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Maintenance and Survival in a Non-Image-Forming Subset of Retinal Ganglion Cells

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MAINTENANCE AND SURVIVAL IN A NON-IMAGE-FORMING SUBSET OF RETINAL GANGLION CELLS
A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

by

Andreea Nistorica

June 2017

The Dissertation of Andreea Nistorica is approved:

_____________________________________
Professor David Feldheim, chair

_____________________________________
Professor Alexander Sher

_____________________________________
Professor Bin Chen

_____________________________________
Tyrus Miller
Vice Provost and Dean of Graduate Studies
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Abstract

Maintenance and Survival in a Non-image-forming Subset of Retinal Ganglion Cells

Andreea Nistorica

The retina detects the visual scene and coverts the information into separate channels of information. These channels then connect to diverse areas in the brain that initiate appropriate visually responsive behaviors. Retinal ganglion cells (RGCs) are the output neurons of the retina and come in ~30 different types, each with distinct receptive field properties generated by circuits within the retina. Each RGC type processes a distinct feature of the visual scene making up a unique channel of visual information and transferring it to the appropriate area of the brain. Intriguingly, all the RGC types arise from one common RGC progenitor cell. One of the questions in vision neuroscience is how does this diversity arise during development? Further, once the different RGC types have been specified, how do they maintain their identity? Answers to these questions will shed light on the mechanisms that set up the parallel channels of visual information and how visual circuitry is established.

Studies on the gene regulatory network that specifies RGCs have so far yielded several transcription factors that are involved. However, many other transcription factors could be involved in specifying, and then maintaining, the diversity of RGC types. A transcription factor called Tbr2 has been demonstrated to be required for
specifying a subset of RGCs. In my thesis work I have tested the hypothesis that Tbr2 is also required for maintaining the identity of that RGC subset. Through loss of function and ectopic expression experiments in adult mice I demonstrate that Tbr2 has an epistatic relationship to melanopsin and is required to maintain the melanopsin-expression aspect of RGCs.

Another aspect of RGC identity is the response to axonal damage. Some RGC types are able to withstand axonal injury and resist death, while other types activate pro-apoptotic pathways within a very short time of the injury and die. The mechanisms responsible for this variation in response to injury is an important factor in understanding the pathogenesis of glaucoma, which is a disease that affects the retinal ganglion cells. There is evidence that the OFF RGCs are more susceptible to apoptosis following optic nerve injury, but still more work needs to be done in classifying RGC types and their response after damage. Melanopsin-expressing RGCs have also been found to survive better than other types following optic nerve injury. Considering that the transcription factor Tbr2 is expressed in all melanopsin RGCs, and that Tbr2 is required for RGC survival during development, I tested the hypothesis that Tbr2-expressing class of RGCs survive better following optic nerve injury as compared to other RGC types. My results show that Tbr2-expressing RGCs do survive better than other RGC types, however not due to Tbr2 expression. This implies the neuroprotective aspect of Tbr2-expressing RGCs is regulated by other factors, and future RNA-seq experiments will help reveal the genes involved.
Acknowledgements

I would like to thank the members of my lab for their support in general. I want to especially thank my advisor David Feldheim for providing me with an environment where I could grow as a scientist, for his thoughtful input throughout the years, and for helping with the stressful task of restraining mice for one of the experiments. I also want to thank Jena Yamada, for not only being a great labmate and keeping the lab running smoothly, but for also being a great friend and making my time in the lab that much more pleasant. Neal Sweeney and Kiely James, who were postdocs in the Feldheim lab, also deserve a big thank you for training me in various techniques and showing me the ropes on how to design experiments.

I want to thank the members of my thesis committee, Sasha Sher and Bin Chen, for their input and guidance. The members of their labs have also been very helpful in discussions about experiments and sharing reagents, and have been great to collaborate with over the years.

I also want to thank my parents, Corina and Gabriel, and my fiance Nick for always being interested in hearing about my experiments and how grad school is going. They’re the best.
Chapter 1: Introduction

1.1 Retinal Structure

Vision originates from a light-sensitive part of the central nervous system called the retina. This tissue is situated at the back of the eye and comprises five major neuronal cell types: photoreceptors, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells (RGCs). The photoreceptors, called rods and cones, are located at the outer part of the retina and are the main way of light input into visual pathways. The rods and cones connect to horizontal cells and bipolar cells. The bipolar cells then connect to amacrine cells and retinal ganglion cells (RGCs). The horizontal, bipolar, and amacrine cells are collectively termed the interneurons of the retina. The RGCs are in the inner part of the retina and they are the only retinal cell type to connect to the brain through the optic nerve (Fig. 1). The RGCs receive the light input from the rods and cones and send light information through the optic nerve to over 20 retinorecipient brain areas (Morin and Studholme, 2014).

One of the goals in the vision neurodevelopment field is to understand the genetic programs that dictate neuronal diversity such that we can harness this knowledge and apply it towards regenerating retinal neurons in vitro for study and for therapeutic purposes. The neurons in the retina can be separated into five major types; however, each neuronal type has subtypes with specific functions to give an estimated
Fig. 1: Schematic of a cross section of the retina.
The light enters the eye through the pupil and goes through the retina to the photoreceptors at the back of the eye. The light information is then passed on to the bipolar cells, and then the ganglion cells before reaching the brain. Figure adapted from Pearson Education Biology textbook.

~55 separate neuronal cell types (Masland, 2001). Intriguingly, they all emerge from one common retinal progenitor (Levine and Green, 2004). Although some progress has been made during the last two decades in gaining insight into how this retinal cell diversity is generated, the mechanisms that drive a retinal progenitor cell towards a particular retinal cell type remain unclear.
1.2 Early Retinal Development

In the mouse retina, neurogenesis begins before birth and is largely completed shortly after birth. The retinal ganglion progenitor cells are the first of these neuron types to be born, and begin a lineage that generates >20 types of RGCs. The RGC progenitors emerge from dividing retinal progenitor cells starting around embryonic day 11 (E11) in the mouse, with a peak birth rate around E13, and continue emerging at a slower rate until postnatal day 0 (P0) (Guerin, 2006). One of the first factors that is part of a progenitor cell’s decision to go down a RGC lineage is the expression of basic helix-loop-helix transcription factor atonal homolog 7 (Atoh7; also known as “Math5”) (Wang, 2002). Mutations in Atoh7 lead to failure of RGC formation, however it is not sufficient for an RGC fate since Atoh7-expressing progenitor cells give rise to all retinal cell types (Brzezinski, 2012; Feng, 2010). Downstream of Atoh7, the POU domain transcription factors Brn3a, Brn3b, Brn3c, and the insulin gene enhancer protein 1 (Isl1) have been found to control commitment of a retinal progenitor cell to an RGC lineage (Badea, 2009; Wu, 2015).

1.3 Retinal Ganglion Cell Type Specification

There are currently ~30 types of RGCs, characterized based on morphology, molecular markers, or functional responses (Sanes and Masland, 2014; Baden,
2016). The RGC morphology consists of the size of their somas, their dendritic arbor, where in the Inner Plexiform Layer (IPL) their dendritic arbor laminates, and what brain areas their axons target. The RGC functional responses are the action potential activity of RGCs in response to light stimuli. The RGC molecular markers are proteins expressed in known RGC classes that can be used to label a particular RGC class for experimental purposes. RGCs have also been broadly categorized into two groups: image-forming, and non-image-forming. The image-forming pathway sends all aspects of the visual scene to the brain and contributes to conscious vision. The ancient non-image-forming pathway enables organisms to detect light as a means for reflexive behaviors such as sleep/wake cycles. Light information carried by the non-image-forming pathway reaches almost all parts of the brain to influence subconscious neural responses, either directly or indirectly (Matynia, 2013). One of the challenges in tying together these categories of RGCs in order to characterize the anatomical and functional data for each RGC type has been the lack of molecular markers. Although RGCs can be distinguished based on their anatomy and their response to stimuli, it is impossible to understand the combinatorial code of molecular factors that are responsible for their identities without first knowing the pattern of gene expression for different RGC types.

To overcome this obstacle, our lab and others have been performing screens for molecular markers of non-overlapping classes of RGCs. In doing so, our lab found three transcription factors expressed in three broad non-overlapping classes of
RGCs, and together account for >90% of the RGCs in the mouse retina (Sweeney, 2017). The LIM-homeodomain transcription factor Isl2 is expressed in ~40% of RGCs and labels image-forming, non-Direction Selective RGCs (Triplett, 2014). The T-box transcription factor Tbr2 labels ~20% of all RGCs. Tbr2 labels most of the RGCs in the non-image-forming class and includes the melanopsin-expressing RGCs (Sweeney, 2014; Mao, 2014). And lastly, the special AT-rich sequence-binding protein 2 (Satb2) is expressed in the Direction-Selective (DS) class of RGCs (Sweeney, 2017). The RGC classes marked by these three transcription factors are further divided into subtypes. Some RGC subtypes can be identified through molecular markers, while others can be identified through transgenic mouse lines.

