal mitophagy following Ucp2-deletion, but the specific cell type and environment from which Ucp2 is deleted will dictate which components of mitophagy machinery are altered.

We measured protein levels of both nonlipidated (LC3B-I) and lipidated (LC3B-II) forms of LC3B (Fig. 4.9A), and found a significant increase in LC3B-II within both PBS and bead-injected retinas Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub> retinas (p=0.029 and 0.0015, respectively, df=15, n=3-4/group, Fig. 4.9B). We also noticed an increase in total LC3B levels in the PBS- and bead-injected retinas of both Ucp2<sup>β/β</sup>; GFAP-creERT<sub>2</sub> (p=0.0002 and 0.014 respectively, df=15, n=3-4) and Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub> (p=0.021 and 0.005 respectively, df=15, n=3-4) mice (Fig. 4.9C). When we studied the effect of Ucp2-deletion on BNIP3L (Fig. 4.9D), we found a significant increase in BNIP3L within both transgenic strains (p=0.033, df=14, n=3-4 for Ucp2<sup>β/β</sup>; GFAP-creERT<sub>2</sub> and p=0.033, df=14, n=3-4 for Ucp2<sup>β/β</sup>; Thy1-creERT<sub>2</sub>), but this increase was exclusive to PBS-injected control eyes (Fig. 4.9E). Notably, the most clearly defined bands in BNIP3L blots migrated at 76 kDa and 100 kDa, respectively corresponding to the BNIP3L dimer (Chen et al., 2010) and a nonspecific band. Analysis of protein levels was performed exclusively on the 76 kDa band, though analysis of the whole lane below 100 kDa yielded almost identical relative protein density values.
Figure 4.9. Ucp2 deletion increases LC3B protein and retinal mitophagy (A,D) Western blot of protein extracts from PBS-injected Ucp2<sup>fl/fl</sup> (black bars, n=3-4/group), Ucp2<sup>fl/fl; GFAP-creER<sup>T2</sup></sup> (red bars, n=3/group) and Ucp2<sup>fl/fl; Thy1-creER<sup>T2</sup></sup> (blue bars, n=3-4/group) retinas, labeled for LC3B-I and LC3B-II (A) as well as BNIP3L/NIX (D). Corresponding quantification of protein levels for both PBS- and Bead-injected retinas are shown in (B-C,E). (B) LC3B-II was significantly elevated in both PBS- and bead-injected Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> retinas. (C) Total LC3B levels were significantly elevated in PBS- and bead-injected retinas from both Ucp2<sup>fl/fl</sup>; Thy1-creER<sup>T2</sup> and Ucp2<sup>fl/fl; GFAP-creER<sup>T2</sup></sup> mice. (E) BNIP3L/NIX was significantly elevated in PBS injected retinas of both cre-expressing transgenic mice, but not with bead injection. *p<0.05, **p<0.01, ****p<0.001.
The RNA and protein expression data yield differing accounts of the molecular events that occur following neural or glial Ucp2 deletion, and we wanted to determine whether these expression changes led to an increase in autophagosomes in retinal tissue. Within this tissue, mitochondria are too dense for an accurate colocalization analysis of a mitochondrial marker with Lc3b, so we determined the density of autophagosomes in retinas from bead-injected Ucp2\(^{+/+}\), Ucp2\(^{0/0}\), GFAP-creER\(^{T2}\) (n=3), and Ucp2\(^{0/0}\), Thy1-creER\(^{T2}\) (n=3) mice. Retinal tissue from Ucp2-deleted mice generally labeled more strongly than Ucp2\(^{0/0}\) tissue, which is consistent with an increase in total LC3B protein. In addition to the increase in

Figure 4.10. Layer Specific Ucp2-deletion dependent retinal autophagy. We labeled LC3B (green) and DNA (Hoechst-33258, blue) in retinal tissue from Ucp2\(^{0/0}\) (n=4), Ucp2\(^{0/0}\), GFAP-creER\(^{T2}\) (n=3), and Ucp2\(^{0/0}\), Thy1-creER\(^{T2}\) (n=3) mice. Example images are on the left, and quantifications of LC3B puncta density are on the right. Compared to the same regions of Ucp2\(^{0/0}\) retinas, LC3B puncta significantly increase in the outer retinas of Ucp2\(^{0/0}\), GFAP-creER\(^{T2}\), and in the inner retinas of Ucp2\(^{0/0}\); Thy1-creER\(^{T2}\) mice. *p<0.05.
background LC3B levels, bead-injected transgenic retinal tissue had significantly more Lc3b puncta relative to bead-injected Ucp2^{fl/fl} controls. The regional distribution of the increase in these puncta was unique for each cre variant. The density of LC3B+ autophagosomes increased to a greater extent within the outer retina in Ucp2^{fl/fl}; GFAP-creERT2 mice (p=0.044, df=5, n=3-4, Fig. 4.10A), though there were trends towards increase in the inner retinas of these mice which may be more clearly resolved with a larger number of replicates. Conversely in bead-injected Ucp2^{fl/fl}; Thy1-creERT2 retinas, the increase in LC3B puncta density was restricted to the inner retina, which contains RGC soma, dendrite, and axon mitochondria (p=0.01, df=4, n=3, Fig. 4.10B).

