SOX2 IS ESSENTIAL FOR THE MATURATION AND MAINTENANCE OF RETINAL MÜLLER GLIA

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Neurobiology in the School of Medicine.

Chapel Hill
2014

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ABSTRACT

Amelia Rose Bachleda: SOX2 is Essential for the Maturation and Maintenance of Retinal Müller Glia
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Müller glia (MG) are the principal glial cell of the vertebrate retina. The last cell to divide from a multipotent retinal progenitor cell, they maintain many stem cell characteristics including the expression of the HMG-box transcription factor Sox2. In this thesis, we explore the role of Sox2, a marker of pluripotency throughout the CNS, in this population of presumptive neural stem cells, the MG. Through glial specific ablation of Sox2 we demonstrate that SOX2 plays an essential role in the maturation and maintenance of MG in the murine retina. Loss of SOX2 at P5 results in aberrant development and extension of MG side processes that ensheathe the neuronal cell bodies and neurites in the retina. Additionally, MG cell bodies are disorganized and their end feet fail to properly form the limiting membranes of the retina. As a result, neuronal processes in the synaptic plexiform layers are disorganized, accompanied by a marked reduction in inner retinal function. These data indicate a role for Sox2 in guiding the structural development of MG, as well as providing new insights into the role of MG in the maturation of the neural retina.

Additionally we address the complex regulation of SOX2 in the retina by examining SOX2 expression in the mildly hypomorphic $Sox2^{COND}$ line in two different strain backgrounds: on the inbred C57BL6/J background and on a mixed, outbred CD1 background. On a CD1 background, mice heterozygous for the $Sox2^{COND}$ allele display only a mild reduction in SOX2 expression and display no phenotypic abnormalities. However,

SOX2 expression is significantly reduced on the C57BL6/J background compared to wild type levels, accompanied by a marked reduction in retinal function that degenerates over the animal's lifetime. Further, Müller glial specific ablation in the C57BL6/J background results in almost complete loss of retinal function and a slow loss of MG cells over time. Together these results demonstrate the essential role of SOX2 in the maturation of MG and the neural retina, as well as pointing to a role for SOX2 in the maintenance of retinal and MG structure and function in the compromised retina.

For Larysa – Your light, love, and enthusiasm continue to be a daily inspiration.

ACKNOWLEDGEMENTS

This dissertation would never have been possible if not for Larysa Pevny. She provided the best possible foundation for my graduate studies. Her encouragement, enthusiasm and wisdom have been a sustaining force throughout the duration of this project.

I would like to thank Dr. Ellen Weiss and Dr. Eva Anton for welcoming me into their labs and providing a scientific home for me. For being so generous with your time and resources and for advising me through a foreign project, I am so grateful.

To the members of my committee, Dr. Terete Borras, Dr. Richard Cheney and Dr. Ken McCarthy: thank you for your guidance and for your faith in me. For encouraging me to finish what I started with Larysa, I am truly appreciative.

Thank you to all the members of the Pevny Lab, past and present, for helping me along every step of the way. I felt welcomed from the moment I first stepped through the laboratory door. Thank you for your patience, and for always being there to answer my questions and lend your advice. A special thank you to Dr. Tessa Crowl and Dr. Natalia Surzenko for puzzling through the biology of Müller glia with me.

To the members of Neurobiology Curriculum, thank you for all your support over the years. Special thanks to the directors, Dr. William Snider and Dr. Aldo Rustioni for ensuring I had the funds, resources and support to finish what I started.

Thank you to Dr. Vladimir Ghukasyan and Ying Hao. Your technical expertise and generosity with your time is greatly appreciated.

And to my friends and family – I could not have completed this adventure without your unwavering support. From coast to coast and the prairies in between, there isn't a day that goes by that I don't feel your love.

To my mother Phoebe, to my father Matthew, and my brother Alexander - you inspire me everyday. Thank you for believing in me, for encouraging me, and for reminding me from a very young age 'it doesn't have to be perfect'. I love you more than words can say.

To my friends in North Carolina, who were there for me every step of the way. Especially my cousin Katie Ferris, and my cohort mate Amanda Lyall; you ladies are the best and you never cease to amaze me.

To Michele Kloda, for your support and your guidance. You and the rest of the crew at the Museum of Life and Science have gone out of your way to welcome me into your little community. Your energy and warmth is contagious.

And to my partner in crime and in life, Adrian Down – thank you for taking a chance on me. You dropped everything to move across the country to embark on this adventure. You have provided unwavering strength and support through the darkest and brightest times. Your capacity for love amazes me everyday.

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LIST OF ABBREVIATIONS

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ascl	Achaete-scute complex-like
ß-gal	ß-galactosidase
bHLH	Basic helix-loop-helix
BK	Big conductance
BL6	C57BL6
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CRALBP	Cellular retinaldehyde-binding protein
DNA	Deoxyribonucleic acid
Dpc	Days postcoitum
E	Embryonic
EFTF	Eye Field Transcription Factors
ERK	Extracellular signal-regulated kinases
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
GABA	Gamma-aminobutyric acid
GAT	GABA transporter
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
GSK-3β	Glycogen synthase kinase 3β
HMG	High mobility group

HZ	Horizontal cell
ICM	Inner cell mass
ILM	Inner limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer
iPSC	Induced pluripotent stem cell
Jak	Janus kinase
Kir	Inwardly rectifying potassium channel
MAPK	Mitogen-activated protein kinase
MG	Müller glia
mGluR	Metabotropic glutamate receptor
NBL	Neuroblast layer
NFL	Nerve fiber layer
NMDA	N-methyl-D-aspartate
NPC	Neural progenitor cell
NSC	Neural stem cell
OLM	Outer limiting membrane
ONL	Outer nuclear layer
P	Postnatal
PVR	Proliferative vitroretinopathy
RGC	Retinal ganglion cell
RPC	Retinal progenitor cell
RPE	Retinal pigmented epithelium
SOX	Sry-related HMG box
SRY	Sex-determining region Y

Stat	Signal transducer and activator of transcription
SVZ	Subventricular zone
Tnfα	Cytokine tumor necrosis factor-α

CHAPTER ONE: INTRODUCTION

Adult neural stem cells (NSC), present in two well-characterized populations in the central nervous system (CNS) – the subventricular zone (SVZ) and the hippocampal dentate gyrus – display markers and characteristics of glial cells. As such, the stem cell potential of glial cells is an area of active research (Robel et al., 2011). The data presented in this thesis explores the maturation of a presumptive neural stem cell, the retinal Müller glia. Müller glia (MG) are the principal macroglia of the vertebrate retina and retain many stem cell characteristics. The last cell to develop from the multipotent retinal progenitor cell (RPC) that gives rise to all six types of retinal neurons, MG preserve their RPC radial morphology, spanning the apical/basal axis of the retina (Bringmann et al., 2006). However, unlike most differentiated glial cells (e.g. astrocytes), MG maintain the expression of SOX2, an HMG-box transcription factor that maintains stem cell pluripotency in the embryonic nervous system and in populations adult NSCs in the SVZ and dentate gyrus (Zappone et al., 2000; Bylund et al., 2003; D'Amour, 2003; Graham et al., 2003). Ablation of Sox2 in the developing CNS leads to premature neuronal differentiation and depletion of progenitor pools (Pevny and Nicolis, 2010). Furthermore, Sox2 is an essential factor in reprogramming fibroblasts into inducible pluripotent stem cells (Takahashi et al., 2007). While many studies have been carried out examining various aspects of MG's neurogenic potential (Dyer and Cepko, 2000; Ooto et al., 2004; Das et al., 2006; Karl et al., 2008), the role of Sox2 in the maturation and maintenance of MG remains to be determined.

Analysis of SOX2 function is an ideal approach to the study the biology of MG in the retina. Like other regions of the CNS, lowering SOX2 expression levels in the retina, leads to premature neuronal differentiation whereas Sox2 deletion results in complete lack of neuronal competence (Taranova et al., 2006; Matsushima et al., 2011). Mutations of the human Sox2 allele have been shown to lead to microphthalmia or anophthalmia (small eye or no eye respectively) (Fantes et al., 2003).

In this thesis I address the role of SOX2 in MG by characterizing the maturation of Sox2 deficient MG. I find that expression of SOX2 is essential for the proper maturation of the extensive side branches MG develop during the first two postnatal months. Disruption of these processes results in abnormal retinal morphology and loss of neuronal function. Further, I report that in a Sox2 hypomorphic background, ablation of Sox2 in MG results in neural degeneration and near complete vision loss. Together, these data provide new insights into the function of Sox2 in the maturation of MG processes. Additionally they indicate Sox2's essential role in the maintenance of retinal cytoarchitecture and function, in both typically developing and hypomorphic retinas.

In the following sections, I provide context for these experiments, describing: the development and function of the retinal (**Section 1.1**); the development and function of Müller glia (**Section 1.2**); and the role of Sox2 in embryonic, neural and retinal development (**Section 1.3**).

1.1 Development and Function of the Neural Retina

We are visual creatures; no other sense so dominates our experience of the world. The number of trite and cliché idioms is seemingly endless: The eye is the window to the soul, an eye for an eye, seeing is believing, love at first sight, eyesore, and on and on. You are, of course, using your eyes at this very moment to efficiently scan the rows and rows (and rows)

of little black squiggles on this page. It takes milliseconds for your eye to refine the contours of the letters, so that higher brain structures can quickly determine that this particular pattern is indeed an ‘R’ or an ‘S ‘or an ‘&’. A brief introduction to this outpost of the CNS follows.

1.1.1 The Retina: A Highly Specialized Sensory Element of the CNS

Light enters the eye globe through the cornea, is refracted onto the lens, which further focuses the light onto the neural retina at the back of the eye globe. In order to reach the light sensitive photoreceptors, light must travel through the entire, dense thickness of the retina. The retina itself is composed of six neural cell types and one glial cell, with two stages of synaptic connections (See Fig. 1.1). Information, in the form of photons flows from the photoreceptors located in the outer retina, to bipolar cells in the inner retina, to ganglion cells whose axons form the optic nerve and carry the stimulus to the brain. Horizontal cells located in the synaptic, outer plexiform layer (OPL), and amacrine cells located in the inner plexiform layer (IPL) modulate the neuronal signaling across these synaptic regions (Neves and Lagnado, 1999; Masland, 2012). The primary glial cell of the retina, the Müller glia, spans the entire width of the neural retina and provides structural and homeostatic support.

While the basic structure is relatively simple, the diversity and specialization of neuronal cells within the retina is striking. Over 55 subtypes of retinal neurons have been identified, each with their specific role in refining the visual stimulus (Masland, 2001). Twelve parallel streams of information leave the population of photoreceptors (composed of rods and cones), and via bipolar and amacrine cells, connect to as many as 15 specific types of ganglion cells (Masland, 2012). Then there is the sheer number of retinal neurons, humans have approximately 100 million rod cells (the second most numerous neurons after cerebellar granule cells) packed into a sheet of tissue only 200 μm thick (Masland, 2012).

With its complex yet still accessible circuit and clear function, the retina truly is an "approachable part of the brain" (Dowling, 1987).

1.1.2 From Photon to Ion, Phototransduction in the Retina

Phototransduction, the process of translating the light stimulus into neural signals begins with the absorption of light by visual pigments. These pigments are located in membrane disks, tightly packed into the outer segments of rod and cone photoreceptors. Visual or photopigments consist of an opsin, a G protein-coupled receptor that is linked to a light absorbing chromophore. The components of these photopigments vary between rods and cones, and contribute to their unique functional specializations. Phototransduction in rods has been characterized in the most detail (Purves et al., 2001; Luo et al., 2008; Yau and Hardie, 2009). Upon absorption of a photon, the chromophore 11-*cis* retinal isomerizes to all-*trans* retinal, initiating a conformational change of the rod opsin (rhodopsin), which results in the activation of the G protein transducin. Transducin activation stimulates a phosphodiesterase that hydrolyzes cGMP and reduces its concentration (Purves et al., 2001; Luo et al., 2008).

In the dark, the concentration of free cGMP is high, which maintains cGMP-gated cation channels in the open state. These cation channels allow for a steady flow of inward current, (or the "dark current"), resulting in a depolarized membrane potential, and sustained release of the synaptic transmitter, glutamate (Yau and Hardie, 2009). Light stimulation therefore results in the cessation of glutamate release by the reduction cGMP concentration and the subsequent polarization of the membrane.

While their functions are somewhat unique, both rods and cones respond to light by hyperpolarizing. Rods are specifically tuned to respond in dim light and outnumber cones by approximately 20 fold (Masland, 2012). While rods have only one type of opsin - rhodopsin,

several types of opsins exist within the cone population. Each cone expresses only one type of opsin that is specifically tuned to absorb a certain wavelength. The combinatorial response of cones forms the basis for color vision (Masland, 2012).

Rods synapse onto one type of bipolar cells, while cones synapse onto eleven in the mouse retina (Wassle et al., 2009). Each subtype of bipolar cell expresses distinct sets of receptors and ion channels, suggesting that each of the multiple bipolar cells that contact a given cone, transmit a different component of the cone's response (Masland, 2012). The classic example of this is the distinction between "ON" and "OFF" responsive bipolar cells. OFF bipolar cells express glutamate-stimulated ion channels that open in response to glutamate (AMPA and kainate). Since photoreceptor cells release glutamate in the absence of light, these bipolar cells respond (depolarize) in the absence of light. ON bipolar cells express the metabotropic receptor mGluR6. Glutamate binding to this receptor activates a G protein that mediates closure of a cation channel (TRPM1) is closed. In this cell then, a decrease in glutamate released in response to light stimulus results in the opening of TRPM1 channels and depolarization (Morgans et al., 2009; Masland, 2012).

Horizontal cells modulate the synapse between the photoreceptors and the bipolar cells in the outer plexiform layer, by providing inhibitory feedback to rods and cones. With their wide receptive field, and gap junction coupling, it is thought that their primary purpose is to provide gain control, adjusting the response of the photoreceptors to be within the operating range of the inner retina (Masland, 2012).

Retinal ganglion cells (RGCs) are a diverse population of cells responsible for the largest degree of processing in the retina. Across species, there are approximately 20 distinct subtypes of ganglion cells, each responding to an optimal, specialized stimulus (Dhande and Huberman, 2014). Retinal ganglion cells receive input from bipolar and amacrine cells, and their axons form the optic nerve, carrying retinal outputs to over two

dozen brain regions (Dhande and Huberman, 2014). Similar to bipolar cells, they also have an ON and OFF response, but subtypes can respond to motion, direction, or differences in contrast and color (Masland, 2012). Additionally, a subset of RGCs is also intrinsically light responsive, expressing the photopigment melanopsin. These RGCs project to brain areas responsible for pupillary light reflexes and the hypothalamic circadian clock (Dhande and Huberman, 2014).

Amacrine cells modulate the synapse between bipolar and ganglion cells. These cells lack a clear polarity, and most have no axons. Their connectivity however is complex; they feed back onto bipolar cells, and synapse on ganglion cells as well as each other (Masland, 2012). Over 29 types of amacrine cells have been identified. The AII subtype is the most common, linking rod bipolar cells with the cone pathway, distributing their purely ON responses to both the ON and OFF channels. Two other subtypes have also been well defined, the starburst amacrine cell, which enhances ganglion cell response to moving stimuli, and the dopaminergic amacrine cell that modulates retinal response to dim or bright light levels (MacNeil and Masland, 1998).

Supporting all of the retinal neurons in their function are the Müller glial (MG) cells. The principal glial cell of the vertebrate retina, MG span the entire thickness of the retina, their end feet forming the inner and outer limiting membranes. Müller glia play an essential role in maintaining retinal homeostasis and facilitating phototransduction (Bringmann et al., 2006). Their structure and function in the homeostatic and gliotic retina is discussed in detail in **Section 1.2**.

1.1.3 Retinal Development

The eye is composed of three major tissues, the cornea, the lens and the neural retina. During development, these structures develop in tandem. As neurulation of the

embryo progresses, the eye field is specified in the medial diencephalon. The first visible stage of this process is the formation of the optic pits or optic sulci on embryonic day 8 (E8) in the mouse and at about 28 days of human gestation (Graw, 2010). As development progresses, the optic pits enlarge to form the optic vesicles at E9. The optic stalks are also present at this stage, connecting the optic vesicles to the developing forebrain (Graw, 2010). The first bundle of nerve fibers that will give rise to the optic nerve develop are visible at E12 in the mouse (Colello and Guillery, 1992). Concurrently, the surface ectoderm thickens and forms the lens placode (E8). At E10, the optic vesicle comes in contact with the lens placode, invaginates and forms the double-layered optic cup (Kaufman, 1992; Hinrichsen, 1993; Graw, 2010). The two layers of the optic cup become distinct structures – the outer layer begins to produce pigment and eventually forms the retinal pigmented epithelium, while the inner layer forms the neural retina. Concurrently, the presumptive lens invaginates from the lens placode, forming the lens pit, which eventually gives rise to the lens (McAvoy et al., 1999).

Neural retinal differentiation begins at E12 in the mouse and continues throughout the first postnatal month. In humans, this process begins at about 47 days of gestation. Unlike the mouse retina, development occurs largely in utero, finishing in the 8th month. The fovea does however undergo further maturation following birth (Hinrichsen, 1993). This prolonged period of development allows for refinement of synaptic connections following sensory input, resulting in a finely tuned and dynamic sensory tissue.

The developing retinal tissue expresses a suite of transcription factors, called the eye field transcription factors (EFTFs) that are conserved among mammals. These EFTFs include Pax6, Rax, Six3 and Lhx2 (Zuber et al., 2003; Heavner and Pevny, 2012) and during early retinal development, promote a period of progenitor proliferation that defines the neuroblast zone (Agathocleous and Harris, 2009). The developing tissue must then undergo

a complex neurogenic shift; a population of progenitor cells must remain multipotent and proliferative, while another begins the process of neural differentiation. Several transcription factors play an important role during this period. Pax6 signaling, one of the early EFTFs, induces the expression of the basic helix-loop-helix (bHLH) family of transcription factors, which are the driving force in neural differentiation (Guillemot, 1999; Marquardt et al., 2001; Hatakeyama and Kageyama, 2004; Oron-Karni et al., 2008; Agathocleous and Harris, 2009). The transcription factor Sox2 has also been shown to play an essential role in retinal development. Loss of SOX2 results in developmental eye defects in humans (Fantes et al., 2003) and in mice (Taranova et al., 2006). Sox2 is required for the establishment of neural progenitor competence (Van Raay et al., 2005; Taranova et al., 2006; Matsushima et al., 2011) yet also suppresses terminal differentiation, maintaining the progenitor pool in a competent, yet undifferentiated state (Bylund et al., 2003; Graham et al., 2003) (The role of Sox2 in embryonic and neural development is discussed in **Section 1.3**). Together, Pax6 and Sox2 work in tandem to coordinate progenitor cell fate in the retina (Smith et al., 2009; Matsushima et al., 2011).

Unlike development in some CNS regions, the different retinal cell types are not specified through spatial patterning, but rather through the changing competence of a single retinal progenitor over time (Cepko et al., 1996; Livesey and Cepko, 2001; Marquardt and Gruss, 2002). This common retinal progenitor cell (RPC) gives rise to all six retinal neurons and one glial cell, the Müller glia (Turner and Cepko, 1987). Different combinations of the bHLH transcription factors result in varying degrees of competencies and deficiencies for retinal neurons (Akagi et al., 2004). Retinal neurons differentiate in a central to peripheral gradient and in stereotypic order that is conserved among mammals (Young, 1985; Prada et al., 1991; Livesey and Cepko, 2001).

Retinal ganglion and horizontal cells are the first to differentiate beginning around embryonic day 12 (E12) in the mouse. Cone photoreceptors and amacrine cells follow. Rod photoreceptors and bipolar cells are born predominately in the first postnatal week. The last cell type to differentiate from the multipotent RPC are the Müller glia, born from P0 to P10 in the postnatal retina (Young, 1985; Ohsawa and Kageyama, 2008).

The majority of retinal synaptogenesis occurs postnatally in the mouse retina and continues throughout the majority of the first month. The retina forms synapses in two distinct synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) (See Fig. 1.1). Beginning at P0 and continuing through P15, ganglion and amacrine cells form synapses in the IPL. Outer plexiform layer synaptic development begins at approximately P5 with the formation of cone-HZ cell synapses followed by cone-bipolar synaptogenesis. Shortly thereafter, around P8, rod-HZ cell and rod-bipolar cell synaptogenesis begins (Olney, 1968; Blanks et al., 1974; Fisher, 1979; Sherry et al., 2003). Spontaneous waves of retinal activity refine these synaptic connections in the early postnatal period (Meister et al., 1991; Penn et al., 1994; Wong, 1999). The majority of synapses are formed by P15. Directly following eye opening and continuing until P25, there is a dynamic increase in specialized bipolar ribbon synapses, which allow for the rapid and sustained release of neurotransmitter required for the transmission of visual sensory information (Fisher, 1979; Dieck and Brandstätter, 2006). Throughout this period of synaptogenesis, Müller glial cells extend lamellar side processes into all neuronal and synaptic layers, supporting and ensheathing the retinal neurons.

1.2 Müller Glia: Principal Glial Cell of the Vertebrate Retina

1.2.1 Development

Müller glia (MG), the principal macroglia of the vertebrate retina, retain many stem cell characteristics. In the mouse, late retinal progenitor cells (RPCs) give rise to Müller glia over a ten-day period spanning the first days of postnatal life (P0-P10). Retinogenesis proceeds in a spacio-temporal manner, with populations in the central retina developing first (Prada et al., 1991). MG are the last cell type to develop from the multipotent progenitor cell and preserve RPC radial morphology spanning the apical/basal axis of the retina (Ohsawa and Kageyama, 2008; Bringmann et al., 2006; Young, 1985). MG also preserve an RPC-like gene expression profile, including the expression of SOX2, an HMG-box transcription factor that maintains stem cell pluripotency in the embryonic nervous system and in populations of adult Neural Stem Cells (NSCs) (Zappone et al., 2000; Bylund et al., 2003; D'Amour, 2003; Graham et al., 2003; Roesch, 2008).

Müller glia mature slowly over the first postnatal month, elaborating an intricate network of processes that extend into all neuronal and synaptic layers of the retina (See Fig. 1.2). Through this complex network, MG facilitate retinal function by supporting glucose metabolism, ion and water homeostasis, and neural signaling via reuptake and recycling of neurotransmitters. Further, in certain species, MG are capable of dedifferentiating and giving rise to neurons in response to retinal injury (Poitry-Yamate et al., 1995; Newman and Zahs, 1998; Bringmann et al., 2006; Reichenbach and Bringmann, 2013; Goldman, 2014) To provide retinal neurons with so many supportive functions, MG must undergo significant differentiation during the first postnatal weeks. This process involves morphological, physiological and biochemical changes (Bringmann et al., 2006). Morphologically, Müller glia extend lamellar processes that envelop neuronal cell bodies and side branches that ensheath the neuronal processes, synapses, and form the inner and outer limiting

membranes (Reichenbach et al., 1989a; García and Vecino, 2003). Physiologically, the Müller glial resting potential changes dramatically over the course of the first few weeks. Initially, the resting membrane potential is quite low, or 'depolarized' – about -20mV – and the open probability of big conductance calcium activated potassium channels (BK) is high. However during maturation, inwardly rectifying potassium channels (Kir) are inserted in to the membrane, dramatically increasing the inward flow of K^+ and bringing MG resting membrane potential to its mature voltage of -80mV (Bringmann et al., 1999a; Kofuji et al., 2002). Biochemically, MG begin to express glutamate uptake transporter molecules and the enzyme glutamine synthetase to convert the glutamate to glutamine (Bringmann et al., 2006). This maturation process enables MG to take on their role as the primary glial cell in the retina, maintaining retinal homeostasis and supporting neurotransmission.

1.2.2 Müller Glia Maintain Retinal Homeostasis

As the principal glial cell of the vertebrate retina, MG provide metabolic and homeostatic support to the neurons. MG ensheath neuronal cell bodies, their processes and retinal blood vessels. Their end feet form the inner and outer limiting membranes, and contribute to the formation of the blood/retina barrier (Tout et al., 1993). MG processes thus provide both a barrier and controlled link between the retina, the vasculature and the vitreous and subretinal space. In this manner, MG facilitate molecular exchange (nutrients, waste, water, ions) between retinal neurons and the compartments of the eye.

Arranged in a columnar fashion, each MG cell forms the core of one functional unit of retinal processing. Each MG in the mouse supports an average of 35 neurons, while the MG of the zebra retina maintain an average of 16 (Reichenbach and Robinson, 1995). During development, radial glial cells establish functional units in the neocortex (Noctor et al., 2001) and there is some evidence to suggest that a single retinal progenitor cell might give rise to

this functional column in the retina, with the MG as the last cell to differentiate (Turner and Cepko, 1987; Reichenbach and Robinson, 1995; Das et al., 2003).

Muller glia, as is the case in many glial cell populations, rely largely on anaerobic glycolysis (Winkler et al., 2000). This provides for metabolic coupling between glial and neural cells. Muller glia take in glucose from the vasculature, process it via glycolysis and release pyruvate, which is then taken up by retinal neurons, allowing for efficient oxidative metabolism (Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996). Neurons generate CO_2 as a waste product of their metabolism, which is then taken up by MG and processed via the glial-specific enzyme, carbonic anhydrase, into HCO_3^- and released into the vitreous or blood vessels (Bringmann et al., 2006). Water is also generated as a waste product of neuronal metabolism and is transported into MG and out to blood vessels and the vitreous via aquaporin-4 water channels. MG express particularly high concentrations of aquaporin-4 channels in the synaptic plexiform layers and the inner limiting membrane that opposes the vitreal surface (Nagelhus et al., 1998).

Aquaporin-4 channel expression is highly colocalized with the expression of the potassium channel Kir4.1, suggesting that water transport is coupled to K^+ siphoning (Nagelhus et al., 1999, 2004). The expression of these inwardly rectifying K^+ channels, results in a high K^+ permeability and the maintenance of a very negative membrane potential of -80mV – almost at the equilibrium potential of K^+ ions (Bringmann et al., 2006). This negative resting potential is required for many of the MG homeostatic functions, including neurotransmitter uptake and spatial potassium buffering (Reichenbach and Bringmann, 2013). Similar to water transport, MG take up flux of K^+ ions that are released during synaptic transmission in the plexiform layers and release it into blood vessel and vitreal sinks.