The current hypothesis is that since these three transcription factors are expressed in different classes of RGCs, they are potentially pushing RGCs towards a particular fate. For example, Tbr2 was demonstrated to be a transcription factor that steers RGCs towards a non-image-forming fate, since it is required during development for the specification of non-image-forming RGC types. When Tbr2 is removed during development, non-image-forming RGC types fail to form and die (Mao, 2008; Mao, 2014, Sweeney 1014). Further, there is a loss of behavioral outputs from the non-image-forming circuitry such as the pupillary light reflex and circadian entrainment (Sweeney, Mao). Considering the effect Tbr2 has on non-image-forming RGC fate, and ultimately on behavioral outputs, it is likely that there are other transcription factors that control the fate of other RGC types. Future loss- and
gain - of function experiments will reveal the transcription factors important for the specification of different RGC types.

1.4 Image-forming RGC Types

There are various types of RGCs in the image-forming RGC class. The image-forming class is ~80% of all RGCs and is marked by the transcription factor Brn3a (Badea, 2011; Quina, 2005). Within this class the RGCs can be separated into two broad groups with little overlap: one in which RGCs are marked by the transcription factor Isl2, and another in which RGCs are marked by the transcription factors Satb1/Satb2 (Sweeney, 2017). Some of the Satb1/Satb2-expressing RGCs are involved in motion detection (Sweeney, 2017; Rousso, 2016). The Isl2-expressing RGCs are labeled by the Isl2-GFP mouse line and include ON and OFF alpha RGCs (Sweeney, 2017; Triplett, 2009). The axons from image-forming RGCs mainly target the dorsal lateral geniculate nucleus (dLGN) and the superior colliculus (SC) (Fig. 2). These are areas that receive a topographic representation of the visual scene and initiate a wide range of visually dependent behaviors. Thus together, these subtypes of image-forming RGCs convey to the brain diverse aspects of the visual scene.
Fig. 2: Schematic of RGC inputs from the retina to the brain. Image forming RGCs from the retina innervate brain areas that lead to conscious vision (orange). Tbr2+ RGCs innervate brain areas that control innate behaviors such as circadian rhythm and pupil reflex (blue). Non-image-forming RGCs that control eye movements innervate the MTN (white). SC= Superior Colliculus, SCN= Suprachiasmatic nucleus, dLGN= dorsolateral geniculate nucleus, vLGN= ventrolateral geniculate nucleus, IGL= intergeniculate leaflet, dLGN= dorsolateral geniculate nucleus, OPN= olivary pretectal nucleus, PPN= posterior pretectal nucleus, MTN= medial temporal nucleus. Adapted from a figure designed by Jena Yamada, Feldheim Lab, UC Santa Cruz.

1.5 Non-image-forming RGC Types

The non-image-forming RGCs are make distinct connections from the image-forming RGC class. The non-image-forming RGCs connect to areas of the brain that are involved in light-dependent reflexive behaviors like sleep, mood, circadian rhythm, pupillary light reflex and eye movements (Schmidt, 2011; Osterhout, 2015;
Most non-image-forming RGCs express Tbr2, except for the ones involved in eye movements which target the medial tegmental nucleus (MTN) and the nucleus of the optic tract (NOT) (Osterhout, 2015; Sun, 2015). Throughout this thesis I will refer to Tbr2-expressing RGCs as “non-image-forming”. The non-image-forming RGCs have similar anatomical features. When Tbr2-positive RGCs are labeled with a Tbr2-GFP or a melanopsin-GFP mouse line (Sweeney, 2014; Mao, 2014), some cells are observed to have dendrites that laminate in the innermost ON and other cells have dendrites that laminate in the outermost OFF laminas of the inner plexiform layer (IPL). Their axons can be visualized in what are thought of as non-image-forming brain areas: the suprachiasmatic nucleus (SCN), the ventral lateral geniculate nucleus (vLGN), intergeniculate leaflet (IGL), the olivary pretectal nucleus (OPN), and the posterior pretectal nucleus (PPN) (Fig. 2).

The RGC diversity within this non-image-forming RGC class has not been well characterized yet. It is currently known that there are several subtypes within the non-image-forming class of RGCs. There are at least 5 types of intrinsically photosensitive RGCs (ipRGCs), a group labeled by Cad3-GFP expression, another group marked by the axonal guidance molecule Unc5d, and others for which we do not have markers yet (Osterhout, 2011; Schmidt, 2011; Sweeny, 2014).

The most well studied non-image-forming RGC type is the ipRGC. These RGCs express the photosensitive pigment melanopsin (Provencio, 2000; Ruby, 2002). Mice that lack melanopsin have defects in circadian rhythm and pupillary reflex
behaviors (Panda, 2002; Lucas, 2003; Hattar, 2003; Guler, 2008; Keenan 2016). This evidence demonstrates that melanopsin mediates persistent light detection in ipRGCs since the mice lacking melanopsin have impaired ability to perform these reflexive behaviors that involve constant light detection.

Considering that there is a variety of subtypes within the non-image-forming RGC class, it is possible that there exists a certain molecular code regulating the morphological identity of this RGC class as a whole such that all the subtypes have similar dendritic and axonal connectivity patterns, and that they are maintained throughout the animal’s life. Yet it is also this same molecular code that imposes distinctions among the subtypes. For example, the M1 subtype of ipRGCs can be divided into two groups, one that expresses the transcription factor Brn3b, and one that does not. The M1 Brn3b+ RGCs project to the OPN shell and therefore aid in mediating the pupil light reflex, while the Brn3b- group project to the SCN and are involved in photoentrainment (Chen 2011; Sonoda 2016). These types of observations provoke questions about the nature of the mechanisms behind the identity of RGCs, and prompt loss and gain of functions experiments in order to disclose the factors that determine their fate.

1.6 Retinal Ganglion Cell Identity Maintenance in Adulthood

Many transcription factors continue being expressed beyond the developmental
stage and into the mouse’s adult life. Although progress has been made in identifying the role of some transcription factors during RGC development, little is known about the reasons for their expression later in the animal’s life. One hypothesis is that some of these transcription factors are master regulators of a particular RGC identity, and they are necessary for maintaining it longterm (Kay and Baier, 2004; Deneris and Hobert, 2014). Another hypothesis is that they are part of cell survival pathways and are involved in keeping the RGCs alive (Pfisterer and Khodosevich, 2017; Alavian 2014).

Recently there have been efforts to better characterize the survival of RGC types after injury such as axotomy or diseases such as glaucoma. Researchers use the optic nerve crush mouse model (Tang, 2011) or intraocular pressure elevation (Gross, 2003) in order to mimic conditions of RGC injury. Reports demonstrate that RGC types differ profoundly in their ability to survive after injury (Della Santina, 2013; Duan, 2015; Cui, 2015). In light of this, yet another hypothesis is that certain factors might confer neuroprotective properties to specific RGC types. Identifying the molecular factors that aid in the survival of RGCs will lead toward potential therapies for retinal injury or diseases such as glaucoma.

In this thesis I explore the question of retinal ganglion cell identity and which genes control different aspects of it in adult mice. My work is focused on the non-image forming group of RGCs and the role Tbr2 plays in their maintenance and survival.
Chapter 2: Experimental Methods

Many of the methods used are common across the projects I worked on, therefore they will be discussed in this separate methods section.

2.1 Mice

Tbr2CreER— Mice were generated as described in Pimeisl et. al., 2013. This mouse has a tamoxifen-inducible Cre knocked-in at the Tbr2 locus. Thus CreER expression will be driven by the Tbr2 promoter, but the CreER will only be able to translocate into the cell nucleus upon binding of tamoxifen. When used in conjunction with the Tbr2 floxed mice (Zhu, 2010), the CreER will bind to the loxP sites and remove the floxed Tbr2 gene. Cre-positive transgenic mice were determined by PCR of tail DNA using the following primers:

Fwd: 5’ GAGGGAGGAAGGGGACATTA
Cre R: 5’ CAGGTTCTTGCGAACCTCAT
WT R: 5’ AGACTGCCCGGAAACTTCTT

Expected band sizes are: 327 bp for widltype, and 202 bp for the CreER.

Tbr2 floxed— Mice were generated as described in Zhu et. al., 2010. The Tbr2 floxed mouse line (stock no. 017293) was acquired from the Jackson Laborarory. This mouse has loxP sites around the Tbr2 gene locus that can be recognized by a Cre enzyme
such that the Tbr2 gene can be excised. Floxed transgenic mice were determined by PCR of tail DNA using the following primers:

5'-AGATGGAAAATTTGGGAATGAA-3' and

5'-GGCTAC- TACGGCCTGAAAC-3'.