Conclusions

When ROS production exceeds the capacity for detoxification by antioxidants, they damage cellular components, which is seen in a variety of pathogenic conditions. Mild ROS levels are signals that mitochondria are damaged, and increase mitochondrial autophagy (also known as mitophagy) (Frank et al., 2012) among other physiological events (Angelova and Abramov, 2016). The data show that Ucp2 deletion enhances ψ_m, provoking an increase in ROS (Fig. 4.2).

The increase in ROS stimulated by Ucp2 deletion was associated with changes to mitochondrial morphology in cultured astrocytes. Damaged mitochondria typically undergo fission and are smaller in size (Kim et al., 2007; Frank et al., 2012). We found that Ucp2-deficiency did not result in the extensive mitochondrial fragmentation we expected, but instead increased the average size (Fig. 4.6), implying that the mitochondria inhabiting Ucp2-deleted cells were healthier. This suggestion is corroborated by the increased respiratory capacity of Ucp2-deficient astrocytes (Fig. 4.2). Such a population of ‘healthier’ mitochondria could be sustained by an increase in mitochondrial biogenesis, which can generate new, undamaged mitochondria, as well as by mitophagy (Lemasters, 2005), which selectively disposes of damaged or dysfunctional mitochondria.

We investigated both possibilities and found that transcription of factors associated with mitochondrial biogenesis were unaltered in both cells and retinal tissue. We next measured mitophagy in Ucp2-sufficient and deficient astrocytes, through colocalization of
the mitochondrial outer membrane protein TOMM20 and the autophagosome component LC3B. We found little colocalization between these two in \( \text{Ucp2}^{\text{fl/fl}} \) astrocytes, though this increased after treatment with FCCP, which at high levels and over 3 hours induced mitochondrial fragmentation and colocalization with LC3B. Elevated marker colocalization in \( \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{T2} \) astrocytes was a positive indication of mitophagy (Fig. 4.7). This is the second study we know of that has found an increase in mitophagy with \( \text{Ucp2} \) deletion, with another group finding that vascular endothelial cells lacking \( \text{Ucp2} \) have greater expression of the mitophagy signaling proteins PINK1 and PARKIN, as well as a greater mitochondrial turnover, diagnosed using a tandem fluorescent marker of LC3B turnover (Haslip et al., 2015). Our cellular data were supported by increases in the expression of several mitophagy-related genes in vivo (Fig. 4.8B), though notably these expression profiles did not always correspond with increases in mitophagy or autophagy protein markers.

\( \text{Ucp2} \) deletion in \( \text{Thy1} \)-expressing neurons had a particularly large impact on protein levels of LC3B-II, and it is unclear why the same effect was undetectable in \( \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{T2} \) retinal tissue, particularly given that levels of BNIP3L do increase in this tissue (Fig. 4.9D-E), and the density of LC3B+ autophagosomes also increases in immunolabeled retinal sections (Fig. 4.10A-B). These data however are based on tissues exposed to elevated IOPs for different periods of time. Protein expression data is based on tissue isolated 3 days post-bead injection and LC3B puncta counts were from tissue isolated 30 days post-bead injection, and together the data suggest that while autophagic and mitophagic markers are always elevated in \( \text{Ucp2}^{\text{fl/fl}}; \text{Thy1-creER}^{T2} \) retinas, their expression is likely much more dynamic in \( \text{Ucp2}^{\text{fl/fl}}; \text{GFAP-creER}^{T2} \) retinal tissue. Of note is the finding that total LC3B levels are always elevated, and if the stimulation of mitophagy in Müller glia or astrocytes is dynamic, that dynamism is likely supported by a larger pool of available LC3B protein. By finding protein-level increases in lipitated LC3B (LC3B-II), we show an increase in retinal autophagosomes (Fig. 4.9-10) (Tanida et al., 2004), and by finding greater levels of the mitochondrial LC3B adaptor BNIP3L (also known as NIX), we show that mitochondrial disposal is increased as well (Sandoval et al., 2008; Chen et al., 2010; Novak et al., 2010; Johansen and Lamark, 2011).
Relative to \( Ucp^2-/- \) control retinas, \( Ucp^2 \)-deletion in neurons and glia of \( Ucp^2-/-; GFAP-\text{creER}^{T2} \) and \( Ucp^2-/-; Thy1-\text{creER}^{T2} \) retinas was protective against an increase in retinal gliosis, the accumulation of oxidative damage, and RGC death in the microbead model of glaucoma (Fig. 4-5). This finding is not unprecedented given that \( Ucp^2 \) deletion in mice can also be protective against neural damage due to cerebral ischemia (de Bilbao et al., 2004). It is however surprising, as \( Ucp^2 \) overexpression is neuroprotective in retinas exposed to excitotoxic factors (Barnstable et al., 2016) and the reverse genetic manipulation was predicted to have the opposite effect. The observed increase in mitophagy stimulated by \( Ucp^2 \)-deletion is a likely source of neuroprotection. Mitophagy is already decreased in the glaucomatous mouse optic nerve (Coughlin et al., 2015), and \( Ucp^2 \)-deletion may be protective by re-balancing baseline levels of mitophagy. Alternatively, overexpressing other factors that increase mitophagy can also protect retinal ganglion cells from glaucomatous death, as has been shown in rats with the E3 ubiquitin ligase Parkin (Dai et al., 2018).