1.2.3 Muller Glia Facilitate Neurotransmission

In addition to K^+, MG rapidly clear synaptic regions of neurotransmitters, process and recycle them back to the neurons. MG express the glutamate/aspartate transporter GLAST. The GLAST is an electrogenic cotransporter. At least two Na^+ ions are transported down their electrochemical gradient with each glutamate molecule (Brew and Attwell, 1987; Cortazar, 1967). Once taken up by the MG, glutamate is converted to glutamine via glutamine synthetase, which is expressed specifically in MG (Linser and Moscona, 1979; Bringmann et al., 2006). MG also take up GABA through GABA transporters GAT-1 and GAT-3 (Biedermann et al., 2002). GABA is then converted to glutamate by GABA transaminase, and then further converted to glutamine via glutamine synthetase (Bringmann et al., 2013). MG then release glutamine back to retinal neurons to be used as a precursors for neurotransmitter synthesis (Pow and Crook, 1996).

In addition, MG play a distinctive role in supporting the photoreceptors. MG contribute to the assembly of photoreceptor outer segments during development (Wang et al., 2005). The photosensitive, photopigment rich discs within photoreceptor cells are recycled in regular intervals. The disc membranes within the cone photoreceptor are continuous with the outer segment membrane, and therefore entire portions of the cone outer segment must be recycled (Anderson et al., 1978). MG contribute to this process by aiding in the phagocytosing cone outer segments (Long et al., 1986). Additionally, MG participate in the cone-specific visual cycle by processing and recycling cone retinal (Wang and Kefalov, 2011).

Müller glia have recently been shown to assist in the transmission of light through the retina. Light must travel through the entire thickness of densely packed neuronal cell bodies and synaptic zones to reach the photoreceptors at the back of the retina. Such dense tissue would, in absence of a clear path, result in scattered light. However MG, with their cylindrical

shape and orientation act as living optical fibers, channeling light efficiently though the retina to the photoreceptors in the back (Franze et al., 2007). Further, the ratio of MG to cones is roughly 1:1, suggesting an optimized coupling between the light guiding units and the light sensing units allowing for high image resolution (Agte et al., 2011). The image is in a sense resolved as 'pixels' with each MG defining one pixel, and a functional unit (Reichenbach and Bringmann, 2013). It was recently demonstrated that MG separate white light according to its wavelengths, channeling medium and long-wavelength light (green-red) to cones and allowing short wavelength (blue and purple) light to 'leak' onto surrounding rods (Labin et al., 2014). Together these results suggest that visual processing in the retina begins with the Müller glial mediated propagation of light through the retina.

1.2.4. Gliosis: Müller Glial Response to Injury

Muller glia are the first responders to retinal insult. Due to their unique metabolism (see **Section 1.2.2**), MG are highly resilient in ischemic, anoxic and hypoglycemic conditions (Winkler et al., 2000). Though resilient and capable of maintaining retinal homeostasis even in the compromised retina, MG do undergo reactive gliosis following injury or during degeneration. The onset of reactive gliosis in MG is defined by a suite of changes that, similar to the changes that occur during MG maturation are morphological, biochemical and physiological. An early, and sensitive indicator of retinal stress is the upregulation of glial intermediate filaments, vimentin and glial fibrillary acidic protein (GFAP). Reactive gliosis can also include MG swelling or hypertrophy, proliferation and de-differentiation (Bringmann and Wiedemann, 2012).

1.2.4.1 Physical and Morphological Changes

An immediate consequence of GFAP upregulation is the stiffening of the neural retina

(Lu et al., 2011). This response allows the MG to maintain the retinal structural integrity in response to insult (Lundkvist et al., 2004), however the increased stiffness may also inhibit retinal repair following injury. It has been demonstrated that in the absence of GFAP and vimentin, grafted neurons are able to efficiently migrate and extend processes throughout the retina (Kinouchi et al., 2003). In addition to intermediate filaments, reactive MG also upregulate the expression of extracellular matrix and cell adhesion molecules that further inhibit neurite outgrowth (Bringmann and Wiedemann, 2012).

Acute, mechanical injury can induce MG proliferation and the generation of significant glial scars. This proliferative response is likely a maladapted and over zealous attempt to heal retinal wounds (Bringmann et al., 2006). Retinal detachment is a mechanical injury that commonly results in proliferative gliosis and has been studied in detail (Fisher et al., 2005). During retinal detachment, a small tear forms in the retina, allowing the fluid from the vitreal humor to seep in between the retina and the retinal pigmented epithelium (RPE). As the retina pulls away from the RPE, rod and cone outer segments degenerate. If not treated within a few days, retinal detachment, which can be caused by head trauma, diabetes, inflammation and other factors, leads to permanent blindness. In most cases, this proliferation leads to harmful glial scars, which greatly impair the recovery process and can lead to formation of a cellular membrane between the photoreceptors and RPE (subretinal fibrosis) and growth of MG end feet in to the vitreous resulting in obscured vision (proliferative vitroretinopathy or PVR) (Anderson et al., 1986; Lewis and Fisher, 2000). Calcium influx, activation of the extracellular signal-regulated kinases (ERKs), upregulation of immediate early gene c-Fos and growth factors that occur following mechanical stress in MG have been implicated in MG proliferative response (Puro et al., 1989; Kodal et al., 2000; Geller et al., 2001; Lindqvist et al., 2010; Bringmann and Wiedemann, 2012).

1.2.4.2 Biochemical Changes

There are numerous biochemical changes that occur during gliosis, many of which are dependent on the particular type of retinal insult. Changes in expression of the glutamate-processing enzyme, glutamine synthetase (GS) is a good example. During acute photoreceptor loss, such as in response to retinal detachment or light invoked injury, GS expression is downregulated. This downregulation is thought to be due to the sudden loss of photoreceptors resulting in a lower concentration of released glutamate (Grosche et al., 1995; Bringmann et al., 2006). However, a decrease in GS expression was not found in the *rd1* mouse model of retinitis pigmentosa (Roesch et al., 2012). Retinitis pigmentosa is characterized by progressive degeneration and apoptosis of photoreceptors, which leads to eventual blindness (Portera-Cailliau et al., 1994). In addition to processing glutamate, GS also is involved in ammonia detoxification in the retina and as a result, expression is maintained to detoxify the compromised retina (Bringmann et al., 2006). In a similar example, cellular retinaldehyde-binding protein (CRALBP), which is involved in cone photopigment recycling, is downregulated during retinal detachment, but not in degeneration (Fisher et al., 2005; Roesch et al., 2012).

In the early phases of gliosis, MG release neurotrophic factors and antioxidants. Glutathione is the major antioxidant released by MG in response to hypoxia, hypoglycemia and oxidative stress, (Schütte and Werner, 1998; Bringmann and Wiedemann, 2012). Antioxidants have been shown to reduce the vulnerability of photoreceptors to light damage (Penn et al., 1987). However, glutathione synthesis is dependent on the availability of glutamate, which may be reduced as degeneration progresses. MG transcription and secretion of neuroprotective basic fibroblast growth factor (bFGF) and ciliary neurotrophic factor (CNTF) also occurs during the early phases of gliosis (Wen et al., 1995; Jin et al., 2005).

As gliosis progresses however, MG release substances that can hasten the degeneration of the retina. In response to retinal detachment, MG upregulate inflammatory factors that recruit macrophages and microglia. The macrophages release free radicals and cytotoxic cytokines, hastening photoreceptor apoptosis and neuronal degeneration (Nakazawa et al., 2007; Bringmann and Wiedemann, 2012). MG also release vascular endothelial growth factor (VEGF) which can also hasten disease progression by inducing vascular leakage and neovascularization (Bringmann et al., 2006). MG also upregulate the expression of nitric oxide synthase (Goureau et al., 1994). In low quantities, nitric oxide can reduce glutamate toxicity by closing NMDA receptor channels. However high concentrations result in the formation of free radicals that induce neurotoxicity (Kashii et al., 1996).

1.2.4.3 Physiological Changes

During MG maturation, inwardly rectifying Kir4.1 channels are inserted into the membrane, securing the MG resting potential at -80mV. This negative resting potential is required for MG role in neurotransmitter uptake and K^+ spatial buffering homeostasis (see **Sections 1.2.2** and **1.2.3**). During gliosis however, Kir4.1 channels are mislocalized and the density of K_{IR} currents is downregulated (Bringmann et al., 2000; Pannicke et al., 2006). Alterations in K_{IR} currents have been observed in animal models of a wide range of retinal diseases including light injury, diabetic retinopathy, ischemia-reperfusion, retinal detachment and proliferative vitreoretinopathy (Bringmann and Wiedemann, 2012). The reduction in K_{IR} currents is most severe in proliferative retinopathies, where the K^+ currents are almost completely gone, coupled with a significant decrease of the resting membrane potential (Bringmann et al., 1999b, 2002). The decreased density of K_{IR} currents and in resting membrane potential mimics the physiological profile of immature MG. This has lead to the hypothesis, that the down regulation of K_{IR} channels during gliosis is the first step in the de-

differentiation of MG that may be required for the return to a proliferative state (Bringmann et al., 2000). In response to retinal injury, MG of several vertebrate species including fish and birds are capable of de-differentiating, proliferating and giving rise to functional neurons (Fischer and Reh, 2001; Bernardos et al., 2007). While mammalian MG are not alone capable of this neurogenic feat, and MG proliferation most often leads to the development of glial scars rather than the generation of new neurons, they do function as a presumptive retinal stem cell.

1.2.5 Müller Glia as Retinal Progenitor Cells

In addition to their role in maintaining retinal homeostasis, MG are capable of de-differentiating, proliferating and giving rise to functional neurons in response to retinal injury in several vertebrate species including fish and birds (Fischer and Reh, 2001; Bernardos et al., 2007). In the mammalian retina, even though proliferation in response to injury and degeneration is extremely limited, MG are considered a presumptive retinal stem cell. Several groups have shown that upon addition of growth factors in combination with retinal injury mammalian Müller glia are capable of proliferating and de-differentiating (Dyer and Cepko, 2000; Ooto et al., 2004; Das et al., 2006; Karl et al., 2008). Moreover, cultured MG, including those harvested from human retina, are capable of multipotential differentiation *in vitro* (Nickerson et al., 2008; Singhal et al., 2012)

Teleost fish, such as the well-studied zebrafish, are capable of complete retinal regeneration. Why the teleost retina is capable of this neurogenic feat and the mammalian retina is not remains unknown. However the teleost retina does have several characteristics that make it uniquely suited for regeneration, namely a retina that continues to grow throughout the animal's life. Rod precursors located in the outer nuclear layer and general retinal progenitor cells located in the ciliary marginal zone at the edge of the retina give rise

to neurons which populate the retina as the fish grows (Johns, 1977).

In response to injury, the MG of zebrafish are capable of de-differentiating and giving rise to all neuronal cell types, which can stably integrate into the retina (Fausett and Goldman, 2006; Bernardos et al., 2007). Fish retinas can regenerate in response to multiple retinal insults, including light damage, toxins and mechanical damage (Goldman, 2014). The task of reprogramming a MG cell and generating retinal progenitors is large, and one that involves multiple steps. Retinal injury or insult must initiate reprogramming of the MG through changes in genomic methylation (Powell et al., 2013). Once reprogrammed, the MG cell must undergo interkinetic migration to the apical (photoreceptor) side of the retina and undergo an asymmetric division (Nagashima et al., 2013). This asymmetric division results in the generation of a retinal progenitor cell which is then tasked with the job of giving rise to retinal neurons, capable of integrating into the retinal circuit (Fausett and Goldman, 2006). Over 20 factors have been implicated in the reprogramming of MG cells including the cytokines (acting through the Jak-Stat pathway), Wnts (through the glycogen synthase kinase 3β (GSK-3β)–β-catenin pathway) and growth factors (MAPK-ERK pathway) (for review see: Goldman, 2014).

A likely candidate for the induction of the MG reprogramming in response to injury is the cytokine tumour necrosis factor-α (Tnfα). Expressed by both MG and neurons in response to injury, Tnfα has been demonstrated to be necessary for MG cell reprogramming in the fish retina through its role in upregulating Achaete-scute complex-like 1a (Ascl1a) and Signal transducer and activator of transcription 3 (Stat3) (Nelson et al., 2013). Ascl1a and Stat3's role in the reprogramming of retinal MG has been well documented (Fausett et al., 2008; Ramachandran et al., 2010; Nelson et al., 2012; Goldman, 2014). It has further been demonstrated that in response to injury-induced Ascl1a expression, Wnt signaling is initiated.

In response to Wnt activation, GSK-3β signaling is inhibited and ß-catenin is stabilized, enabling the activation of downstream targets and MG dedifferentiation (Ramachandran et al., 2011). One such downstream target is the RNA binding protein lin28, which has been shown to be activated by Ascl1 activity (Ramachandran et al., 2010). Lin28 is an important regulator of stem cell growth, metabolism and self renewal and has been used in the induction of pluripotent stem cells (Yu et al., 2007; Melton et al., 2010; Shyh-Chang and Daley, 2013). Interestingly, it has recently been demonstrated that the transcription factor Sox2, which is constitutively expressed in mammalian MG, regulates proliferation and neurogenic potential of neural precursors through its maintenance of lin28 expression (Cimadamore et al., 2013). Further study of lin28's role in both teleost and mammalian MG may reveal mechanisms underlying the regenerative potential of this cell.

Unlike the teleost retina, the regenerative potential of the mammalian retina is quite limited. Mammalian MG do however retain the expression of many progenitor cell genes, including Sox2, Pax6, Sox9, Notch and Cyclin D3 (Roesch, 2008; Jadhav et al., 2009). Many different combinations of injury and growth factors have been employed in an attempt to stimulate proliferation and regeneration in the mammalian retina with limited results (Ooto et al., 2004; Osakada et al., 2007; Karl et al., 2008). One recent study found that overexpression of ASCL1, a TF that is not normally expressed in mammalian MG but is required in the regenerative response of fish and chicks, was capable of reprogramming mouse MG in culture (Fausett et al., 2008; Pollak et al., 2013).

It has also been demonstrated that primary human MG cell cultures are capable of generating ganglion cells and photoreceptor cells, and when transplanted into an injured rodent retina, show some ability to integrate into a functional circuit (Singhal et al., 2012; Jayaram et al., 2014).

Further research into both the regenerative mechanisms of the teleost retina and the regenerative potential of the mammalian retina will be required to fully understand the intricate and complex injury response mechanism of Müller glia.

1.3 Sox2: Master Regulator of the Pluripotent State

Müller glia (MG), retain many stem cell characteristics (see **section 1.2**). Unlike most differentiated glial cells, MG constitutively express SOX2, an HMG-box transcription factor that maintains stem cell pluripotency in the embryonic nervous system and in populations of adult NSCs in the SVZ and dentate gyrus (Zappone et al., 2000; Bylund et al., 2003; D'Amour, 2003; Graham et al., 2003). Ablation of Sox2 in the developing CNS leads to premature neuronal differentiation and depletion of progenitor pools (Pevny and Nicolis, 2010). Furthermore, Sox2 is an essential factor in reprogramming fibroblasts into inducible pluripotent stem cells (Takahashi et al., 2007).

Like other regions of the CNS, lowering SOX2 expression levels in the retina leads to premature neuronal differentiation, whereas Sox2 deletion results in complete lack of neuronal competence (Taranova et al., 2006; Matsushima et al., 2011). Mutations of the human Sox2 allele have been shown to lead to microphthalmia or anophthalmia (small eye or no eye respectively) (Fantes et al., 2003). Moreover, the retina provides an ideal system in which to dissect Sox2's function as it is the only one of three highly related SoxB1 factors expressed in the retina (Taranova et al., 2006). Analysis of SOX2 function is therefore an ideal approach to the study of both the biology and neurogenic potential of MG in the retina.

1.3.1 The Sox2 Allele

The family of *Sox* genes was originally identified based on sequence similarity to Sry, the sex-determining gene located on the mammalian Y chromosome (Gubbay et al., 1990). It is

from this gene that the group takes its name, the Sry-type HMG-box containing or Sox genes. The Sox group shares a conserved ~35kb element encoding a 79 amino acid HMG (high mobility group) DNA-binding domain with the Sry gene. Twenty different Sox genes have been identified in mice and humans (Schepers et al., 2002). Sox proteins that share at least 80% sequence homology of their HMG domain have been divided into groups A-H. Due to their sequence homology, group members share biochemical properties and have overlapping functions. Sox2 is a member of the SoxB1 group together with Sox1 and Sox3 (Wegner, 2010). While all three members of this group are expressed in the cortex, only SOX2 is expressed in the retina (Taranova et al., 2006). This makes the retina an ideal system in which to dissect Sox2's function.

SOX proteins bind to a specific DNA sequence (A/T A/T CCA A/T G), via their HMG domain. Unlike most transcription factors, the HMG domain binds with the minor groove of the DNA, which widens the minor groove, causing the DNA to bend toward the major groove. The SoxB1 group has a short N-terminal sequence, followed by the HMG box domain and a the long C-terminal sequence which includes a distal transcriptional activation domain (Love et al., 1995; Pevny and Lovell-Badge, 1997; Kamachi and Kondoh, 2013). This transcriptional activation domain is not cell specific or dependent on binding partners. Target specificity is instead achieved through co-DNA-binding partner factor which binds at the proximal end of the C-terminal domain. In fact, the SOX proteins bind DNA very weakly, creating weak bond, not stable enough to initiate transcriptional activation in the absence of a stabilizing partner factor (Kamachi et al., 1999, 2000).

Multiple Sox2 binding partners have been identified. During embryonic development, SOX2 interacts with the POU transcription factor Oct3/4 to initiate transcription of *Fibroblast growth factor 4* (FGF4) – which plays an essential role in embryonic development (Ambrosetti et al., 2000). Sox2 and Oct3/4 also initiate expression of undifferentiated

embryonic cell transcription factor 1, itself a transcriptional co-activator expressed in pluripotent embryonic stem cells (Nishimoto et al., 1999). Brn2, another member of the POU transcription factor family, couples with Sox2 to drive the expression of nesting via its neural enhancer nestin (Tanaka et al., 2004). The first Sox2 binding partner identified was δEF3, which partners with SOX2 to activate the expression of δ-crystallin and drive induction of the lens (Kamachi et al., 1998). The same group also identified Pax6 (an important regulator of eye development – see section 1.1.4) as a Sox2 binding partner. The Sox2/Pax6 complex similarly drives δ-crystallin expression, further aiding in the initiation of lens development (Kamachi et al., 2001). Together with its binding partners, SOX2 plays an essential role in embryonic, neural and sensory development.

1.3.2 Sox2 in Embryonic Development

The first lineage specification event that occurs in the mammalian embryo is the formation of the trophectoderm (TE) and inner cell mass (ICM) within the blastocyst. The ICM contains a population of pluripotent cells that will give rise to the epiblast and subsequently all the embryonic lineages, while the TE has a population of multipotent cells that form the extra embryonic ectoderm and give rise to the placenta (Sarkar and Hochedlinger, 2013). SOX2 is initially expressed in both of these tissues [3.5 days postcoitum (dpc) in the mouse], but then becomes refined to the epiblast in the ICM. By dpc 7, SOX2 expression becomes further restricted to the presumptive neuroectoderm. At dpc 9, Sox2 RNA is present throughout the brain, neural tube, sensory placodes, branchial arches, and gut endoderm and germ cells (Avilion et al., 2003). Embryos null for Sox2 abort early due to failure in the formation of the pluripotent epiblast and improper implantation of the embryo (Avilion et al., 2003; Keramari et al., 2010). Deletion of Sox2 in already established mouse embryonic stem cells (ESCs) results in their differentiation into TE-like cells, pointing

to Sox2's role in maintaining ESCs pluripotency (Masui et al., 2007).

Throughout embryonic development, SOX2 continues to be expressed in ectodermal, endodermal, and mesodermal tissues as well as in primordial germ cells, where it plays an essential role in specifying cell fate. SOX2 role in the developing the CNS, foregut, and in skin and bone tissues is particularly important (Sarkar and Hochedlinger, 2013). SOX2 specifies cell fate by antagonizing transcription factors of other lineages. For example, in the developing embryo, the neural plate (which will give rise to the neural tube) and the paraxial mesoderm (which gives rise to somites) come from a common population of bipotential axial stem cells (Wilson et al., 2009). In the absence of competing transcription factors, SOX2 expression drives the entire population of stem cells towards a neural fate. Expression of the mesodermal T-box transcription factor Tbx6 (Chapman et al., 1996) is required for the formation of paraxial mesoderm. Tbx6 represses Sox2 by inactivating the N1 enhancer region, inhibiting neural development in the axial stem cells and allowing for mesodermal specification (Takemoto et al., 2011). Similarly, in the developing foregut, in the absence of the transcription factor Nkx2.1, Sox2 drives cell fate towards esophageal at the expense of the future trachea (Que et al., 2007). Finally, our laboratory demonstrated that in the developing neural retina, Sox2 antagonizes Pax6 to define the neurogenic zone (Matsushima et al., 2011).

Across development, Sox2 functions in a dose dependent and context dependent manner. Work in both the foregut and in the retina have demonstrated that SOX2 positive stem and progenitor cells in these tissues are highly sensitive to SOX2 dose (Taranova et al., 2006; Que et al., 2007). Sox2's function is highly dependent on both cooperative and antagonistic factors. It follows then that it is the delicate balance between these factors and SOX2 levels that allow for the proper specification of cell fate (Pevny and Placzek, 2005;

Sarkar and Hochedlinger, 2013). Sox2 also functions in a tissue specific manner. Deletion of Sox2 from neural progenitors results in cell cycle exit, whereas Sox2 deletion in the trachea, tongue and esophagus results in abnormal differentiation without changes in cell cycle dynamics (Sarkar and Hochedlinger, 2013). Sensitivity to SOX2 dosage also appears to vary across species. While heterozygous mutations in mice have resulted in only mild defects, humans with heterozygous defects display severe phenotypes (Fantes et al., 2003; Pevny and Placzek, 2005; Taranova et al., 2006). Further, data presented in this thesis provides evidence that SOX2 sensitivity varies within species in a background specific manner (See **Chapter Four**). These observations point to the complexity with which Sox2 interacts with and upon its environment (Pevny and Placzek, 2005).

1.3.3 Sox2 in CNS Development: Neural Stem Cells and Induced Pluripotency

SOX2 is expressed throughout the cells of the early neural tube and continues to be expressed by proliferating CNS stem and progenitors up to their final cell cycle exit (Zappone et al., 2000; Graham et al., 2003). While forced expression of SOX2 promotes the differentiation of mouse embryonic stem cells into neuroectoderm at the expense of mesoderm and endoderm (Zhao et al., 2004), Sox2's main role in the developing CNS is the maintenance of neural progenitor identity, rather than the induction of neural fate (Graham et al., 2003; Pevny and Placzek, 2005). Loss of SOX2 in neural progenitor cells results in their premature exit from the cell cycle and depletion of the progenitor pool. Conversely, forced, constitutive SOX2 expression inhibits neuronal differentiation (Bylund et al., 2003; Graham et al., 2003).

SOX2 is also expressed in the populations of adult neural stem cells in the dentate gyrus of the hippocampus, and in the subventricular zone (SVZ), surrounding the adult lateral ventricles. Further, SOX2 positive cells isolated from these regions can form

multipotent neurospheres in culture (Ellis et al., 2004). Mice with hypomorphic levels of Sox2 (below 50% of wild type) display significant brain defects including reduction in cortical volume, epilepsy and motor and neurological problems including neural degeneration. These gross abnormalities are accompanied by a reduction in the number of neural stem cells in the hippocampal dentate gyrus, as well as a reduction in newborn neurons the dentate gyrus, SVZ, and olfactory zone (Ferri et al., 2004; Cavallaro et al., 2008; Pevny and Nicolis, 2010). Humans with Sox2 mutations also present with brain and eye defects, accompanied by neurological problems (Fantes et al., 2003). Recent evidence suggests that Sox2 mutations may leads to neural degeneration in human populations as well (Ragge et al., 2013).

In addition to the role of Sox2 in the maintenance of neural stem cell identity, numerous studies have demonstrated an essential role for Sox2 in the induction of pluripotency. Together with Oct3/4, Klf4, and c-Myc, Sox2 is capable of reprogramming both mouse and human fibroblasts into inducible pluripotent stem cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Recent evidence suggests that Sox2 alone is capable of reprograming mouse and human fibroblast cells into multipotent neural stem cell (Ring et al., 2012). The role of Sox2 in the maintenance of adult neural stem cell populations, combined with its role in the induction of pluripotency, place Sox2 as a key player in regenerative therapy research.

1.3.4 Sox2 in Retinal Development

Unlike other CNS regions, Sox2 is the only member of the highly related Sox2B1 family that is expressed in the retina (Kamachi et al., 1998). The retina therefore offers a unique environment in which to study the role of Sox2 in neural development, as well as being particularly susceptible to its loss. Our laboratory has demonstrated an essential role

for Sox2 in proper eye and retinal development (Taranova et al., 2006; Matsushima et al., 2011; Langer et al., 2012; Surzenko et al., 2013). Further, mutations in the human Sox2 allele have been shown to lead to microphthalmia or anophthalmia (small eye or no eye, respectively) (Fantes et al., 2003; Bakrania et al., 2007).

During retinal development, a multipotent, SOX2 positive retinal progenitor cell (RPC) systematically gives rise to all six retinal neurons in a conserved order from embryonic day 12 to postnatal day 10 (Turner and Cepko, 1987; Livesey and Cepko, 2001). Similar to the human population, global reduction in murine SOX2 levels result in microphthalmia and anophthalmia (Taranova et al., 2006; Langer et al., 2012). Neural-specific loss of SOX2 in the embryonic retina results in a neural-to-epithelial fate shift and loss of neural competence in the progenitor population (Matsushima et al., 2011). As retinal development proceeds, neural progenitors down regulate SOX2 as they exit the cell cycle and terminally differentiate (Taranova et al., 2006). SOX2 expression is maintained in Müller glia (MG), the last population of cells to develop from the RPCs (Roesch, 2008; Nelson et al., 2011). However, when Sox2 is ablated ubiquitously in the early postnatal retina, nascent MG re-enter the cell cycle and migrate to the apical lamina of the retina to divide (Surzenko et al., 2013). MG that have undergone this terminal division do not survive, leading to their eventual depletion and retinal degeneration. These data point to a role for Sox2 in the maintenance of nascent MG quiescence.

The role of Sox2 specifically in MG maturation and maintenance however was previously unknown. Data in this thesis uncover an essential role for SOX2 in the maturation of MG. Here we report that ablation of Sox2 specifically in MG at P5 *in vivo* not only results in the disruption of MG maturation, but also morphological and functional abnormalities in the neural retina. These data highlight both the critical role for SOX2 in MG maturation, as well as the role of MG in guiding the final stages of retinal maturation. Further, we provide

the first evidence of retinal degeneration in a SOX2 hypomorphic retina, as well as indicating a role for SOX2 in the maintenance of a stable population of MG in the degenerating retina.