Expected band sizes are 621 bp for the wildtype band, and 731 bp for the floxed band.

ROSA26tdTomato— Mice were generated as described in Madisen et. al., 2009. These mice have a lox-stop-lox-tdTomato cassette knocked-in to the ROSA26 locus. Upon Cre activation, the stop signal will be excised and the cell will express tdTomato throughout the cytoplasm. Positive transgenic mice were determined by PCR of tail DNA using primers against tdTomato:

WT F: 5’AAGGGAGCTGCAGTGGAGTA

WT R: 5’ CCGAAAATCTGTGGGAAGTC

Tom F: 5’ CTGTTCCTGTACGGCATGG

Tom R: 5’ GGCATTAAAGCAGCGTATCC

Expected band sizes are: 297 bp for the wildtype band, and 196 bp for the tdTomato band.

Isl2GFP— Cryopreserved sperm from Isl2-GFP transgenic mice (Stock Tg (Isl2-EGFP)LW124Gsat/Mmuce) was obtained from the Mutant Mouse Regional Resource Center (MMRRC), an NIH funded strain repository, and was donated
to the MMRRC by the NINDS-funded GENSAT BAC transgenic project. In vitro fertilization was performed at University of California, Santa Cruz (UCSC). Positive transgenic mice were determined by using GFP-exciting flashlight and detecting filter glasses to visualize expression in newborn pups (P0–P4) (BlueStar model Bls2, Lexington, MA) or by PCR of tail DNA using primers against GFP:

5′-CCTACGGCGTGCAGTGTTCAGC-3′

and 5′-CGGCGAGCTGCACGCTGCGTCCTC-3′.

Expected band sizes are: no band for wildtype, and 350 bp for GFP.

C57Bl/6 wildtype mice: Mice were obtained from Charles River Laboratories (Wilmington, MA).

This study was approved by and performed in accordance with the Institutional Animal Care and Use Committees at UCSC.

2.2 Immunohistochemistry

Postnatal mice were sacrificed and intracardially perfused with ice-cold PBS followed by ice-cold paraformaldehyde (PFA) (pH 7.4, 4% in PBS). Eyes were dissected out and fixed in 4% PFA in PBS for 30 min at room temperature. The retinas were isolated, and either placed in block solution for whole mount staining, or placed in 30% sucrose in PBS overnight. Block solution is made of 5% donkey serum,
0.1% TritonX100 and diluted in phosphate buffered saline (PBS). The retinas that were sunk in sucrose solution were embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) the following day, frozen, and cryosectioned at 20 um on a CM1520 Cryostat (Leica Microsystems, Buffalo Grove, IL) and collected on glass plus slides. Slides were allowed to dry overnight at room temperature and immediately used for immunostaining or frozen at −80°C until used. Immunostained sections and whole retinas were blocked for 1-3 hrs in 5% heat inactivated donkey serum in PBS+ 0.1% Triton-X100. Primary and secondary antibodies were diluted in this blocking medium, and 3 washes were done with in a solution of PBS+0.1%Triton-X100 in between the primary and secondary antibody solution, for 5mins each wash. For immunostained sections 4’,6-diamino-2-phenylindole, dihydrochloride (DAPI) (Sigma-Aldrich, St. Louis, MO, D8417) was included in a penultimate PBS rinse at a dilution of 1:1,000 to visualize nuclei.

2.3 Brain staining

Tissue was harvested then brains were fixed overnight in 4% PFA at 4°C, then placed in 30% and allowed to sink over 2 days. The brains were then sectioned at a thickness of 100 um on a freezing HM430 sliding microtome (Thermo Fisher). Free floating brain sections were then immunostained in 24-well plates (up to 5 sections per well). Primary and secondary antibodies were diluted in this blocking medium. Sections were left in block+primary antibody solution overnight at 4°C, and three 15 minute
washes were done with PBS+0.1% Triton-X100 the next day, then block+secondary antibody solution was added to the wells. The sections were left in block+secondary antibody solution overnight at 4°C or at room temperature for 4 hours. Then 3 15-min PBS washes were done and the sections were mounted on glass plus slides in Fluoromount-G mounting medium (SouthernBiotech).

### 2.4 Antibodies

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<td><strong>Antigen</strong></td>
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<td>Tbr2</td>
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<td>Tbr2</td>
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<tr>
<td>Melanopsin (Opn4)</td>
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<td>Brn3a</td>
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<td>Unc5d</td>
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<td>Satb2</td>
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<td>RFP</td>
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<td>GFP</td>
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<td>ChAT</td>
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### 2.5 Tamoxifen delivery through gavage

To dissolve the tamoxifen in corn oil, the solution was left at 50°C for several hours (depending on volume) on a rotator. After use, the solution was stored at 4°C. Before each use the solution was warmed to 50°C and rotated to make sure the tamoxifen was dissolved.
Mice were held by hand and a 24 Gauge feeding needle was inserted down their esophagus (Fine Science Tools Inc., cat# 18061-24) to feed the mice tamoxifen (150mg/kg) dissolved in corn oil (Sigma cat# C8267). If using a 60mg/mL tamoxifen in corn oil solution, average-size adult mice can be given a 50 uL dose of this solution. In this case, it is easier to insert the tip of the gavage needle or the tip of a P20 pipette about 0.5-1 cm into the mouth of the mouse, at the back of the tongue. The mice swallow this amount easily, and it avoids possible complications such as perforating the stomach or drowning that can come from the usual gavage method. For the experiments discussed in this document, adult mice aged P35 to 3 months were gavaged for 4 days in a row.

2.6 Pupillometry

For all experiments, mice were unanesthetized and restrained by hand. To measure the pupillary light response, mice of both sexes were dark adapted for 5 mins under infrared illumination. Still under infrared illumination, mice were filmed using a Sony Handycam HDR-SR11 for 10 s to obtain a baseline reading, followed by exposure of 460 nm LED light to the contralateral or ipsilateral eye for 30 s followed by 30 sec-1 min in darkness. This protocol was repeated three times, at each light condition, giving three readings per mouse at each light level. The light levels were: low-light condition: 0.20 mW/cm², medium-light condition: 0.55 mW/cm², and the high-light condition: 6 mW/cm². Light levels were controlled using neutral
density filters. These light conditions roughly correspond to dawn/dusk at the lowest light condition, to very bright sunlight at the highest light condition. Movies were converted into still photos using Apple iMovie and the percent of pupil constriction was calculated using ImageJ by measuring the diameter of the pupil at different light conditions and dividing it by the pupil diameter of that same mouse in the dark.

2.7 Day/Night Locomotor Activity

Mice were placed in cages with free running wheels that can track the wheel revolutions (Columbus Instruments). Mice were placed in these cages a few days before the start of the experiment to get them accustomed to running on the wheel. Mice were left in the cages for 3-4 weeks. The cages were placed in a room with white light that functioned on a 12-hour light/12-hour dark cycle.

Analysis of the wheel revolution data was done using the Actogram plugin in Image J.

2.8 Viral infection

Viral constructs used: AAV2-CMV-myrRFP-2A-mEomes; AAV2-CMV-mCherry, both made to order from Vector Biolabs (Malvern, PA). Constructs were at a concentration on the order of $10^{13}$gc/mL. The two constructs have slightly different red fluorescent proteins: one is a myristoylated RFP which labels mostly the cell membrane, while the other is an mCherry that is found throughout the cytoplasm. They are not the same due to the conservative price of purchasing these constructs.
versus purchasing constructs with the exact same red fluorescent reporter. However, the slightly different fluorescent reporters should not have any effect on the results of the experiment.

Mice of both sexes were anesthetized with ketamine/xylazine (100mg/kg and 10mg/kg, respectively) administered through intra-peritoneal injection. Adult mouse eyes were perforated with a 30-gauge needle near the edge of the cornea close to where it meets the sclera. The needle must make a hole through the RPE as well. Then a pulled glass needle containing AAV solution was inserted into the hole made with the 30-gauge needle, and 1-2uL of AAV solution was pumped into the intravitreal space with a Picospritzer III set at 30 psi and ~15 ms pulse duration. The mice were monitored for the hours after the procedure and the following few days to make sure there is no infection or pain.

For all the experiments, mice were sacrificed at least 2 weeks post infection to ensure robust expression of the viral construct.