Therefore, the RGC-intrinsic increases in mitophagy following \( Ucp^2 \) deletion are probably directly protective, though our investigations in \( Ucp^2-/-; GFAP-\text{creER}^{T2} \) mice lead us to the critical question of how glial mitophagy can protect RGCs from oxidative stress and cell death. Retinal glia are phagocytic (Bejarano-Escobar et al., 2017), and astrocytes of the optic nerve head are able to dispose of damaged RGC mitochondria under physiological conditions (Davis et al., 2014), which leads us to the hypothesis that a \( Ucp^2 \)-deletion dependent increase in glial mitophagy increases the trans-cellular disposal of RGC mitochondria. To determine whether this is the case in future studies; we will need a sophisticated methodology that combines transgenic \( Ucp^2 \) deletion in glia with a probe specifically targeted to retinal ganglion cell mitochondria. In this setup we could determine whether deletion of glial \( Ucp^2 \) increases the density of RGC mitochondria engulfed by glia.

Overall, while the in vivo role of uncoupling proteins appears to be the regulation of ROS production, uncoupling protein inactivity may also work as a regulated or perhaps coincidental trigger of mitophagy (Frank et al., 2012), specifically selecting dysfunctional mitochondria for degradation (Lemasters, 2005; Kim and Lemasters, 2011). This activity likely enriches the mitochondrial population within cells, thus decreasing the burden of
mitochondrial dysfunction and consequent tissue level oxidative stress, proposed to mediate
death in glaucoma.

Although it is well known that mitochondrial autophagy is a well controlled process
that is linked to decreasing membrane potential (Narendra et al., 2008), the physiological
triggers that stimulate it are still not entirely resolved. As a mediator of damage, ROS is one
of these (Frank et al., 2012). While the interaction between $Ucp2$ and ROS is well known, the
role of $Ucp2$-dependent ROS in the control of mitophagy that we have uncovered here is a
novel process that deserves further investigation.
Chapter 5. Overall Conclusions and Discussion

This dissertation is entitled “Ucp2-Dependent Changes in Mitochondrial Dynamics Protect the Retina From Glaucoma”, and our data show that both the membrane potential and autophagic dynamics of mitochondria are shifted with manipulation of Ucp2 levels, in most cases improving retinal ganglion cell survival. Our overall hypothesis was that “Mitochondrial Uncoupling Protein 2 Protects Retinal Ganglion Cells from Death in Glaucoma”, and through this hypothesis we tested whether the observed shifts in mitochondrial dynamics are protective from oxidative damage and retinal ganglion cell death in the microbead model of glaucoma. The data show this to be the case, as bidirectional Ucp2

Figure 5.1. Model for Retinal Neuroprotection through bidirectional manipulations of Ucp2

Hypothesis Supported by Completed Experiment(s)

Supported in scientific literature but not experimentally determined here

#Cell autonomous protection, as astrocytes dispose of neural mitochondria (suggested by Davis et. al., 2014)
expression-dependent changes in $\Psi_m$ and ROS either directly protect RGCs or increase mitochondrial autophagy (mitophagy) to the same end. This hypothetical mechanism of neuroprotection is pictured in Figure 5.1.

The first sub-aim of this dissertation was to test the hypothesis that elevated Ucp2 expression is neuroprotective against retinal ganglion cell loss during chronic neurodegeneration. We addressed this in Chapter 3 and show that artificial increases in Ucp2 decrease mitochondrial membrane potential as well as oxidative stress, which translates to ganglion cell-intrinsic protection over the 30 day period of elevated IOP. Despite being one of many studies to show a protective effect (Diano et al., 2003; Mattiasson et al., 2003; Andrews et al., 2005; Deierborg et al., 2008; Barnstable et al., 2016), the aim is unique in that it demonstrates protection from not only acute, but also more chronic neurodegenerative circumstances. However, despite the simpler view of mitochondrial ROS production presented in the introduction, the positive correlation between $\Psi_m$ and ROS only exists under a unique bioenergetic circumstance known as reverse electron transport (RET), which may or may not be relevant to glaucoma (Korshunov et al., 1997; Shabalina and Nedergaard, 2011).

During RET, the mitochondrial electron carrier coenzyme Q$_{10}$ (CoQ$_{10}$) is sufficiently reduced to (A) saturate activity of the Coenzyme Q: cytochrome C reductase (complex III) and (B) provide electrons from succinate oxidation flow to the NADH: Ubiquinone oxidoreductase (complex I), which then becomes a massive source of superoxide (ROS) production (Robb et al., 2018). This particular form of ROS production occurs at high levels of proton-motive force ($\Delta\rho$), and has been best studied in isolated mitochondria respiring on succinate. The deleterious effects of RET are alleviated by decreases in mitochondrial membrane potential ($\Psi_m$) or pH (Korshunov et al., 1997; Robb et al., 2018), which is why the proposed functional role of Ucp2 as an uncoupler is thought to be protective.

