Microglia and Progranulin regulate neurogenesis in the developing vertebrate retina

by

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I dedicate this work to my parents, Betsy and John Walsh.
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LIST OF FIGURES

Figure
2.1 Quantification of retinal microglia at 72hpf.................................................. 86
2.2 Developmental neurogenesis is altered in the retinas of panther mutant embryos.......................................................... 88
2.3 Retinal phenotype of 8 days post fertilization panther mutant and PLX5622-treated embryos.................................................. 90
2.4 Treatment with PLX5622 results in altered neurogenesis in the developing zebrafish retina.................................................. 92
2.5 Expression of pgm-a in the developing retina and Western blot of Pgrn-a expression at 24hpf.................................................. 94
2.6 pgm-a 5'UTR-targeting morpholinos block Pgm-a translation and gross morphology of pgm-a morphants.................................................. 96
2.7 pgm-a splice site-targeting morpholinos cause intron retention and knockdown Pgrn-a................................................................ 98
2.8 Pgm-a knockdown results in microphthalmia and diminished neuronal differentiation.................................................. 100
2.9 Aspects of Pgm-a knockdown retinal phenotype recover by 8 days post fertilization.................................................. 102
2.10 No difference in atoh7 expression and TUNEL-positive cell counts between pgm-a morphant and control embryos.......................... 104
2.11 Co-injection of 5'UTR MO and zf pgm-a, zf pgm-b, or hGRN mRNA rescues most aspects of knockdown retinal phenotype...... 106
2.12 Knockdown of pgm-a alters cell cycle kinetics in retinal progenitors........................................................................... 108
2.13 Mitotic index in 28 and 48hpf panther mutant and PLX5622-treated embryos.......................................................... 110
3.1 Zebrafish sortilin receptors.................................................................. 152
3.2 Canonical Wnt signaling is upregulated following Pgrn-a knockdown........................................................................ 153
3.3 Notch signaling pathway gene expression is altered following Pgrn-a knockdown.................................................. 154
3.4 Representative maps of recombineered pgma:nfsB-EGFP and pgma:nfsB-mCh BAC constructs.................................................. 155
3.5 Confirmation of BAC constructs.................................................. 156
3.6 CRISPR/Cas-9 genome engineering system for pgm-a mutant line........................................................................ 158
3.7 Characterization of F0 pgm-a mosaic mutants.................................................. 159
3.8 Characterization of F3 pgm-a homozygous (pgm-a'') mutants........... 160
LIST OF TABLES:

Table

<table>
<thead>
<tr>
<th></th>
<th>Antibody List</th>
<th>112</th>
</tr>
</thead>
</table>
| 2.1| Restriction Enzymes and RNA Polymerases for 
|    | pgrn-a, atoh7, and fms Riboprobe Synthesis                                   | 113 |
| 2.2| Morpholino Oligonucleotide Sequences                                           | 114 |
| 2.3| Primer Sequences for mRNA Rescue Experiments                                  | 115 |
| 2.4| Primer Sequences for qRT-PCR                                                   | 116 |
| 2.5| List of Antibodies                                                            | 161 |
| 2.6| Restriction Enzymes and RNA Polymerases for axin2 and sort1a/b Riboprobe Synthesis | 162 |
| 3.1| Primer Sequences for sort1a/b Riboprobe Synthesis                             | 163 |
| 3.2| Primer Sequences for BAC Recombineering and Screening                         | 164 |
| 3.3| pgrn-a-targeting sgRNA Sequences                                               | 165 |
| 3.4| Primer Sequences for CRISPR/Cas-9 Genome Editing of pgrn-a                    | 166 |
| 3.5| Status of pgrn-a mutant lines                                                 | 167 |
Chapter I

Introduction

The Vertebrate Retina as a Model for Studying CNS Development

The central nervous system (CNS) is remarkably complex. There are approximately 86 billion neurons and 84 billion non-neuronal cells in the human brain (Azevedo et al., 2009). Given that individual neurons form hundreds to thousands of synaptic connections, the neocortex contains an estimated 0.15 quadrillion synapses (Pakkenberg et al., 2003). Neuronal heterogeneity adds to this complexity. There are many different types of neurons; each subtype is characterized by different morphological, physiological, molecular, and synaptic properties. Normal CNS development and function requires specific cell types to be generated at the correct time and ratio, migrate, form precise connections, and integrate into networks. Therefore, understanding how the CNS develops is a very complicated task.

The retina is a thin piece of light sensing tissue (~0.5mm thick), derived from the neural tube and located at the back of the eye. The retina is an anatomical extension of the brain. However, compared with the brain, the retina is a relatively simple and approachable part of the CNS. Moreover, the retina is the most widely studied and well-characterized part of the vertebrate brain (Dowling, 2012). Among vertebrates, the retina is precisely laminated and the
cytoarchitecture is evolutionarily very highly conserved. Retinal cells are stereotypically positioned and have distinct morphologies. Therefore, different cell types are distinguishable and subtle changes in developmental programs are easily detected as structural changes (Cepko et al., 1996). Importantly, retinal development and function are conserved across vertebrate species (Dowling, 2012; Stenkamp, 2015). The retina is easily accessible for experimental manipulations, and many of the intrinsic and extrinsic factors that temporally and spatially regulate all stages of retinal development are well characterized (Levine and Green, 2004; Yang, 2004; Agathocleous and Harris, 2009). For these reasons, the vertebrate retina has become a well-established and tractable model for investigating the cellular and molecular mechanisms that regulate developmental neurogenesis (Agathocleous and Harris, 2009).

**Basic Retinal Anatomy and Function**

The anatomy of the vertebrate retina was first described by Ramon y Cajal in the late 19th century. Using the Golgi staining technique, Cajal compared retinal histology and morphology across different species and noted the presence of distinct cell types in a conserved structure and organization (Cajal et al., 1972; Piccolino et al., 1989). There are five major types of neurons in the retina: photoreceptor cells, bipolar cells, horizontal cells, amacrine cells, and ganglion cells. The neural retina has a laminar structure with three distinct stratified cell/nuclear layers separated by two synaptic/plexiform layers. Each nuclear layer contains the cell bodies of specific cell types. The outer nuclear layer (ONL) contains the cell bodies of photoreceptor cells. The inner nuclear layer (INL)
contains cell bodies of bipolar, horizontal and amacrine interneurons. The ganglion cell layer (GCL) contains cell bodies of ganglion cells, which are the output neurons of the retina. Photoreceptors are photosensitive; they detect photons of light and carry out phototransduction, which is a process that converts light to biochemical then electrical signals for image-forming vision. There are two types of photoreceptor cells: rods and cones. Rods are extremely sensitive and detect dark, dim light (mostly at night). Cones are less sensitive than rods, detecting and distinguishing between a spectrum of light wavelengths, which provides the basis of daytime color vision. Some retinal ganglion cells are intrinsically photosensitive and are responsible for non-image-forming vision, such as measuring ambient light for synchronizing circadian rhythms and controlling pupil size (Fu et al., 2005).