2.9 Optic nerve crush

Mice were anesthetized with ketamine/xylazine (100mg/kg and 10mg/kg, respectively) administered through intra-peritoneal injection. Jeweler’s fine forceps (Dumont #55, FST) were used to expose the optic nerve intraorbitally and crushed for 5 seconds at a distance approximately 2 mm from the posterior pole of the eye. The mice were carefully monitored for any damage to the ophthalmic artery and any signs
of bleeding during the period after the crush and the several following days. Ointment containing atropine sulfate (Bausch and Lomb, NDC 24208-825-55) was applied pre-operatively to protect the cornea from drying. Any mice with vascular damage in the eye after optic nerve surgery were euthanized immediately after and were not included in the dataset.

2.10 Imaging

Images were taken on a Zeiss Axioimager Z2 Widefield Microscope. For some figures, brightness and contrast were changed in ImageJ and Photoshop to standardize the appearance of cells and enhance figure legibility.

2.11 Data analysis

For experiments done on the adult Tbr2 mutants (discussed in Chapter 3) 4 fields of view (624.7 um by 501.22 um) from each whole mount retina were imaged. Cells were then manually counted with ImageJ by using the multi-point counting function and manually clicking on each cell. For optic nerve crush experiments (discussed in chapter 4), 5-8 fields of view from each whole mount retina were imaged. Cells were automatically counted using the ImageJ Binary-->Analyze Particles feature. The threshold for size was anything above a cell area of 20 um². GraphPad’s Prism software program was used to calculate and graph means, standard deviations and standard errors, following cell counts.
Chapter 3: Tbr2 is required for maintaining non-image-forming RGC identity in the adult mouse

3.1 Introduction

The non-image-forming RGC class includes several known subclasses of RGCs. One is the melanopsin-expressing subclass of intrinsically photosensitive RGCs (ipRGCs), another is the RGCs labeled by the axon guidance molecule Unc5d, and a third is the RGCs labeled by the Cad3-GFP mouse line (Sweeney 2014; Mao, 2014; Schmidt, 2011; Osterhout; 2011). There are also other RGC types in this class that have not been molecularly categorized. However, most non-image-forming RGCs express the T-box transcription factor Tbr2 (Sweeney, 2014; Sweeney, 2017; Mao, 2014). Removal of Tbr2 during development negatively affects this class of RGCs and their behavioral outputs: ipRGCs fail to form, and there is a loss of inputs to the non-image-forming brain nuclei they normally target. Consequently, these mice have a pupil reflex defect at all light levels and exhibit an inability to photoentrain their circadian activity cycle (Sweeney, 2014; Mao, 2014).

After specification and integration into visual circuits, RGCs maintain their identity throughout the lifetime of the animal. How neuronal maintenance is executed is not well understood, but in some cases, a gene important for developing a cell type is also required for its survival. Tbr2 expression persists in adult mouse RGCs, which
prompted the hypothesis that it plays a role in maintaining a non-image forming RGC fate or survival. In this study I tested this hypothesis by removing Tbr2 from adult mouse RGCs and observing the effects on non-image forming RGC fate by assaying molecular marker expression, morphology, and reflexive visual behaviors associated with the non-image-forming neural circuit. I find that when Tbr2 is removed from the adult retina the mutant RGCs remain alive and do not display overt changes in dendritic or axonal morphology. However, Tbr2 mutant RGCs fail to maintain melanopsin expression. Consistent with this result and known melanopsin knockout phenotypes, the melanopsin deficiency caused by loss of Tbr2 leads to an impaired pupil light reflex at high irradiances, but no defect in circadian photoentrainment (Lucas, 2003; Altimus, 2010; van Diepen, 2013; Keenan 2016). Additionally, I found that ectopic introduction of Tbr2 is sufficient for melanopsin expression in image-forming RGCs. These results are the first to demonstrate the requirement of a transcription factor for the maintenance of RGC identity.

3.2 Results

3.3.1 Tbr2 is required for melanopsin expression in an adult mouse retina.

It has been previously shown that Tbr2 is required for expression of Tbr2+ RGC subtypes during development (Sweeney, 2014; Mao, 2014). To determine whether Tbr2 plays an active role in the maintenance of melanopsin expression in adulthood, I
**Fig. 3:** Schematic of the genetic strategy to conditionally remove Tbr2 and get control and mutant mice.

I used a mouse genetics approach. I crossed mice containing a floxed Tbr2 allele and a ROSA26-lox-stop-lox-tdTomato reporter gene with mice that express a tamoxifen-inducible Cre from the Tbr2 promoter (Fig. 3). The reporter gene will express tdTomato and label the cell bodies, dendrites, and axons of cells with active Cre. Thus, the control mice will be Tbr2 hets and express tdTomato in Tbr2 cells, and the mutants will have one copy of Tbr2 rendered null by the CreER insertion, and the other Tbr2 copy removed by the Cre-lox system.

Tamoxifen was administered to Tbr2<sub>cre/+</sub> and Tbr2<sub>cre/fl</sub> adult mice and the animals were sacrificed one week after the last tamoxifen dose. I then immunostained the retinas and compared the number of tdTomato cells in Tbr2<sub>cre/</sub> and Tbr2<sub>cre/+</sub> retinas that also co-expressed melanopsin, since an epistatic relationship of Tbr2 to melanopsin has been suggested by previous studies (Sweeney, 2014; Mao, 2014). I found that the number of tdTomato+ cells that expressed melanopsin decreased by 74% as soon as one week post tamoxifen administration (Fig. 4A, D, G, p<0.0002, n=5 control animals, 4 mutant animals, counted 200-400 cells/animal).
Fig. 4: Control and mutant retinas 1 week post tamoxifen.

(A-F) Whole mount view of retinas with ganglion cell side up from control and mutant mice 1 week post tamoxifen. (A,D) Tbr2 expression is lost in mutant retinas. (B,E) The number of cells expressing melanopsin is decreased in mutant retinas. (C,F) The Cre-expressing cell bodies are labeled by tdTomato. (G) Quantification of melanopsin-expressing cells in control and mutant retinas. (H) Quantification of tdTomato-expressing cells in control and mutant retinas. Scale bar= 25 um.

Since the Tbr2 mutant retinas displayed a drastic loss in melanopsin-expressing RGCs, it prompted me to investigate whether removing Tbr2 from RGCs lead to RGC death, or just no melanopsin expression. To distinguish between these possibilities, I first compared the average number of tomato-labeled RGCs in Tbr2creER/+ to Tbr2creER/fl retinas one week post tamoxifen administration. I observed that tomato-labeled cell bodies could still be visualized in the Tbr2 mutant retinas, and found
**Fig.5:** Brain targets of RGCs in control and mutant mice.
Coronal brain sections of control and mutant brains 3 weeks post tamoxifen showing axonal projections to non-image-forming nuclei. Scale bar= 100 um. 
SCN= Suprachiasmatic nucleus, dLGN= dorsolateral geniculate nucleus, vLGN= ventrolateral geniculate nucleus, IGL= intergeniculate leaflet, OPN= olivary pretectal nucleus, POPN= posterior OPN, NOT= nucleus of the optic tract, PPN= posterior pretectal nucleus.

there was no statistical difference between the numbers of tdTomato+ cell bodies counted (Fig. 4C, F, H, n=5, p<0.06, n=5 control animals, 4 mutant animals, counted 200-400 cells/animal). I then compared the innervation of non-image forming brain nuclei in Tbr2cre/+ and Tbr2cre/fl brains three weeks post tamoxifen administration. The three weeks timepoint was chosen because it allows for enough time for the axons to degenerate in the case that Tbr2 mutant RGCs were undergoing apoptosis. I compared whether known targets of Tbr2-expressing RGCs were innervated by tdTomato-
labeled axons in control and mutant mice. Since there is variation in the orientation of brain sectioning, it is a tough task to quantify the innervation in each of these brain areas. Therefore I used the following criteria for assaying the brain area innervation: 1) I used control and mutant retinas with a density of tdTomato-labeled RGCs that was not statistically different when I counted the tdTomato cell bodies in the retina, and 2) I noted whether tdTomato-labeled axons innervate a particular brain area throughout consecutive brain sections. I found that in Tbr2 mutant brains the SCN, IGL, vLGN, OPN and PPN were still innervated by tomato-labeled axons (Fig. 5, n= 3 control, n= 4 mutant). I did not observe any noticeable differences between the brain area innervation of control and Tbr2 mutant brains.

Additionally, I also investigated whether the lack of Tbr2 had any effect on the dendritic stratification of Tbr2 mutant RGCs. However, the dendritic stratification of Tbr2 mutant cells was still observed in S1 and S5 of the IPL as in the control (Fig. 6). This data implies that upon removal of Tbr2, non-image-forming RGCs exhibit a

![Fig.6: IPL dendrite stratification of RGCs in control and Tbr2 mutant mice. Retinal sections of control and mutant retinas 3 weeks post tamoxifen showing similar lamination patterns of tdTomato-labeled cells. Scale bar= 25 um.](image-url)
down-regulation in melanopsin expression, but persist and maintain their connections rather than apoptose. Therefore Tbr2 is not necessary for maintaining the survival of non-image forming RGCs in adult mice, but it is required for maintaining melanopsin expression. Although Tbr2 is responsible for some of the gene expression that maintains an ipRGC identity in adulthood, it is not a master regulator of all aspects of this RGC fate.