Our data show the dependence of mitochondrial superoxide generation on $\Psi_m$ during an attempt to mimic RET. We did so with 10 nM FCCP as a ‘mild’ uncoupling agent, and stimulated RET with Antimycin A (AA). AA prevents complex III-mediated transport of electrons from CoQ$_{10}$ to cytochrome C and thus increases the pool of reduced CoQ$_{10}$. Although Ježek et al. somewhat unclearly argues that complex III dysfunction cannot cause RET (Ježek et al., 2018), we show that while 10 nM FCCP potentiated rotenone-stimulated
superoxide production, it decreased AA-stimulated superoxide production (Appendix, Fig. D1). This suggests that if *Ucp2* functions as a neuroprotective agent and decreases ROS by virtue of its uncoupling properties, it does so in an environment that at least somewhat favors RET. Unfortunately, some have argued that RET is not at all relevant to mitochondrial physiology in vivo (Shabalina and Nedergaard, 2011). Thankfully for this dissertation, Michael Murphy’s group demonstrated otherwise by showing that RET occurs *in vivo* during ischemia-reperfusion (Chouchani et al., 2014), and is fueled by the buildup of succinate. Mice expressing both mouse and human *Ucp2* variants are protected from neuronal damage following middle cerebral artery or more global ischemia and reperfusion (Mattiasson et al., 2003; Deierborg et al., 2008), although *Ucp2* is sufficient for protection, its protective effects seem to span multiple bioenergetic conditions (Diano et al., 2003; Andrews et al., 2005; Barnstable et al., 2016), and it is unknown whether in glaucoma the retina also suffers from ischemia-reperfusion or downstream RET. Notably, purine nucleotide levels are decreased following optic nerve crush (Sato et al., 2018), which could potentially fuel transient elevations in succinate sufficient for RET (Chouchani et al., 2014), but there is currently insufficient support for this hypothesis. One way to determine whether RET occurs in glaucoma would be to measure either retinal succinate levels or the redox status of retinal CoQ₁₀, both normally and during glaucoma. These readouts of retinal bioenergetic state would determine whether the retina fits the metabolic/redox “profile” of RET. Possibly in favor of the notion that RET does occur in glaucoma, exogenous CoQ₁₀ (1% in the diet) successfully decreases glaucomatous retinal ganglion cell loss (Lee et al., 2014), which perhaps occurs by shifting the redox state of CoQ₁₀ away from being “too reduced”. Regardless this notion would need to be supported by a determination of the redox state with or without dietary CoQ₁₀. Overall, a determination of retinal redox status in health and with glaucoma could be a critical step understanding the mechanism of UCP2 function, but also for a better understanding of the source of ROS in glaucoma, which may dictate the nature of an anti-oxidative therapeutic treatment.

If UCP2-mediated neuroprotection cannot be accounted for by RET, UCP2 function could be explained by a different hypothesis. Although this has yet to be widely adopted, one group has proposed a “metabolic” hypothesis, in which UCP2 is a solute carrier protein that
utilizes $\Delta \rho$ to import $P_i$ and export mitochondrial aspartate, malate, or oxaloacetate (Vozza et al., 2014; Bouillaud et al., 2016). Glucose-derived oxaloacetate export from mitochondria is proposed to decrease the proportion of citric acid cycle intermediates originating from glucose, and shift the metabolic source of these intermediates to glutamine or fatty acids, which can fuel NADH reduction even with decreased oxaloacetate levels. Under these circumstances, UCP2 would catalyze a decrease in ROS by lowering overall citric acid cycle and electron transport activity (Vozza et al., 2014); providing a mechanism of oxidative stress relief that is less dependent on $\Delta \rho$ and that does not require RET.

This hypothesis is somewhat in conflict with our own data, as primary cortical astrocyte oxygen consumption is increased with $Ucp2$ overexpression (Appendix, Fig. E1). If the metabolic hypothesis fully describes UCP2 function, overexpression should instead decrease citric acid cycle activity and consequent oxygen consumption below control levels, though this experimental evidence alone is insufficient to fully disprove the metabolic hypothesis.

Part of the difficulty in determining the mechanism of UCP2 action have been the ubiquity of protein level mechanisms regulating activity. Many of these are determined by cellular redox chemistry, as UCP2 activity appears to be regulated by lipid peroxides (4-hydroxynonenal), mitochondrial matrix superoxide, and modification by glutathione (Echtay et al., 2002; Echtay et al., 2003; Mailloux et al., 2011). Given that superoxide levels are drastically increased during RET, and that UCP2 is stimulated by oxidative stress, the regulatory milieu of RET may favor UCP2 protein activation, as a form of negative feedback to prevent the deleterious build-up of ROS (Mailloux and Harper, 2011). Overall, while we have evidence to support a relationship between $\Psi_m$ and ROS during RET, and know that UCP2 bears structural similarity to the “true” uncoupling protein Ucp1 and can even transport protons, we are still unsure of how UCP2 decreases ROS in vivo.

In chapter 3, we also addressed the hypothesis that increasing glial $Ucp2$ expression will protect RGCs in a non-cell autonomous manner. Ultimately we found that RGC loss was not altered in a statistically significant manner with increasing glial $Ucp2$ expression, suggesting that although both müller glia and astrocytes can each regulate the bioenergetic and oxidative status of ganglion cells (Kawasaki et al., 2000), $Ucp2$ expression in these cells
does not have a strong effect on these particular functions. In support of this finding, rosiglitazone (RSG) treatment also did not appear to have an effect on neuroprotection from glaucoma, conflicting with some previous studies that used very different model systems of optic nerve damage (Zhu et al., 2013; Zhang et al., 2017). Although RSG treatment acutely increased Ucp2 expression, the receptor for rosiglitazone, PPAR-γ, is mainly expressed in glia, and even if Ucp2 expression resulted in translation of a functional protein, elevations in glial Ucp2 still do not appear to benefit RGCs, which will perhaps serve as a warning for those who tout elevated Ucp2 expression as a panacea. These data do not suggest that glial Ucp2 is nonfunctional. In fact when we tested our third major hypothesis, that decreasing Ucp2 expression in a chronic model of neurodegeneration will increase RGC loss, we found that glia did in fact play a major role, as Ucp2 deletion in both neurons or glia protected retinal ganglion cells. This was clearly contrary to our expectations, particularly given the vulnerability of retinal ganglion cells to death from hypoxia (AMES and GURIAN, 1963) or hypoxia generated ROS (Tezel and Yang, 2004). However, we found mounting support for a neuroprotective role for decreases in UCP2 function (de Bilbao et al., 2004; Zhao et al., 2018; Zhong et al., 2018), and we investigated the mechanism of RGC protection.