Visual perception originates in the eye. The retina receives and processes images, ultimately sending information collected from the visual scene to the vision centers in the brain. Visual processing begins when light is focused on the retina, traveling through the GCL and INL and activating the photoreceptors at the back of the retina. The signal is transduced back through the retina: photoreceptors synapse with bipolar cells (and horizontal cells) in the outer plexiform layer (OPL), and bipolar cells (and amacrine cells) synapse with ganglion cells in the inner plexiform layer (IPL). Horizontal and amacrine cells laterally regulate the input to and output of bipolar cells, respectively. The axons of ganglion cells form the nerve fiber layer or optic nerve, which transmits visual information to cortical areas in the brain.
The human retina contains three types of glial cells: Müller glia and astroglia (macroglia), and microglia (Vecino et al., 2016). Though both are neuroectodermal in origin, Müller glia are derived from retinal progenitor cells, while astroglia are not (Turner and Cepko, 1987; Wetts and Fraser, 1988). Müller glia are the primary glial cell of the retina; they provide structural, metabolic and homeostatic support and can serve as intrinsic stem cells (Bringmann et al., 2006; Bernardos et al., 2007). The cell bodies of Müller glia are located in the INL, but the processes span radially across the entire retina from the inner limiting membrane to the outer limiting membrane (Bringmann et al., 2006). Astroglia enter the retina via the optic nerve and their presence is highly correlated with retinal vasculature patterning, such that avascular retinas do not contain astrocytes (Stone and Dreher, 1987; Watanabe and Raff, 1988; Vecino et al., 2016). With cell bodies and processes located exclusively in the nerve fiber layer, astroglia regulate ganglion cell and blood retinal barrier homeostasis (Vecino et al., 2016). A major distinction between the human and fish retina is that the fish retina does not contain astroglia. Microglia are derived from mesodermal origins, located throughout the nuclear and synaptic layers of the retina, and act as immune cells (see more below; Vecino et al., 2016).

**Zebrafish as a Model Organism**

The zebrafish (*Danio rerio*, previously *Brachydanio rerio*) is a small tropical freshwater teleost fish of the Cyprinidae family. In the 1970s, George Streisinger and colleagues at the University of Oregon identified and developed zebrafish as a model organism (Streisinger et al., 1981). For a number of
reasons, the zebrafish is a powerful model organism to study vertebrate CNS development. First, zebrafish embryos are fertilized and develop externally inside a transparent chorion. Second, the embryos are relatively robust. Third, the eggs and embryos are also transparent throughout early development, allowing for direct visualization (observation and imaging) in vivo and in situ. Fourth, zebrafish are born in relatively large clutches. Zebrafish are photoperiodic breeders and mate soon after sunrise. Optimal breeding pairs can generate ≥200 embryos per spawn. Fifth, the rate of zebrafish development is relatively rapid. When raised at 28.5°C, zebrafish progress from a fertilized egg to fully formed embryo within twenty-four hours. Within three days, larvae are hatched, swimming, and hunting and feeding. By three months, zebrafish are sexually mature adults. Hence, the generation time is relatively short (only 3-4 months). Sixth, zebrafish are easily maintained in the laboratory setting. Due to their relatively small size and propensity for living in large colonies, small spaces can house high densities (Brand et al., 2002). And, zebrafish husbandry is less expensive than rodent models. Seventh, zebrafish can be manipulated using a wide range of biochemical, genetic and molecular tools. They are well suited for large-scale genetic or mutagenesis and chemical screens. Finally, the zebrafish genome is fully sequenced and annotated (see ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA_000002035.3_GRCz10/). There is at least one known zebrafish orthologue for 70% of all human genes (Howe et al., 2013). Therefore, the zebrafish is an experimentally amenable model organism and powerful resource for genetic and developmental research.
The teleost retina is developmentally, morphologically, and functionally similar to other vertebrate retinas, including humans. And, many zebrafish mutants exist that model human retinal diseases. By studying the zebrafish retina, significant insights into the mechanisms regulating CNS development and disease can be made (Malicki, 1999; Glass and Dahm, 2004; Hitchcock and Raymond, 2004; Stenkamp, 2007; Fadool and Dowling, 2008). Advantageously, the zebrafish retina matures in a stereotyped spatial and temporal pattern, from a simple neuroepithelium to a functional retina by 72 hours post fertilization (hpf) (Easter and Nicola, 1996).

Zebrafish Retinal Development

Development of the retina is similar in all vertebrates (Chow and Lang, 2001). However, compared with warm blooded vertebrates, retinal morphogenesis in zebrafish is very rapid (Schmitt and Dowling, 1994; Malicki, 1999; Easter and Malicki, 2002). The zebrafish retina develops in three stages. The first stage begins at the end of gastrulation with the specification of retinal domains from a single field of dividing cells in the anterior neural keel that express eye field transcription factors, such as odd-paired-like (opl/zic1), retinal homeobox (rx1, rx2 and rx3), paired box protein (pax6), and sine oculis (Six3) (Chuang, 2001; Chuang and Raymond, 2002; Hitchcock and Raymond, 2004). The neural keel is a solid mass of cells akin to the neural tube in humans, and is also referred to as the neural plate. Around 12hpf, the eye field is split into two bilaterally evaginating optic primordia by Nodal and Sonic hedgehog signals emanating from the ventral midline (Macdonald et al., 1995; Masai et al., 2000).
At about 13hpf, the optic primordia flatten, proliferate and extend laterally, taking on a wing-like structure, and begin posteriorly detaching from the neural plate. Then the optic primordia, remaining anteriorly attached to the neural plate via the optic stalk, rotate from a horizontal to vertical orientation, and, around 15hpf, begin the transformation into eyecups through invagination (Easter and Malicki, 2002). By 24hpf, eyecups have formed, and the retina is a layer of proliferating neuroepithelial cells (Schmitt and Dowling, 1994; 1999).

The second stage of retinal development encompasses a series of neurogenic events, including cell proliferation, exit from the cell cycle, and differentiation. Retinal progenitors are a uniform population of multipotent cells that give rise to multiple distinct cell types (Agathocleous and Harris, 2009). From 24 to 36hpf, retinal progenitors rapidly proliferate, expanding the retinal tissue (Schmitt and Dowling, 1999). The retina matures in a stereotyped spatiotemporal pattern. Post-mitotic, newly differentiated cells are incorporated into a precise and highly conserved laminar structure (Chow and Lang, 2001). At approximately 28hpf, neuronal differentiation begins adjacent to optic stalk in a precocious ventronasal patch (Hu and Easter, 1999). Neuronal differentiation and lamination progress sequentially in inside-out, circumferential waves that move dorsally, then temporally (Schmitt and Dowling, 1996; 1999; Hitchcock and Raymond, 2004). There are three waves of neurogenesis, each separated by a delay of approximately 10hrs (Hu and Easter, 1999; Stenkamp, 2007). First, between 28 and 38hpf, ganglion cells exit the cell cycle. Then, from 38 to 48hpf, bipolar, horizontal, and amacrine cells in the INL terminally differentiate. Finally, between
48 and 60hpf, photoreceptor cells in the ONL are generated. Cones differentiate first, followed by rods. The zebrafish retina contains four different opsin-expressing cone photoreceptors that differentiate in the order of their spectral subtype (red-green double cones, then UV and blue cones) and are arranged in a mosaic pattern (Raymond et al., 1993; 1995). Rods differentiate initially in a dense ventral patch, then become more sporadically interspersed between cones, and continue to accumulate throughout the life of the zebrafish from proliferating rod precursor cells in the ONL (Raymond, 1985). Müller glia are the last cells to differentiate. This histogenic birth order of retinal cells is largely conserved (Stenkamp, 2015). Relatively little cell death takes place in the embryonic zebrafish retina (Biehlmaier et al., 2001; Cole and Ross, 2001). By 72hpf, the initial phase of retinal neurogenesis is largely complete, and the retina is fully laminated and functional: newly hatched, 72hpf embryos respond to visual stimuli (Easter and Nicola, 1996).