3.3.2 Tbr2 is required for a normal pupillary light reflex in the adult mouse.

The pupillary light reflex is known to be a behavior output of a subset of the non-image forming RGC circuit. It has been shown that when Tbr2 is removed from developing retinas, the pupil reflex is diminished (Sweeney, 2014; Mao, 2014; Hattar, 2016). In order to determine the effect of Tbr2 on the pupillary light reflex circuitry in adult mice I administered tamoxifen to Tbr2creER/fl and control mice. I recorded the pupillary light reflex 3 weeks post tamoxifen administration under low, medium, and high light intensities, and measured the percentage of pupil constriction. I found that there was no significant difference in the pupil constriction at the lowest light level of 0.2 mW/cm² (Fig. 7B,C, n= 5 control and n=4 mutant mice). Similarly, at a light intensity of 0.5 mW/cm² there was no significant difference in pupil constriction (Fig. 7D,E, n= control mice and n=6 mutant mice). However, at the high light intensity of 6 mW/cm² there was an impairment in the pupil constriction of mutant mice (Fig. 7D-G, Student’s t-test p<0.01, n=5 control mice and n=6 mutant mice). Yet even
Fig. 7: Pupil reflex in control and mutant mice.

(A) Representative pupil images at 25s post light illumination for each light condition.
(B, D, F) Kinetics of transient pupil constriction under low, medium, and high light conditions respectively. (C, E, G) Relative pupil area 25s post light illumination under low, medium, and high light conditions respectively.

though the pupil reflex under higher light was impaired, it was only a mild impairment (Fig. 7A,F,G). I hypothesize that the remaining pupillary response is due to the leftover ipRGCs that did not have an active Cre, as well as the input from the rod and cone pathways which also feed into the pupil reflex circuit.

3.3.3 Tbr2 is not required for night/day activity cycle entrainment.

To further investigate the potential reflexive behaviors affected by loss of Tbr2, I assayed the ability of Tbr2 mutant mice to entrain to the night/day cycle. I set control and Tbr2 mutant mice into free wheel running cages that record the number of revolutions the wheel has turned. The mice were subjected to a 12 hours light,
Fig. 8: Day/night activity cycles of control and Tbr2 mutant mice. Actograms representing the average activity of 3 mice of each genotype. Each line represents one day of a 12 hour light/dark cycle. 

followed by 12 hours dark cycle. Wildtype mice are nocturnal and can entrain to the onset of light such that they only run on the wheel during the hours in the dark. I compared the locomotor activity of the control and Tbr2 mutant mice over a period of 3 weeks. I found that there was no significant difference in their ability to entrain to the light/dark cycle, and both genotypes of mice remained active during the 12 hour dark period (Fig. 8). This evidence suggests that loss of Tbr2 affects the reflexive pupil behavior, but not the ability of the mice to photoentrain.
3.3.4 Tbr2 is sufficient for melanopsin expression in image forming RGCs.

The epistatic relationship of Tbr2 to melanopsin has been suggested by data from this study as well as others (Sweeney, 214; Mao, 2014). In order to determine whether Tbr2 is sufficient to regulate expression of melanopsin I ectopically expressed Tbr2 in RGCs through intravitreal injection of AAV2-CMV-myrRFP-2A-Tbr2 (Tbr2-AAV) or AAV2-CMV-mCherry (control AAV) in the adult eye.

I first tested the expression of the Tbr2 viral constructs in vivo to make sure Tbr2 was being expressed. I intravitreally injected 1-2 uL of virus into adult mouse eye and allowed 2 weeks for construct expression before harvesting the retinal tissue. I found that with this method I was able to get hundreds of RGCs infected (Fig. 9A-B). The Tbr2 and control AAV constructs express a red fluorescent reporter

**Fig.9:** Retinal infection with Tbr2-AAV construct. (A) Whole mount retina with ganglion cell side up showing infected cells in the RGC layer labeled by the fluorescent reporter. (B) Zoom in of section in (A) outlined in the black box. (C) Infected cells denoted by RFP labeling also express Tbr2. (D) AAV also infects image forming RGCs marked by Brn3a (arrows). (E) Some infected RGCs also express melanopsin (arrows). Scale bar= 25 um.
protein so that the soma, dendrites, and axon of infected cells will be labeled (Fig. 9B). The RGCs infected with the Tbr2-AAV construct also expressed the Tbr2 protein (Fig. 9C). The virus also infected image-forming RGCs marked by Brn3a, thus image-forming RGCs were able to ectopically express Tbr2 (Fig. 9D). Some of the Tbr2-AAV infected RGCs also expressed melanopsin (Fig. 9E). This could be endogenous melanopsin expression, or it could be a cell that began expressing melanopsin due to the ectopic Tbr2 expression.

To quantify whether the ectopic Tbr2 expression was inducing melanopsin expression I compared the numbers of infected cells that expressed melanopsin in retinas infected with Tbr2-AAV or mCherry-AAV. I found that 2 weeks after AAV injection, ~20% of cells expressed melanopsin when infected with the control-AAV (Fig. 10). This number agrees with previous findings that the melanopsin-expressing RGCs are ~20% of RGCs (Sweeney, 2014; Mao, 2014) and it implies that the virus infects random RGC types and is not biased towards infecting only melanopsin cells. In retinas infected with Tbr2-AAV, ~40% of infected cells expressed melanopsin (Fig. 10B). To analyze whether there was an increase of RGCs co-expressing melanopsin specifically in an image-forming RGC subtype, I infected retinas from the Isl2-GFP mice which have the image forming Isl2+ RGCs labeled by GFP (Triplett, 2014). The Isl2-expressing group of RGCs comprises most of the alpha RGCs labeled by the phosphoprotein SMI-32, and Isl2-expressing RGCs mostly do not overlap with melanopsin-expressing RGCs (Triplett, 2014). I found that in Isl2-GFP mouse retinas
Fig. 10: Ectopic expression of Tbr2 in adult mouse eyes. (A) Whole mount retinas infected with either AAV2-myrRFP-2A-Tbr2 (Tbr2-AAV) or AAV2-mCherry (RFP-AAV). Infected cells are denoted by RFP expression. Yellow arrows point to RGCs that co-express RFP, melanopsin and Isl2. Magenta arrows point to an infected cell that expresses melanopsin, but not Isl2. (B) The percent of infected cells that co-express melanopsin is significantly increased in cells infected with Tbr2-AAV. The percent of image-forming RGCs co-expressing melanopsin is significantly increased (n=4 RFP-AAV, n=5 Tbr2-AAV, *** p<0.0001, Student’s t-test). Scale bar = 20 um.

infected with control-AAV only ~7% of infected Isl2+ RGCs co-expressed melanopsin, while in retinas infected with Tbr2-AAV ~22% of infected Isl2+ RGCs co-expressed melanopsin (Fig. 10B). This evidence demonstrates that Tbr2 is sufficient to reprogram various image forming RGC types to activate melanopsin expression. However, it is important to note that not all the infected RGCs activated melanopsin expression. This implies that there are other mechanisms involved in the regulation of melanopsin expression in different types of RGCs, and environments in some RGC types are more permissible to melanopsin expression activation while others are not. However, it is possible that there were many other infected cells that might have induced melanopsin expression but at low levels such that they are undetectable by immunofluorescent staining. This is the case with the M4 type of
3.3.5 **Tbr2 ectopic expression in adult RGCs did not affect their axonal targets.**

Since Tbr2 is regulating the melanopsin expression aspect of non-image-forming RGC identity, it was possible that it was also regulating other factors such as molecules involved in maintaining axonal connections, and it could be involved in the plasticity of RGC axons. To investigate this, I sectioned the brains of mice that had retinas infected with control or Tbr2 virus. I did this at two time points, the first 2 weeks post infection and the second 4 weeks post infection. I did the second time point to account for the case where axons might take longer in rewiring the adult brain. I compared the brain areas innervated by RGCs infected with control and with Tbr2 virus. However, I did not see any differences in the brain areas innervated by both viruses after 2 (n=4 mice control AAV, n=5 mice Tbr2 AAV) and not even after 4 weeks (n=3 mice each virus). The axons of cells infected with either virus were innervating all the known retinorecipient targets, image-forming and non-image-forming. I also did this experiment in the Isl2-GFP mouse line. I showed that Tbr2 can induce melanopsin expression in Isl2-GFP RGCs, and a follow-up question was whether these image-forming cells are now also changing their targets to non-image-forming brain areas. To answer this I compared the innervation of non-image-forming
**Fig. 11:** Brain targets of AAV infected RGCs in Isl2-GFP mice. 
Coronal sections of brains from mice infected with either control or Tbr2-AAV 4 weeks post infection (n=3 mice each virus type). Green color represents axons from Isl2-GFP RGCs. Red color represents axons from infected RGCs. Scale bar= 100 um. vLGN= ventrolateral geniculate nucleus, dLGN= dorsolateral geniculate nucleus, OPN= olivary pretectal nucleus, MTN= medial terminal nucleus, SC= superior colliculus.
brain areas by GFP+ axons in retinas that were infected with control and Tbr2-AAV. I did not see infected GFP+ axons going to non-image-forming areas 4 weeks post infection (Fig. 11, select retinorecipent targets shown). A small percentage (~6.5%) of Isl2-GFP RGCs are melanosin-positive and innervate the OPN. But I observed these axons in mice infected with both viruses. I did not observe other GFP axons in non-image-forming brain areas. This data suggests that Tbr2 is not sufficient to the plasticity of RGC axons, and it does not affect that aspect of a non-image-forming RGC identity in the adult mouse.