RGCs are particularly vulnerable to suboptimal mitochondrial function, and this concept is illustrated through the data in Table 9, which details the most common disorders associated with mitochondrial mutations, and shows that they all overlap in some manner with retinal dysfunction, seeming sometimes to be particularly targeted to retinal ganglion cells, possibly due in part to the generation of free radicals by light hitting the inner retina (Del Olmo-Aguado et al., 2016). As we noted in the introduction, mutations in some genes that interact with mitochondrial function are causative of glaucoma. For example, Optineurin and Tbk1 directly interact with the mitochondrial quality control process (Sirohi et al., 2015). Even increases in IOP are sufficient to increase the mutational load of retinal ganglion cell mitochondria (Zhang et al., 2016). What seems to be an unavoidable conclusion is that mitochondrial dysfunction and consequent oxidative stress are central to the pathophysiology of glaucoma and are detected in both animal and human tissue (Izzotti et al., 2003; Moreno et al., 2004; Abu-Amero et al., 2006; Malone and Hernandez, 2007; Feilchenfeld et al., 2008; Lee et al., 2011; Mousa et al., 2015; Khawaja et al., 2016; Chidlow et al., 2017). We have
recapitulated this finding in the microbead model of glaucoma, as have others (Coughlin et al., 2015; Williams et al., 2017; Harun-Or-Rashid et al., 2018).

### Table 10. Mitochondrial Disorders that Affect the Retina.

<table>
<thead>
<tr>
<th>Mitochondrial Disorder</th>
<th>Principal Phenotype</th>
<th>Cell Type or Region Affected</th>
<th>Gene(s) Involved</th>
<th>Polymorphism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kearns-Sayre/Pearson Syndrome</td>
<td>Ophthalmoplegia, RP, Ptosis</td>
<td>Outer retina/RPE</td>
<td>Cytochrome C oxidase (I-III)</td>
<td>Deletion</td>
</tr>
<tr>
<td>Neuropathy, ataxia, and retinitis pigmentosa</td>
<td>Retinitis Pigmentosa</td>
<td>Outer retina/RPE</td>
<td>ATPase 6</td>
<td>m.8993T&gt;G or T&gt;C</td>
</tr>
<tr>
<td>Chronic progressive external ophthalmoplegia</td>
<td>Very mild vision loss or pigmentary retinopathy</td>
<td>Outer retina/RPE</td>
<td>tRNA or protein coding genes, POLG</td>
<td>Multiple (nDNA or mtDNA)</td>
</tr>
<tr>
<td>Maternally inherited diabetic and deafness/mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes</td>
<td>Macular dystrophy, Perifoveal atrophy</td>
<td>RNFL/RPE</td>
<td>tRNA-Leu</td>
<td>m.3243A&gt;G</td>
</tr>
<tr>
<td>Leber’s Hereditary Optic Neuropathy</td>
<td>Optic Neuropathy</td>
<td>RGCs/RNFL</td>
<td>Complex I, ND-1/4/6</td>
<td>m.3460G&gt;A, m.11778G&gt;A, m.14484T&gt;C</td>
</tr>
<tr>
<td>Leigh Syndrome</td>
<td>Optic Atrophy</td>
<td>RGCs/RNFL</td>
<td>ATP synthase, Complex I, ND1-6, COXIII, tRNA-encoding genes</td>
<td>m.8993T&gt;G</td>
</tr>
<tr>
<td>Myoclonus, Epilepsy with Ragged Red Fibers</td>
<td>Pigmentary Retinopathy</td>
<td>RGCs/RNFL</td>
<td>tRNA-Lys</td>
<td>m.8344A&gt;G</td>
</tr>
<tr>
<td>Dominant Optic Atrophy</td>
<td>Optic Atrophy</td>
<td>RGCs/RNFL</td>
<td>OPA1</td>
<td>nDNA, chr3q28</td>
</tr>
<tr>
<td>Charcot Marie Tooth Disease</td>
<td>Optic neuropathy, Juv. Glaucoma</td>
<td>RGCs/RNFL</td>
<td>COX6a1, Mitofusin 2, GDAP1, etc. multiple variants</td>
<td>nDNA</td>
</tr>
</tbody>
</table>

(Yu-Wai-Man et al., 2002; Yu-Wai-Man et al., 2011; Gentil and Cooper, 2012)