Unique to teleosts and amphibians, the third and final stage of retinal development is characterized by persistent retinal neurogenesis (Marcus et al., 1999; Otteson and Hitchcock, 2003; Hitchcock et al., 2004). After the initial neuronal differentiation and lamination is complete, the retina continues to grow by the addition of new neurons from the ciliary marginal zone (CMZ)/circumferential germinal zone (CGZ) located at the peripheral edge between the retina and iris (Hitchcock et al., 2004; Hitchcock and Raymond, 2004). Throughout the life of the zebrafish, new neurons are added to the retina in concentric annuli (Otteson and Hitchcock, 2003; Hitchcock et al., 2004;
Raymond et al., 2006). Remnant retinal stem cells in the CMZ/CGZ generate all retinal neurons, except rod photoreceptors, which are derived from rod progenitors in ONL and Müller glia in INL (Raymond, 1985). A fundamental feature of the zebrafish retina is that Müller glia are at the apex of the rod photoreceptor lineage. In the zebrafish retina, Müller glia act as retinal stem cells that can re-enter the cell cycle and divide asymmetrically to produce late-stage rod progenitors that in turn give rise to either rod or cone photoreceptors (Raymond et al., 2006; Bernardos et al., 2007).

**Eukaryotic Cell Cycle**

The eukaryotic cell cycle is a highly regulated process divided into phases based on cellular activity (Lodish et al., 2000). There are four sequential phases in the eukaryotic cell cycle: gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M). During G1-phase, progenitors progress from M- to S-phase or permanently exit from the cell cycle and enter the arrested phase (G0). During S-phase, DNA is replicated. G2-phase of the cell cycle falls between DNA synthesis and the onset of cell division, and during M-phase cell division occurs. Cyclin/cyclin-dependent kinase (CDK) complexes and CDK inhibitors, such as CDK inhibitory protein/kinase inhibitor protein (Cip/Kip) family members p27 and p57, regulate the progression through the different phases of the cell cycle (Dyer and Cepko, 2001a; Bilitou and Ohnuma, 2010; Lim and Kaldis, 2013). Cyclin-CDK complexes promote cell cycle progression, whereas, CDK inhibitors prevent cell cycle progression and promote cell cycle exit (Dyer and Cepko, 2001a; 2001b; Bilitou and Ohnuma, 2010).
Retinal progenitors progress through and exit from the cell cycle in a spatially and temporally controlled manner (Li et al., 2000). Retinal progenitor proliferation, cell cycle exit, and differentiation are coordinated by complex interactions between intrinsic (competence) factors, such as transcriptional programs, and extrinsic (environmental) factors, such as soluble growth factors (Yang, 2004; Levine and Green, 2004). There are two main classes of transcription factors that intrinsically regulate retinal progenitor proliferation and differentiation: homeobox-containing, which includes retinal homeobox (rx), cone-rod homeobox (crx), and paired box gene 6 (pax6), and basic helix-loop-helix (bHLH)-containing, which includes atonal homologue 5 (ath5/atoh7) and neuroD. Both rx and crx genes are expressed by mitotically active retinal progenitors, and promote differentiation of retinal progenitors, including photoreceptors (Chuang et al., 1999; Shen and Raymond, 2004). pax6 is expressed by proliferating retinal progenitors and retinal stem cells, and necessary for S-phase re-entry (Otteson and Hitchcock, 2003; Yi-Wen Hsieh, 2009). ath5/atoh7 is expressed by retinoblasts and required for the initiation of neurogenesis and ganglion cell differentiation (Masai et al., 2000; Kay et al., 2001; Kay, 2005). In the zebrafish retina, neuroD is expressed by mitotically active photoreceptor progenitors and nascent photoreceptors, and promotes cell cycle exit and photoreceptor genesis via the Notch signaling pathway (Hitchcock and Kakuk-Atkins, 2004; Ochocinska and Hitchcock, 2007; Taylor et al., 2015).

In addition, multiple soluble or membrane-bound factors extrinsically regulate the rate at which zebrafish retinal progenitors progress through the cell
cycle. Notch/Delta signaling promotes retinal progenitor proliferation and inhibits neuronal differentiation (Scheer et al., 2001; Bernardos et al., 2005). Sonic hedgehog (Shh) signaling promotes cell cycle exit and is required for differentiation of multiple retinal neurons (Shkumatava and Neumann, 2005). Wnt signaling promotes progression through the cell cycle and maintains retinal progenitors in a proliferative state (Kubo, 2003; Kubo et al., 2005; Yamaguchi et al., 2005; Sánchez-Sánchez et al., 2010; Meyers et al., 2012). Further, there are numerous growth factors that act on the cell cycle during retinal neurogenesis. For example, the secreted growth factor, midkine-a (mdka), functions upstream of id2a to regulate cell cycle kinetics in retinal progenitors by negatively regulating Notch signaling (Luo et al., 2012; Uribe et al., 2012).

There are multiple tools and assays available to study cell cycle kinetics in zebrafish (Shepard et al., 2004). Bromodeoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU) are thymidine analogues used to label cells during S-phase of the cell cycle. The DNA content of a cell can be measured using flow cytometry. Phospho-histone H3 (pH3) labels cells in M-phase of the cell cycle. Further, qRTPCR, Western blot, in situ hybridization and immunohistochemistry are used to assay the expression of cell cycle regulatory genes that promote cell cycle progression or cell cycle exit.

**Microglia**

There are two major classes of glia in the vertebrate nervous system: macroglia—oligodendrocytes, schwann cells, and astrocytes, derived from neuroectoderm and also referred to as neuroglia—and microglia. In the 1920s,
the Spanish neuroscientist, Pío Del Rio-Hortega, introduced the concept of microglia, which were recognized at the time as part of a morphologically distinct “third element” of the CNS, in addition to neurons and neuroglia (Del Rio-Hortega, 1937). From cytological observations, Rio-Hortega postulated microglia were a discrete macrophage population that derived from mesodermal origins and immigrated into nerve tissue during embryonic development. He noted microglia reside ubiquitously in the parenchyma of the mature brain and occupy discrete, non-overlapping spaces. Rio-Hortega discerned two distinct microglial morphologies: amoeboid, found in early developmental stages, and ramified, found at later stages. Following injury or under pathological conditions, he observed that microglia change back to an amoeboid-like morphology, migrate to the damaged area, proliferate, and become phagocytic. He concluded that the main physiological function of microglia is to uptake cellular debris and eliminate waste (Del Rio-Hortega, 1939). Almost all of Rio-Hortega’s initial seminal observations still hold true today.

Origins and Development of Microglia

Unlike neurons and neuroglial cells, microglia derive from non-neuronal lineages. Though the ontogeny of microglia was debated for many years (Cuadros and Navascués, 2001; Ginhoux and Prinz, 2015), it is now established in fish (Herbomel, 2001), birds (Cuadros et al., 1993), and rodents (Ginhoux et al., 2010) that embryonic microglia originate as primitive yolk sac macrophages. In zebrafish, prior to the onset of blood circulation, macrophage precursors arise from ventro-lateral mesoderm and migrate into the yolk sac and differentiate; a
subset then enters the blood circulation, while a large proportion invade the cephalic mesenchyme between 22-40hpf (Herbomel et al., 1999). Once in the head, early macrophages invade nervous system tissues, including the brain and retina, between 30-48hpf, and then concurrently undergo a phenotypic transformation to become microglia around 60hpf (Herbomel, 2001). During embryonic development, migrating microglial precursors have an amoeboid morphology and are phagocytic (Svahn et al., 2012). The migration of microglial precursors into the developing CNS is highly conserved across vertebrate species (fish: Herbomel, 2001; rodents: Swinnen et al., 2013; humans: Verney et al., 2010).