3.3 Discussion

Although much work has been done to characterize the gene expression steps that lead to a particular retinal ganglion cell fate during development, the molecular factors that maintain RGC identity in the adult mouse remain a mystery. In this study I investigated which aspects of RGC identity are regulated by the transcription factor Tbr2 in adult mice. I found that Tbr2 is indeed required for maintaining aspects of a non-image-forming identity. These results are the first to demonstrate the requirement of a molecular factor for the maintenance of RGC identity.

3.3.1 Tbr2 is required for maintaining the melanopsin expression aspect of non-image-forming RGC identity
A crucial identity aspect for ipRGC function is their expression of melanopsin. Data from previous studies suggests that there is a stringent epistatic relationship between Tbr2 and melanopsin and that Tbr2 is involved in regulating melanopsin (Sweeney, 2014; Mao, 2014). ChIP experiments in embryonic stem cells have also demonstrated that Tbr2 does bind to the melanopsin promoter, strengthening the evidence for Tbr2 regulation of melanopsin (Teo, 2011). My study adds further evidence of Tbr2 regulation of melanopsin, even after development. Thus Tbr2 is required not only to specify ipRGCs during development, but also to maintain their intrinsic photosensitivity later in the animal’s life.

However, the role of Tbr2 in maintaining survival of non-image-forming RGCs differs from development to adulthood. If Tbr2 is lacking during developmental RGC specification the non-image-forming RGC types do not survive, and their cell bodies, dendrites and axons are missing (Sweeney, 2014; Mao, 2014; Mao, 2008). But in the adult, the RGCs lacking Tbr2 continue to survive and no changes were observed in their connectivity patterns. However, it is possible that the integrity of their synapses or their function in general is compromised and I did not assay for it in this study. One challenge to pursuing the potential changes in identity of Tbr2 mutant cells is the lack of markers for Tbr2 RGC subtypes. Another challenge is that very little is known about the function of Tbr2 subtypes other than the ipRGCs. Thus this study only reveals part of the possible Tbr2-dependent aspects of non-image-forming RGC identity.
3.3.2 Tbr2 is required for maintaining a functional pupillary light reflex in the adult mouse

The pupillary light reflex is one of the behavioral outputs of non-image-forming RGCs. Non-image-forming RGCs (both ipRGCs and conventional) target the OPN and are a key part of the neural circuit that controls pupil diameter (Berson, 2003; Sweeney, 2014). The M1 Brn3b-positive ipRGCs connect to the OPN shell and are necessary components of the pupil light reflex circuit (Chen, 2011). If these ipRGCs are ablated there is a severe deficit in the pupil reflex (Chen, 2011). However, the pupil reflex is not completely impaired. This is true even in mice that have melanopsin knockout from all types of ipRGCs (Lucas, 2003; Keenan, 2016). This is due to the rods and cones which also contribute to the OPN input (Hattar, 2003; Keenan, 2016; Jain, 2016). In this study, removing Tbr2 yields a phenotype similar to the melanopsin knockout mice: the pupil reflex is partially impaired at high light irradiances (0.35 mW/cm², and 5662 mW/cm²). The melanopsin knockout mice also do not have a pupil reflex defect at low light irradiance since the light input through the rod and cones are sufficient to induce a pupil reflex (Lucas, 2003; Mao, 2014; Keenan, 2016; Jain, 2016). Similarly, in the Tbr2 adult knockout mice, the pupil reflex in the mutant mice is not significantly different from control mice at the low light level (0.04 mW/cm²). Taken together, this data suggest that by removing Tbr2 the maintained light-detection pathway upstream of melanopsin is affected, and that Tbr2 is a key component of maintaining this part of the ipRGC identity functional.
In melanopsin knockout mice the circadian photoentrainment is not impaired and mice can still photoentrain due to the light input from the photoreceptors (Panda, 2002; Ruby, 2002; van Diepen, 2013). My results from the Tbr2 mutant mice are similar and the mutant mice do not show any defect in entraining to night/day cycles. Melanopsin knockout mice are known to have some phase-shifting defects (Panda, 2002; Ruby, 2002), however in this study I did not test the phase-shifting of Tbr2 mutant mice.

3.3.3 Tbr2 is sufficient for melanopsin expression

The data so far suggesting that Tbr2 is a regulator of melanopsin (Sweeney, 2014; Mao, 2014; Teo, 2011) is fully supported by the finding that Tbr2 is sufficient to activate melanopsin expression in the image-forming RGCs labeled by the Isl2-GFP mouse line. Notably, not all the cells that were ectopically expressing Tbr2 activated melanopsin expression. This suggests that there are other regulatory mechanisms in place that either enable melanopsin activation by Tbr2 or suppress it. One model would be that the mechanism is dependent on the level of Tbr2 being expressed. I have observed through immunostaining that some non-image-forming RGCs express higher levels of Tbr2 while others look much dimmer and therefore have lower Tbr2 levels (data not shown). This could correlate to how much melanopsin is expressed. Another model could be that there are co-activating proteins that aid Tbr2 in enabling melanopsin expression and only certain RGC types express these proteins. Future
studies will shed light on this mechanism. But for now, the epistatic relationship of Tbr2 to melanopsin can be exploited to yield light-sensitivity in RGCs that might otherwise lack it, whether for experimental purposes or for gene therapy.
Chapter 4: The role of Tbr2 in RGC survival in an RGC injury model

4.1 Introduction

Glaucoma is the second leading cause of blindness worldwide (Quigley and Broman, 2006). In glaucoma the most affected retinal cell type is the retinal ganglion cell (Greco, 2016). It is currently not clear what the mechanism is that initiates the cascade of cellular damage, but an elevated intra-ocular pressure (IOP) is associated with optic nerve damage, and more elevated IOP results in an increased rate of glaucoma progression (Mantravadi and Vadhar, 2016). IOP-lowering agents can slow down the progression of RGC death, however this is not a cure (Mantravadi and Vadhar, 2016). Elucidating the pathogenesis of glaucoma and the mechanisms that lead to RGC death can reveal ways to inhibit them and lead towards a cure.

The underlying cause of blindness in all types of glaucoma is the RGC death. However, there are separate molecular pathways for the degeneration of different anatomical parts of the RGC (Fernandes, 2014; Libby, 2005). If an axon is severed, the portion distal to the injury degenerates by a process called Wallerian degeneration, while the portion proximal to the injury is eliminated by a process called dying back (Struebing and Geisert, 2015; Yaron, 2016). The RGC soma dies by apoptosis (Nickells, 1996; Janssen, 2013). When the pro-apoptotic protein Bax is knocked out in a glaucoma mouse model, the RGC soma was protected but axonal degeneration
was not prevented (Libby, 2005). The JNK (c-Jun, N-terminal kinase) pathway however is involved in both axonal and soma degradation following optic nerve injury, and in mice lacking \textit{Jnk2} and \textit{Jnk3} the RGC death after optic nerve injury is delayed (Fernandes, 2012). Additionally, the dual leucine kinase (DLK) regulates the JNK pathway in somal but not axonal degeneration of RGCs after optic nerve injury; its deficiency was shown to yield lower numbers of dying RGCs post optic nerve injury (Fernandes, 2014). Thus it is important to understand these distinct molecular pathways of RGC death and how they play a role in glaucoma.