We also know that the disposal of damaged mitochondria is a potential means of rescuing overall tissue mitochondrial function. A strong example of this can be seen in individuals with heteroplasmic mitochondrial DNA (mtDNA). In these people some mtDNA strands carry deleterious mutations, while others are normal. Each mitochondrion can carry between 2-10 copies of mtDNA (Wiesner et al., 1992), so within a single cell, mitochondria
can span a spectrum of mtDNA-mutation-induced dysfunction. Mitochondrial heteroplasmy however can have a self-purifying effect, as mutated mtDNA produces dysfunctional mitochondria that are more quickly disposed of by mitophagic machinery than their healthy counterparts (Suen et al., 2010; Emperador et al., 2018). Therefore to an extent, dysfunctional mitochondria may be able to purify themselves out of a mixed population to erase potentially deleterious mtDNA mutations (Suen et al., 2010; Kandul et al., 2016). In a mixed population of mitochondria, this can be accomplished when an individual damaged mitochondrion completely loses its polarity, or on the other end of the spectrum when they generate excessively high levels of ROS (Narendra et al., 2008; Frank et al., 2012). *Ucp2* deletion adds to baseline ROS generation, and it is our belief that the increase in ROS generation brings *Ucp2*-deleted mitochondria closer to some sort of damage-sensing threshold necessary to activate mitophagy. This signaling may be mediated in part through mtDNA. The mitochondrial genome is not protected by histones (Bogenhagen, 2012) and is therefore highly susceptible to damage from ROS. In our samples we observe that *Ucp2*-deletion leads to an increase in mtDNA copy number, and high copy number heterogeneity (Appendix, Fig. F1). mtDNA copy number is proposed to be causatively associated with increases in autophagy (Clay Montier et al., 2009). A better understanding of whether and how ROS may affect mtDNA copy number could be key in understanding how *Ucp2* deletion increases mitophagy.

**Unanswered Questions**

Although the present dissertation finds a role for *Ucp2* as a protective factor from glaucoma, the use of this factor as a therapeutic in a clinical setting will require a deeper understanding of protein function. This would involve experimentation to determine what the mechanism of UCP2 activity actually is. This needs to be approached from several directions. The first of these is to answer the questions, what regulates UCP2 protein levels and activity? In our own studies, we use transgenic animals that overcome the difficulty of dealing with endogenous regulatory mechanisms, but for an accurate view of in vivo UCP2 function, one would need to know how to stimulate protein expression and activate protein function, which may be cell type specific and would need to be determined through a
combination of assays to measure protein levels (western blot) and activity (ROS as the mechanism of it’s effect on $\Psi_m$ are under debate). This has unfortunately been difficult due in part to the lack of a sensitive or specific UCP2 antibody, and may need to be pursued either by generating a new antibody or by using a tagged protein.

Additional complications come from the other neural uncoupling proteins UCP4 and UCP5, both of which are expressed in neurons and glia. As with UCP2, there are data to suggest that they do and data to suggest they may or may not uncouple mitochondria (Ramsden et al., 2012). It is not clear however whether they are regulated at the translational level, as Ucp2 appears to be. This is important, because although we have shown that while manipulation of Ucp2 does not clearly alter Ucp4 or Ucp5 expression (Appendix, Fig. G1), we cannot observe an effect of Ucp2 on translation of these factors in our samples. One potential solution that would allow for the study of UCP2 or any uncoupling protein in isolation would be to generate a Ucp knockout animal or cell line where none of the 5 Ucps are expressed. This approach may be more difficult than necessary, as one could alternatively study UCP2 in cell or tissue that do not normally express the protein or another uncoupling protein. For example, birds only express a single uncoupling protein, and deletion of that Ucp (the AvUcp) could be followed by re-expression of a mouse or human Ucp2 or another uncoupling protein for functional studies.

As we have covered earlier in the discussion, one could also focus on understanding the mechanism of mitophagy in Ucp2-deleted mice, by determining whether mtDNA damage is altered by Ucp2 (sequencing or a PCR-based mutation assay) and ROS, as well as by determining the extent (if any) to which Ucp2 deletion activates mtDNA damage sensing pathways or mitochondrial protein damage sensing pathways. For those more biased towards understanding cellular interactions, axonal RGC mitochondria are normally disposed of by optic nerve and optic nerve head astrocytes. Whether this is altered by elevations in IOP, some other facet of glaucoma, or by Ucp2 deletion is still unknown, and findings resulting from such a study could have high impact for an understanding of retinal biology in glaucoma.
Appendix A. Glutamine Dependent Leak Respiration is Ucp2-independent

Glutamine is thought to increase mitochondrial UCP2 protein levels by preventing binding of an inhibitory upstream open reading frame (uORF) (Hurtaud et al., 2006; Hurtaud et al., 2007). The uORF is sensitive to levels of glutamine >0.5 mM, and we tested whether glutamine could (A) stimulate mitochondrial leak respiration (uncoupling), and (B) do so in a Ucp2-dependent manner.

We measured oxygen consumption in primary cortical astrocytes using a seahorse extracellular flux assay, and found that treating cells with glutamine at the indicated concentrations did indeed increase leak (Oligomycin-insensitive) respiration, compared to treatment with varying levels of glucose, particularly at and above 0.5 mM glutamine (Fig. A1), and suggested either that glutamine could stimulate this form of respiration through uncoupling of mitochondria. We tested whether Ucp2 was involved in this uncoupling by incubating control and 4-OHT-pretreated primary cortical astrocytes isolated from Ucp2^fl/fl;
GFAP-creER\textsuperscript{T2} mice in 1 mM glucose, 2 mM glutamine, or 2 mM pyruvate (a control for directly stimulating the citric acid cycle).