Studies using mice suggest that microglia derive from a single yolk sac origin (Ginhoux et al., 2010; Schulz et al., 2012). The prevailing view is that CNS microglia are established embryonically, and virtually no new microglia arise from exogenous sources, but are sustained through self-renewal in situ throughout adulthood instead (Ajami et al., 2007; Wohl et al., 2011; Bruttger et al., 2015). However, more recent evidence unambiguously demonstrates that, in zebrafish, embryonic/larval and adult microglial populations located in the brain and retina are derived from different progenitor pools, thus challenging the concept that microglia originate from a single embryonic source. Using light-induced high-resolution fate mapping, researchers showed that in zebrafish embryonic/larval microglia arise from yolk sac macrophages produced by the rostral blood island (RBI), and adult microglia originate from progenitors in the ventral wall of the
dorsal aorta (VDA; Xu et al., 2015). Whether these findings hold true in mammals or are due to variations between species remains to be determined.

Although the complex mechanisms that regulate macrophage migration and microglial maturation are not completely understood, these processes depend on multiple factors and are critical for normal CNS development and function. During CNS development, both cell autonomous factors expressed by the microglial lineage, including receptors and transcriptional programs, and non-cell autonomous factors produced by neuroepithelial tissues, such as cell death signals and chemokines, can stimulate macrophage chemotaxis, drive differentiation, and promote microglial survival (Polazzi and Contestabile, 2002; Prinz and Priller, 2014; Schwarz and Bilbo, 2014). Below is a brief overview of key known factors.

The colony-stimulating factor 1 receptor (CSF1R) is tyrosine kinase transmembrane receptor expressed by macrophages and CNS microglia (Byrne et al., 1981; Sawada et al., 1990). In mice, CSF1R is expressed throughout embryonic development, peaking postnatally and significantly reduced in the adult brain (Nandi et al., 2012). Csf1r knockout mice (Csf1r−/−) completely lack CNS microglia (Ginhoux et al., 2010; Erblich et al., 2011). Zebrafish panther mutants lack a functional fms gene, which encodes Csf1r (Parichy et al., 2000). In panther embryos, macrophages fail to migrate from the yolk sac into the embryonic brain and retina (Herbomel, 2001). Therefore, the migration of microglial precursors to the brain and retina is dependent on Csf1r (Pixley, 2012). There are two CSF1R ligands, CSF-1 and interleukin-34 (IL-34); both cytokines
are differentially expressed by neurons in distinct yet complementary brain regions (Lin et al., 2008; Wei et al., 2010; Nandi et al., 2012). CSF-1 is a known chemoattractant for macrophages (Webb et al., 1996; Pixley, 2012). Compared with CSF-1, IL-34 is more broadly expressed in the mouse brain and binds with greater affinity to CSF1R (Chihara et al., 2010; Nandi et al., 2012). In mice, knockout of either CSF-1 (Csf1op/op) or IL-34 results in decreased CNS microglia in distinct brain regions, but both phenotypes are less severe than the Csf1r<sup>−/−</sup> (Yoshida et al., 1990; Wegiel et al., 1998; Greter et al., 2012; Wang et al., 2012). A CSF-1/IL-34 double knockout mouse has not been generated. Whether CSF-1 and IL-34 promote the differentiation and/or survival of microglia has been speculated (Metcalf, 1985; Lagasse and Weissman, 1997), but remains unknown.

Microgliogenesis also requires Pu.1, Irf8, and Runx1 transcription factors. PU.1 is an ETS transcription factor encoded by the SPI1 gene (Rosenbauer and Tenen, 2007), expressed by hematopoietic cells, and required for microglial development; Pu.1 knockout mice (Pu.1<sup>−/−</sup>) completely lack CNS microglia (Beers et al., 2006; Kierdorf et al., 2013). In zebrafish, pu.1<sup>G242D</sup> mutants lack early RBI-derived microglia, but not later VDA-derived microglia, suggesting pu.1 is differentially required for embryonic/larval and adult microglial development (Jin et al., 2012; Xu et al., 2015). Interferon regulatory factor-8 (IRF8) is a heterodimeric partner of Pu.1 that functions downstream of Pu.1; CNS microglia are significantly reduced in Irf8-deficient mice (Irf8<sup>−/−</sup>; (Kierdorf et al., 2013). The lack of microglia in both Pu.1<sup>−/−</sup> and Irf8<sup>−/−</sup> mice is due to decreased survival and maturation of yolk sac myeloid progenitors (Kierdorf et al., 2013). Runt-related
transcription factor 1 (RUNX1) binds to the Pu.1 promoter and inhibits Pu.1 expression in a negative feedback loop (Jin et al., 2012). In zebrafish, overexpression of Runx1 inhibits macrophage/microglial precursor formation, whereas the number of microglia is significantly increased in \textit{runx1}^{w84x} mutants (Jin et al., 2012). Further, in zebrafish, Runx1 is required for the development of the VDA-derived adult microglial population (Xu et al., 2015). In mice, Runx1 regulates microglial proliferation and homeostasis in the postnatal brain (Ginhoux et al., 2010; Zusso et al., 2012). And, recent studies demonstrated microRNAs (miRs), such as miR-124, post-transcriptionally regulate microglial development and functional maturation (Svahn et al., 2015).

Zebrafish mutant screens are a powerful resource for identifying microgliogenesis factors \textit{in vivo} and have recently yielded several novel regulators of microglial precursor migration and differentiation. The Leucine/Arginine transporter, \textit{scl7a7}, is expressed by a distinct sub-lineage of primitive macrophages in the anterior lateral plate mesoderm precommitted to give rise to microglia, and necessary for macrophage colonization of the zebrafish brain and retina (Rossi et al., 2015). The zebrafish orthologue of phosphate exporter XPR1, \textit{xpr1b}, is autonomously required for primitive macrophages colonize the CNS parenchyma and differentiate to microglia (Meireles et al., 2014). Further, noncanonical NOD-like receptor (\textit{nlrc3-like}) acts cell autonomously and is required for migration of primitive macrophages into the zebrafish CNS (Shiau et al., 2013). Zebrafish \textit{scl7a7}, \textit{xpr1b}, and \textit{nlrc3-like}
mutants all lack microglia (Shiau et al., 2013; Meireles et al., 2014; Rossi et al., 2015).

**Microglial Function**

Microglia are mononuclear cells that are ubiquitously, but not uniformly, distributed throughout the CNS parenchyma, occupying discrete territories, and comprising between 5-12% of all cells in the CNS (Lawson et al., 1990). The two main functions of microglia are CNS immune defense and homeostasis. Microglial function in the retina is the same as in the brain (Provis et al., 1996; Cuoghi and Mola, 2007; Langmann, 2007; Dick, 2008; Karlstetter et al., 2010; Fischer et al., 2014; Noailles et al., 2014; Fischer et al., 2015; Li et al., 2015).