Mouse models have been very useful for studying glaucoma. In some models mice have been engineered to have mutations that lead to elevated IOP (Struebing and Geisert, 2015). In other models glaucoma is experimentally induced by injecting microbeads into the eye, which leads to blockage of the trabecular meshwork and elevated IOP (Sappington, 2010; Bunker, 2015; Gross, 2013). The elevated IOP puts mechanical stress on the optic nerve head and causes RGC axon damage and disruption in axonal transport (Weinreb, 2014). Another experimentally induced mouse model of glaucoma is the optic nerve crush, where the optic nerve is crushed using fine forceps (Templeton and Geisert, 2012). This model has the advantage that it causes mass synchronized RGC death, thus the effect can be studied in a large number of cells at the same time. Each glaucoma mouse model has its own benefits, but they all simulate the end result of glaucoma which is RGC death.

One area of research in the RGC degeneration field has focused on
understanding how different RGCs types are affected by optic nerve damage in glaucoma rodent models. Studies comparing the structural changes of dendrites in ON versus OFF RGCs have found that RGCs with dendrites in the OFF lamina are prone to structural changes after elevated IOP, while RGCs with dendrites in the ON lamina are more resistant to structural changes due to elevated IOP (El-Danaf and Huberman, 2015; Della Santina, 2013, Della Santina and Ou, 2016). The melanopsin-expressing RGCs and ON-alpha RGCs are more resistant to optic nerve damage compared to conventional RGCs (Cui et al., 2015; Vidal-Sanz, 2017). More than 70% of M1 ipRGCs and more than 80% of alpha-RGCs preferentially survive an optic nerve crush injury (Duan et. al, 2015). This is evidence that some RGC types are more resistant to nerve injury than others, and understanding what their mechanism is for survival could improve current glaucoma treatments.

It is possible that the RGC types that seem to preferentially survive after optic nerve damage all have something in common. The melanopsin-expressing RGCs are known to also express the transcription factor Tbr2 (Sweeney, 2014; Mao, 2014). Alpha-RGCs have dendrites that laminate either in the ON or in the OFF IPL sublamina, and about 50% of alpha-RGCs have been identified as the M4 type of ipRGC, which have dendrites in the ON sublamina, and melanopsin levels that are undetectable by immunofluorescence (Schmidt, 2014). Thus half of the alpha-RGCs that survive likely also express Tbr2. Additionally, alpha-RGCs were able to regrow axons and re-innervate their correct brain targets upon visual and chemogenetic
stimulation post optic nerve crush injury (Lim, 2016). This suggests that most of the RGCs that survive a nerve crush injury also express Tbr2.

Tbr2 has been shown to be implicated in neuronal survival in other areas of the CNS such as the cortex and hippocampus (Sessa, 2008; Hodge, 2012). In the retina, lack of Tbr2 during development leads to non-image-forming RGC death (Sweeney, 2014; Mao, 2008). In this study I sought to understand the role Tbr2 is playing in maintaining the survival of RGCs. To explore this I used the mouse optic nerve crush as a model for mechanical injury to axons. This injury leads to the initiation of caspase cascades and RGC death and degeneration (Niwa, 2016). I found that Tbr2-expressing cells preferentially survive such an injury, even for up to 4 weeks following optic nerve crush. I further tested the requirement and sufficiency of Tbr2 in RGC survival and found that although Tbr2 itself does not seem to be responsible for the neuroprotective effect, its expression can be used as a cell-type marker to identify a group of RGCs that have unique survival properties.

4.2 Results

4.4.1 Tbr2-expressing RGCs selectively survive after optic nerve injury in adult mice.

The murine optic nerve crush injury is one of the methods used to study RGC death due to axon damage at the optic nerve head, as it is believed to occur in
Recent work showed that ipRGCs preferentially survive after an optic nerve crush in mouse (Duan, 2015). I hypothesized that other non-image forming RGCs also survive after nerve crush. To test this hypothesis I performed optic nerve crushes in adult mice and used immunohistochemistry to determine which RGC classes survive. Since the transcription factors Tbr2, Isl2, and Satb2 label almost all of the RGCs in an adult retina with very little overlap between the types (Sweeney, 2017), I used these factors as markers for the 3 major RGC classes. I also used Brn3a as a pan-image-forming RGC marker (Sweeney, 2017). Additionally I used melanopsin as a marker since melanopsin-expressing RGCs have been previously reported to survive this injury better (Duan, 2015; Lim, 2016). Consistent with previous work (Duan, 2015; Vidal-Sanz, 2017) I found that image forming RGCs marked by Brn3a decrease by ~80% by 2 weeks post nerve crush (Fig. 12A, C, p<0.0001, n=5 mice). The group of RGCs marked by Satb2, which includes the Direction-Selective RGC class, decrease by ~90% by 2 weeks post nerve crush (Fig. 12A, C, p<0.0001, n=5 mice). The RGC class marked by the Isl2-GFP mouse line decrease by ~90% (Fig. 12A, C, p<0.0001, n=5 mice). But surprisingly, the majority of non-image forming RGCs marked by Tbr2 stay alive, with only a ~30% decrease by 2 weeks post nerve crush (Fig. 12A, C, p< 0.001, n=5 mice). A majority (~60%) of the melanopsin positive cells also stay alive, confirming previous findings (Fig. 12A, C, p< 0.001, n=5 mice). After a month following optic nerve crush the number of surviving RGC of all types is decreased
**Fig. 12:** Survival of different RGC types post optic nerve crush.

(A,B) Whole mount view of retinas from control and the corresponding optic nerve crushed eye. Retinas are labeled with the following RGC markers: Tbr2, Brn3a, Satb2, Isl2, Melanopsin. Images in (A) are from 2 weeks post nerve crush, while images in (B) are from 4 weeks post nerve crush. (C, D) Quantification of the number of cells present labeled by each marker 2 weeks (C) and 4 weeks (D) post nerve crush.
(Fig. 12B). The Brn3a, Isl2, and Satb2 expressing RGCs are decreased by ~90%, 95%, and ~99% decreased respectively (Fig. 12B, D, p<0.0001, n=4 mice). The melanopsin-expressing RGCs were slightly further decreased, with ~50% of them surviving 4 weeks after the nerve crush (Fig. 12B, D, p<0.0001, n=4 mice). The Tbr2 class as a whole was slightly decreased, although it was not statistically significant (Fig. 12B, D). This data shows that not only the melanopsin-expressing subset of RGCs, but a larger class of non-image-forming RGCs that express Tbr2 are resistant to cell death after axonal injury. This leads to the hypothesis that Tbr2 might play a neuroprotective role for RGCs.

4.4.2 Tbr2 is not necessary for the survival of non-image-forming RGCs after nerve crush.

In order to understand the role Tbr2 might be playing in the survival of non-image-forming RGCs I sought to test the hypothesis that Tbr2 is necessary for these RGCs to stay alive after optic nerve crush. I crossed mice containing a floxed Tbr2 allele (Zhu, 2010) with mice that express a tamoxifen-inducible Cre from the Tbr2 promoter (Pimeisl, 2013). These mice also harbor a ROSA26-lox-stop-lox-tdTomato reporter gene so that cells with active Cre will express tdTomato, which can be visualized under a fluorescent microscope. The mice received tamoxifen for 4 consecutive days in order to activate the Cre in as many Tbr2 cells as possible, and
**Fig. 13:** Survival of Tbr2-expressing RGCs post optic nerve crush in a Tbr2 knockout mouse model.

(A) Whole mount view of Tbr2creER/fl retinas from the control and the corresponding nerve crushed eye. Cells with active cre are labeled by tdTomato. Scale bar= 25 um. (B) Quantification of the numbers of cells present labeled by tdTomato in control and nerve crushed (ONC) retinas. Each dot represents one field of view. The wide range stems from the gradient of Tbr2-expressing RGCs in the retina. The control and nerve crushed retinas from 4 animals did not have significantly different numbers of tdTomato-labeled cells by one-way ANOVA.

I waited one week to ensure the removal of the Tbr2 gene and degradation of the protein. Then I performed an optic nerve crush on one eye and waited two weeks before sacrificing the mice. I analyzed matched comparisons of the number of tdTomato-labeled cells in the retina with the crushed nerve to those in the retina without the crushed nerve in each mouse. I found that there was no significant difference in the survival of tdTomato-labeled cells in the nerve crushed retina versus
the control (Fig. 13A-B, n=4, one-way ANOVA). This result indicates that Tbr2 is not necessary for the survival of the spared non-image-forming RGCs.