I performed all of the experiments described in this thesis, with the exception of the western blots described in **Figure 4.1**, which were completed by Tessa Crowl, PhD.

Figure 1.1

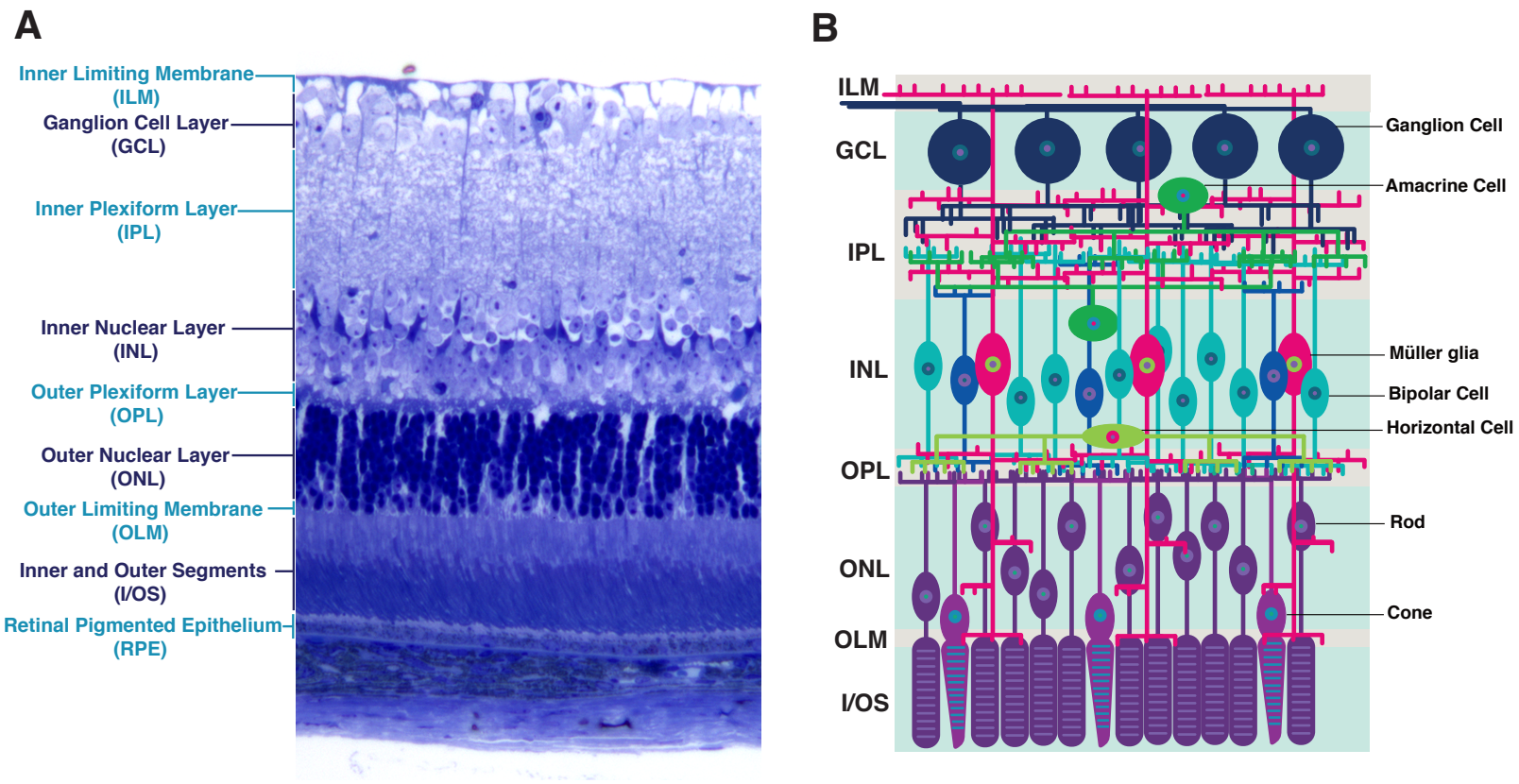

A
Inner Limiting Membrane (ILM)
Ganglion Cell Layer (GCL)
Inner Plexiform Layer (IPL)
Inner Nuclear Layer (INL)
Outer Plexiform Layer (OPL)
Outer Nuclear Layer (ONL)
Outer Limiting Membrane (OLM)
Inner and Outer Segments (I/OS)
Retinal Pigmented Epithelium (RPE)
B
ILM
GCL
IPL
INL
OPL
ONL
OLM
I/OS
Ganglion Cell
Amacrine Cell
Müller glia
Bipolar Cell
Horizontal Cell
Rod
Cone

Figure 1.1 Functional organization of the retina.

(A) Thin section of the central mouse retina. (B) Retinal Schematic. Rod and cone inner and outer segments are located in the outer retina. Rod and cone cell bodies are located in the outer nuclear layer (ONL). Bipolar cells, located in the inner nuclear layer (INL), transmit the signals from the photoreceptors to ganglion cells located in the ganglion cell layer (GCL). The axons of the ganglion cells, located in the nerve fiber layer (NFL) make up the optic nerve. There are two main synaptic layers, the outer plexiform layer (OPL), composed of synapses between photoreceptors and bipolar cells, and the inner plexiform layer (IPL) composed mainly of synapses between bipolar and ganglion cells. Horizontal cells mediate the synaptic activity in the OPL and amacrine cells mediate synaptic activity in the IPL. Muller glial cells, the principal glial cells of the vertebrate retina, extend apical-basal processes throughout the entire thickness of the retina. Their end feet form the inner and outer limiting membrane (I/OLM). Branching side processes extend into all synaptic and neuronal layers, providing architectural and trophic support to all the neurons of the retina.

Figure 1.2

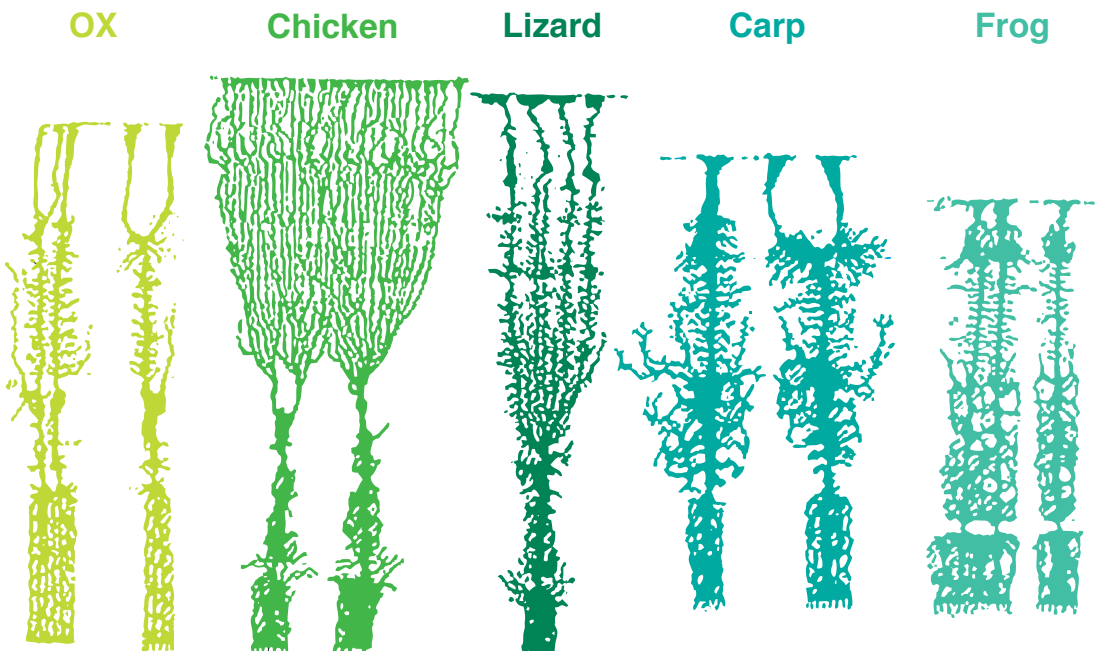


Figure 1.2 Müller glia have a complex morphology.

Muller glia send branching side processes into all neuronal and synaptic layers of the retina. While MG morphology varies among species, the basic network of processes that support the retina and form the inner and outer limiting membrane are conserved among vertebrates. Representative drawings from ox, chicken, lizard, carp and frog retinas are depicted.

Adapted from Cajal, 1892.

Chapter Two: Materials and Methods

2.1 Mouse Lines

All animal work was carried out in accordance with the University of North Carolina IACUC and DLAM regulations. Generation of the $Sox2^{COND}$ mouse line was previously described (Taranova et al., 2006). GLASTCreER mice were a gift from Dr. Jeremy Nathans (Nathans, 2010; Melo et al., 2012; Wang et al., 2012). The Rosa26Reporter strain (R26R; Soriano et al., 1999) was obtained from Jackson Laboratories. All mouse lines in Chapter Three were maintained on a mixed CD1;C57BL6/J background, mouse lines in Chapter Four are on the backgrounds specified therein. Genotyping primers and protocols were described previously (Surzenko et al., 2013).

2.2 Sox2 Ablation

The $Sox2^{COND}$ line was crossed to the glial specific, tamoxifen (TAM) inducible GLASTCreER line, and to the R26R reporter line. For the majority of the experiments, $Sox2^{COND/+}$;GLASTCreER mice were crossed to $Sox2^{COND/+}$;R26R/R26R mice. Some experiments were performed by crossing a pair of $Sox2^{COND/+}$;GLASTCreER mice. Pregnant dams were monitored to determine pups date of birth (P0). P5 $Sox2^{MUTANT}$ ($Sox2^{COND/COND}$;GLASTCreER;R26R) and $Sox2^{CONTROL}$ ($Sox2^{COND/+}$;GLASTCreER;R26R or $Sox2^{+/+}$;GLASTCreER;R26R) pups were given a 60μl intragastric injection of 8mg/ml tamoxifen (Sigma) prepared in a 1:10 EtOH:corn oil solution.

2.3 Immunohistochemistry

Retinas were harvested at P15, P25 and P60. Eyes were removed from the animal immediately following cervical dislocation and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 minutes. Eyes were then removed from the PFA solution and placed in PBS for dissection. An incision was made in the cornea, through which the lens was gently removed. Eyecups were returned to 4% PFA in PBS overnight. Eyecups were sequentially immersed in 10%, 20% and 30% Sucrose in PBS, mounted in OCT medium (Tissue-Tek) and frozen at -80º C. Horizontal 14-16 μm cryostat sections were blocked in 10% goat serum in PBS, 1.0% Triton-X solution for at least 2 hours and then incubated with primary antibodies in a solution containing 5% goat serum and 0.1 % Triton-X in PBS overnight at 4ºC. Following three 5-minute washes in PBS, tissue was incubated with secondary antibodies for one hour at room temperature.

The following antibodies and stains were used at the noted dilutions for this study: Sox2, rabbit polyclonal (Millipore, 1:2000), SOX2 mouse monoclonal (R&D Systems, 1:100), CRALBP (Abcam, 1:500), Glutamine Synthetase (Millipore, 1:1000), ß-galactosidase (Molecular Probes, 1:10000), Sox9 (Millipore, 1:1000), Calretinin (Millipore, 1:500), Neurofilament (Hybridoma Bank, 1:5000), PKCα (Santa Cruz, 1:500), GFAP (DAKO, 1:500), Cleaved Caspase 3 (Cell Signaling, 1:250), Chx10 (Gift from Cepko Lab, 1:500), Islet1 (Hybridoma bank, 1:50), Goat Anti-Mouse IgG1 (Alexa Fluor® 488 conjugate, 1:2000), Goat Anti-rabbit IgG (Alexa Fluor® 488 conjugate, 1:2000), Goat Anti-Mouse IgG2a (Alexa Fluor® 546 conjugate, 1:1000), Goat Anti-Rabbit (Alexa Fluor® 546 conjugate, 1:1000), Hoechst 33258 (Invitrogen, 1:10000).

Z-stack images were collected on a Zeiss LSM 710, collapsed, and processed using Adobe Photoshop software.

2.4 Electron Microscopy

P60 eyecups were prepared as described above and fixed in a solution of 2% glutaraldehyde, 2% paraformaldehyde in 0.1% cacodylate buffer, pH 7.2. Semi-thin 0.5 μm sections through the central retina were stained with 1% methylene blue. Images were collected on a Leica DMIRB inverted microscope with a Retiga (SRV-1394) camera. Electron microscopy specimens were post fixed in a solution of 2% Osmium tetraoxide in 0.1% cacodylate buffer and embedded in Epon 812 resin. Sections were cut at 65nm thick using a Leica EM CU7 (Leica Microsystems) and contrast stained with a 2% uranyl acetate, 4% lead citrate solution. Ultrathin sections were visualized on a JEM-1400 transmission electron microscope (JEOL) using an ORIUS SC1000 camera. Digital camera Model: ORIUS CCD 35mm port.

2.5 Histological Statistical Analysis

At least 4 retinas from independent litters were analyzed for every molecular marker used for experiments in Chapter Three, with the exception of GFAP and PKCα, which were analyzed in 3 retinas for each age and experimental group. For the MG cell body analysis, X,Y coordinates of Sox9 positive cells were obtained from 6 non-consecutive sections in the central retina of 5 $Sox2^{MUTANT}$ and 5 $Sox2^{CONTROL}$ retinas at P25. We measured disorganization in the retina using a modified least squares analysis in which we measured the distance, if any, of each Sox9 positive MG cell body from a band representing a theoretical organized retina. The "organizational band" was defined as the region 19.5 μm (approximately the width of four MG cell bodies) above and below the quadratic least squares fit to the measured X,Y coordinates of the MG cell bodies in each section of each retina. Mean squared distance from the edge of the organizational band was computed for each section and pooled to create a disorganization metric for each retina. Disorganization

of control and mutant retinas was compared using a Student's *t-test* (two-tailed, heteroscadastic). Retinal thickness was measured in three, non-consecutive horizontal sections in 4 mutants and 4 controls. ImageJ was used to determine the length of the nasal and temporal retina from optic nerve head to the edge of the neural retina, where it meets the ciliary epithelium. The length of the retina was then bisected and the thickness of the nasal and temporal neural retina was measured at that point. Nasal and temporal measurements for each of the 3 sections per retina were averaged and the values from the 4 $Sox2^{CONTROL}$ and $Sox2^{MUTANT}$ retinas were compared using an unpaired Student's *t-test* (two-tailed, homoscedastic).

2.6 Electroretinography

In response to light stimulus, retinal neurons generate a field potential that can be measured on the cornea. The resulting stereotypical waveform has two main components that compose the Electroretinogram: the negative a-wave that arises from the photoreceptor response, and the positive b-wave that is generated in the inner retina. Electroretinogram (ERG) responses were recorded using the Espion E^2 system and ColorDome Ganzfeld Stimulator (Diagnosys, LLC). P25 and P60 $Sox2^{MUTANT}$ and $Sox2^{CONTORL}$ animals were dark-adapted overnight, then anesthetized by an i.p. injection of a 2.5% Avertin solution at 0.018 ml/g and maintained at 37ºC on a heated platform. Pupils were dilated with drops of 2.5% phenylephrine hydrochloride and 1% tropicamide. Hydroxypropyl methylcellulose was applied to the eyes to prevent dehydration. ERGs were recorded under scotopic (or dark-adapted) conditions in response to 12, 4ms flashes of light intensities ranging from 0.001 to 100 $cd \cdot s/m^2$. Traces from each eye were averaged across four flashes per intensity. The a-wave was calculated as the amplitude of the negative peak following the flash. The b-wave was calculated as the amplitude of the positive peak from the negative trough of the a-wave.

The data were graphed using Graphpad Prism. P25 wave amplitudes from 14 $Sox2^{MUTANT}$ and 12 $Sox2^{CONTROL}$ eyes were compared using a Student's *t-test* at each step; statistical significance was determined using the Holm-Sidak method, with alpha = 5.000%.

CHAPTER THREE: SOX2 DEFICIENT MÜLLER GLIA DISRUPT THE STRUCTURAL AND FUNCTIONAL MATURATION OF THE MAMMALIAN RETINA

Müller glia (MG), the principal glial cells of the vertebrate retina, display the characteristics of a quiescent progenitor cell. Müller glia maintain a progenitor-like morphology and express key progenitor markers, including the HMG-box transcription factor *Sox2*. In the embryonic and mature CNS, SOX2 functions to maintain neural stem cell identity. In this study, we use inducible, MG specific genetic ablation of *Sox2 in vivo* to analyze its function in murine retinal maturation Müller glia. *Sox2* ablation at postnatal day 5 (P5) results in disorganization of Müller glial processes in the synaptic inner plexiform layer (IPL) of the retina and mislocalized cell bodies in the retinal nuclear layers by P25. This disorganization leads to thinning of the neural retina, as well as disruption of neuronal processes in the inner and outer plexiform layers. Functional analysis by electroretinography reveals a decrease in the amplitude of the b-wave, indicating reduction in inner retina function and visual sensitivity. This disruption is maintained at P60. Together these results demonstrate the essential role for Müller glia in the development and maintenance of retinal cytoarchitecture and provide new insights into the maturation of MG processes.

3.1 Introduction

Müller glia (MG), the principle macroglia of the vertebrate retina, retain many stem cell characteristics. They are the last cell type to develop from multipotent retinal progenitor cells (RPCs). RPCs give rise to all six types of retinal neurons in a distinct spacio-temporal order spanning embryonic day (E) 12 to postnatal day (P) 10 in the murine retina (Turner and

Cepko, 1987; Livesey and Cepko, 2001). Born from P0 to P10, MG preserve RPC radial morphology spanning the apical/basal axis of the retina (Ohsawa and Kageyama, 2008; Bringmann et al., 2006; Young, 1985). MG also preserve an RPC-like gene expression profile, including the expression of SOX2, an HMG-box transcription factor that maintains stem cell pluripotency in the embryonic nervous system and in populations of adult Neural Stem Cells (Zappone et al., 2000; Bylund et al., 2003; D'Amour, 2003; Graham et al., 2003; Roesch, 2008). *Sox2* ablation in the developing CNS leads to premature neuronal differentiation and depletion of progenitor pools (Pevny and Nicolis, 2010). Furthermore, *Sox2* is an essential factor in reprogramming fibroblasts into inducible pluripotent stem cells (Takahashi et al., 2007). While numerous studies have examined aspects of MG's neurogenic potential (Dyer and Cepko, 2000; Ooto et al., 2004; Das et al., 2006; Karl et al., 2008), the role of SOX2 in the maturation and maintenance of MG has not been studied.

We previously demonstrated that SOX2 is required for the maintenance of neurogenic potential in RPCs as well as for maintaining early postnatal RPC/MG quiescence (Matsushima et al., 2011; Surzenko et al., 2013). In addition to the unexplored role of SOX2 specifically in MG, little is known about postnatal maturation of MG processes and the maintenance of their complex morphology (Reichenbach and Reichelt, 1986; Reichenbach et al., 1989b; Willbold et al., 2000). Over the first postnatal month, MG processes develop an intricate network that provides architectural support and enables MG to maintain retinal homeostasis. Müller glia's lamellar processes envelop neuronal cell bodies, and their side branches ensheath the neuronal processes, synapses, and form the inner and outer limiting membranes (Reichenbach et al., 1989a; García and Vecino, 2003). Through this network, MG facilitate neuronal transmission by supporting glucose metabolism, ion and water homeostasis, recycling neurotransmitters, channeling light to the photoreceptors, and even retinal regeneration (Poitry-Yamate et al., 1995; Newman and Zahs, 1998; Bringmann et al.,

2006; Reichenbach and Bringmann, 2013; Goldman, 2014; Labin et al., 2014).

In this study we address the role of SOX2 in MG by characterizing the maturation of *Sox2* deficient MG. We find that expression of SOX2 is essential for the proper maturation of the extensive side branches MG develop during the first two postnatal months. Disruption in these processes results in loss of neuronal function as well as disruption of amacrine and horizontal cell neurites in the inner and outer plexiform layers (IPL and OPL). These data indicate SOX2's essential function in the maturation of MG processes, and provide new insights into the role of MG in the maintenance of retinal cytoarchitecture and function.

3.2 Results

3.2.1 Müller Glia Mature Throughout the First Postnatal Month

We analyzed the maturation process of Müller glia over the first two postnatal months, using the Müller marker cellular retinaldehyde-binding protein (CRALBP), an essential protein involved in chromophore transport (Das et al., 1992) (Fig. 3.1A). At P5, the main apico-basal processes are well established and the MG end feet have formed the inner and outer limiting membranes (I/OLM). Müller glia have not yet extended processes into the synaptic and neuronal layers. This is especially striking in the inner plexiform layer (IPL) (Fig. 3.1A, see inset) and the outer nuclear layer (ONL). At this developmental stage, SOX2 is specifically expressed in MG cell bodies in the inner nuclear layer (INL, arrow) and a subpopulation of amacrine cells (arrowheads) in the INL (Fig. 3.1F). The SOX2 staining reveals that at P5, MG retain an immature elongated cell body morphology. At P10, just prior to eye opening, retinal synapses are forming (Fisher, 1979). At this stage, Müller glia have begun to extend side processes into the outer plexiform layer (OPL, arrowhead) and to define the outer boundary of the inner plexiform layer (IPL, arrow) (Fig. 3.1B). The SOX2 staining in the Müller cell bodies is well defined (Fig. 3.1G). By P15 there is a considerable

increase the density of processes (Fig. 3.1C) and the bands of MG fibers that ensheathe neuronal processes in the IPL are beginning to form. In the ONL, the lamellar processes that envelope the photoreceptor cell bodies are evident for the first time. During this period in development, specialized ribbon synapses form between the bipolar cells and photoreceptors, and retinal synapses are refined based on light-induced neuronal transmission (Fisher, 1979; Tian, 2004). At P25, synaptogenesis is complete and Müller glial process in the IPL and ONL are well defined (Fig. 3.1D) (Fisher, 1979). However, further maturation of MG process is observed from P25 to P60. Concomitant with a slight decrease in the thickness of the retina is an increase in density of the MG processes in both the IPL and ONL (Fig. 3.1E). SOX2 expression in the INL reveals that the morphology and localization of MG cell bodies remains relatively stationary from P10 to P60 (Fig. 3.1G-J). These data point to an active and extensive period of postnatal MG maturation in the murine retina.

3.2.2 Robust Müller Glial Specific Sox2 Ablation

To determine the function of SOX2 in the maturation and maintenance of MG *in vivo*, we genetically ablated *Sox2* in postnatal MG using a Sox2 conditional mouse line previously generated by our laboratory (Taranova et al., 2006). The $Sox2^{COND}$ allele contains the *Sox2* open reading frame flanked by loxP sites such that Cre-mediated recombination results in removal of the entire, single exon, SOX2 coding sequence and generates a null allele. The $Sox2^{COND}$ line was crossed to a glial specific, tamoxifen (TAM) inducible Cre line, GLASTCreER, which in the retina, drives CRE expression specifically in MG (Melo et al., 2012). The $Sox2^{COND}$ line was also crossed to the Rosa26LacZ reporter line (Soriano, 1999), which reports the cells in which CRE has been activated. Administration of a systemic dose of TAM to the $Sox2^{COND/COND}$;GLASTCreER;R26R pups, defined as "$Sox2^{MUTANT}$", results in

efficient, MG specific, *Sox2* ablation in the postnatal retina (Fig. 3.2A, D). To assess the role of SOX2 in the maturation of Müller glia, we induced *Sox2* ablation at P5, at the height of Müller glial genesis in the murine retina (Young, 1985). Retinas were harvested at P15 during the maturation of Müller glial processes, at P25 when the retinal is functionally mature and synaptogenesis is complete, and at P60, when the animal is sexually mature (Fig. 3.2B). Induction of CRE expression by tamoxifen administration at P5 results in robust, Müller glial-specific CRE expression at P15, as shown by co-localization of β-galactosidase (β-gal) and the MG marker glutamine synthetase (Fig. 3.2C). Sox2 is efficiently and specifically ablated in $Sox2^{MUTANT}$ MG cell bodies located in the INL (arrow) (Fig. 3.2D"). A population of SOX2 positive amacrine cells in the IPL maintains SOX2 expression in both $Sox2^{MUTANT}$ and $Sox2^{CONTROL}$ retinas (arrowheads) (Fig. 3.2D', 2D''). The establishment of this line allows for the genetic dissection of SOX2's role in postnatal Müller glial maturation.

3.2.3 Loss of SOX2 Disrupts the Maturation of MG Branching Side Processes

To examine Müller glia over the course of their postnatal maturation, the CRE positive MG population was identified via ß-galactosidase staining, at P15, P25 and P60. At P15, $Sox2^{MUTANT}$ and control Müller glia processes in the IPL display similar morphologies (Fig. 3.3A, D, insets). However, by P25, a striking disorganization of MG branching side processes in the $Sox2^{MUTANT}$ IPL is evident (Fig. 3.3B, E, insets). Müller glial end-feet that compose the ILM are disrupted and decreased in density in the mutant (arrowheads). At P60 MG processes in the IPL and ILM remain diffuse and disorganized compared to the control (Fig. 3.3C, F, insets). These data indicate that loss of SOX2 in Müller glia at P5 results in aberrant maturation and development of processes by P25.

We next examined the entire population of Müller glia to visualize the density of processes in the synaptic layers and integrity of the inner and outer limiting membranes. The

expression pattern of MG specific glutamine synthetase, an enzyme that converts glutamate to glutamine, was analyzed to assess MG morphology in the $Sox2^{MUTANT}$ retina. As shown in Figure 3.1, MG processes in the IPL undergo significant maturation during the first two postnatal months. At P15, MG apico-basal processes in the IPL of the in $Sox2^{MUTANT}$ retinas are similar in morphology to the control (compare the insets in Figs. 3.3G and 3J). However, $Sox2^{MUTANT}$ processes that extend into the outer plexiform layer (OPL) are not as refined, expanding into a greater area compared to control (arrowheads, Figs. 3.3G, J). At P25, the $Sox2^{MUTANT}$ MG processes in the OPL are more refined; yet remain disorganized compared to control. There is a marked decrease in density and complexity of MG processes in IPL of the $Sox2^{MUTANT}$ compared to control retinas (Fig. 3.3H, K). Particularly evident in the insets is the decreased density of processes that define the INL border and synaptic bands in the IPL (arrows, insets for Fig. 3.3H,K). Breaks in the ILM, also seen in the ß-galactosidase staining, are clearly present in the $Sox2^{MUTANT}$ (arrowheads, Fig. 3.3H, K). Further, the central retina is significantly reduced in thickness in the $Sox2^{MUTANT}$ compared to $Sox2^{CONTROL}$ (average retinal thicknesses of 206.6 μm and 252.5 μm respectively). The reduced density of processes in the $Sox2^{MUTANT}$ IPL and disrupted OPL and ILM are maintained at P60 (Fig. 3.3I, L) suggesting that once disrupted, Müller glial morphology remains constant.