Figure A2. Glutamine-stimulated leak respiration is not Ucp2 dependent. Control and 4-OHT-pretreated Ucp2\textsuperscript{fl/fl}; GFAP-creER\textsuperscript{T2} astrocytes were treated with 1 mM glucose, 2 mM glutamine, or 2 mM pyruvate and leak respiration (oxygen consumption following exposure to 2.5 ug/mL Oligomycin A) measured. 4-OHT-stimulated Ucp2 deficiency did not appear to alter leak respiration under any of the tested circumstances. *=p<0.05, **=p<0.01.

In these cells, Ucp2 deficiency did not clearly alter glutamine-stimulated respiration, suggesting that the effects of glutamine, and similar effects of pyruvate, are not Ucp2 dependent. This suggests that Ucp2 translation may not be controlled by glutamine in primary cortical astrocytes, or if it is, than the translated protein was not active during the test.
Appendix B. *Ucp2* Expression is Elevated Early in Glaucoma and Depressed Late in Glaucoma

*Ucp2* expression and activity is regulated at every level by oxidative stress (Pecqueur et al., 2001; Zhou et al., 2012; Donadelli et al., 2014). We therefore tested whether glaucoma induced sufficient stress to increase *Ucp2* transcription, using publically available data generated in DBA2/J models of pigmentary glaucoma by (Howell et al., 2011). In this study, mice were scored as ‘early’, ‘moderate’, or ‘severe’ glaucoma based on axon loss at 10.5 months of age and compared to D2-Gpnmb+ controls, which do not develop elevations in IOP. Early glaucoma was categorized as <10% axon loss, moderate was categorized as 10-50% axon loss, and severe was categorized as >50% axon loss.

Figure B1. *Ucp2* Expression in the Glaucomatous DBA2/J Retina. Retinal RNA expression was analyzed in a microarray of 10.5 month old DBA2/J mice with differing levels of disease severity (n=10-20). *=p<0.05

*Ucp2* gene expression appears to negatively correlate with damage levels, which could suggest that (A) *Ucp2* is protective and there is less RGC damage as a consequence of elevated *Ucp2* expression, (B), *Ucp2* is upregulated during all stages of glaucoma, and decreases in expression as *Ucp2*-expressing retinal ganglion cells are lost. Given that there is no causal test to determine whether (A) is true, we suggest that at least (B) is likely, as ‘early’ glaucoma mice with undetectable to little damage are the best comparison to D2-Gpnmb+ controls and express *Ucp2* to a greater extent.
Appendix C. Diurnal IOP Variation is Unaffected by Bead Injection

IOP changes at different times of day, and in mice increases at later hours (Savinova et al., 2001). We show this in our own survey of C57BL6/J mice IOP early in the day (10 AM, eastern standard time) and later at 6 PM (Fig. C1A). We also show that the diurnal increases occur not only in healthy PBS-injected eyes, but also in bead-injected eyes of the same mice (Fig. C1B). These data suggest that in our model of glaucoma, the greatest IOP levels and thus possibly the greatest extent of damage may occur at a later point than we would test, using a normal work schedule.

Figure C1. IOP Changes at Different Times of Day. (A) Raw IOP values at 10 AM and 6 PM in PBS and Bead-injected C57BL6/J mouse eyes, and (B) IOP at different times of day following internal normalization of an eye to itself earlier on the same day (n=6).
Appendix D. FCCP Decreases ROS when Complex III is Inhibited*

*This experiment generated the data shown in Figure 3.8B and uses overlapping data. It is presented separately here to provide additional context to arguments made in the discussion.

The mitochondrial NADH:Coenzyme Q oxidoreductase (complex I) is the start of the electron transport chain and a major site of superoxide production, either through forward electron transport, or more potently by reverse electron transport (RET, electrons move from CoQ\textsubscript{10} to NADH, powered by Δψ (Pryde and Hirst, 2011). Dissipation of Δψ slows or stops RET, which can be achieved through the addition of a chemical uncoupler such as FCCP. To see if we could show a protective effect of FCCP in cultured astrocytes (presumably by mimicking features of RET), we measured the generation of superoxide by pre-loading cells with the superoxide indicator MitoSox (5 uM), and observing superoxide generation with succinate (to drive forward electron transport without altering Δψ), rotenone (an inhibitor of complex I that is commonly used to increase superoxide production), or Antimycin A (an inhibitor of complex III, also known to increase superoxide generation). These cells are being images in a 1x PBS assay medium containing 1 mM glucose. Addition of 10 nM FCCP to

Figure D1. Superoxide Production Following Electron Transport Chain Activation or Inhibition. Astrocytes were treated with succinate, rotenone, Antimycin A, or any of the above inhibitors combined with 10 nM FCCP. The dotted line represents superoxide generation by untreated control astrocytes, which is negligible, given that MitoSox fluorescence decrease over time in these cells. n=6, **=p<0.01
these electron transport chain factors has different effects. It does not significantly alter ROS production with succinate, and further enhances rotenone-stimulated superoxide production (p<0.01), but appears to decrease superoxide production following complex III inhibition. n=6 biological replicates/treatment. Although this experiment is insufficient to show Antimycin-stimulated reverse electron flow, we do show that when complex III is inhibited, small decreases in Δρ (see Fig. 3.8A for details on the extent to which Ψ_m) blunt superoxide generation, which may relate to the circumstances of UCP2 action normally or during pathological circumstances.
Appendix E. *Ucp2* Overexpression Increases Oxygen Consumption