**4.4.3 Tbr2 is not sufficient to confer neuroprotection during optic nerve crush.**

Although Tbr2 was found not to be required for the survival of the spared RGCs, there was the possibility that this was due to redundant unknown factor factors that were enough to sustain cell survival even without Tbr2. Thus I hypothesized that if this was the case, and Tbr2 did still confer neuroprotective properties to RGCs, it should be sufficient to do so when ectopically expressed in image-forming RGCs and rescue them from cell death. As shown in the data from figure xx, the image forming RGCs undergo dramatic death after nerve crush. So I ectopically expressed Tbr2 in

![Graph showing survival of RGCs ectopically expressing Tbr2 post optic nerve crush](image)

**Fig.14:** Survival of RGCs ectopically expressing Tbr2 post optic nerve crush. Comparison of the numbers of surviving infected RGCs (denoted by RFP expression) that co-express either Brn3a or Isl2. n=5 mouse eyes infected with control virus, n=4 mouse eyes infected with Tbr2 virus. No statistically significant difference was found through one-way ANOVA.
RGCs of adult mice through an intravitreal injection of an adeno-associated viral (AAV) that expresses both Tbr2 and a red fluorescent reporter. After two weeks, the optic nerves of infected eyes were crushed. Two weeks post crush, the AAV-infected retinas were immunostained, and infected cells expressing the red fluorescent reporter were labeled with various RGC markers and counted. Retinas infected with the Tbr2-AAV were compared with retinas infected with a control AAV, which only encodes the red fluorescent reporter. I counted 100-200 infected cells from each retina. When comparing the numbers of surviving cells in these retinas, if Tbr2 was conferring neuroprotective properties, then a greater number of infected image-forming RGC types would survive in retinas infected with Tbr2-AAV than with the control. However, I found that there was no significant difference in the percentage of surviving Brn3a- or Isl2- positive RGCs infected with Tbr2 and control virus (Fig. 14). Additionally, there were extremely low numbers of Satb2-expressing RGCs in retinas infected with either virus. This evidence shows that Tbr2 is not sufficient to confer neuroprotection when it is ectopically expressed in RGCs in the adult mouse.

4.3 Discussion

4.4.1 Some RGC types survive preferentially following optic nerve injury

Recent evidence supports the idea that particular RGC types are able to survive survive longer after axonal injury (Della Santina, 2013; El-Danaf and Huberman,
2015; Vidal-Sanz, 2017). The melanopsin-expressing RGCs in particular have been shown to be more resistant to injury (Duan, 2015; Vidal-Sanz, 2017). This study reveals that in addition to the melanopsin-expressing RGCs, a significant portion of all RGCs (~20%) are resistant to degeneration caused by axonal injury. Additionally, most of these surviving RGCs express Tbr2. Tbr2 is expressed in ~28% of all RGCs in the mouse retina, and all ipRGCs express Tbr2 (Sweeney, 2017; Mao, 2014). Of the Tbr2-positive surviving RGCs, some of them express melanopsin, which is in accordance with previous findings (Duan, 2014; Lim, 2016). However, although the majority (~80%) of Brn3a-positive RGCs die, there are still some left that persist even 4 weeks post optic nerve crush. This group of RGCs do not express any of the other proteins I assayed for in this study. This is surprising because Tbr2, Isl2, and Satb2 account for virtually all the RGCs in the mouse retina. And yet, by 4 weeks post optic nerve crush, there are almost no Isl2- or Satb2-positive RGCs left, whereas the surviving Brn3a-positive RGCs are about half as abundant as the Tbr2-positive ones. The surviving Brn3a-positive RGCs do not co-express Tbr2, Isl2, or Satb2, thus these are different RGC populations. One explanation could be that these surviving Brn3a RGCs used to be either Satb2- or Isl2-positive, but under injury conditions they have turned off various markers of their identity. Further experiments using mouse lines that label specific RGC types with fluorescent reporters can help trace the fate of RGCs after injury and could help reveal what types of RGCs they were in case they are turning off expression of known protein markers.
4.4.2 Tbr2 is not required for the neuroprotection of Tbr2-expressing RGCs post injury

Considering that Tbr2 is necessary for the survival and/or maintenance of non-image-forming RGCs during development (Sweeney, 2014; Mao, 2014; Mao, 2008), and that the Tbr2-expressing RGCs have a unique survival ability, it lead to the hypothesis that Tbr2 is also required in this situation for the RGCs to survive. However, the RGCs from which Tbr2 was deleted continued to survive after the nerve crush. Combined with the findings that Tbr2 is not required for non-image-forming RGC maintenance in the adult mouse and that some non-Tbr2-expressing RGCs also survive after optic nerve crush, I conclude that Tbr2 is not required for the neuroprotection of RGCs after optic nerve crush.

4.4.3 Tbr2 is not sufficient to activate anti-apoptotic mechanisms post injury

Although Tbr2 is not required for the survival of the Tbr2 RGCs after nerve crush, I hypothesized that ectopic expression of Tbr2 could confer neuroprotection to RGCs. For example, Tbr2 could start a cascade that leads to the expression of proteins involved in neuroprotection, but then no longer require Tbr2 to maintain their expression. There could also be other redundant factors working in combination with Tbr2 to activate the cell survival pathways such that deleting Tbr2 yielded no effect but ectopic expression of Tbr2 could be sufficient for neuroprotection. However, I found that ectopic expression of Tbr2 did not rescue RGCs from axonal damage-
induced death. Future work (outlined below) can take advantage of our ability to fluorescently label surviving RGCs to isolate them in pure populations and determine which genes these RGCs express. This approach could reveal the genes in these RGCs that are providing neuroprotection.
Chapter 5: Conclusions and Future Work

My thesis work has focused on the role the transcription factor Tbr2 has in the maintenance of non-image-forming RGC identity and survival. My first project expounds on a biological pathway for melanopsin expression, and provides evidence that Tbr2 is a key part of the maintenance mechanism for non-image-forming RGC identity and behavioral outputs. I discovered that Tbr2 is required for melanopsin expression in non-image-forming RGCs in the adult mouse and when Tbr2 is lacking, the pupillary reflex behavior is also impaired. Additionally, I discovered that Tbr2 is sufficient to induce melanopsin expression even in image-forming RGCs.

These results are important because even though much work has been done in classifying RGCs and understanding key factors that contribute toward their specification during development, there is still much to be learned about what factors contribute to maintaining that specified fate in the adult life of the mouse. These results reveal a transcription factor that is actively regulating an aspect of RGC identity in the adult mouse. One of the goals of vision neuroscience is to understand the basis of neurodegenerative diseases such as glaucoma and work towards a cure. Since diseases such as glaucoma mostly affect adults (Mantravadi and Vadhar, 2015), it is important to know which factors play a role in maintaining a functional RGC in adulthood. If these factors are known, then researchers can use that information to employ gene therapy methods to prevent disease progression.
Future experiments:

One future experiment will focus on rescuing the pupil reflex defect in the Tbr2 knockout mice by ectopically expressing Tbr2 using the Tbr2-AAV construct. Tbr2 mutant mice will be given tamoxifen, then 3 weeks later their pupil reflex will be measured. Then, one eye of the mouse will undergo intravitreal injection of either Tbr2-AAV or control AAV. Two weeks post injection the mice will have their pupil reflex recorded again. Considering that Tbr2 can activate melanopsin expression, the hypothesis is that melanopsin expression that had disappeared from RGCs that had Tbr2 removed can now be activated again, and since the connections of these cells are still correct the pupil reflex can be rescued.

Additionally, multi-electrode-array experiments in conjunction with the Sher lab at UC Santa Cruz performed on mice that have channel-rhodopsin expressed in Tbr2 RGCs will help characterize the functional properties of Tbr2 RGCs. The multi-electrode-array experiments can also be performed on Tbr2 mutant mice, and comparing these responses to the wild type functional responses will elucidate even further the role Tbr2 plays in maintenance of non-image-forming RGC functions.

My second project sheds light on the types of RGCs that survive in a glaucoma mouse model. I discovered that the Tbr2-expressing group of RGCs preferentially survives compared to other RGC classes even 4 weeks post injury. This means that they have the potential to regrow their axons, and a recent study shows how this was
possible on a subset of Tbr2 RGCs (Lim, 2016). Clinical studies in individuals with glaucoma have found a strong correlation between glaucoma and pupillary light reflex defects, sleep disorders, and depression (Oba, 2016; Onen, 2000; Drouyer, 2008). These Tbr2-expressing RGCs are necessary for the body’s circadian rhythm, and attempts to get them to regrow their connections to the brain are important for improved quality of life in individuals where these connections no longer exist.

Future experiments:

Even though Tbr2 itself does not seem to be involved in the survival mechanism of these RGCs, it is useful that I found Tbr2 can be used as a marker for this population. This can be exploited in future experiments where the Tbr2CreER;tdTomato mouse line (Pimeisl, 2013) can be used to label the surviving cells, then isolate them for RNA-Seq. Their gene expression patterns will reveal what factors they express that could be involved in their advantageous survival mechanism.

In conclusion, my research identifies for the first time a transcription factor required for RGC maintenance in adult mice, and points future research towards a path in better understanding of how retinal ganglion cells maintain their function and survival upon injury and disease.
Chapter 6: Bibliography


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