3.2.4 $Sox2^{MUTANT}$ Müller Glial Cell Bodies are Disorganized

To further define and quantify the disorganization of the MG cell bodies, we examined SOX9 expression, which specifically labels MG cell bodies in the INL. Compared to the control, the $Sox2^{MUTANT}$ Müller glia have mislocalized cell bodies, with some nuclei located ectopically in the outer plexiform layer (OPL) and outer nuclear layer (ONL), amidst the photoreceptor cell bodies (Fig. 3.4). At P15, there is limited disorganization of $Sox2^{MUTANT}$ MG cell bodies, consistent with the limited disorganization of processes in the IPL at this

stage (Fig. 3.4A, D, arrowheads). At P25, there is a marked disorganization of Sox2^{MUTANT} MG cell bodies (Fig. 3.4B, E, arrowheads). We quantified this disorganization using a modified least squares regression and found significant disorganization ($P < 0.042$, Student's *t-test*) of the Sox2^{MUTANT} MG cell bodies compared to control. The exported X,Y coordinates of a representative P25 mutant and control pair are overlaid in Fig. 3.4G. As maturation progresses, SOX9 positive MG cell bodies in the Sox2^{MUTANT} retinas may be displaced from the INL when the appropriate architecture and density of lamellar processes fail to develop. At P60, SOX9 positive cells remain in the ONL of the Sox2^{MUTANT} retina (Fig. 3.4C, F), however they are decreased in number compared to P25. A population of the ectopic MG may have undergone apoptosis, although analysis of cleaved caspase 3 staining at P25 and P60 does not reveal a significant increase in cell death (data not shown). This suggests that if Sox2^{MUTANT} MG undergo apoptosis, it is a small fraction of the population and occurs at a slow rate. The mislocalization of Sox2^{MUTANT} MG cell bodies phenocopies MG disorganization that occurs during retinal degeneration (Joly et al., 2011). Therefore we analyzed GFAP expression, which is up-regulated in gliotic MG of degenerating retinas (Ekström et al., 1988). However, we saw no marked up-regulation of GFAP at P15, P25 or P60 in the Sox2^{MUTANT} (Fig. 3.5D, E, F) compared to Sox2^{CONTROL} (Fig. 3.5A, B, C). This provides evidence that the Sox2^{MUTANT} phenotype is a result of aberrant maturation, rather than degeneration.

3.2.5 Neuronal Processes and Cell Bodies are Disrupted in Sox2^{MUTANT} Retinas

The disorganized MG processes in the IPL and OPL suggest that the neuronal axons and dendrites in the plexiform layers may also be disrupted. We therefore examined amacrine cell neurites in the IPL and horizontal cell neurites in the OPL. A subset of amacrine cells and their processes are labeled specifically with an antibody to the calcium

binding protein, calretinin. From P15 to P25 in the control retina, we find there is notable refinement of the three synaptic bands that define the ON and OFF synaptic regions within the IPL (Fig. 3.6A, B, see inset). By 25, this synaptic band is fully mature and there is no additional refinement between P25 and P60 (Fig. 3.6B, C, see inset). In contrast, $Sox2^{MUTANT}$ retinas exhibit a mild disruption of the fasciculation of the three synaptic bands at P15 (Fig. 3.6D). At P25 in the $Sox2^{MUTANT}$, consistent with the pattern of MG disruption, there is marked disorganization and further defasciculation of the processes compared to control retinas (Fig. 3.6B, E). The disorganization of the amacrine cell processes is maintained at P60 (Fig. 3.6C, F), suggesting a stable phenotype from P25 onward. This phenotypic progression provides evidence that loss of SOX2 not only affects MG maturation, but also results in the abnormal development of neuronal processes and points to an extended period of neuronal maturation and plasticity in the postnatal retina. Horizontal cells regulate the photoreceptors response and input to bipolar cells and extend their processes in the OPL (Fig. 3.6G, arrowhead)(Masland, 2012). Neurofilament staining (Haverkamp and Wässle, 2000), reveals disruption of horizontal cell neurites, extending into the inner and outer nuclear layers in the $Sox2^{MUTANT}$ at P15 (Fig. 3.6J arrow). This disruption is maintained at P25 and P60 in the $Sox2^{MUTANT}$ retinas compared to control (Fig. 3.6H, K arrow and I, L arrow). In the mammalian retina, PKCα positive bipolar cells transmit neuronal signals from rods and cones in the outer retina to their synaptic partners in the inner retina (Masland, 2001; Vardi et al., 2002; Ruether et al., 2010). Their axons display extensive arborization in the synaptic IPL. Beginning at P15 and continuing through P60, there is a marked increase in the density of bipolar axonal terminals in $Sox2^{MUTANT}$ retinas, resulting in an expanded area within the IPL that is innervated by PKCα positive processes (see brackets in insets, Fig. 3.6M-R). The disruption of amacrine, horizontal and bipolar processes point to the role of MG in stabilizing the synaptic plexiform layers and indicate a potential disruption in retinal

function.

The retinal tissue ultrastructure was next assessed through semi-thin section histology and electron microscopy. At P60, semi-thin retinal sections indicate a decrease in the density of processes in the $Sox2^{MUTANT}$ IPL compared to control (Fig. 3.7A, B). At the ultrastructural level, electron micrographs reveal neurites in the $Sox2^{MUTANT}$ IPL are swollen in diameter compared to control (Fig. 3.7C, D, * denotes swollen neurites).

In the murine ONL, photoreceptor (PR) cell bodies form linear columns that are surrounded by MG lamellar processes (Fig. 3.7E, inset). However in the $Sox2^{MUTANT}$, the columns of PR cell bodies are bent and disorganized (Fig. 3.7F, inset). Furthermore, the outer limiting membrane (OLM, arrows), formed by zonula adherens junctions between MG and photoreceptor outer segments, is irregular in the $Sox2^{MUTANT}$, compared to the linearly formed OLM in the $Sox2^{CONTROL}$. At the ultrastructural level, the adherens junctions that form the OLM are identified as short (~ 0.3 μm), dark densities between MG lamellar processes and photoreceptor outer segments (OS) (arrowheads, Fig. 3.7G). In the $Sox2^{MUTANT}$, adherens junctions are visibly shorter and oriented parallel to the apical basal axis of the retina as opposed to perpendicular in the control (arrowheads, Fig. 3.7H). In the $Sox2^{CONTROL}$, MG processes maintain rod nuclei (RN) at a distance of approximately 3 μm from the OLM. However, PR cell bodies (RN and cone nuclei, CN) in the $Sox2^{MUTANT}$ have collapsed basally to the edge of OLM. These thin-section and ultrastructural analyses provide strong evidence that the loss of SOX2 causes a disruption of processes that extends throughout the entire width of the retina.

3.2.6 Loss of SOX2 in Müller Glia Results in Decreased Retinal Function

Electroretinograms (ERGs) were performed at P25 to assess retinal function in $Sox2^{MUTANT}$ and $Sox2^{CONTROL}$ retinas. ERG responses were recorded under scotopic (or dark-

adapted) conditions over 12, 4ms light flashes that covered a 10^5 fold range in intensity. Representative traces from $Sox2^{CONTROL}$ and $Sox2^{MUTANT}$ eyes are shown in black and red, respectively (Fig. 3.8A). At P25, ablation of *Sox2* in Müller glia results in a significant decrease in a-wave amplitude at low light levels (Fig. 3.8B). The a-wave measures the photoreceptor specific current, and at low light levels this decrease in amplitude indicates a minor decrease in rod function. Also notable is a decreasing trend in a-wave amplitude at the highest flash intensity, indicating a possible reduction in cone function. A highly significant reduction in b-wave amplitude, which is generated in the inner retina, was observed at a range of low and high flash intensities, indicating significant functional disruption of the inner retina (Fig. 3.8C). ERGs performed at P60 were consistent with histological data showing no further degeneration after P25; P60 retinas maintained the functional deficit present at P25 (data not shown). These data indicate that loss of SOX2 in MG disrupts not only the morphology of the neural retina and plexiform layers, but also results in a significant decrease in retinal function across a range of light intensities. Furthermore, these results point to an essential function of MG to establish and stabilize the neuronal circuitry of the inner retina.

3.3 Discussion

3.3.1 Loss of SOX2 in Müller glia Disrupts Neuronal Structure and Function

In the postnatal retina, the stem cell transcription factor *Sox2* plays an essential role in MG maturation. Between P10 and P15, Müller glia begin to extend processes into the synaptic plexiform layers of the retina. The extension of branching side processes continues throughout the first postnatal month. Loss of SOX2, specifically in MG at P5, results in their aberrant maturation. In the $Sox2^{MUTANT}$, disrupted Müller and neuronal processes are evident throughout the retina. Neurites of amacrine and bipolar cells in the inner plexiform and

horizontal cells in the outer plexiform layer are disorganized. The outer and inner limiting membranes formed by MG end feet are disrupted. And, in the outer nuclear layer, photoreceptor cell bodies are disorganized. This phenotypic disorganization progresses over the first postnatal month.

Though *Sox2* is ablated at P5 in MG and absent from MG cell bodies by P10 (data not shown), at P15 there is a mild disruption of Müller and neuronal processes. However by P25, striking disruption of both Müller and neuronal processes is evident. Analysis of Müller side branching in the rabbit retina identified P10 to P25 as the period of the greatest extension of MG side branching and increase in cytoplasmic volume (Reichenbach and Reichelt, 1986). In the mouse retina, starting at P0 and continuing through P15, ganglion and amacrine cells form synapses in the IPL. Outer plexiform layer synaptic development begins at approximately P5 with the formation of cone-HZ cell synapses followed by cone-bipolar synaptogenesis. Shortly thereafter, around P8, rod-HZ cell and rod-biploar cell synaptogenesis begins (Olney, 1968; Blanks et al., 1974; Fisher, 1979; Sherry et al., 2003). Spontaneous waves of retinal activity refine these synaptic connections in the early postnatal period (Meister et al., 1991; Penn et al., 1994; Wong, 1999). The majority of synapses are form by P15. Directly following eye opening and continuing until P25, there is a dynamic increase in specialized bipolar ribbon synapses (Fisher, 1979).

It is likely that the extension of processes in the $Sox2^{CONTROL}$ retina and the disruption of MG that develops from P15 to P25 in the $Sox2^{MUTANT}$ retina directly follows this period of synaptogenesis. It is well established that glial sheaths form around active synapses (Eroglu and Barres, 2010), and in many regions of the nervous system glia play an active role in synaptogenesis (Ullian et al., 2001; Christopherson et al., 2005; Allen et al., 2012; Corty and Freeman, 2013), although the role of glia in synapse formation has regional variation (Steinmetz et al., 2006). In the murine retina, photoreceptor circuits are established well

before MG lamellar processes reach them (Williams et al., 2010). Interestingly, it has been suggested that the onset of neuronal activity may guide or stimulate the MG outgrowth, possibly through the local influx of K^+ across MG membranes (Reichenbach and Reichelt, 1986; Williams et al., 2010). These light-evoked K^+ currents are specifically localized to the synaptic plexiform layers (Karwoski et al., 1985), where the MG morphology shows the greatest degree of disruption in the $Sox2^{MUTANT}$. Indeed, a hallmark of Müller glia maturation is a dynamic change in potassium regulation. During the second postnatal week there is concomitant decrease in the open probability of Ca^{2+}-dependent K^+ channels of big conductance (BK channels) and increase in insertion of the inwardly rectifying potassium channel Kir4.1, resulting in the mature MG resting potential of -80 mV (Kofuji et al., 2002; Bringmann et al., 2006). Loss of SOX2 in MG may prohibit or disrupt their maturation program, resulting in the maintenance of an immature structural and functional state.

In addition to the structural abnormalities, there is a decrease in the b-wave amplitude in the $Sox2^{MUTANT}$. ON bipolar cells are likely to be the major contributor to the formation of the stereotypic b-wave of the electroretinogram (Gurevich and Slaughter, 1993; Masu et al., 1995; Tian and Slaughter, 1995). PKCα staining reveals the morphology of ON bipolar cells to be only mildly disrupted in the IPL in the $Sox2^{MUTANT}$, and we observed no decrease in number of bipolar cell bodies. However, the b-wave arises from an ensemble response of the inner retina and several studies have demonstrated a reduction in b-wave similar to the $Sox2^{MUTANT}$ that is Müller glial in origin. Deletion of the water channel Aquaporin-4 (AQP4), which is specific to MG in the retina, results in a reduction in b-wave amplitude (Li et al., 2002) similar to the phenotype in the $Sox2^{MUTANT}$ retinas. Potassium siphoning and water transport in MG are mediated by the coenrichment of the inwardly rectifying potassium channel, Kir4.1 and AQP4 (Nagelhus et al., 1999). This suggests that AQP4 deletion may reduce b-wave amplitude by disrupting K^+ currents in MG. Further, AAV-mediated,

optogenetic ablation of a subset of MG leads to a reduction in b-wave amplitude (Byrne et al., 2013).

3.3.2 Defining a New Role For Sox2 in Retinal Development

Together, these data describe a new role for Sox2 in postnatal retinal maturation. Our laboratory and others have established that SOX2 is required at all major stages of retinal development for the proper formation of the mammalian eye. Global reduction in Sox2 levels result in microphthalmia and anophthalmia (small and no eye respectively) (Taranova et al., 2006; Langer et al., 2012). Neural-specific loss of SOX2 in the embryonic retina results in a neural-to-epithelial fate shift and loss of neural competence in the progenitor population (Matsushima et al., 2011). As retinal development proceeds, neural progenitors down regulate SOX2 as they exit the cell cycle and terminally differentiate (Taranova et al., 2006). When *Sox2* is ablated ubiquitously in the P0 retina, nascent MG re-enter the cell cycle and migrate to the apical lamina of the retina to divide (Surzenko et al., 2013). This points to a role for SOX2 in the maintenance of nascent MG quiescence. Recently, it was demonstrated that SOX2 plays a specific and essential role in the positioning and dendritic stratification of amacrine cells in the neural retina (Whitney et al., 2014). Here we report that ablation of *Sox2* specifically in MG at P5 *in vivo* results in disruption of MG maturation. In conjunction, these studies define a critical period for MG cell cycle entrance. SOX2 has numerous functions during development and it is conceivable that this critical period defines a shift in the role of SOX2 towards the development and maintenance of retinal cytoarchitecture in the postnatal retina.

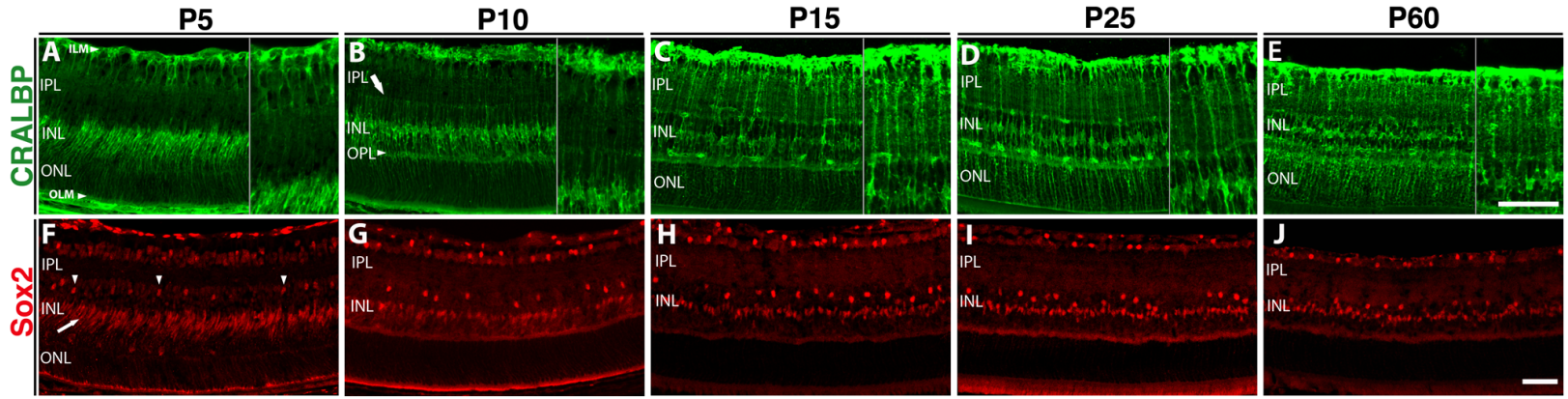
In the early postnatal retina, multiple factors contribute an environment that is permissive to cell cycle entrance. Varying levels of Notch expression contribute to the ability of MG to reenter the cell cycle (Ghai et al., 2010; Nelson et al., 2011). The changing cell

cycle dynamics of MG are also likely to be affected by WNT signaling in the postnatal retina (Liu et al., 2006b). It is well established that Sox factors interact with the WNT signaling pathway during development (Kormish et al., 2010); SOX2 has been shown to inhibit WNT signaling by binding WNT's downstream mediator, ß-catenin (Seo et al., 2011). The balance of these two transcription factors is essential for the proper specification of the neural retina (Agathocleous and Harris, 2009). In the postnatal retina, upregulation of WNT (Osakada et al., 2007; Sanges et al., 2013) leads to a more permissive environment for MG proliferation. It is conceivable that loss of SOX2 in nascent MG results in the de-repression of ß-catenin, resulting in the proliferative phenotype of nascent MG at P5. As retinal development proceeds, WNT expression is down regulated (Liu et al., 2006b) and ß-catenin expression is maintained largely in the synaptic plexiform layers (data not shown). ß-catenin has a well-established connection to cell adhesion, stabilizing N-cadherin at the membrane. This shift in WNT function from influencing cell cycle dynamics to a primarily regulating cell-cell adhesion may mirror SOX2's changing role in retinal development. SOX2 has been linked to N-cadherin expression in the developing lens (Kamachi et al., 2001). And both N-cadherin and N-CAM have been shown to be important in MG-neuronal adhesion (Drazba and Lemmon, 1990). Ablation of *Sox2* at P5 in Müller glia could result in the disruption of the MG's ability to adhere correctly to neural processes in the plexiform layers.

Together, these data establish a role for SOX2 in the development and maintenance of retinal cytoarchitecture and provide new insights into the role of SOX2 in the maturation of MG processes. The structural and functional disruption of neuronal and glial architecture at postnatal stages also highlights the extended period of dynamic maturation of the mammalian retina. Understanding the functional and structural plasticity of the postnatal and

mature retina is essential for understanding mechanisms of retinal regeneration. The role of SOX2 during this dynamic period provides new groundwork for the establishment of therapeutic regenerative strategies in the neural retina.

Figure 3.1


P5
P10
P15
P25
P60
CRALBP
Sox2
A
B
C
D
E
F
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H
I
J
ILM
IPL
INL
ONL
OLM
OPL

Figure 3.1 Müller glia mature slowly over the first postnatal month

(A-E) The Müller-specific marker CRALBP highlights morphological maturation. (A) At postnatal day 5 (P5), MG apico-basal processes are established and end feet form the inner and outer limiting membranes (I/OLM). (B) At P10, MG extend side processes into the outer plexiform layer (OPL, arrowhead) and define the boundary of the inner plexiform layer (IPL, arrow). (C) At P15, MG display a marked increase in the density of processes: bands of MG fibers ensheathing neuronal processes in the IPL form, lamellar processes enveloping photoreceptor cell bodies are visible in the outer nuclear layer (ONL). (D) At P25, Müller glial process in the IPL and ONL are well defined. (E) At P60, concomitant with slight decrease in retinal thickness, MG processes in IPL and ONL increase in density. **(F-J)** Sox2 stains MG and a subset of amacrine cells in the mature retina. (F) At P5, SOX2 is specifically expressed in the immature and elongated MG cell bodies in the inner nuclear layer (INL, arrow) and a subpopulation of amacrine cells (arrowheads) in the INL. (G) At P10, MG cell body morphology is mature and SOX2 staining is well defined. (G-J) SOX2 localization and expression in the INL remains static from P10 to P60. Scale bar: 50 μm.

Figure 3.2

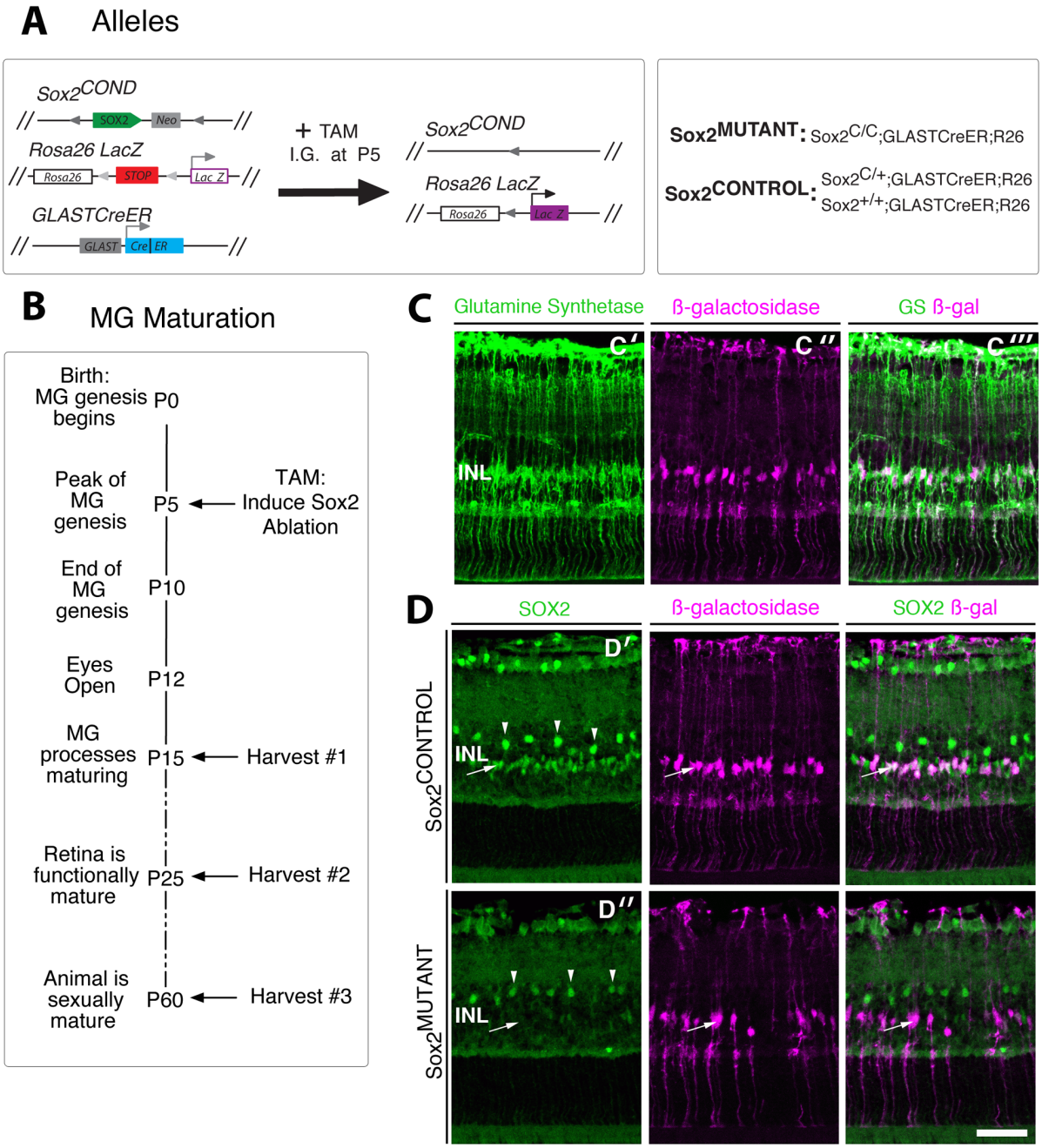

A
Alleles
Sox2COND
SOX2
Neo
Rosa26 LacZ
Rosa26
STOP
Lac Z
GLASTCreER
GLAST
Cre ER
+ TAM
I.G. at P5
Sox2COND
Rosa26 LacZ
Rosa26
Lac Z
Sox2MUTANT: Sox2C/C;GLASTCreER;R26
Sox2CONTROL: Sox2C/+;GLASTCreER;R26
Sox2+/+;GLASTCreER;R26
B
MG Maturation
Birth: MG genesis begins P0
Peak of MG genesis P5
TAM: Induce Sox2 Ablation
End of MG genesis P10
Eyes Open P12
MG processes maturing P15
Harvest #1
Retina is functionally mature P25
Harvest #2
Animal is sexually mature P60
Harvest #3
C
Glutamine Synthetase
ß-galactosidase
GS ß-gal
C′
C″
C‴
INL
D
SOX2
ß-galactosidase
SOX2 ß-gal
D′
D″
Sox2CONTROL
Sox2MUTANT
INL

Figure 3.2 Robust Müller glial specific Sox2 ablation

(A) Genetic ablation of *Sox2* in postnatal MG using the *Sox2* conditional mouse. The $Sox2^{COND}$ allele contains the *Sox2* open reading frame flanked by loxP sites. The $Sox2^{COND}$ line was crossed to a glial specific, tamoxifen (TAM) inducible Cre line, GLASTCreER, and to the Rosa26LacZ reporter line. TAM was administered via intra gastric injection at postnatal day 5 (P5), resulting in deletion of the Sox2 coding sequence and expression of ß-galactosidase in $Sox2^{MUTANT}$ populations. (B) Experimental timeline; *Sox2* ablation was induced at P5; retinas were harvested at P15, P25 and P60. (C) GLASTCRE expression is MG specific; the MG marker glutamine synthetase (C') and Cre induced ß-galactosidase staining (C") are coexpressed in P15 MG (C"'). (D) Following TAM administration, SOX2 staining is largely absent from $Sox2^{MUTANT}$ MG cell bodies in the INL (D'', arrow), but maintained the population of Sox2 positive amacrine cells (typified by arrowheads) in both $Sox2^{MUTANT}$ (D'') and $Sox2^{CONTROL}$ (D') retinas. Scale bar: 50 μm.