Historically considered the resident macrophages or primary immune cells of the CNS (Butovsky et al., 2006; Walton et al., 2006; Hanisch and Kettenmann, 2007; Peri and Nüsslein-Volhard, 2008; Saijo and Glass, 2011; Shigemoto-Mogami et al., 2014), microglia are very sensitive to their environment. Detection of homeostatic disturbances, such as invading pathogens or damaged and dying neurons, induces rapid microglial ‘activation’. In the context of brain injury and disease, microglia retract their processes and take on an amoeboid morphology. In this activated or reactive state, microglia proliferate, migrate to and invade the site of injury, increase surface antigen expression, phagocytose cellular debris, and secrete numerous diffusible factors, such as chemokines, cytokines and growth factors as part of the immune response (Kettenmann et al., 2011; Tremblay et al., 2011; Sieger et al., 2012; Prinz and Priller, 2014). Activated microglia are pathological markers of many neurodegenerative diseases,
including Amyotrophic Lateral Sclerosis (ALS), Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Brouwers et al. 2007 & 2008; Sleegers et al. 2008; Viswanathan et al. 2009).

Whether microglial activation is a beneficial or harmful response to CNS injury or disease has been debated for many years (Streit et al., 1999; Kempermann, 2003; Hanisch and Kettenmann, 2007; Ekdahl et al., 2009; Tremblay and Sierra, 2014). Some studies demonstrate that activated microglia secrete neurotoxic factors, exacerbating neuronal damage, and that microglial activation negatively correlates with neurogenesis (Streit et al., 1999; Hanisch, 2002; Ekdahl et al., 2003; Monje et al., 2003; van Rossum and Hanisch, 2004; Rice et al., 2015). While, contrary evidence reveals activated microglia secrete neurotrophic factors, and microglial activation is a response to neuronal damage, not a cause, and positively correlates with neurogenesis, playing a necessary neuroprotective and/or regenerative role (Miwa et al., 1997; Nakajima et al., 2001; Streit, 2002; Nakajima and Kohsaka, 2004; Battista et al., 2006; Butovsky et al., 2006; Simard and Rivest, 2006; Ziv et al., 2006; Fischer et al., 2014; Shigemoto-Mogami et al., 2014). Nevertheless, microglial activation is variable, context-dependent, and adaptable (Colton, 2009), and more research is needed to understand the heterogeneity of microglial responses to CNS injury and disease.

In the normal adult CNS, microglia are classified as inactive or resting. However, unlike the name suggest, resting microglia are not passive or dormant cells, but in fact dynamically extend and retract their ramified processes,
continuously surveying their environment (Davalos et al., 2005; Nimmerjahn, 2005; Svahn et al., 2012). Resting microglia are emerging as critical components of CNS homeostasis and plasticity. In zebrafish, in vivo time-lapse imaging demonstrates that resting microglia respond to increases in neuronal activity by directing their processes toward and making contact with the cell bodies of active neurons (Li et al., 2012); and microglial contact subsequently leads to downregulation of spontaneous activity in the contacted neuron (Li et al., 2012; Ji et al., 2013). In adult mice, microglial depletion attenuates performance on learning and memory tasks and learning-dependent synapse remodeling (Parkhurst et al., 2013). Further, in vivo two-photon imaging of visual cortex in monocularly deprived mice demonstrated that microglial processes interact with synapses in an activity-dependent manner (Wake et al., 2009; Li et al., 2012; Sipe et al., 2016); and pharmacologically or genetically blocking microglial process motility inhibits ocular dominance plasticity (Sipe et al., 2016). These studies strongly implicate resting microglia in homeostatic regulation of neuronal activity and synaptic plasticity in the adult brain.

Evidence of the neurogenic role microglia play in the adult brain is increasing. Mutations in microglia-specific genes are associated with neurological disorders, including AD, FTD, and MS, suggesting microglia are important regulators of normal brain physiology (De Jager et al., 2009; Adam C Naj et al., 2011; Hollingworth et al., 2011; Griciuc et al., 2013; Guerreiro et al., 2013). In the adult mammalian brain, microglia are elements of the neurogenic stem cell niche (Ninkovic and Götz, 2007). In adult mice, microglia promote neural progenitor
survival and migration in the subventricular zone (Ribeiro Xavier et al., 2015), and are required for exercise-induced neural precursor cell activation in the hippocampus (Vukovic et al., 2012).

In addition to their homeostatic and neurogenic functions in the adult brain, microglia regulate multiple aspects of CNS development. *In vivo*, microglia regulate the size of the neural progenitor pool in embryonic rat SVZ (Cunningham et al., 2013). Microglia promote neuronal survival in the developing post-natal brain by releasing neurotrophic factors (Ueno et al., 2013). During CNS development, microglia promote programmed cell death (Marín-Teva et al., 2011), and actively regulate activity-dependent synaptic pruning, maturation and remodeling (Paolicelli et al., 2011; Schafer et al., 2012; Bilimoria and Stevens, 2014; Sato, 2015). Multiple developmental brain abnormalities are found in *Csf1r*−/− mice that lack microglia, including small brains, increased neuronal density and decreased cortical thickness (Nandi et al., 2012). *Csf1r*−/− mice do not live beyond one month of age (Dai et al., 2004). *In vitro*, proliferation is reduced in embryonic neural precursors from *Pu.1*−/− mice (Antony et al., 2011). *In vitro*, microglia can induce neurogenesis (Walton et al., 2006). An increase in neurogenesis occurs when neural progenitors are either co-cultured in media-connected chambers with microglia (Nikolakopoulou et al., 2013) or cultured with microglia-conditioned medium (Morgan et al., 2004; Walton et al., 2006; Antony et al., 2011), which strongly implicates that factors expressed and secreted by microglia regulate neurogenic events. Further investigations into the role microglia and/or their
secreted factors play during early neurogenic events *in vivo* are an important and necessary area of research.

**Progranulin**

Progranulin (PGRN) is an evolutionarily conserved secreted growth factor/pleiotropic glycoprotein precursor (Baba et al., 1993; Cadieux et al., 2005). PGRN was identified by several research groups working independently, and as a result has several synonymous names. PGRN is also known as granulin-epithelin precursor (Bhandari and Palfree, 1992; Plowman et al., 1992), proepithelin (Plowman et al., 1992), acrogranin (Baba et al., 1993), and PC cell-derived growth factor (Zhou et al., 1993). Mammalian genomes contain one progranulin gene (*GRN/Grn*), which, in humans, is located on chromosome 17 and contains twelve protein-coding exons (Bhandari and Palfree, 1992). Structurally related to the epithelin growth factor family, *GRN* is made up of seven and a half tandem repeats of twelve-cysteine granulin/epithelin motifs (Bhandari and Palfree, 1992; Bateman and Bennett, 1998). Once secreted, the 68kDa PGRN protein is heavily glycosylated to 90kDa, then enzymes, such as protease-3, elastase, and metalloproteinases, can proteolytically cleave PGRN into smaller (6kDa) peptide fragments called granulins (Zhou et al., 1993; Zhu et al., 2002; Kessenbrock et al., 2008; Suh et al., 2012). Secreted leukocyte protease inhibitor (SLPI) can bind to and inhibit proteolysis of secreted PGRN (Zhu et al., 2002). Both PGRN and individual granulins have biologically functional properties (Shoyab et al., 1990), though the majority of research, including this thesis, focuses on the intact precursor (Toh et al., 2011).
There are two putative receptors for Pgrn: tumor necrosis factor receptor (TNFR) and sortilin (Hu et al., 2010; Tang et al., 2011). Sortilin was identified through unbiased ligand binding assays and expression cloning as a neuron-specific PGRN receptor (Hu et al., 2010), though microglia-specific Sortilin expression has been demonstrated both in vitro (Dicou et al., 2004; Hu et al., 2010) and in vivo (Reuter et al., 2015). As an endocytic and intracellular sorting receptor, Sortilin is a known mediator of protein trafficking between the cell surface and various intracellular compartments (Hermey, 2009). Sortilin-mediated endocytosis and lysosomal targeting of PGRN is thought to regulate extracellular and intracellular PGRN levels (Hu et al., 2010). A study using yeast two-hybrid screening identified direct binding between PGRN and TNFR1 and TNFR2 (Tang et al., 2011). Further, when bound to the TNFR, PGRN antagonizes TNFα signaling and results in anti-inflammatory effects (Tang et al., 2011).