Many of our cellular experiments utilize 4-hydroxytamoxifen (4-OHT) treatment to stimulate creER<sup>T2</sup>-mediated *Ucp2* deletion. To address the concern that 4-OHT alone could influence oxygen consumption in C57BL6/J primary cortical astrocytes, we pretreated these cells with 4-OHT for 48 hours and measured oxygen consumption rate (OCR) as a part of a

![Graph A](image)

**Figure E1. Ucp2 Overexpression Increases Oxygen Consumption** (A) Control and 4-OHT-pretreated C57BL6/J primary cortical astrocytes, treated with (1) 4-OHT, (2) 2.5 ug/mL Oligomycin (3) 1 uM FCCP, and (4) 1 uM rotenone+1 uM Antimycin A. These data show that 4-OHT has neither a transient nor a long-term effect on mitochondrial oxygen consumption (B) Treatments (2-4) were also applied to control and 4-OHT-pretreated Ucp2<sup>KI</sup>; GFAP-creER<sup>T2</sup> astrocytes in the same order, and show that *Ucp2* overexpression appears to increase basal oxygen consumption rate.
normal mitochondrial stress test. We treated cells with several metabolic inhibitors, as described in the figure caption. There are no differences in OCR between control and 4-OHT-pretreated cells, and there is no difference before or after transient exposure to 4-OHT (1), suggesting that mitochondrial function is not influenced by 4-OHT at the concentrations used to stimulate Ucp2 deletion or overexpression (Fig. E1A).

If the metabolic hypothesis of Ucp2 function is true, increasing Ucp2 expression should increase the export of citric acid cycle factors from mitochondria and decrease oxygen consumption rate. We tested this hypothesis using untreated or 4-OHT-pretreated Ucp2\textsuperscript{KI}; Cre\textsuperscript{ERT2} primary cortical astrocytes, and show that Ucp2 appears to enhance oxygen consumption (Fig. E1B), suggesting that metabolic hypothesis may not fully explain UCP2 function.
Appendix F. *Ucp2* Deletion Increases mtDNA/nDNA

The mtDNA/nDNA ratio is a correlate of mitochondrial mass (Quiros et al., 2017), and we measured this ratio using qPCR to amplify the mitochondrial *CytB* and nuclear β-actin genes. We found that *Ucp2* deletion has a drastic effect on this ratio, as neural *Ucp2* deletion almost doubled retinal mtDNA/nDNA, despite RGCs (the population in which this transgene is active) being only 1-2% of nuclear material in the retina (Fig. F1A). Glial *Ucp2* deletion did not alter retinal mtDNA/nDNA, which we believed to be a consequence of the relatively low mitochondrial density in glia, combined with their status as a minority cell population in the retina, even when compared to RGCs. We next determined whether glial mtDNA is affected by *Ucp2* deletion using *Ucp2*°/° and *Ucp2*°/°; *GFAP-creERT2* primary cortical astrocytes. *Ucp2*°/° mice.

![Graph A](image1)

![Graph B](image2)

Figure F1. *Ucp2* Deletion Increases mtDNA/nDNA. (A) mtDNA/nDNA in PBS- and bead-injected retinas from *Ucp2*°/°, *Ucp2*°/°; *GFAP-creERT2*, and *Ucp2*°/°; *Thy1-creERT2* mice. (B) mtDNA/nDNA in primary cortical astrocytes from *Ucp2*°/° and *Ucp2*°/°; *GFAP-creERT2* mice.
deletion also increased mtDNA/nDNA in these cells, though to a much less drastic extent. To determine whether this increase in mtDNA/nDNA was tied to mitochondrial membrane potential, we treated $Ucp2^{fl/fl}; GFAP-creER^{T2}$ cells with a low concentration (16 pM) of FCCP, which minimally uncouples mitochondria, in an attempt to restore a ‘normal’ $\Psi_m$. (Fig. F1B) 16 pM FCCP significantly decreases mtDNA/nDNA back to $Ucp2^{fl/fl}$ levels, suggesting that membrane potential or consequent ROS production may control some aspect of mtDNA synthesis or degradation. Because of the direct effects Ucp2 may exert on mtDNA/nDNA, we did not use this measure as a determinant of retinal mitochondrial mass in transgenic animals.
Appendix G. *Ucp2* Deletion Does Not Increase Expression of Other Uncoupling Proteins

If manipulation of *Ucp2* expression in turn regulates or alters the regulation of expression for other uncoupling proteins, these alternative uncoupling proteins may interfere with accurate measurements of any *Ucp2*-dependent effects. To determine whether Ucp2 regulates other uncoupling proteins, we probed reverse transcribed RNA from bead-injected *Ucp2*-sufficient and *Ucp2*-deleted retinal tissue with oligonucleotide primers from (Perreten Lambert et al., 2014). As we show in Figure G1, Ucp2 deletion does not appear to substantially alter the expression of any other uncoupling protein gene, though notably *Ucp1* expression was left out from this figure as it was undetectable in the retina.

![Expression Relative to Bead-injected Ucp2^+/+](chart.png)

- *Ucp2^+/+
- *Ucp2^+/+; GFAP-creERT2
- *Ucp2^+/+; Thy1-creERT2
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Curriculum Vitae

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