Figure 3.3

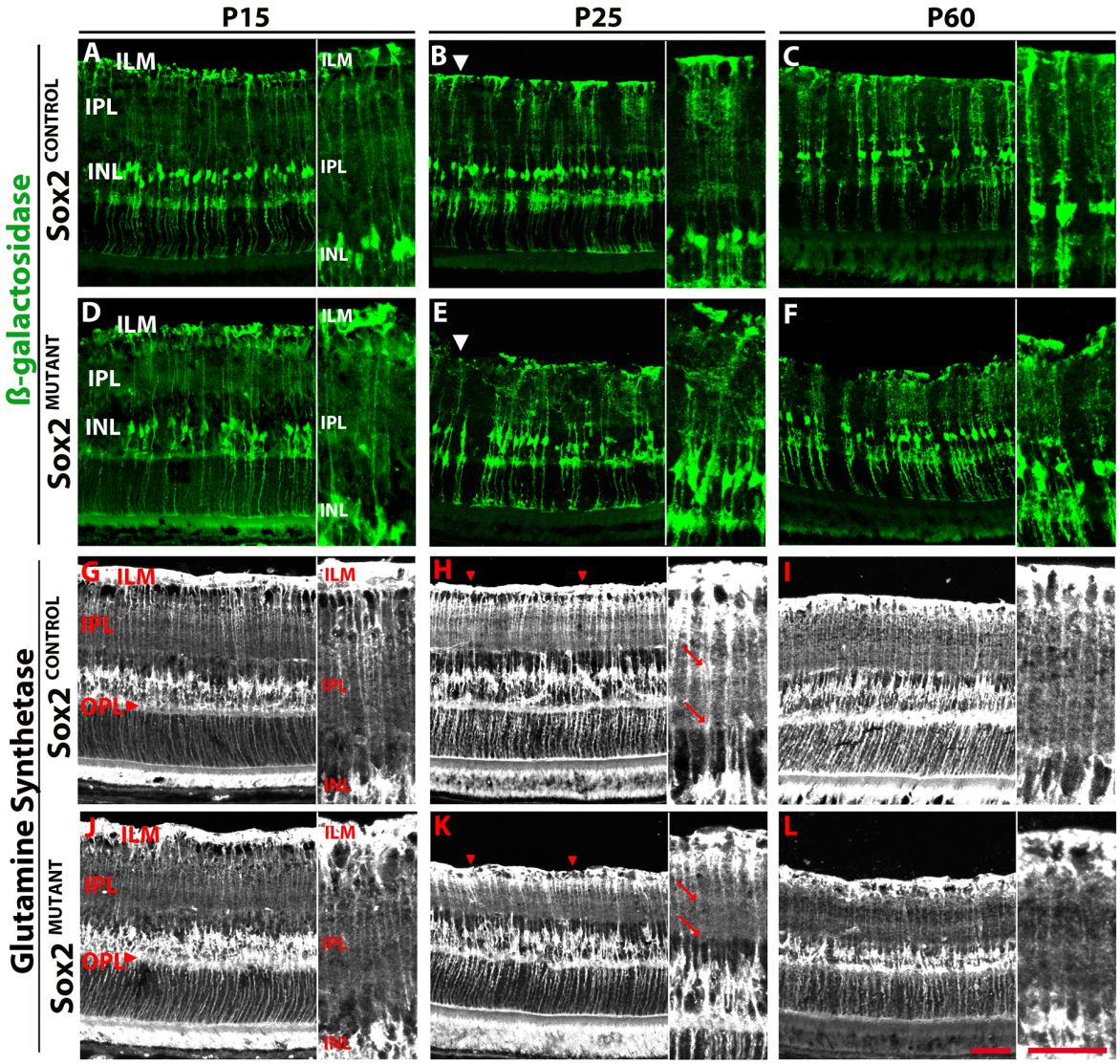

P15
P25
P60
ß-galactosidase
Sox2 CONTROL
Sox2 MUTANT
A
B
C
D
E
F
ILM
IPL
INL
Glutamine Synthetase
Sox2 CONTROL
Sox2 MUTANT
G
H
I
J
K
L
OPL

Figure 3.3 Sox2 loss disrupts MG maturation

(A-F) CRE positive Müller glia express ß-galactosidase. (A, D) At P15, $Sox2^{MUTANT}$ (D, inset) and $Sox2^{CONTROL}$ (A, inset) MG processes in the inner plexiform layer (IPL) display similar morphologies. (B, E) At P25, $Sox2^{MUTANT}$ (E, inset) MG side processes are disorganized in the IPL compared to $Sox2^{CONTROL}$ (B, inset). The inner limiting membrane (ILM) is disrupted and decreased in density in the mutant (E, arrowhead). (C, F) At P60, MG processes in the IPL and ILM remain diffuse and disorganized in the $Sox2^{MUTANT}$ (F, inset) compared to $Sox2^{CONTROL}$ (C, insets). **(G-L)** Glutamine synthetase labels the entire MG population. (G, J) At P15, the $Sox2^{MUTANT}$ (J, inset) and $Sox2^{CONTROL}$ (G, inset) processes in the IPL have a similar morphology. $Sox2^{MUTANT}$ processes in the outer plexiform layer (OPL) expand into a greater area compared to control (G, J arrowheads). (H, K) At P25, $Sox2^{MUTANT}$ MG processes in IPL (K, inset) are decreased in density compared to control (H, inset); arrows denote a decreased density of processes that define the inner nuclear layer (INL) border and the synaptic bands in the IPL. Breaks in the ILM are present in the $Sox2^{MUTANT}$ (arrowheads). The central retina is significantly reduced in thickness in the $Sox2^{MUTANT}$ compared to the $Sox2^{CONTROL}$ (average retinal thicknesses of 206.6 μm and 252.5 μm respectively, $P<0.017$, n=4 eyes). At P60, disruptions in the $Sox2^{MUTANT}$ (L, inset) IPL, OPL and ILM compared to $Sox2^{CONTROL}$ (I, inset) are maintained. Scale bar: 50 μm.

Figure 3.4

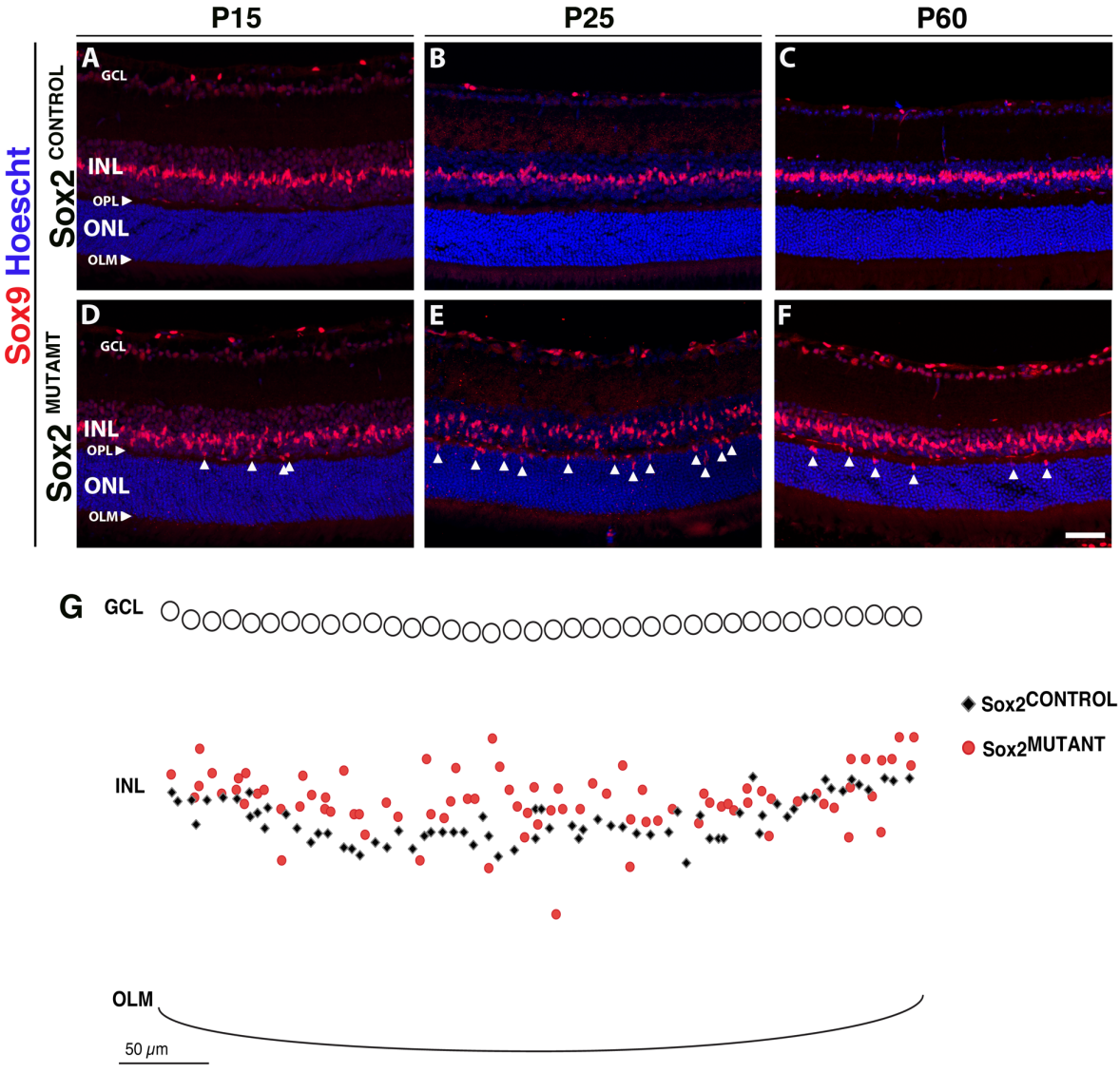

P15
P25
P60
Sox9 Hoescht
Sox2 CONTROL
Sox2 MUTAMT
A
B
C
D
E
F
GCL
INL
OPL
ONL
OLM
G
GCL
INL
OLM
Sox2CONTROL
Sox2MUTANT
50 μm

Figure 3.4 $Sox2^{MUTANT}$ Müller glial cell bodies are disorganized

(A-F) MG cell bodies in the INL are specifically labeled with Sox9. (A, D) At P15, $Sox2^{MUTANT}$ MG (D) have mislocalized cell bodies compared to $Sox2^{CONTROL}$ (A), with some nuclei located ectopically in the outer plexiform layer (OPL) and the outer nuclear layer (ONL) (D, arrows). (B, E) At P25, $Sox2^{MUTANT}$ MG cell bodies (E, arrows) display increased disorganization compared to $Sox2^{CONTORL}$ (B). Significant INL disorganization of $Sox2^{MUTANT}$ MG cell bodies was identified by a least squares regression analysis ($P < 0.042$, n=5 eyes). (G) Exported and overlaid (X,Y) coordinates of a representative P25 $Sox2^{MUTANT}$ and $Sox2^{CONTROL}$ pair. (C, F) At P60, $Sox2^{MUTANT}$ Sox9 positive MG remain disorganized (F, arrows) compared to $Sox2^{CONTROL}$ (C), and are decreased in number compared to the P25 $Sox2^{MUTANT}$ (E). Scale bar: 50 μm.

Figure 3.5

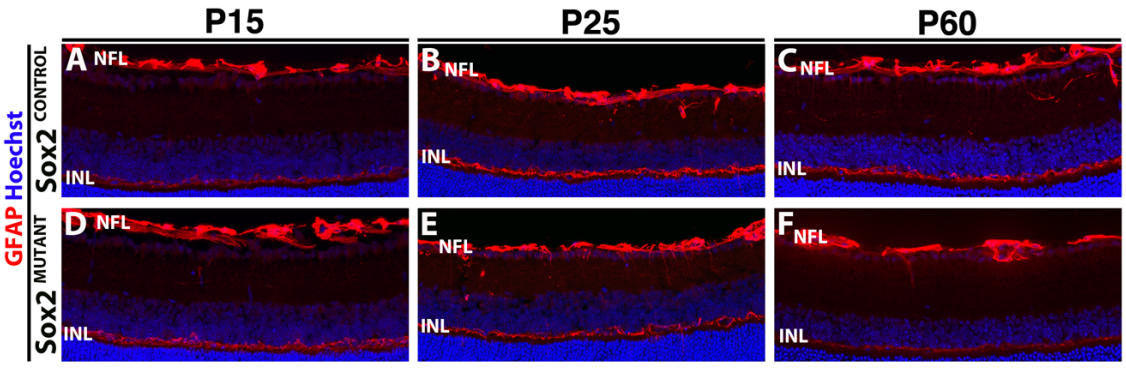


Figure 3.5 Gliosis is not observed in Sox2^{MUTANT} retinas

(A-F) GFAP staining is not upregulated in the Sox2^{MUTANT} retina. (A-C) Sox2^{CONTROL} retinas display GFAP staining only in the inner nuclear layer (INL) and nerve fiber layer (NFL) at P15 (A), P25 (B) and P60 (C). (D-F) GFAP is not up regulated in Sox2^{MUTANT} retinas at P15 (D), P25 (E) or P60 (F).

Figure 3.6

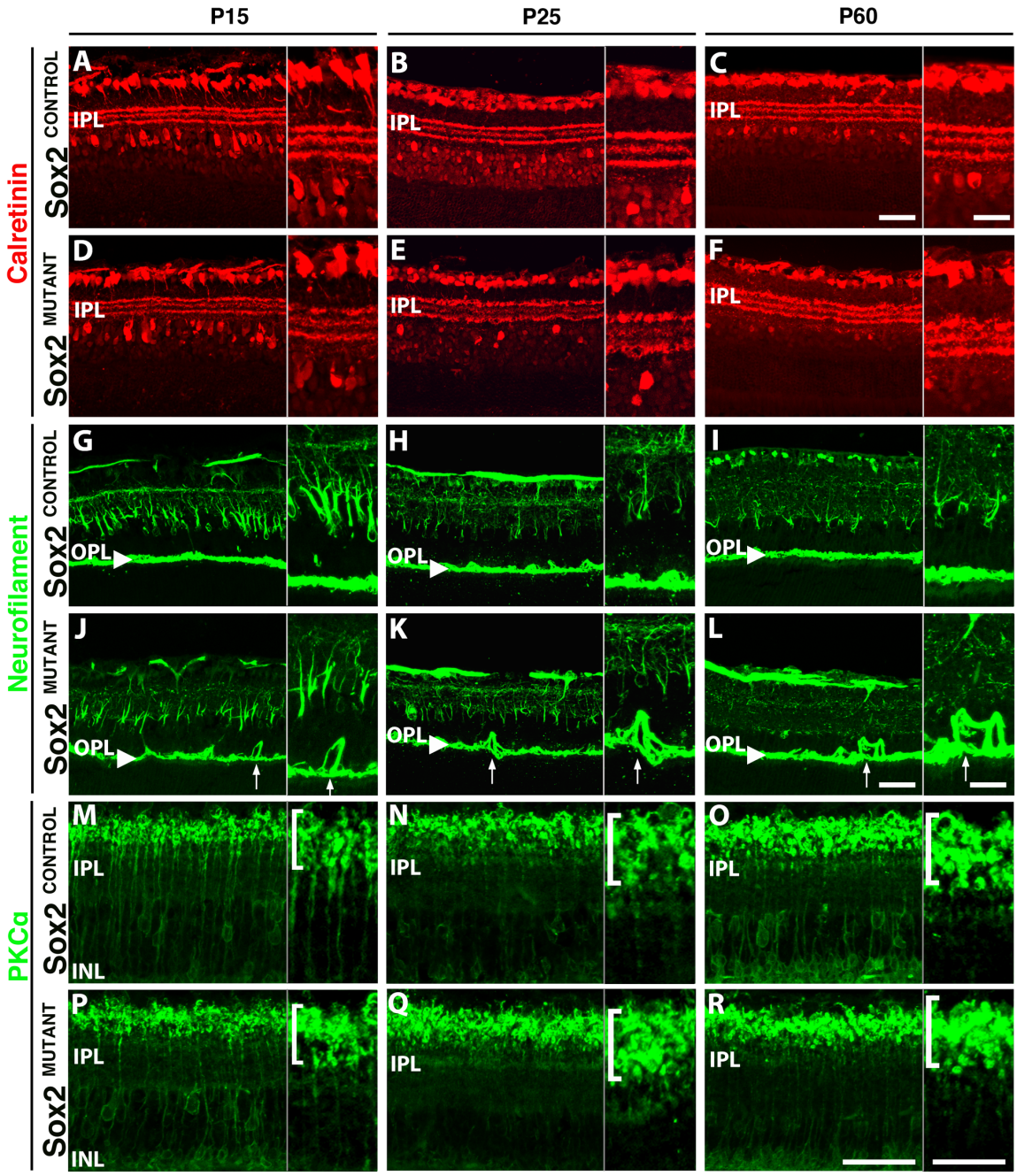

P15
P25
P60
Calretinin
Neurofilament
PKCα
Sox2 CONTROL
Sox2 MUTANT
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
IPL
OPL
INL

Figure 3.6 Sox2^{MUTANT} neuronal processes are disrupted

(A-F) Calretinin positive amacrine cell neurites are disrupted in the inner plexiform layer (IPL) of Sox2^{MUTANT} retinas. (A, D) At P15, Sox2^{MUTANT} (D, inset) retinas exhibit mild disruption of three synaptic IPL bands compared to Sox2^{CONTROL} (A, inset). (B, E) At P25 Sox2^{MUTANT} (E, inset) retinas display marked disorganization of amacrine neurites compared Sox2^{CONTROL} (B, inset). (C, F) At P60, disruption of the IPL is maintained in Sox2^{MUTANT} compared to Sox2^{CONTROL} retinas. **(G-L)** Neurofilament-positive horizontal cell processes (G, arrowhead) are disrupted in the outer plexiform layer (OPL) of Sox2^{MUTANT}. (G, J) At P15, Sox2^{MUTANT} neurites (J, inset) are disorganized compared to Sox2^{CONTROL} (G), extending into the inner and outer nuclear layers (I/ONL, arrow). OPL disruption is maintained in P25 and P60 Sox2^{MUTANT} (K, L; arrows) retinas compared to Sox2^{CONTROL} (H, I). **(M-R)** PKCα positive ON bipolar cell arborization in the IPL is disrupted in the Sox2^{MUTANT}. At P15, 25 and 60, bipolar cell axon terminals in the IPL (insets, brackets) are increased in density in Sox2^{MUTANT} retinas (P, Q, R; insets) compared to Sox2^{CONTROL} (M, N, O), resulting in a decreased area in the IPL that is not invaded by PKCα positive processes. Scale bar: main figures, 50 μm: insets, 25 μm.

Figure 3.7

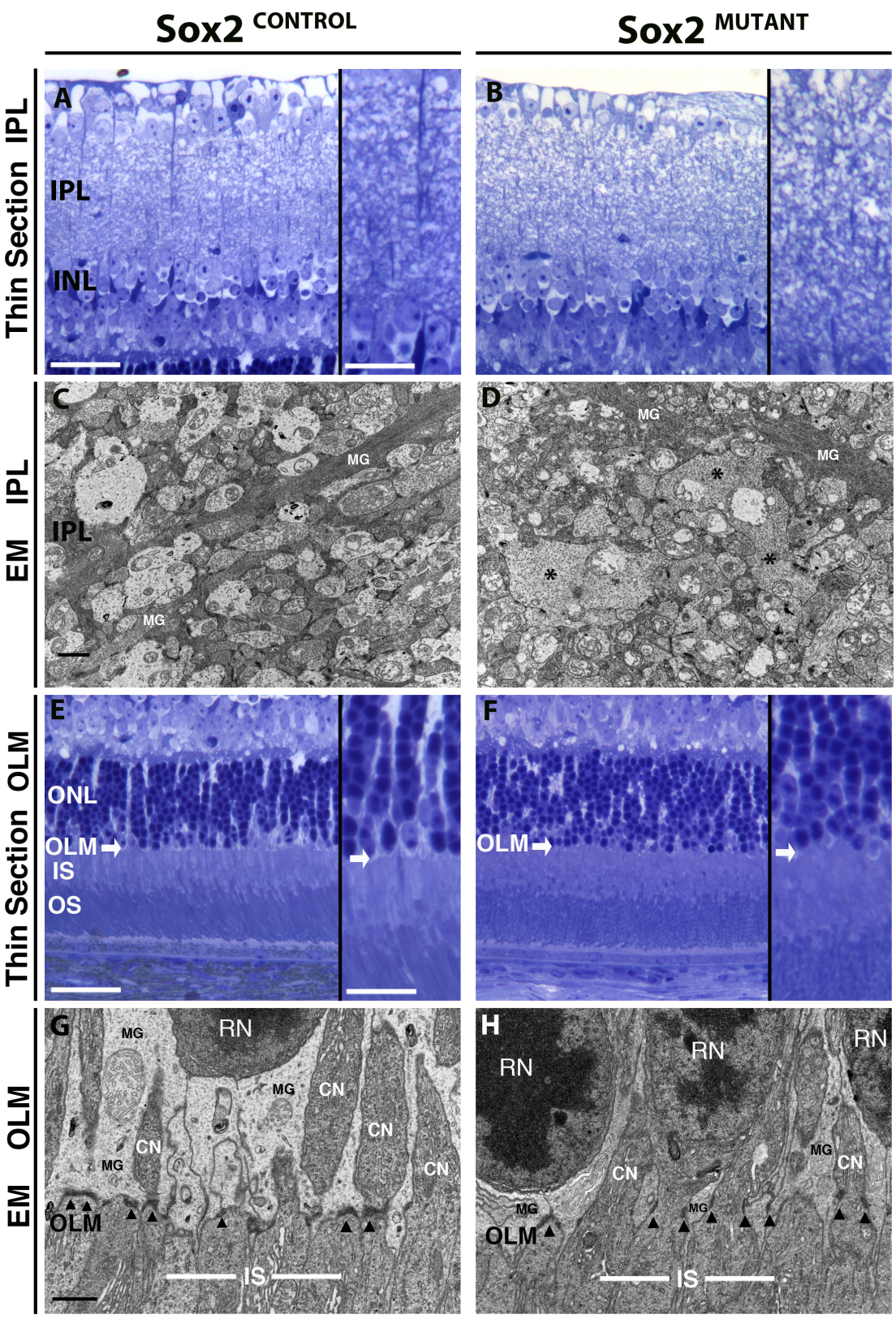

Sox2 CONTROL
Sox2 MUTANT
Thin Section IPL
A
IPL
INL
B
EM IPL
C
IPL
MG
MG
D
MG
MG
Thin Section OLM
E
ONL
OLM
IS
OS
F
OLM
EM OLM
G
MG
RN
MG
CN
CN
CN
MG
OLM
IS
H
RN
RN
RN
CN
MG
CN
MG
MG
OLM
IS

Figure 3.7 Sox2^{MUTANT} retinas display disrupted retinal ultrastructure

(A-D) IPL neurites are swollen in the P60 Sox2^{MUTANT}. (A, B) Semi-thin sections highlight neurite density in the Sox2^{CONTROL} (A, inset) IPL, which is reduced in the Sox2^{MUTANT} (B, inset). (**C, D**) Electron micrographs show Sox2^{MUTANT} (D) neurites in the IPL are swollen in size (* denotes swollen neurites) compared to Sox2^{CONTROL}. (**E-H**) The outer neural retina is disrupted in the Sox2^{MUTANT}. (**E, F**) Semi-thin sections through the outer retina highlight linear rows of photoreceptor (PR) nuclei in the ONL of the Sox2^{CONTROL} (E, inset). In the Sox2^{MUTANT} (F, inset) the rows of PR nuclei are formed irregularly. The outer limiting membrane (OLM, white arrows) formed between MG and photoreceptor inner segments (IS) is warped in the Sox2^{MUTANT} compared to Sox2^{CONTROL}. Slightly darker in stain, the photoreceptor outer segments (OS) are also visible. (**G, H**) Electron micrographs show a reduced area of MG lamellar processes in Sox2^{MUTANT} (H) ONL, resulting in rod and cone nuclei (RN, CN) located approximately 2μm closer to the OLM compared to control (G). Zonula adherens junctions (black arrowheads) between MG and PR are irregularly formed in the Sox2^{MUTANT} (H) compared to Sox2^{CONTROL} (G). Thin Sections: Scale bars main figures- 50 μm, Thin Section inserts- 25 μm, N=4 eyes for each condition; EM: Scale bar 1.0 μm, N=3 Sox2^{MUTANT} eyes and N=2 Sox2^{CONTROL} eyes.

Figure 3.8

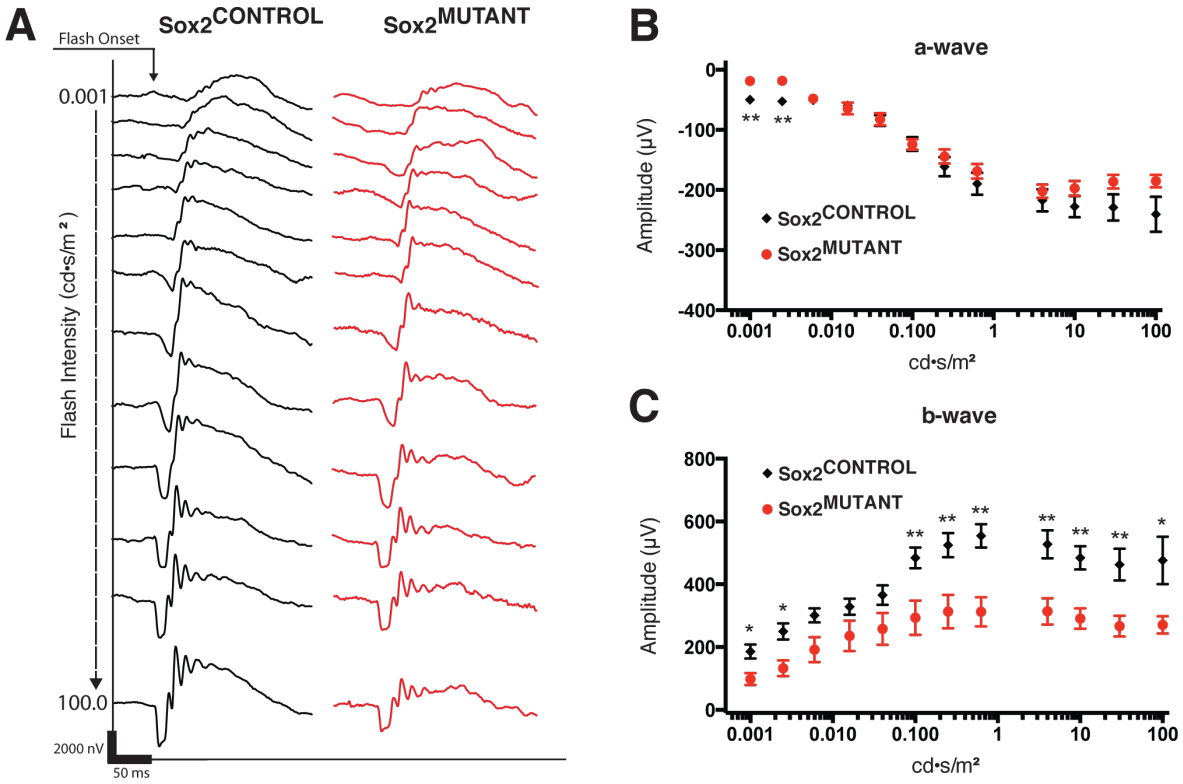

A
Sox2CONTROL
Sox2MUTANT
Flash Onset
0.001
100.0
Flash Intensity (cd•s/m²)
2000 nV
50 ms
B
a-wave
Amplitude (µV)
0
-100
-200
-300
-400
Sox2CONTROL
Sox2MUTANT
0.001
0.010
0.100
1
10
100
cd•s/m²
C
b-wave
Amplitude (µV)
800
600
400
200
0
Sox2CONTROL
Sox2MUTANT
0.001
0.010
0.100
1
10
100
cd•s/m²

Figure 3.8 Loss of Sox2 in MG results in a deficit in retinal function

Electroretinography at P25 demonstrates reduced function in $Sox2^{MUTANT}$ retinas (**A-C**). (A) Representative traces from $Sox2^{MUTANT}$ (n=14) and $Sox2^{CONTROL}$ (n=12) eyes recorded under scotopic conditions in response to 12, 4-ms flashes of light intensities ranging from 0.001 to 100 cd•s/m^2. (B) $Sox2^{MUTANT}$ retinas show a significant decrease in a-wave amplitude at low light levels, indicating a decrease in rod function (Step 1, $P<0.0001$; Step 2, $P<0.0018$). Impaired cone function is suggested by a trend towards a decreased a-wave amplitude at the highest flash intensity ($P< .068$). (C) $Sox2^{MUTANT}$ retinas display a highly significant reduction in b-wave amplitude over a range of low and high flash intensities, indicating functional disruption of the inner retina (Step 1, $P<0.0059$; Step 2, $P<0.0034$; Step 3, $P<0.0316$; Step 4, $P<0.1228$; Step 5, $P<0.0922$; Step 6, $P<0.0083$; Step 7, $P<0.0047$; Step 8, $P<0.0006$; Step 9, $P<0.0019$; Step 10, $P<0.0006$; Step 11, $P<0.0029$, Step 12, $P<0.0124$). * $P<0.05$; ** $P< 0.01$.