**The Function of Progranulin in Peripheral Tissues**

PGRN is a multifunctional protein, with well-established roles in vertebrate embryogenesis, tumorigenesis and wound healing (He and Bateman, 2003; He et al., 2003; Ong and Bateman, 2003). PGRN was first identified as a novel autocrine growth factor in transformed cell lines (Zhou et al., 1993). Subsequent studies established that in peripheral tissues and tumor cells, PGRN is mitogenic, stimulates cell proliferation and migration, and promotes cell survival (He et al., 2002; He and Bateman, 2003; Ong and Bateman, 2003). PGRN is primarily expressed by gametes, hematopoietic cells, and mitotically active epithelial cells
Ex vivo studies demonstrated that PGRN is secreted by the blastocyst and regulates early embryo formation and development in mammals (Díaz-Cueto, 2000; Qin, 2005). In vitro, exogenous application of recombinant PGRN to mouse embryos eight-cell stage accelerates cavitation onset, stimulates blastocoel expansion, increases trophoderm cell number, and promotes blastocyst hatching, adhesion, and outgrowth during implantation (Díaz-Cueto, 2000; Qin, 2005). Conversely, inhibition of PGRN with function-blocking antibodies significantly inhibits embryo development to the blastocyst stage (Díaz-Cueto, 2000; Qin, 2005).

PGRN is highly expressed in many different types of tumors, including renal (Donald et al., 2001), liver (Cheung et al., 2004; Ho et al., 2008), gastrointestinal (Demorrow, 2013), breast (Lu and Serrero, 2001; Serrero and Ioffe, 2003; Tangkeangsirisin and Serrero, 2004; Koo et al., 2012), ovarian (Jones et al., 2003; Han et al., 2011), prostate (Pan et al., 2004), bladder (Monami et al., 2006), esophageal (Chen et al., 2008), and neuroglial (Liau et al., 2000; Wang et al., 2011). The level of PGRN expression is directly proportional to proliferation rate of transformed cells in vitro and tumorigenicity in vivo, such that increased PGRN levels accelerates cell division (He and Bateman, 1999) and decreasing PGRN levels attenuates tumor growth (Zhang and Serrero, 1998). In vitro, PGRN promotes migration and invasiveness of transformed cells (Tangkeangsirisin and Serrero, 2004; Monami et al., 2006; Swamydas et al., 2011; Dong et al., 2015). Further, PGRN inhibits apoptosis of tumor cells (Tangkeangsirisin and Serrero, 2004; Monami et al., 2006); in vitro, inhibition of
PGRN with neutralizing antibodies increases ovarian cancer cell apoptosis (Kamrava et al., 2005). For these reasons, PGRN may be used as a prognostic biomarker in tumorigenesis (Han et al., 2011; Koo et al., 2012).

Following cutaneous wounding, PGRN, which is normally at undetectable levels, is strongly induced in injured fibroblasts and endothelial cells and upregulated in infiltrating neutrophils and macrophages (Zhu et al., 2002; He et al., 2003). *In vitro*, PGRN stimulates proliferation and migration of endothelial and fibroblast cells; *in vivo*, application of PGRN to a cutaneous wound results in an increase in macrophage, neutrophil, and blood vessel accumulation in the wound (He et al., 2003). These findings suggest PGRN may also function as a paracrine growth and chemotactic factor. *In vivo*, administration of recombinant PGRN inhibits inflammation and promotes re-epithelialization and wound healing (Zhu et al., 2002; Kessenbrock et al., 2008; Tang et al., 2011). Factors that regulate cell proliferation must ultimately exert their effects on the cell cycle. Acting as both a competence and progression factor, PGRN promotes mitosis in embryonic and adult epithelial cells by stimulating classic growth factor signal transduction cascades, such as mitogen-activated protein kinase (MAPK)/extracellular-signal-related kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways (Zanocco-Marani et al., 1999; Ong and Bateman, 2003; Bateman and Bennett, 2009).

**The Function of Progranulin in the CNS**

Much is known about PGRN activity in non-neuronal tissues, however the function of PGRN within the CNS is not well understood. In the normal adult
mammalian CNS, neurons and microglia express PGRN at relatively low levels (Daniel et al., 2000). Evidence of the neurogenic role PGRN plays in the adult brain is increasing. In vivo, PGRN increases voluntary exercise-induced neurogenesis in the hippocampus of adult mice (Asakura et al., 2011). In the mouse middle cerebral artery occlusion model of ischemia, overexpression of PGRN in the brain rescues motor and neurological deficits (Tao et al., 2012). Further, in the adult mouse retina, PGRN regulates retinal progenitor proliferation and photoreceptor differentiation following retinal damage (Kuse et al., 2016).

In humans, diminished PGRN production due to heterozygous mutations in the \textit{PGRN} gene (\textit{hGRN}) is a major cause of the neurodegenerative disease, Frontotemporal Lobar Degeneration (FTLD) (Baker et al., 2006; Cruts et al., 2006). FTLD leads to Frontotemporal Dementia (FTD). FTD is the second most common cause of dementia in individuals under 65 years of age (http://www.theaftd.org/understandingftd/diagnosis). Characterized by bilateral atrophy of the prefrontal and anterior temporal neocortical lobes and non-Alzheimer disease-type pathology, FTD results in progressive cognitive and motor deficits (Neary et al., 2005). FTD is a behavioral disorder with an early insidious onset and clinical diagnostic features including lack of social and personal awareness, emotional blunting, mental rigidity and inflexibility, and altered speech (Brun et al., 1994). FTD affects men and women equally and is strongly heritable. Approximately 40\% of FTD patients have a family history of FTD or other related dementia; 5-10\% show an autosomal dominant inheritance pattern (Chow et al., 1999; Bird et al., 2003; Rosso, 2003; Goldman, 2005;
Seelaar et al., 2008). Autosomal dominant FTD is most commonly linked to mutations in *microtubule associated protein tau* (*MAPT*) or *GRN*, located one megabase apart on chromosome 17 (Baker et al., 2006; Cruts et al., 2006). Scientists have identified over 60 mutations in the *GRN* gene, accounting for 10% of all FTD patients and approximately 20% of familial FTD (Baker et al., 2006; Cruts et al., 2006; Gass et al., 2006). Mutations in *GRN* often create null alleles, resulting in rapid degradation of *GRN* mRNA, reduced levels of PGRN and haploinsufficiency (Cruts et al., 2006). Missense mutations in *GRN* can result in the production of truncated, non-functional or unstable PGRN (van der Zee et al., 2007). Interestingly, FTLD patients with *GRN* mutations also display early disease retinal thinning that precedes FTD onset (Ward et al., 2014). A mouse model of PGRN haploinsufficiency (*Grn*+/− and *Grn*−/−) recapitulates aspects of FTD, including abnormal behavior, lipofuscinosis and neurodegeneration, inflammation, gliosis, and retinal degeneration in adults (Kayasuga et al., 2007; Ahmed et al., 2010; Ward et al., 2014) Yin et al., 2010; Wils et al., 2012; Ghosal et al., 2012; Filiano et al., 2013; Gotzl et al., 2014). However, CNS development has yet to be studied in *Grn*+/− and *Grn*−/− mice. The preponderance of research on the function of PGRN within the CNS focuses on understanding how life-long reductions in PGRN leads to neurodegeneration and dementia later in life. Yet, the mechanisms remain unknown.