CHAPTER FOUR: SOX2 EXPRESSION IS REGULATED IN A STRAIN DEPENDENT MANNER

A high degree of genetic variability exists between and within the mouse strains used in biomedical research. In this study we analyze the effect of strain background on expression levels of the transcription factor *Sox2* in a floxed transgenic line. On a mixed, outbred CD1/BL6 background, SOX2 expression levels in homozygous floxed retinas, $Sox2^{C/C}$, were comparable to those recorded during initial characterization of the line on a mixed 129/BL6 background. However on an inbred C57BL/6 background, SOX2 retinal protein in the $Sox2^{C/C}$ line is reduced to 50% of wild type levels. Further, C57BL/6 $Sox2^{C/C}$ retinas exhibit abnormal morphology and display loss of retinal function. $Sox2^{C/C}$ on the mixed CD1 background are phenotypically normal. Targeted ablation of *Sox2* in MG using a glial specific inducible Cre results in retinal degeneration on the C57BL/6 background and near complete vision loss. This phenotype is distinct from the previously characterized MG specific ablation of *Sox2* on a CD1/BL6 background. Together, these results demonstrate the powerful affect strain background can exert on phenotypic expression and point to the necessity to characterize these strain dependent influences.

4.1 Introduction

Genetic diversity is key to evolutionary success. As is true in any population, genetic variability exists between and within the many mouse strains used in biomedical research. In this study we investigated the affect of strain background on the baseline expression of SOX2 in the retina of a *Sox2* floxed mouse ($Sox2^{COND}$) previously developed in the lab

(Taranova et al., 2006). Originally developed from 129/svPas ES cells carrying the floxed transgene injected into a C57BL/6J blastocyst, this line like many transgenics, was developed on a mixed background. Inbred 129 ES cell lines are commonly used for the generation of transgenics for their particular ability to integrate into the germ line (Robertson et al., 1986; Limaye et al., 2001). C57BL/6 mice, with their fully sequenced genome, good breeding abilities and extensive record as a research model animal are the default host. This method for producing floxed mice results in the generation of a line of mixed genetic background. In our laboratory, the $Sox2^{COND}$ line has been maintained on the C57BL/6 background for over six years. However, the initial characterization was completed soon after the mouse was developed, in the 129/BL6 mixed background (Taranova et al., 2006).

Genetic and phenotypic differences between strains have been well characterized, including classical studies on differing response to pain, alcohol consumption and anxiety (He et al., 1997; Mogil et al., 1999; Voikar et al., 2001; Bothe et al., 2004). Even within the same strain – including the most commonly used inbred mouse, C57BL/6 – there are reports of variability (Mekada et al., 2009; Zurita et al., 2011). Strain differences are an important factor when selecting a mouse line for use in experiments. For example, in vision research, certain strains within the C57BL/6 line should be avoided, due to a contamination with the *rd8* mutation, which results in slow but significant retinal degeneration over the animal's lifetime (Mattapallil et al., 2012).

Here we report that $Sox2^{COND}$ mice homozygous for the floxed allele ($Sox2^{C/C}$) on a primarily C57BL/6 background show a baseline reduction in SOX2 expression. These reduced levels of SOX2 in the murine eye result in marked thinning of the retina and severe reduction in visual function by postnatal day 25 (P25). When outbred to the CD1 line for two generations, SOX2 expression in the mixed background $Sox2^{COND}$ line is restored to near

wild type levels. Further, no differences in retinal morphology or function are observed between $Sox2^{C/C}$ and wild type populations on this background.

Sox2, an HMG-box transcription factor and member of the highly related SoxB1 family (Collignon et al., 1996), maintains stem cell pluripotency in the embryonic nervous system and in populations of adult neuronal stem cells (NSCs) (Zappone et al., 2000; Bylund et al., 2003; D'Amour, 2003; Graham et al., 2003). *Sox2* ablation in the developing central nervous system (CNS) leads to premature neuronal differentiation and depletion of progenitor pools (Pevny and Nicolis, 2010). Unlike other CNS regions, *Sox2* is the only member of the highly related Sox2B1 family that is expressed in the retina (Kamachi et al., 1998). The retina therefore offers a unique environment in which to study the role of SOX2 in neural development, as well as being particularly susceptible to its loss. Our laboratory has demonstrated an essential role for SOX2 in proper eye and retinal development (Taranova et al., 2006; Matsushima et al., 2011; Langer et al., 2012; Surzenko et al., 2013). Further, mutations in the human *SOX2* allele have been shown to lead to microphthalmia or anophthalmia (small eye or no eye, respectively) (Fantes et al., 2003).

During retinal development, a multipotent, SOX2 positive retinal progenitor cell (RPC) systematically gives rise to all six retinal neurons in a conserved order from embryonic day 12 to postnatal day 10 (Turner and Cepko, 1987; Livesey and Cepko, 2001). Ablation of *Sox2* during embryonic retinal development results in a loss of neural competence and a drastic reduction in retinal area (Taranova et al., 2006; Matsushima et al., 2011). SOX2 expression is maintained in Müller glia (MG), the last population of cells to develop from the RPCs (Roesch, 2008; Nelson et al., 2011). In postnatal RPCs, *Sox2* ablation results in a population of nascent MG reentering the cell cycle and undergoing a terminal division which leads to their eventual depletion (Surzenko et al., 2013). Loss of SOX2 specifically in Müller glia results in their aberrant maturation, leading to disrupted retinal morphology and function

(see Chapter Three). Here we report that MG specific ablation carried out in the hypomorphic C57BL/6 background results in near total loss of retinal function, depletion of MG and retinal degeneration. Together, these results demonstrate the powerful affect that genetic background can exert on phenotypic expression and point to the necessity to characterize background strain effects independently of experimental manipulations.

4.2 Results

4.2.1 Strain Background Influences Sox2 Expression Levels in the Sox2^{COND} Line

SOX2 is required for the maintenance of a pluripotent state and is expressed in all neural stem cell populations (Avilion et al., 2003; Ellis et al., 2004; Pevny and Nicolis, 2010). Because embryos homozygous null for *Sox2* abort prior to gastrulation, a *Sox2* floxed allele (Sox2^{COND}) was previously generated to examine SOX2 loss in a temporally controlled, tissue specific manner (Taranova et al., 2006). The *Sox2* allele is located on chromosome three, and the open reading frame is encoded by a single exon (Stevanovic et al., 1994; Collignon et al., 1996; Wiebe et al., 2000). The Sox2^{COND} line was developed by introducing a floxed *Sox2* targeting vector into ES cells on the inbred 129/SvPas background. In the targeting vector, the full *Sox2* mRNA and minimal or 'core' promoter region was flanked by two loxP sites. A neomycin resistant cassette was inserted into the targeting vector to allow for a chemical selection step in the identification of ES cells that had undergone homologous recombination and incorporated the transgene. ES cells containing the transgene were injected into C57BL/6J blastocysts, and transferred to a pseudo pregnant female. Once developed, the Sox2^{COND} line (Fig. 4.1A), was maintained on the C57BL/6J background for over six years. However, the initial characterization was completed soon after the line was established, on a 129/BL6 mixed background. At the time of development, mice homozygous for the Sox2^{COND} allele (Sox2$^{C/C}$) were born in the expected Mendelian ratios,

and no phenotypic differences were observed between homozygous and wild type retinas (Taranova et al., 2006).

Seven years after the $Sox2^{COND}$ line was generated in the lab, SOX2 protein levels in P5 retinas were analyzed in two different background strains by western blot: an inbred strain on the C57BL/6 background and a mixed strain, outbred two generations to the CD1 line. All experiments carried out in the outbred, CD1 line in this chapter were conducted in the second generation. This mixed CD1 line (referred from this point onward as CD1) recapitulates the background in which the line was developed. $Sox2^{C/C}$ retinas from the mixed CD1 background expressed SOX2 protein at levels 80% of wild type (Fig. 4.1B). When the line was initially developed, SOX2 levels were not reported, but projected to have a mild reduction in SOX2 expression (Taranova, 2006). However, $Sox2^{C/C}$ retinas on the inbred C57BL/6 background expressed SOX2 protein at 50% of wild type levels, indicating marked hypomorphic SOX2 expression (Fig. 4.1C). This striking 30% difference in SOX2 levels occurred over only two generations, pointing to a strong effect of strain background on protein expression levels.

4.2.2 $Sox2^{C/C}$ Retinas on a C57BL/6 Background Exhibit Abnormal Development

To determine if the reduction in SOX2 levels leads to a disruption of retinal morphology, we examined the population of principal retinal glial cells, the Müller glia. Müller glia (MG) express SOX2 constitutively and span the entire width of the neural retina, sending out lamellar processes into all synaptic and neuronal layers. Therefore, MG morphology provides a good measure of retinal organization in a SOX2 compromised background. Glutamine synthetase, a critical glial enzyme, labels the entire population of MG. On the mixed CD1 genetic background, $Sox2^{C/C}$ retinas at P25 are phenotypically normal, as previously described during the development of the conditional allele (Taranova et al., 2006).

In the CD1 background, $Sox2^{C/C}$ MG display normal morphology (Fig. 4.2A). MG cell bodies reside in the inner nuclear layer (INL) and MG apico-basal processes span the thickness of the neural retina. The inner and outer limiting membranes (ILM/OLM) of the retina are formed by MG end feet. MG lamellar processes extend into the synaptic inner and outer plexiform layers (IPL/OPL) and envelope neuronal cell bodies in the inner and outer nuclear layers (INL/ONL) (Fig. 4.1A). On the inbred C57BL/6 background however, $Sox2^{C/C}$ retinal morphology is disrupted. Glutamine synthetase staining reveals a distinct reduction in retinal thickness compared to the CD1 background at P25 (Fig. 4.2C).

Though severe, this phenotype does not display complete penetrance. Retinas harvested from the same mouse stochastically exhibit monocular morphological disruption. In Figure 4.2, the right (R) eye of a P25 C56BL/6 mouse is severely reduced in thickness (C), while the left (L) eye is phenotypically normal (B). *SOX2* mutations in humans also result in abnormal eye development, and, in severe cases, the complete lack of an eye, known as anophthalmia (Fantes et al., 2003). As in this population of mice, the human phenotype is variable and can present monocularly (Reis et al., 2010). This ocular disparity is not uniformly lateralized. The left (D) and right (F) retinas from a P60 mouse in Figure 4.2 show an opposite lateralization of the phenotype. From P25 to P60 (Fig. 4.2B and C compared to Fig. 4.2D and E), C57BL/6 $Sox2^{C/C}$ retinas with no morphological defects remain stable and do not degenerate.

While the entire retina is reduced in thickness, the inner plexiform layer (IPL) is the most severely affected region, thinning disproportionately to the ONL, where the photoreceptor cell bodies are located. To examine the IPL, retinas were stained with calretinin, which labels amacrine neurites in this layer. In the P25 CD1 $Sox2^{C/C}$ mice, calretinin positive amacrine neurites form three distinct, fasciculated bands of processes in the IPL (Fig. 4.2F). In the C57BL/6 background, $Sox2^{C/C}$ mice that display marked retinal

thinning (P25R and P60L), the three, calretinin positive bands have collapsed, nearly forming a single band (Fig. 4.2H, I). P25L and P60R C57BL/6 Sox2$^{C/C}$ retinas that are not reduced in thickness maintain organized and distinct calretinin positive amacrine processes in the IPL (Fig. 4.2G, J).

4.2.3 Retinal Function is Decreased in Sox2$^{C/C}$ Retinas on a C57BL/6 Background

To determine if, in addition to morphological defects, Sox2$^{C/C}$ mice display defects in visual function, retinal response to light was assessed in the two strain backgrounds. An electroretinogram (ERG) was used as a measure of retinal function, recording the neural retina's stereotypic response to light as a field potential on the cornea. ERG responses were recorded under scotopic (or dark adapted) conditions, with a series of 12, 4-ms flashes of increasing light intensity (0.001 to 100 cd•s/m^2). At P25, when the retina is functionally mature, CD1 Sox2$^{C/C}$ display normal retinal function. ERG traces from a representative P25 CD1 wild type eye (Sox2$^{+/+}$) and Sox2$^{C/C}$ left and right eyes are shown in black in Figure 4.3A. Left and right eye traces from the Sox2$^{C/C}$ mouse are highly correlated and show increasing amplitude of the photoreceptor response, the negative a-wave, and inner retina response, the positive b-wave, as the intensity of the flashes increase. However, at P25 the inbred C57BL/6 Sox2$^{C/C}$ retinas display a functional deficit. ERG traces from the left and right eyes of a representative P25 C57BL/6 mouse are shown in blue (Fig. 4.3A). While the left eye displays a typical response, the right eye shows a marked decrease in a-wave amplitude and near extinction of the inner retinal, b-wave response. This monocular disparity is representative of the incomplete penetrance of this phenotype. Of six Sox2$^{C/C}$ mice, retinal function was disrupted binocularly in two mice, monocularly in one mouse and was not affected in two mice. To determine if the C57BL/6 Sox2$^{C/C}$ retinas degenerate further with age, we assessed retinal function five weeks later, at P60 in a separate cohort.

Representative left and right eye traces are shown in red in Figure 4.3A. In this mouse, it is the left eye in which the hypomorphic phenotype is manifested, showing a complete loss of retinal function. The right eye however does not display a functional deficit.

Although the phenotype does not display complete penetrance, analysis of a-wave and b-wave amplitudes of C57BL/6 P25 and P60 $Sox2^{C/C}$ retinas show a significant decrease in neuronal responses compared to P25 CD1 $Sox2^{C/C}$ and $Sox2^{+/+}$ retinas. Analysis of the photoreceptor-generated a-wave show a notable trend towards a decrease in amplitude of B57BL/6 P25 $Sox2^{C/C}$ retinas at low, rod responsive light intensities (Fig. 4.3B, blue). B57BL/6 P60 $Sox2^{C/C}$ retinas show significant reduction in responses across a wide range of light intensities, suggesting a deficit in both rod and cone function (Fig 4.3B, red). The b-wave response of both P25 and P60 B57BL/6 $Sox2^{C/C}$ retinas are significantly reduced in amplitude across a-wide range of light intensities compared to the CD1 $Sox2^{+/+}$ and $Sox2^{C/C}$ response (Fig. 4.3C; blue, red v. black). At P60, no further reduction in B57BL/6 $Sox2^{C/C}$ b-wave amplitude is measured compared to P25 B6 $Sox2^{C/C}$ b-wave amplitude. These results demonstrate a striking and significant decrease in retinal function when the $Sox2^{COND}$ allele is maintained on a C57BL/6 background.

4.2.4 Müller Glial Specific Sox2 Ablation Results in an Independent Phenotype on the C57BL/6 Background

To further characterize the strain dependent differences of the $Sox2^{COND}$ allele, we analyzed the specific ablation of *Sox2* in MG on the C57BL/6 background. MG-specific loss of SOX2 on the CD1 background is described in detail in Chapter Three. Briefly, conditional ablation of *Sox2* at P5 during the peak of MG genesis is achieved by crossing the glial specific, tamoxifen inducible GLASTCreER line to the $Sox2^{COND}$ line. A single dose of tamoxifen at P5 results in efficient, MG specific Sox2 ablation. (Chapter Three, Fig. 3.2).

Loss of SOX2 ($Sox2^{MUTANT}$) results in aberrant MG maturation, and retinal disorganization. $Sox2^{MUTANT}$ MG extend fewer lamellar processes into the nuclear and plexiform layers, resulting in thinning of the neural retina, neurite disruption and a concomitant decrease in inner retinal function as measured by ERG.

$Sox2^{MUTANT}$ retinas on the hypomorphic C57BL/6 background were stained with glutamine synthetase to examine the MG population. When *Sox2* is ablated in specifically in MG on the C57BL/6 background, the P25 $Sox2^{MUTANT}$ retina is strikingly reduced in thickness compared to $Sox2^{CONTROL}$, similar to the $Sox2^{C/C}$ (Fig. 4.4A-C). However, in addition to the thin neural retina, the $Sox2^{MUTANT}$ retina also displays a decrease in MG density compared to $Sox2^{CONTROL}$ (Fig. 4.4A,B), whereas the $Sox2^{C/C}$ does not (Fig. 4.2A-E). By P60 the $Sox2^{MUTANT}$ MG are further reduced in density, whereas the $Sox2^{C/C}$ MG density is stable from P25 to P60 (Fig. 4.2A-E). Additionally, the reduction in density and organization of $SOX2^{MUTANT}$ MG lamellar processes that extend into the IPL and OPL reported in the CD1 background (Chapter Three, Fig. 3.3) is recapitulated in the C57BL/6 background (Fig. 4.4B,C).

The reduction in the number of MG as the $Sox2^{MUTANT}$ retinas mature is particularly striking in the Cre positive population, labeled with ß-galactosidase. GLASTCreER is efficiently expressed in the P25 $Sox2^{CONTROL}$ MG (Fig. 4.4D). In the P25 $Sox2^{MUTANT}$, Cre positive MG are decreased in number compared to control (Fig. 4.4E). By P60, there is a striking further reduction in MG number, indicating a marked loss of $Sox2^{MUTANT}$ MG from P25 to P60 (Fig. 4.4E,F). Severe reduction in the number of MG is not observed in $Sox2^{C/C}$ retinas on the C57BL/6 background alone. This suggests that in the hypomorphic C57BL/6 background, SOX2 functions in MG to maintain the population, perhaps identifying a new role for SOX2 in degenerating retinas.

In the CD1 $Sox2^{MUTANT}$ population, MG cell bodies in the INL, labeled specifically with Sox9 are significantly disorganized (Chapter Three, Fig. 3.4). The C57BL/6 $Sox2^{MUTANT}$ retinas phenocopy the CD1 result. Sox9 positive MG cell bodies are disorganized compared to control, with some cell bodies migrating into ONL (arrowheads, Fig. 4.4G, v. H, I). At P60, the number of disorganized $Sox2^{MUTANT}$ cell bodies is reduced compared to the P25 $Sox2^{MUTANT}$ (Fig. 4.4H, I). A reduction in the number of disorganized MG cell bodies from P25 to P60 is also observed in the CD1 background (Chapter Three, Fig. 3.4). In conjunction with the decreasing density in glutamine synthetase and ß-galactosidase positive MG from P25 to P60, it is likely that the Sox9 positive MG cell bodies that migrate out of the inner nuclear layer undergo apoptosis. Previous work in the lab demonstrated that loss of SOX2 in nascent MG results in their migration through the ONL to the apical edge of the retina where they undergo a terminal division (Surzenko et al., 2013). Over the course of several days, this process leads to a near complete depletion of the MG population and collapse of the retina. It is likely that this mechanism is also acting on the mature C57BL/6 $Sox2^{MUTANT}$ retinas.

$Sox2^{C/C}$ retinas on the C57BL/6 background and $Sox2^{MUTANT}$ retinas on the CD1 background, exhibit a thinning of the IPL. In the $Sox2^{MUTANT}$ retinas, neurites that extend through this layer, like those from the calretinin positive amacrine cells, are also disorganized (Chapter Three, Fig. 3.5). However in the $Sox2^{C/C}$ population on the C57BL/6 background, these amacrine cell processes collapse within the thinning IPL, but maintain their organization (Fig. 4.2H, I). In the C57BL/6 $Sox2^{MUTANT}$ retinas, amacrine cell processes in the IPL undergo both collapse and disorganization, resulting in virtually indistinguishable bands of neurites extending throughout the layer (Fig. 4.4K,L arrows).

The marked decrease in MG density in $Sox2^{MUTANT}$ retinas compared to $Sox2^{C/C}$ retinas on the C57BL/6 background, combined with the increase in retinal disorganization of

the $Sox2^{MUTANT}$ compared to $Sox2^{C/C}$, provides further evidence for the role of SOX2 in MG maturation. However, MG-specific *Sox2* ablation on the hypomorphic background significantly increases the severity of the phenotype. These results point to the influence of strain on phenotypic expression and highlight the importance of thoroughly characterizing background effects independently of experimental manipulations.

4.2.5 $Sox2^{MUTANT}$ Retinas on the C57BL/6 Background Display Near Complete Vision Loss

Histological analysis suggests that deletion of *Sox2* specifically in MG on the already compromised C57BL/6 background results in retinal degeneration from P25 to P60. To confirm these results we preformed ERGs on the P25 and P60 $Sox2^{MUTANT}$ population. No significant difference in a-wave amplitude was identified between P25 $Sox2^{C/C}$ and P25 $Sox2^{MUTANT}$ retinas (Fig. 4.5A). However, P60 $Sox2^{MUTANT}$ retinas displayed significantly decreased a-wave amplitudes compared to P25 $Sox2^{C/C}$**,** P60 $Sox2^{C/C}$ and P25 $Sox2^{MUTANT}$ populations. This provides further evidence for SOX2's essential role in maintaining MG morphology and function in the mature retina. The b-wave response, already severely decreased in amplitude in the $Sox2^{C/C}$ population did not exhibit a further, significant decrease in amplitude in the $Sox2^{MUTANT}$ retinas. However the P60 $Sox2^{MUTANT}$ response does display a degenerative trend (Fig. 4.5B).

4.3 Discussion

Background strain can exert a profound effect on phenotypic expression. On an inbred C57BL/6 background, $Sox2^{C/C}$ retinas express SOX2 protein at 50% of wild type levels, compared to 80% of wild type levels on a mixed CD1 background. This difference in protein expression was observed after only two generations of breeding the C57BL/6 line to

the CD1 line, highlighting the powerful affect of background on protein expression in this particular transgenic line. The reduction in SOX2 expression in the C57BL/6 background results in marked thinning of the neural retina by P25 in the $Sox2^{C/C}$ mice, with particular severity in the synaptic, inner plexiform layer. The thinning of the neural retina is likely due to a reduction in the population of proliferating progenitor cells during retinal development, resulting in a decrease in the production of retinal neurons. Sox2 ablation in neural progenitor cells results in their premature exit from the cell cycle (Bylund et al., 2003). Further, reduction in SOX2 expression results in a decrease in the number of proliferating cells during retinal development and alters the differentiation capacity of retinal progenitors (Taranova et al., 2006).

A significant reduction in retinal function is also observed at P25 with almost complete loss of inner retinal response. By P60, there is a significant decrease in the photoreceptor-generated response across all light intensities. The sequential loss in retinal function suggests that reduction in SOX2 levels not only affects retinal specification and maturation, but also leads to a gradual retinal degeneration as the animal ages. Interestingly, there is some evidence to suggest that both humans and mice with hypomorphic Sox2 expression levels present with neurodegenerative phenotypes (Ferri et al., 2004; Ragge et al., 2013).

It is important to note that the $Sox2^{C/C}$ phenotype displays a penetrance of approximately 50%. In some cases, C57BL/6 $Sox2^{C/C}$ mice display one eye that is phenotypically normal and one of disrupted morphology and function. No significant lateralization with respect to left or right side was identified. Severe reduction in Sox2 levels (20 to 40%) also results in the development of a cleft palate. In the generation of this phenotype, one sidewall of the palate fails to close, though again no significant sidedness was found (Langer et al., 2014). Importantly, humans with *SOX2* mutations also display

monocular, or one-sided small eye (microphthalmia) or lack of eye (anophthalmia) (Bakrania et al., 2007; Reis et al., 2010). The reason for this is unknown and it would be interesting to examine the lateralization of SOX2 expression in the hypomorphic background.

The $Sox2^{COND}$ allele was initially developed on a mixed C57B6J/129svPas background. Once established, our laboratory, as well as many others, typically moves their lines to a more homogeneous background. While the new line is often bred for several generations onto the C57BL/6 background, a true inbred line as classically defined by brother/sister matings is reached only at 20 generations (Silver, 1995), approximately 3 and half years of breeding. There is an active discussion in the literature concerning potential problems using this strategy in the generation of transgenic animals, including the issue of flanking genes – extra genetic material, proximal to the transgene that is passed along during homologous recombination, which may result in off target effects (Gerlai, 1996; Montagutelli, 2000; Doetschman, 2009). Ultimately however, the problem Eisener-Dorman and colleagues write is that “the two strains used most often in the generation of knockout mice – C57BL/6 and 129 – are quite unlike each other genetically, physiologically, and behaviorally” (Eisener-Dorman et al., 2009).

At the time of initial characterization, the $Sox2^{COND}$ line was on the mixed BL6/129 background. No morphological phenotypes distinguishable from the wild type lines were identified. While no morphological differences between wild type and $Sox2^{C/C}$ retinas were identified, SOX2 expression was projected to be mildly reduced from wild type levels (Taranova, 2006). This reduction in protein expression is likely due to the inclusion of the neo cassette used to confer neomycin resistance in the chemical selection phase of transgene vector development.

It has been shown that the neo cassette contains cryptic splice sites that interfere with mRNA levels of the target gene, and are capable of producing a hypomorphic allele

(Jacks et al., 1994; Carmeliet et al., 1996; Nagy et al., 1998; Dragatsis et al., 2000; Holzenberger et al., 2000a, 2000b). Multiple studies have documented decreased expression of the gene product upon inclusion of the neo cassette (Jacks et al., 1994; Wassarman et al., 1997; Dragatsis et al., 2000; Holzenberger et al., 2000a; Meyers et al., 2012). Standard practice for the development of transgenic mice now includes the flanking of the neo cassette with FRT or loxP sites, such that the neo cassette can be permanently removed (Cohen-Tannoudji and Babinet, 1998; Hall et al., 2009; Meyers et al., 2012). It is likely therefore that the remaining neo cassette in the $Sox2^{COND}$ line results in the decrease in SOX2 expression in both the CD1 and C57BL/6 background.

While *Sox2* is a single exon gene, it is embedded within an intron of a long noncoding RNA, called the *Sox2* overlapping transcript (*Sox2ot*). The *Sox2ot* contains at least five exons and is transcribed in the same orientation of as *Sox2* into an mRNA of about 3.4 kb (Lin et al., 2011). The *Sox2ot* was first described in a study reporting the involvement of *SOX2* mutation in human anophthalmia, and suggested the *Sox2ot* is involved in regulating SOX2 expression (Fantes et al., 2003). Recent work has shown that *Sox2ot* is dynamically expressed during different developmental processes including embryonic and neural stem cell differentiation (Amaral et al., 2009). Further suppression of slice variants of the *Sox2ot* have been shown to alter cell cycle dynamics (Shahryari et al., 2014). These studies suggest SOX2 expression is dynamically regulated through alternative splicing of the *Sox2ot.*

In addition the possibility of interfering alternative splice sites, it is also possible that the physical addition of the Neo cassette reduces protein expression. In the $Sox2^{COND}$ line, the Neo cassette was inserted in between the mRNA polyA signal and the enhancer region, SRR2 (See Fig. 4.1A). Interference with this regulatory region that is important the expression of Sox2 in both ES cells and neural stem cells could also provide a likely

explanation for the reduction of SOX2 in this line (Tomioka et al., 2002; Taranova, 2006; Sikorska et al., 2008).

Less clear however is why protein expression levels would vary so drastically with strain background. If alternative splicing of the *Sox2* RNA product results in lower protein levels in both backgrounds, it is plausible then that these splicing events are differentially regulated in the strain backgrounds. Alternative splicing plays a large role in human phenotypic diversity and disease, with estimates that as many as 90% of protein coding genes undergo alternative splicing (Gamazon and Stranger, 2014; Li et al., 2014; Matera and Wang, 2014). Notably, in a 2011 study Tanackovic and colleagues determined that the human retina expresses the highest amount of snRNAs – small nuclear RNAs that comprise the macromolecular splicesomal complex. Further, the retinal spliceosome processes the largest volume of pre-mRNAs (Tanackovic et al., 2011). Indeed, the human retina has been shown to be particularly susceptible to alternative splicing defects. Systemic mutations in several spliceosomal proteins cause the heritable retinal degenerative disease, retinitis pigmentosa (Pena et al., 2007; Tanackovic et al., 2011; Utz et al., 2013; Matera and Wang, 2014). Alternative splicing of many proteins have been identified between mouse strains (Kleyn et al., 1996; Buchner et al., 2003; Floyd et al., 2003; Shavit et al., 2009; Wheeler et al., 2009; Yu et al., 2009). These data point to the differential splicing of the transgenic *Sox2* allele between CD1 and C57BL/6 backgrounds as a likely cause of the varying degrees of protein expression in the retina. Interestingly, a recent study uncovered a mutation in the C57BL6/J line within the coding region of the Sox2 allele, which is similar in structure to a mutation associated with anophthalmia in a human patient (Schneider et al., 2009; Whitney et al., 2014). This region would have been replaced in the development of the $Sox2^{COND}$ allele, and therefore is not likely the cause of the variation in protein expression between

background strains reported here. However, it provides further evidence of the high degree of variability between and among commonly used strains.

To further characterize the strain dependent differences of the $Sox2^{COND}$ allele, we analyzed the MG specific ablation of *Sox2* on the C57BL/6 hypomorphic background. MG specific *Sox2* ablation on outbred CD1 background is described in detail in Chapter Three. Briefly, ablation of *Sox2* in MG results in aberrant MG maturation, retinal disorganization and a concomitant decrease in inner retinal function as measure by ERG.

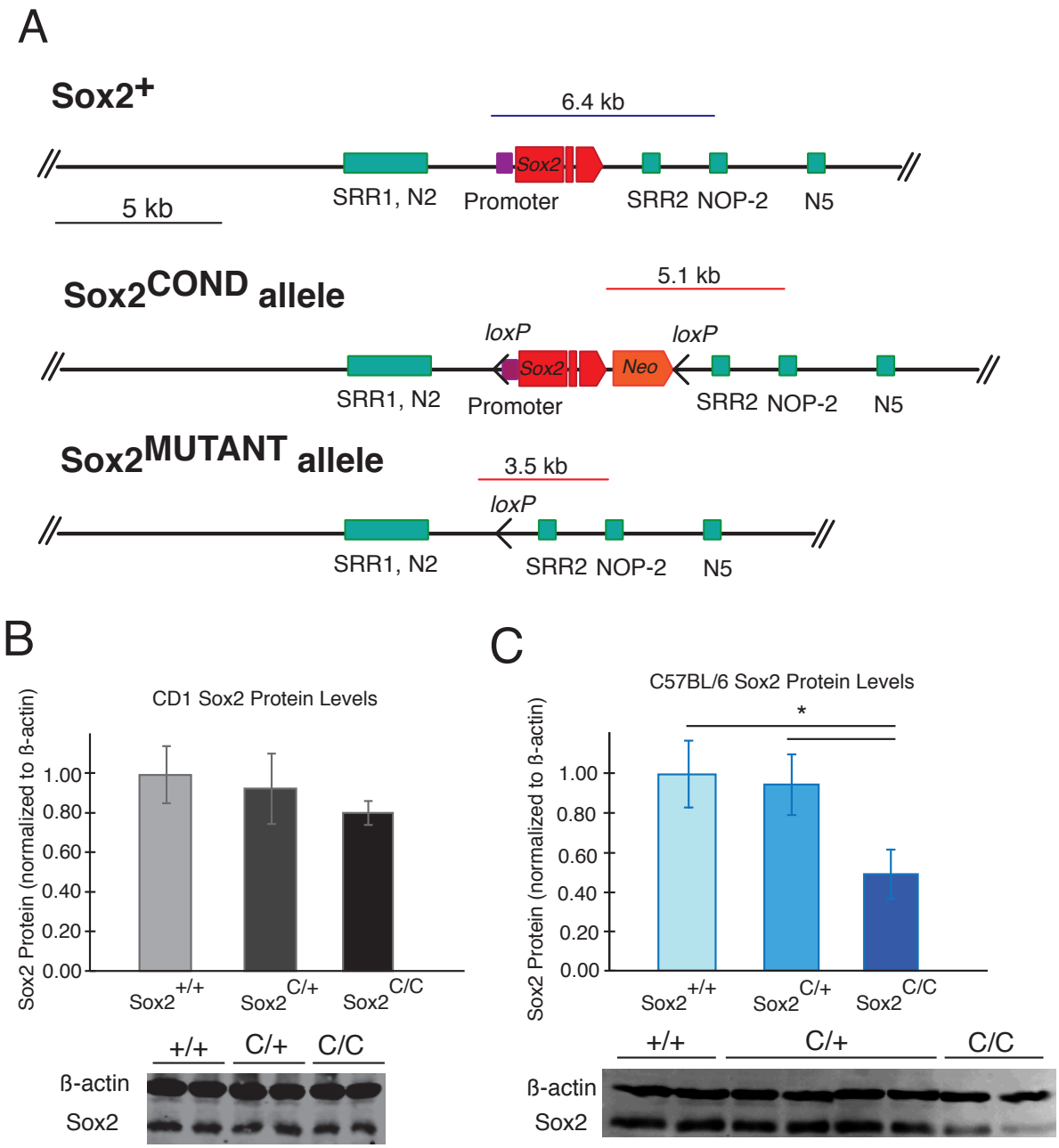
On the C57BL/6 background, disorganization of $Sox2^{MUTANT}$ MG processes is observed, as is disruption of neurites, especially in the synaptic, IPL. This phenotype is however much more severe compared to the CD1 background due to the global reduction in SOX2 levels throughout retinal development. On the C57BL/6 background, ERG analysis shows $Sox2^{MUTANT}$ retinas exhibit almost total loss of function by P60, whereas CD1 retinas show a moderate decrease in retinal function localized to the inner retina. While $Sox2^{C/C}$ retinas on the C57BL/6 background do exhibit a reduction in retinal function, ablating *Sox2* in MG results in further loss. Additionally, while the CD1 $Sox2^{MUTANT}$ phenotype is stable from P25 to P60, on the C57BL/6 background, the $Sox2^{MUTANT}$ retina degenerates, likely driven by a striking loss in MG cells during this period.

Analysis of the $Sox2^{MUTANT}$ phenotype in the CD1 and C57BL/6 lines reveals marked differences. The severity of the MG specific *Sox2* ablation is greatly enhanced in the hypomorphic C57BL/6 background. Had analysis been completed on the C57BL/6 background alone, the affect of a MG specific ablation would have been largely overshadowed by the $Sox2^{C/C}$ hypomorphic phenotype. These results highlight the strong influence strain background can assert on phenotypic expression of a transgenic line and the importance of carefully selecting control populations. It has been well documented that different strains respond distinctively in varied tests of behavior, anxiety, pain tolerance, and

drug consumption (He et al., 1997; Mogil et al., 1999; Voikar et al., 2001; Bothe et al., 2004). It follows then that mice from the same genetic background should be used as controls, a practice that is widely followed. Less frequently discussed and studied however, is the breeding of multiple transgenic strains to create the varied mouse lines commonly used in biomedical research, and in particular, neuroscience research. Valuable mice, with complex genetic components (floxed alleles, inducible Cre drivers, reporter alleles) are commonly shared among labs. While these collaborations are essential to science and the sharing of valuable resources is nothing if not a good thing, often little attention is paid to the background of these mice. Each lab has its own methods for maintaining various strains and this interbreeding creates a genetically diverse population, often of mixed background. And once a colony is established in a laboratory, the tendency is to interbreed the mice, resulting in a colony within which a certain degree of genetic drift is likely compared to the original line.

A more genetically diverse population of animals in a laboratory is not without its benefits, as it likely provides a better model for human development and disease states. However these results demonstrate that special care should be exercised when selecting controls, including efforts made to characterize the phenotypic profile of the strain's background prior to further genetic manipulation.

Figure 4.1


A
Sox2+
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Sox2
5 kb
SRR1, N2
Promoter
SRR2 NOP-2
N5
Sox2COND allele
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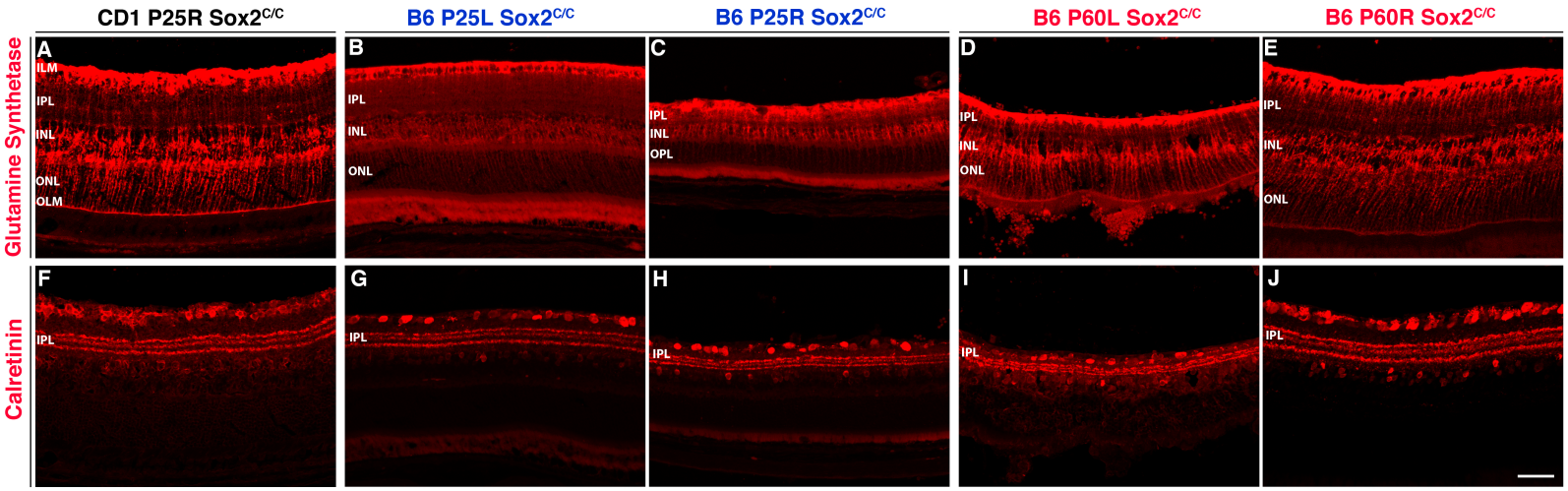
loxP
loxP
Sox2
Neo
SRR1, N2
Promoter
SRR2 NOP-2
N5
Sox2MUTANT allele
3.5 kb
loxP
SRR1, N2
SRR2 NOP-2
N5
B
CD1 Sox2 Protein Levels
Sox2 Protein (normalized to ß-actin)
1.00
0.80
0.60
0.40
0.20
0.00
Sox2+/+
Sox2C/+
Sox2C/C
+/+
C/+
C/C
ß-actin
Sox2
C
C57BL/6 Sox2 Protein Levels
*
Sox2 Protein (normalized to ß-actin)
1.00
0.80
0.60
0.40
0.20
0.00
Sox2+/+
Sox2C/+
Sox2C/C
+/+
C/+
C/C
ß-actin
Sox2

Figure 4.1 Strain background influences SOX2 expression levels in the Sox2^{COND} line

(A) The previously developed Sox2^{COND} line. The full Sox2 coding sequence and minimal or 'core' promoter sequence is flanked by loxP sites, such that upon exposure to Cre-recombinase, a null allele is established (Sox2^{MUTANT}). **(B)** Postnatal day 5 (P5) homozygous floxed retinas Sox2$^{C/C}$ on an outbred, mixed CD1 background express SOX2 protein at 80% of wild type (Sox2$^{+/+}$) levels as measured by western blot, n=4 eyes per genotype. **(C)** P5 Sox2$^{C/C}$ on an inbred C57BL/6 background express SOX2 protein at 50% of wild type (Sox2$^{+/+}$) levels as measured by western blot, n=6 eyes per genotype. Figure 4.1A was modified from (Taranova, 2006). Figure 4.1B was modified from (Crowl, 2014). Enhancers: SSR1, SSR2, NOP2, N2, N5

* Indicates significant difference of at least $p<0.05$

Figure 4.2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B6 P25L Sox2C/C
B6 P25R Sox2C/C
B6 P60L Sox2C/C
B6 P60R Sox2C/C
Glutamine Synthetase
Calretinin
A
ILM
IPL
INL
ONL
OLM
B
IPL
INL
ONL
C
IPL
INL
OPL
D
IPL
INL
ONL
E
IPL
INL
ONL
F
IPL
G
IPL
H
IPL
I
IPL
J
IPL

Figure 4.2 $Sox2^{C/C}$ retinas on a C57BL/6 background exhibit abnormal development

(A-E) Glutamine synthetase specifically labels MG and highlights retinal morphology. (A) MG display typical morphology in P25 CD1 $Sox2^{C/C}$ retinas: MG cell bodies reside in the inner nuclear layer (INL) and MG apico-basal processes span the thickness of the neural retina. The inner and outer limiting membranes (ILM/OLM) of the retina are formed by MG end feet. MG lamellar processes extend into the synaptic inner and outer plexiform layers (IPL/OPL), and envelope neuronal cell bodies in the inner and outer nuclear layers (INL/ONL). (B-E) C57BL/6 $Sox2^{C/C}$ retinas are reduced in thickness at P25 (C) and P60 (D). The phenotype is not 100% penetrant; retinas harvested from the same mouse stochastically exhibit monocular morphological disruption. The left (L) (B) and right (R) eye (E) of C57BL/6 mice at P25 and P60 respectively are phenotypically normal. **(F-J)** Calretinin-positive amacrine neurites form three distinct, bands of processes in the IPL that are phenotypically normal in the P25 CD1 $Sox2^{C/C}$ (F). (G-J) In the C57BL/6 $Sox2^{C/C}$ P25R eye (H) and P60L eye (I) calretinin positive bands have collapsed. C57BL/6 inbred $Sox2^{C/C}$ P25L (G) and P60R (J) maintain organized calretinin positive amacrine processes in the IPL. Scale bar: 50 μm

Figure 4.3

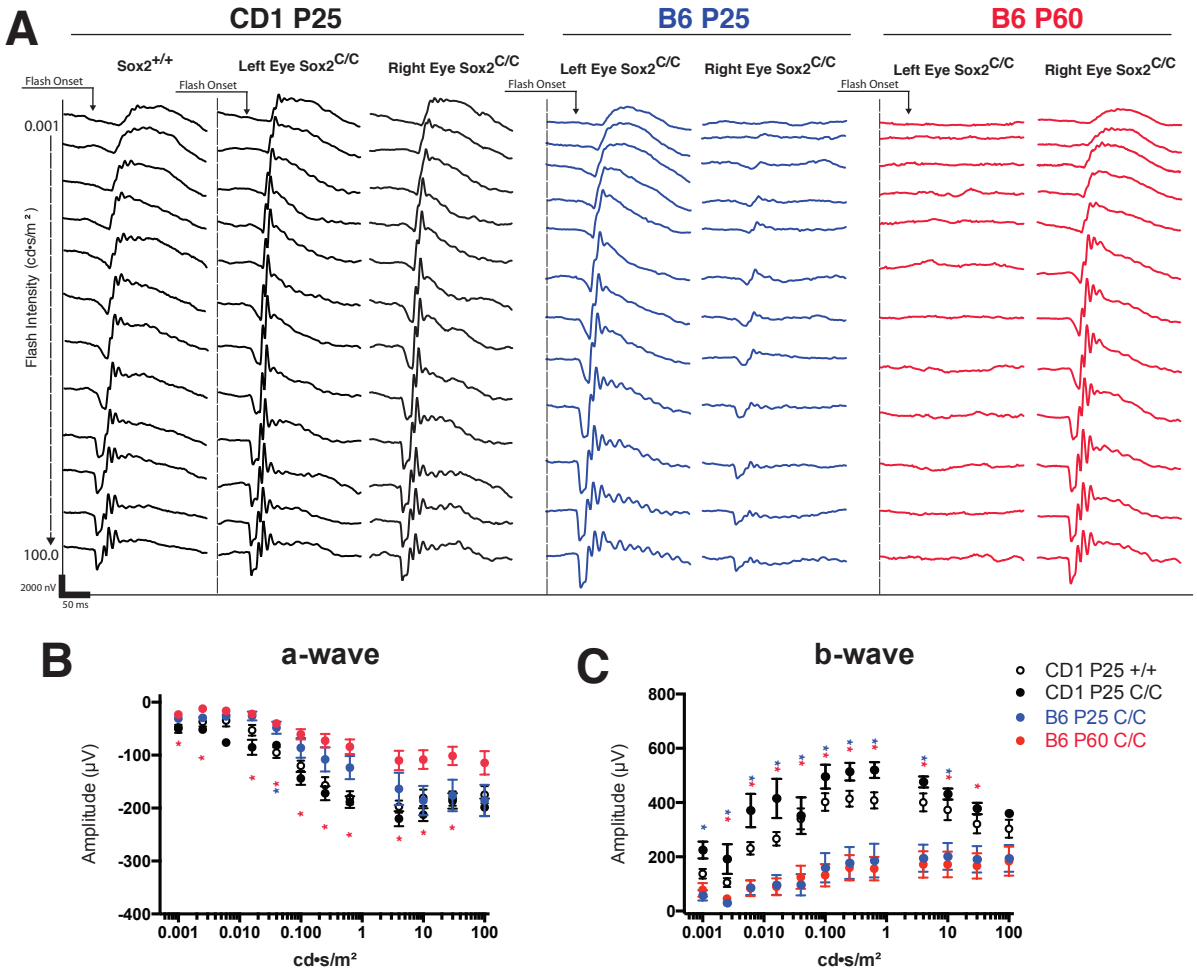
A
CD1 P25
B6 P25
B6 P60
Sox2+/+
Left Eye Sox2C/C
Right Eye Sox2C/C
Flash Onset
0.001
100.0
Flash Intensity (cd•s/m²)
2000 nV
50 ms
B
a-wave
Amplitude (µV)
cd•s/m²
C
b-wave
CD1 P25 +/+
CD1 P25 C/C
B6 P25 C/C
B6 P60 C/C

Figure 4.3 $Sox2^{C/C}$ retinas vary in retinal function depending on genetic background. **(A)** ERG traces from P25 CD1 wild type ($Sox2^{+/+}$) and traces from the left and right eye of a $Sox2^{C/C}$ mouse display normal retinal response. ERG traces from the left and right eyes of a P25 C57BL/6 $Sox2^{C/C}$ mouse shown in blue display different responses. The left eye displays a typical response while the right eye shows a marked decrease in a-wave amplitude and near extinction of the b-wave response. Representative left and right eye traces from a P60 C57BL/6 $Sox2^{C/C}$ are shown in red. The P60 left eye shows a near complete loss of retinal response while the P60 response remains intact. (**B**) The a-wave displays a decreasing trend in amplitude of P25 B57BL/6 $Sox2^{C/C}$ retinas at low, rod responsive light intensities (blue, n=12 eyes) compared to the P25 CD1 $Sox2^{+/+}$ and $Sox2^{C/C}$ response (black, n=8 eyes for both genotypes). P60 B57BL/6 $Sox2^{C/C}$ retinas show significant reduction in responses across a wide range of light intensities (red, n=10 eyes). (**C**) The b-wave of P25 and P60 B57BL/6 $Sox2^{C/C}$ retinas is significantly reduced in amplitude across a wide range of light intensities compared to the P25 CD1 $Sox2^{+/+}$ and $Sox2^{C/C}$ response (blue, red v. black).

* Indicates significant difference of at least $p < 0.05$

Figure 4.4

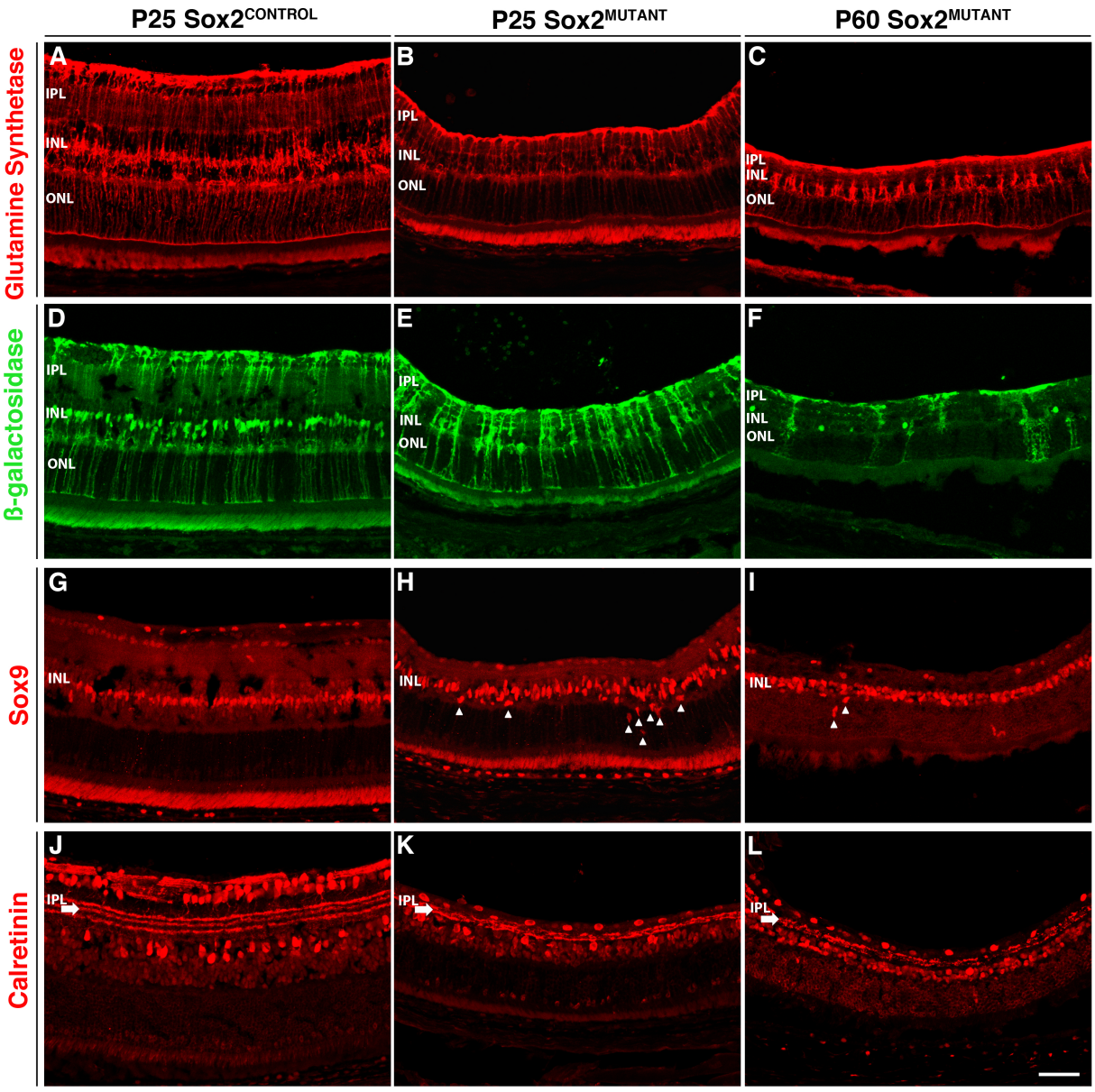
P25 Sox2CONTROL
P25 Sox2MUTANT
P60 Sox2MUTANT
Glutamine Synthetase
ß-galactosidase
Sox9
Calretinin
A
B
C
D
E
F
G
H
I
J
K
L
IPL
INL
ONL

Figure 4.4 $Sox2^{MUTANT}$ retinas display an independent phenotype on a C57BL6/J background

(A-C) The P25 C57BL/6 $Sox2^{MUTANT}$ retina (B) is strikingly reduced in thickness similar to the $Sox2^{C/C}$ retina (See Fig. 4.2C) but also displays a decrease in glutamine synthetase-positive MG density compared to $Sox2^{CONTROL}$ (A). By P60, the $Sox2^{MUTANT}$ MG (C) are further reduced in density. P25 and P60 $SOX2^{MUTANT}$ MG lamellar processes that extend into the inner and outer plexiform layers (IPL/OPL) are disorganized compared to MG processes in $Sox2^{CONTROL}$ retinas. **(D-F)** ß-galactosidase labels the GLASTCre positive population. (D) GLASTCreER is efficiently expressed in the P25 $Sox2^{CONTROL}$ MG. (E) In the P25 $Sox2^{MUTANT}$, ß-galactosidase-positive MG are decreased in number compared to $Sox2^{CONTROL}$. (F) In the P60 $Sox2^{MUTANT}$, there is a striking further reduction in MG number. **(G-I)** MG cell bodies are labeled specifically with Sox9. (G) In the $Sox2^{CONTROL}$, MG cell bodies are neatly organized in the INL. (H) P25 $Sox2^{MUTANT}$ MG cell bodies are disorganized compared to control, with some cell bodies migrating into ONL (arrowheads). (I) At P60, the number of disorganized $Sox2^{MUTANT}$ cell bodies is reduced compared to the P25 $Sox2^{MUTANT}$. (**J-L**) Calretinin-positive amacrine cells extend neurites in the Inner Plexiform Layer (IPL). (J) $Sox2^{CONTROL}$ retinas extend three distinct organized bands of amacrine cell processes. Amacrine processes in the (K) P25 and (L) P60 $Sox2^{MUTANT}$ retinas are disorganized and collapse in the IPL (arrow).