While decreased PGRN results in FTLD, upregulation of PGRN in the CNS is associated with brain cancer and injury. In human glioma tumor cells, PGRN expression levels are consistently and significantly increased (up to 30
fold) compared with normal brain tissue (Liau et al., 2000). Similar to peripheral
tissue tumors, elevated PGRN expression is correlated with increased
recurrence and decreased survival rate in patients with glioblastoma (Wang et
al., 2011). PGRN is a molecular signature of activated microglia (De Muynck and
Van Damme, 2011); CNS injury and disease induces a microglia-specific
upregulation of progranulin message and protein expression (Craig et al., 2008;
Moisse et al., 2009; Naphade et al., 2009; Philips et al., 2010). Furthermore, in
vitro, PGRN acts as a microglial chemoattractant (Pickford et al., 2011).
Therefore, modulation of PGRN levels in the brain plays an important role in CNS
disease pathogenesis.

Evidence of the neurogenic role PGRN plays in the normal developing
brain is increasing. In mice, PGRN is initially highly expressed by embryonic
neuroepithelial cells, and then becomes restricted to fetal forebrain, olfactory
bulbs, retinal ganglion cells, and spinal cord (Daniel et al., 2000; 2003). Early
expression by neuroepithelial cells suggests PGRN plays a role in CNS
development. In rats, PGRN is expressed in the neonatal hypothalamus and
regulates sexual dimorphism and differentiation of the brain (Suzuki and
Nishiahara, 2002). In vitro, in rat cortical and spinal motor neurons, recombinant
PGRN enhances cell survival and increases neurite outgrowth length in a dose-
dependent manner (Van Damme et al., 2008). Compared with wild type, neurite
outgrowth and branching is significantly reduced in primary neuronal cultures
from Grn<sup>−/−</sup> mice (Gass et al., 2012). Transfection of hGRN into NSC-34 motor
neuron-like cells increases the formation of neurite processes and cell survival;
shRNA knockdown of PGRN decreased neuronal cell proliferation and survival (Ryan et al., 2009). PGRN-induced neurite outgrowth in primary mouse hippocampal and cortical neuron cultures requires glycogen synthase kinase 3β (GSK3β) signaling (Gao et al., 2010). Similarly, PGRN-dependent phosphorylation of GSK3β induces proliferation of mouse cultured neural progenitor cells (Van Damme et al., 2008; Ryan et al., 2009; Gao et al., 2010; Nedachi et al., 2011). Extracellular PGRN protects rat primary cortical neuron cultures from glutamate- or oxidative stress-induced cell death by activating MEK/ERK/p90RSK and PI3K/Akt signaling pathways (Xu et al., 2011). Together, these studies highlight the potential neurotrophic effects of PGRN. However, much remains to be determined about the function of PGRN in vivo in the developing CNS.

**Zebrfish Progranulin Gene Family**

Cadieux and colleagues performed initial characterization of the zebrafish progranulin gene family (Cadieux et al., 2005). The zebrafish genome contains four protein-encoding progranulin genes: progranulin-1 (pgrn-1), progranulin-2 (pgrn-2), progranulin-a (pgrn-a), and progranulin-b (pgrn-b). These four paralogues display two distinct structures. The first is exemplified by pgrn-1 and pgrn-2, both of which contain five exons that encode one and a half granulin domains. In addition, the predicted Pgrn-1 and Pgrn-2 protein sequences do not contain glycosylation sites. The second structural type is represented by pgrn-a and pgrn-b, which are made up of eighteen and fifteen coding exons that encode ten and nine granulin domains, respectively. pgrn-a and pgrn-b share
approximately 49% sequence homology, and are at least 45% and 43% homologous to *hGRN*, respectively. Therefore, *pgrn-a* and *pgrn-b* are both co-orthologues of mammalian *GRN*. The fact that zebrafish possess two co-orthologues of mammalian *GRN* is likely due to a pan-genomic duplication event (Taylor et al., 2003). Based on chromosomal location, only *pgrn-a* is syntenically conserved, making it the true orthologue of *hGRN*. RT-PCR analysis demonstrates *pgrn-a* and *pgrn-b* transcripts are maternally provided and continue to be expressed throughout development, from cleavage to 96hpf. In adult zebrafish, *pgrn-a* and *pgrn-b* transcripts are detected in many tissues, including skin, gills, heart, blood, intestine, pancreas, spleen, pronephros, and brain (Cadieux et al., 2005).

There are several studies published on the function of Pgrn-a in zebrafish. *pgrn-a* is upregulated in injured heart muscle, suggesting it may play a regulatory role in heart regeneration (Lien et al., 2006). Our lab identified *pgrn-a* in the zebrafish retina through an unbiased screen for genes modulated during neuronal regeneration in the adult retina; *pgrn-a* is expressed exclusively by microglia in the adult zebrafish retina, and is significantly upregulated in retinal microglia after photolytic lesion and during photoreceptor regeneration (Craig et al., 2008). These data suggest that *pgrn-a* may also regulate retinal regeneration. *pgrn-a* message and protein are expressed in caudal primary motor neurons and purportedly regulates motor neuron development (Chitramuthu et al., 2010). Knockdown of Pgrn-a attenuates motor neuron axonal outgrowth and branching, and Pgrn-a overexpression rescues the knockdown-induced axonopathy (Laird et
Pgrn-a knockdown also results in decreased hepatic outgrowth, suggesting Pgrn-a may potentially play a regulatory role in liver morphogenesis (Li et al., 2010). Pgrn-a is expressed in myogenic progenitor cells (MPCs), and reduction of Pgrn-a decreases MPC proliferation during postembryonic muscle growth and following muscle injury, therefore Pgrn-a regulates aspects of muscle growth and regeneration (Li et al., 2013). However, single and double pgrm-a/b mutant (pgrna−/−, pgrnb−/−, and pgrna−/−pgrnb−/−) fish lines generated using zinc finger nuclease genome editing technology did not recapitulate spinal motor neuron axonopathy and MPC phenotypes reported in the aforementioned knockdown experiments (Solchenberger et al., 2015). Loss of Pgrn-b function produces no developmental phenotype (Shankaran et al., 2008). The function of Pgrn-a in the developing CNS remains unknown and is an interesting and important area of research.