Scale bar: 50 μm

Figure 4.5

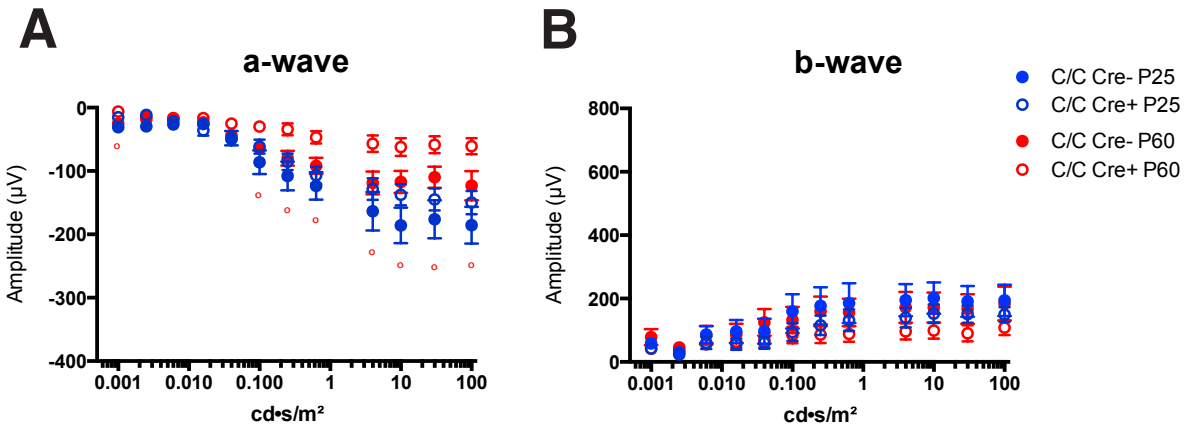
A
a-wave
Amplitude (µV)
0
-100
-200
-300
-400
0.001
0.010
0.100
1
10
100
cd•s/m²
B
b-wave
Amplitude (µV)
800
600
400
200
0
0.001
0.010
0.100
1
10
100
cd•s/m²
C/C Cre- P25
C/C Cre+ P25
C/C Cre- P60
C/C Cre+ P60

Figure 4.5 Sox2^{MUTANT} retinas on the C57BL/6 background display near complete vision loss

(**A**) ERG analysis does not show a significant difference in a-wave amplitude between P25 Sox2$^{C/C}$ (blue dot, n=12 eyes) and P25 Sox2^{MUTANT} retinas (blue circle, n=12 eyes). P60 Sox2^{MUTANT} retinas (red circle, n=18 eyes) display a significantly decrease in a-wave amplitudes compared to P25 Sox2$^{C/C}$ (blue dot, n=12 eyes) P60 Sox2$^{C/C}$ (red dot, n=10 eyes) and P25 Sox2^{MUTANT} (blue circle, n=12 eyes) populations. (**B**) The P25 Sox2$^{C/C}$ (blue dot, n=12 eyes) and P60 Sox2$^{C/C}$ (red dot, n=10 eyes) b-waves display a response that is severely decreased in amplitude. P25 Sox2^{MUTANT} (blue circle, n=12 eyes) and P60 Sox2^{MUTANT} retinas (red circle, n=18 eyes) do not exhibit a further significant decrease in amplitude. The P60 Sox2^{MUTANT} response does display a degenerative trend.

º Indicates significant difference of at least $p < 0.05$

CHAPTER FIVE: DISCUSSION

Work in this thesis investigates the role of the stem cell transcription factor *Sox2* in the maturation and maintenance of the retinal Müller glial cell. We demonstrate that SOX2 deficient Müller glia (MG) undergo aberrant maturation, resulting in the disruption of MG processes that extend throughout the neural retina. Further, we uncovered a critical role for MG in retinal maturation. Retinas in which *Sox2* has been ablated specifically in MG also display morphological and functional deficits in the neuronal population. Additionally, we describe the physiological phenotype of retinas with global reduction in SOX2 protein levels. Hypomorphic SOX2 expression results in severe functional deficits in the retina that worsen over time. While previous studies in the laboratory have analyzed the developmental deficits associated with SOX2 hypomorphism in the developing embryo and the retina, this work is the first to provide evidence of retinal degeneration associated with reduced SOX2 levels. Retinal degeneration in the hypomorphic background is exacerbated by MG specific *Sox2* ablation. Together these results point to Sox2s essential role in the maturation and maintenance of both MG and the retina.

5.1 Loss of Sox2 in Müller glia Disrupts Neuronal Structure and Function.

Müller glia, the principle glial cell of the vertebrate retina, undergo a long period of extensive maturation. In the mouse, MG - the last cell to be born from a pluripotent retinal progenitor cell - are born from postnatal day 0 (P0) to P10. Beginning between P10 and P15, and continuing throughout the first postnatal month, MG extend processes into the synaptic plexiform layers of the retina. Glial specific Sox2 ablation ($Sox2^{MUTANT}$) at the peak of MG

genesis (P5), results in their aberrant maturation. In the $Sox2^{MUTANT}$, disrupted Müller and neuronal processes are evident throughout the retina. Neurites of amacrine and bipolar cells in the inner plexiform and horizontal cells in the outer plexiform layer are disorganized. The outer and inner limiting membranes formed by MG end feet are disrupted. And, in the outer nuclear layer, photoreceptor cell bodies are disorganized. This phenotypic disorganization progresses over the first postnatal month. In addition to the structural abnormalities in the $Sox2^{MUTANT}$, there is a deficit in inner retinal function, as measured by a decrease in electroretinogram recorded b-wave amplitude.

The postnatal period is an active phase of maturation in the mouse retina. The majority of retinal synapses are formed between P0 and P15 (Olney, 1968; Blanks et al., 1974; Fisher, 1979; Sherry et al., 2003). Throughout this period, spontaneous waves of retinal activity refine these synaptic connections (Meister et al., 1991; Penn et al., 1994; Wong, 1999). In this study, while Sox2 is ablated at P5, there is only a mild disruption of Müller and neuronal processes at P15. From P15 to P25 however, there is a marked increase in MG and retinal disorganization and a concomitant decrease in retinal function. This ten day period directly follows eye opening at P12 and is concurrent with the development of specialized bipolar ribbon synapses that enable rapid signal transduction and temporal contrast in the retina (Fisher, 1979; Oesch and Diamond, 2011). The overlap of these two periods suggests that MG may play an essential role in synaptic refinement that occurs after eye opening.

Glial sheathes form around active synapses in the CNS and PNS (Eroglu and Barres, 2010), and numerous studies have pointed to glial cells' active role in synaptogenesis (Ullian et al., 2001; Christopherson et al., 2005; Allen et al., 2012; Corty and Freeman, 2013). In the PNS, there is some evidence for the role of glial Schwann cells in the maintenance of presynaptic structure in the mature neural muscular junction (Reddy et al., 2003; Feng and

Ko, 2008). In the retina, photoreceptor circuits in the outer plexiform layer are established before MG lamellar processes reach them (Williams et al., 2010). Interestingly, it has been suggested that the onset of neuronal activity may guide or stimulate the MG outgrowth (Reichenbach and Reichelt, 1986; Williams et al., 2010). It is possible that the local influx of K^+ across MG membranes following neuronal activity stimulates this growth (Reichenbach and Reichelt, 1986). Light-evoked K^+ currents are specifically localized to the synaptic plexiform layers (Karwoski et al., 1985). Further, neuronal stimulation, which increases K^+ influx, has been shown to increase Ca^{2+} levels in MG (Wakakura and Yamamoto, 1994; Rillich et al., 2009). Increased Ca^{2+} levels result in an increase in MG DNA synthesis and Ca^{2+} signaling in regions of the CNS has been shown to be essential for the maintenance of glial ensheathment of neuronal synapses (Iino et al., 2001; Moll et al., 2002).

During Müller glia maturation, potassium currents undergo a dynamic change in the retina. In the second postnatal week there is concomitant decrease in the open probability of Ca^{2+}-dependent K^+ channels of big conductance (BK channels) and an increase in insertion of the inwardly rectifying potassium channel Kir4.1. This results in a MG resting potential of -80 mV, which is required for many of their mature functions including potassium buffering and neurotransmitter recycling (Kofuji et al., 2002; Bringmann et al., 2006). Morphological disruptions in SOX2 deficient MG are noted shortly after this period. It is possible then that deletion of Sox2 may lead to the abnormal development of potassium channels in the maturing MG.

It is notable that during reactive, proliferative gliosis, there is a reversal of K^+ activity that mirrors the K^+ conductance in the immature retina. Inward rectifying potassium currents are down regulated in the gliotic retina and there is a marked increase in the BK channel activity, which results in unstable MG membrane potential (Bringmann et al., 2000). Loss of Sox2 in MG may disrupt their maturation program, resulting in the maintenance of an

immature structural and functional state.

Additionally, the disruption of the inner and outer limiting membrane, as well as the defasiculated neuronal processes in the $Sox2^{MUTANT}$ retina suggest that there could be a disruption in the regulation of cell adhesion. In the retina, MG express many cell adhesion factors, including multiple members of the cadherin family (Li and Sakaguchi, 2002; Etzrodt et al., 2009). Sox2 has been linked to N-cadherin expression in the developing lens (Kamachi et al., 2001). And both N-cadherin and N-CAM have been shown to be important in MG-neuronal adhesion (Drazba and Lemmon, 1990).

It was recently demonstrated that neural regeneration in the zebrafish retina requires N-cadherin to regenerate retinal neurons (Nagashima et al., 2013). In response to loss of retinal neurons, MG in the zebrafish retina partially de-differentiate and re-enter the cell cycle. The MG then undergo interkinetic nuclear migration; while still maintaining their network of processes; the MG nucleus migrates to the apical side of the retina and undergoes an asymmetrical division to produce a progenitor cell. This progenitor cell then gives rise to a tightly packed cluster of neural progenitors that give rise to neurons. In response to injury, MG upregulate N-cadherin, which is required for the generation of stable neurogenic clusters (Nagashima et al., 2013). This study points to the importance of cell adhesion in retinal regeneration and in the development of neuronal populations in the retina. It is possible that Sox2 ablation in Müller glia results in the disruption of MG's ability to adhere correctly to neural processes in the plexiform layers.

5.2 Sox2 Maintains the Stability of Müller glia in the Degenerating Retina

Morphological disruptions in the retina associated with global reductions in SOX2 protein levels below 50% of wild type levels were previously described in the laboratory, including a thinning of the neural retina (Taranova et al., 2006). Here we identify similar

morphological disruptions when Sox2 protein levels are 50% of wild type. Additionally we demonstrate that hypomorphic SOX2 levels in the retina result in severe a severe functional deficit that worsens as the animal ages. This is the first work to describe retinal function in the SOX2 hypomorphic eye. By P25, mice hypomorphic for SOX2 display severe reductions in inner retinal function, as measured by a reduction in the b-wave component in an electroretinogram (ERG). The photoreceptor-generated response (the a-wave component of the ERG) shows a slight reduction in function at this stage. By P60 both the a-wave and b-wave components of the ERG show a marked reduction in function. This indicates that in addition to developmental defects, SOX2 hypomorphism also results in retinal degeneration. Global reduction in SOX2 levels have been shown to cause impaired neurogenesis and neurodegeneration in the adult mouse brain (Ferri et al., 2004). Recently a case study also reported deterioration of neurological function in a human patient with Sox2 mutations (Ragge et al., 2013). Work in this thesis demonstrates that retinal degeneration also occurs as a result of global reductions in SOX2 levels.

Further reduction in Sox2 levels through the MG specific ablation of Sox2 ($Sox2^{MUTANT}$ retinas) in the hypomorphic background result in an increased rate of retinal degeneration. By P60 mice $Sox2^{MUTANT}$ retinas on the hypomorphic background display near complete vision loss. Notably, there is also a marked decrease in the number of $Sox2^{MUTANT}$ MG, while no decrease in the number of MG is observed in the SOX2 hypomorphic background during the same time period. This suggests that SOX2 may play some role in maintaining or supporting MG when the retina is under stress.

Previous work completed in the laboratory using an *in vitro* culture system to ablate Sox2 specifically in Müller glia at P5 showed an increase in MG cell cycle entrance that correlated with areas of GFAP upregulation (Crowl, 2014). GFAP, an intermediate filament, is an early marker of retinal stress that is upregulated by MG following injury or during retinal

degeneration (Bringmann and Wiedemann, 2012). No evidence of MG cell cycle entrance was identified *in vivo* following ablation of Sox2 in MG on a mixed CD1 background (Chapter Three, data not shown). Likewise, little to no upregulation of GFAP was identified in $Sox2^{MUTANT}$ MG on the CD1 background. However GFAP upregulation in MG was identified in both SOX2 hypomorphic retinas and $Sox2^{MUTANT}$ retinas in the hypomorphic background (data not shown).

In the *in vitro* experiments and those conducted in the hypomorphic background, presence of widespread GFAP upregulation correlates with nascent MG cell cycle entrance *in vitro*, and a reduction in MG number and retinal degeneration *in vivo*. A similar phenotype was identified in a population of nascent MG following the deletion of Sox2 in postnatal retinal progenitor cells (RPCs) (Surzenko et al., 2013). In these retinas, nascent MG re-entered the cell cycle and underwent a terminal division that resulted in their depletion. It is likely that the population of $Sox2^{MUTANT}$ MG in the hypomorphic background is reduced via a similar mechanism. Indeed the RPC experiments were also performed using an *in vitro* system, suggesting retinal stress may also have influenced cell cycle dynamics in these experiments. Together, these data suggest that Sox2 plays an essential role in maintaining the MG population, possibly through regulating their quiescence during retinal stress.

5.3 The Dynamic Role of SOX2 in Retinal Development

Previous work in the laboratory demonstrated that loss of *Sox2* at P0 in postnatal RPCs results in a population of nascent MG re-entering the cell cycle and undergoing a terminal division. This aberrant cell cycle entrance eventually results in depletion of MG and subsequent retinal degeneration. In this thesis, we demonstrate that MG specific ablation of *Sox2* just five days later *in vivo* results instead in a MG maturation phenotype. This changing response to Sox2 ablation in the retina perhaps defines a critical period. During retinal

development the entire population of retinal progenitor cells express SOX2. As neural progenitors exit the cell cycle and give rise the neurons of the retina, SOX2 is downregulated (Taranova et al., 2006). Only MG and a small population of amacrine cells maintain SOX2 expression constitutively. During embryonic retinal development, loss of Sox2 results in the depletion of progenitor pools, changes in cell cycle dynamics and loss of neural competence (Matsushima et al., 2011; Heavner, 2013). Loss of Sox2 in postnatal RPCs results in terminal cell division and the loss of nascent MG quiescence (Surzenko et al., 2013). Here we demonstrate that loss of Sox2 at P5 in MG results in the disruption of glial maturation, resulting in structural and functional abnormalities in the mature retina. Similarly, recent work found that Sox2 regulates amacrine cell position and dendritic stratification during retinal development (Whitney et al., 2014). These data provide further evidence of Sox2's role in guiding structural development of the postnatal retina. In conjunction, these studies define a critical period for the role of Sox2 in the maturing retina, highlighting a shift from master regulator of progenitor pools, to guiding the structural maturation of the retina.

Sox2 is unique in that it is widely expressed and performs so many functions from embryogenesis to regulation of adult neural stem cell populations. Further, SOX2 proteins bind DNA weakly, relying on a host of binding partners that vary between developmental time point and tissue type to initiate transcription. Sox2's role in the proper induction of tissues during embryogenesis is also dependent on the presence of antagonistic factors, which modulate SOX2 expression. Further, some antagonistic factors act as binding partners in other contexts, including Pax6, a master regulator of eye development (for more information on Sox2 see **Section 1.3**). Sox2s function is therefore highly dependent on the environment, opening up near boundless regulatory possibilities.

As development progresses the role of SOX2 in the retina shifts from that of maintaining progenitor pools and defining neural competence, to maintaining glial

quiescence, to a role in the structural maturation of both MG and retinal neurons. Additionally, we provide preliminary evidence that in the degenerating retina, SOX2 plays a role once again in maintaining the population of MG cells, perhaps through regulating their quiescent state. During gliosis, and in response to the changing retinal environment, MG undergo a number of changes, including the release of growth factors, and a return to a more physiologically immature state via the downregulation of inwardly rectifying Kir4.1 channels (See **Section 1.2.4**). As the retinal environment changes, the role of Sox2 changes.

An example of the changing environment is the dynamic regulation of WNT signaling during retinal development. During early retinal development, WNT signaling plays a role in maintaining a proliferative population of retinal progenitor cells (Agathocleous and Harris, 2009). As retinal development proceeds, WNT expression is down regulated (Liu et al., 2006b) and ß-catenin expression, WNT's downstream mediator, is maintained largely in the synaptic plexiform layers. ß-catenin has a well-established connection to cell adhesion, stabilizing N-cadherin at the membrane. This shift from regulating cell cycle dynamics to ß-catenin's role in primarily regulating cell-cell adhesion may mirror SOX2's changing role in retinal development. It is well established that Sox factors interact with the WNT signaling pathway during development (Kormish et al., 2010). During retinal development, Sox2 antagonizes WNT signaling by binding ß-catenin (Seo et al., 2011). And the balance of SOX2 expression and WNT signaling guides the specification of the neural retina. (Agathocleous and Harris, 2009). Notably, upregulation of WNT signaling in the mammalian retina has been demonstrated to increase MG proliferative potential and aid in retinal regeneration (Das et al., 2006; Liu et al., 2006a; Osakada et al., 2007; Sanges et al., 2013).

It is conceivable that loss of SOX2 in RPCs results in the de-repression of ß-catenin, resulting in the proliferative phenotype of nascent MG at P5 previously reported in our

laboratory (Surzenko et al., 2013). However, as retinal development proceeds, WNT signaling is downregulated and ablation of *Sox2* at P5 in Müller glia results instead in the disruption of the MG's structural development.

5.4 Strain-dependent Variation in SOX2 Expression Levels

In Chapter Four we report that the $Sox2^{COND}$ allele previously developed in our laboratory results in global reduction of SOX2 levels that are strain dependent. On a mixed background of the outbred CD1 line and the inbred C57BL/6 line, SOX2 levels in the retina of homozygous $Sox2^{COND}$ mice ($Sox2^{C/C}$) are 80% of wild type. This reduction in SOX2 protein levels does not result in morphological or physiological deficits. However on an inbred C57BL/6 background, SOX2 protein levels in the retina are 50% of wild type. This reduction in SOX2 results in marked physiological and morphological defects as well as retinal degeneration. The $Sox2^{COND}$ line was originally developed in a mixed 129(SvPas)/BL6 background. While SOX2 levels in the $Sox2^{C/C}$ mice were not reported, they were estimated to be 80% of wild type (personal communication) and no morphological defects were found (Taranova et al., 2006). These results point to a clear, strain dependent regulation of SOX2 protein levels.

Notably, a mutation was recently discovered in the Sox2 gene of C57BL6/J mice. The mutation results in the addition of two extra glycines to a polyglycine tract located adjacent to the DNA-binding HMG box on the *Sox2* allele. While the function of this tract has not been thoroughly characterized, it is commonly present in transcription factors. A similar mutation in a human case study that resulted in the addition of one extra glycine to this same polyglycine tract was attributed as the cause of bilateral anophthalmia (Schneider et al., 2009; Whitney et al., 2014). This mutation is not however the likely to be the cause of the reduced levels of SOX2 protein identified in the $Sox2^{COND}$ line on the C57BLC/J background,

as the mice were developed from targeted 129 ES cells. However it does highlight the variation present between different strains.

These results point to the importance of considering strain dependent effects when characterizing a given phenotype. This recent discovery of the mutation in the Sox2 gene of C57BL/6 line is of particular importance to retinal biologists. Sox2 is the only member of the highly related SoxB1 factors that is expressed in the retina (Taranova et al., 2006). This makes the retina both an ideal model system in which to study Sox2, as well as being particularly susceptible to its loss. Our laboratory has noted the stochastic presence of microanophthalmia in wild type populations of C57BL6/J mice received from the vendor. The mutation to the Sox2 allele is the likely cause. Should retinal biologists then avoid this line? Other C57BL6 lines pose problems as well. For example the C57BL6/N line contains a mutation, *rd8*, which results in the slow degeneration of the retina over time (Mattapallil et al., 2012). Great care should be exercised when choosing a mouse line for study of the retina.

It is notable however, that strain dependent differences appear to extend beyond the regulation of protein levels. Previous work in the laboratory reported that on a mixed 129/BL6 background, no phenotypic differences were observed in retinas that contained SOX2 levels 50% of wild type (Taranova et al., 2006). At the time, it was noted that this was a unique finding, as humans with 50% protein reductions (caused by the presence of a null allele for example) display much more severe phenotypes, including anophthalmia (Fantes et al., 2003). Ultimately it was concluded that mice are less sensitize to reduction in SOX2 levels than humans (Taranova et al., 2006; Pevny and Nicolis, 2010). However, here we report that the same reduction in protein levels (50% of wild type) on a C57BL6/J background results in marked morphological and physiological deficits, more similar to those seen in human patients. These results highlight the dramatic affect strain background can

exert on phenotypic expression. Originally, it was suggested that the differences between human and mouse sensitivity to SOX2 levels point to the 'complex interactions of Sox2 with other regulatory factors, which variably affect the outcome of Sox2 deficiency' (Pevny and Nicolis, 2010). This hypothesis can be widened to include variations in strain and background, rather than just interspecies variations.

5.5 Brief Summary of Findings

In this thesis, we demonstrate the essential role of SOX2 in Müller glial maturation, maintenance and retinal development.

5.5.1 Future Directions

Further work to define the mechanism through which SOX2 regulates MG development would provide insights into the poorly understood role Sox2 plays in the structural development of the retina. Isolating a population of Sox2 deficient MG and conducting RNA sequencing (RNA-seq) would yield valuable data about changes in the MG transcriptome in response to loss of SOX2. An RNA-seq experiment would also be interesting to compare the transcriptomes of homozygous $Sox2^{COND}$ retinas in the C57BL/6 background and the CD1 background. If the changes in protein expression level are due in part to alternative splicing of the $Sox2^{COND}$ allele or the *Sox2ot* as suggested in Chapter Four, these transcripts could be identified.

While no retinal degeneration in the $Sox2^{MUTANT}$ population on the CD1 background is detected between P25 and P60, it would be interesting to see if these retinas do degenerate slowly. Assessing retinal morphology and function at 3 months and 6 months would likely answer this question. Further information about the role of SOX2 in the mature retina could be attained through a MG specific ablation of Sox2 in the mature retina. MG specific ablation of Sox2 at P5 results in aberrant maturation, therefore ablation of Sox2 in the mature retina

is unlikely to yield the same phenotype. However, MG specific loss of Sox2 in the degenerating $Sox2^{COND}$ hypomorphic retina results in depletion of the MG population. Ablating Sox2 in MG in mouse model of retinal degeneration, such as the retinitis pigmentosa model, *rd10* could provide further insights into Sox2's role during gliosis. Through these experiments, it would be possible to determine if SOX2 ablation results in depletion of the MG population in a clinically relevant model of retinal degeneration. Likewise if MG specific ablation of Sox2 in the mature retina does not yield a phenotype, it would be interesting to follow Sox2 ablation with induction of a gliotic state through a controlled injury to the retina.

5.5.2 Conclusion

Work in this thesis describes the essential role SOX2 plays in the maturation and maintenance of the murine retina. SOX2 expression during MG development is required for the organized elaboration of processes that extend into the plexiform layers, and for the development of intact inner and outer nuclear layers (IPL/OPL). Disorganization of neuronal processes in the IPL and OPL and a marked decrease in inner retinal function correlate with aberrant MG maturation. These data suggest that MG maturation in the postnatal mouse retina plays an essential role in guiding the final stages of retinal development. Further, we present data that suggests SOX2 plays an important role in maintaining MG quiescence during retinal degeneration.

We also present evidence that highlights the dynamic nature of SOX2 regulation in the eye. SOX2 protein levels and the resulting phenotypic manifestations vary widely between and within mouse strains. Finally we demonstrate that hypomorphic SOX2 levels result in functional degeneration of the retina, implicating Sox2 not only as an essential factor in embryonic and neural development, but also in the maintenance of neural populations throughout an organism's lifespan.

Work in this thesis exploring the role of SOX2 in Müller glia is the final chapter in a body of work that has made undeniable contributions to the field. I am honored to have had the opportunity to study under the careful, wise and enthusiastic direction of Dr. Larysa Pevny. She is truly missed.

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