The main goal of my thesis research is to use zebrafish retinal development as a model to test the hypothesis that microglia and the factors they express and secrete play a fundamental role in vertebrate neurogenesis. A second goal is to generate tools, specifically transgenic and mutant fish lines, to enable further study of the function of microglia and Pgrn-a in the developing and injured/regenerating retina. In chapter two, I present data that demonstrate the requirement of microglia for early neurogenic events in the retina, using genetic and pharmacological models. I also describe the temporal and spatial expression pattern of pgrm-a during embryonic retinal development, and characterize the function of Pgrn-a in developmental neurogenesis using a loss-of-function
approach. This chapter is a largely unabridged version of the manuscript submitted to the journal Glia titled, “Microglia and Progranulin regulate neurogenesis in the developing vertebrate retina” by Caroline E. Walsh and Peter F. Hitchcock. In chapter three, I present data on the downstream signaling mechanisms of Pgrn-a. I also summarize the rationale behind and progress made towards generating 1) a BAC transgenic line to use as a Pgrn-a reporter and to study the function of microglia in the zebrafish retina, and 2) a CRISPR mutant line to study the function of pgm-a in retinal development and regeneration. Finally, in chapter four, I discuss concluding insights from my work on retinal development in vertebrates, microglia and progranulin, summarize the limitations of the methodology/technology and model, highlight remaining questions, and propose possible future directions to address these questions.
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Chapter 2: Microglia and Progranulin Regulate Neurogenesis in the Developing Vertebrate Retina

ABSTRACT

We used independent genetic and pharmacological approaches to determine the role of microglia and their secreted factors during developmental neurogenesis in the embryonic retina of zebrafish. When microglia are excluded from the retina, neurogenesis is significantly delayed - retinal progenitors fail to exit the cell cycle at the appropriate developmental time, and postmitotic cells do not acquire markers of terminal differentiation. We also evaluated the expression and function of the microglia-specific growth factor, Progranulin-a (Pgrn-a). At 24 hpf pgrn-a is expressed throughout the forebrain, but by 48 hpf pgrn-a is exclusively expressed by microglia and/or microglial precursors within the brain and retina. Knockdown of Pgrn-a does not alter the onset of neurogenic programs or increase cell death, however its absence prevents microglial precursors from colonizing the retina, and results in a concomitant delay in neuronal differentiation. Given the link between Progranulin and cell cycle regulation in peripheral tissues and transformed cells, we analyzed cell cycle kinetics among retinal progenitors following Pgrn-a knockdown. Depleting Pgrn-a results in a significant lengthening of the cell cycle. These data suggest that Pgrn-a plays a dual role during nervous system development by both attracting microglial progenitors into the embryonic brain and retina and by governing the
rate at which progenitors progress through the cell cycle. Collectively, these data show that the presence of microglia in the embryonic CNS and/or the molecules they secrete govern neurogenesis by regulating cell cycle kinetics and the transition from proliferation to cell cycle exit and differentiation.

INTRODUCTION

Microglia were first described by Pio del Rio Hortega in the 1920s (Del Rio Hortega, 1939). Though classically thought of as resident macrophages or immune cells of the central nervous system (CNS), microglia are morphologically and functionally heterogeneous (Kettenmann et al., 2011; Prinz and Priller, 2014). Microglia are highly ramified, motile cells that actively survey the CNS parenchyma (Nimmerjahn et al., 2005; Hanisch and Kettenmann, 2007). In the context of brain injury and disease, microglia respond by adopting an amoeboid ‘activated’ phenotype, phagocytosing cellular debris, and secreting numerous cytokines and growth factors as part of the inflammatory response (Garden and Möller, 2006; Hanisch and Kettenmann, 2007). Unlike neurons and other glial cells, which originate from the neuroepithelium, microglia derive from a subset of primitive yolk sac macrophages that migrate into neuroepithelial tissues, colonize the CNS parenchyma and undergo a phenotypic transformation (Herbomel et al., 1999; Herbomel et al., 2001; Ginhoux et al., 2010). In addition to their homeostatic functions in the adult brain, microglia also regulate aspects of CNS development and function, including the proliferation of neural progenitors, neuronal survival, neural patterning, programmed cell death, and the pruning,
maturation and function of synapses (Reviewed in Bilimoria and Stevens, 2014; Sato, 2015). Further, microglia in vivo are elements of the stem cell niche (Ninkovic and Götz, 2007), and microglia in vitro can induce gliogenesis and neurogenesis (Butovsky et al., 2006; Walton et al., 2006; Shigemoto-Mogami et al., 2014). Interestingly, an increase in neurogenesis occurs when neural progenitors are either cultured with microglia in media-connected chambers (Nikolakopoulou et al., 2013) or cultured with microglia-conditioned medium (Morgan et al., 2004), demonstrating that factors secreted by microglia regulate neurogenic events.

Progranulin (PGRN), also known as granulin-epithelin precursor, acrogranin, proepithelin and PC cell-derived growth factor, is an evolutionarily conserved secreted growth factor/pleiotropic glycoprotein precursor with a well established role in embryogenesis, tumorigenesis and wound healing (He and Bateman, 2003; He et al., 2003; Ong and Bateman, 2003). In peripheral tissues and tumor cells, PGRN is mitogenic, stimulates cell proliferation and migration, and promotes cell survival (He et al., 2002; He and Bateman, 2003; Ong and Bateman, 2003). In the mammalian CNS, PGRN is expressed by neurons and microglia (Daniel et al., 2000). In humans, diminished PGRN production due to heterozygous mutations in the PGRN gene (hGRN) is a major cause of the neurodegenerative disease, Frontotemporal Lobar Degeneration (FTLD) (Baker et al., 2006; Cruts et al., 2006). In contrast, malignancy of glioblastomas is correlated with elevated PGRN levels (Liau et al., 2000; Wang et al., 2011). In vivo, PGRN increases exercise-induced neurogenesis in the hippocampus of
adult mice (Asakura et al., 2011). *In vitro*, PGRN enhances neuronal survival, increases neural progenitor proliferation, and promotes neurite outgrowth and neuronal differentiation (Van Damme et al., 2008; Ryan et al., 2009; Gao et al., 2010; Nedachi et al., 2011). PGRN is a molecular signature of activated microglia (De Muynck and Van Damme, 2011); CNS injury induces a microglia-specific upregulation of progranulin message and protein expression (Craig et al., 2008; Moisse et al., 2009; Naphade et al., 2009; Philips et al., 2010). Interestingly, *in vitro* PGRN acts as a microglial chemoattractant (Pickford et al., 2011). Much is known about PGRN activity in non-neuronal tissues and the injured CNS, however the function of PGRN within the developing CNS is not well understood.

We use the zebrafish retina as a model tissue for studying brain development. The vertebrate retina is a well-established and tractable model for investigating the cellular and molecular mechanisms that regulate both developmental and regenerative neurogenesis (Agathocleous and Harris, 2009). Among vertebrates, the cytoarchitecture of the retina is precise and evolutionarily very highly conserved. Even subtle changes in developmental programs are easily detected as structural changes (Cepko et al., 1996). Zebrafish have four protein-encoding progranulin genes, two of which, *pgrn-a* and *pgrn-b*, are co-orthologues of mammalian gene (*GRN*) (Cadieux et al., 2005). *pgrn-a* is syntenically conserved, making it the true orthologue of *hGRN*. We first identified *pgrn-a* as a microglia-specific growth factor in the retina of the adult zebrafish, where it is strongly upregulated following photoreceptor death and during photoreceptor regeneration (Craig et al., 2008).
To test the hypothesis that microglia and the factors they secrete regulate developmental neurogenesis in the vertebrate retina, we used independent genetic and pharmacological approaches to prevent microglia from colonizing the embryonic brain and retina and evaluated the consequences of this on retinal neurogenesis. The results show that independent of the method used, when microglia fail to colonize the retina, there is a delay in retinal development; retinal progenitors remain in the cell cycle at times when they are normally postmitotic, and there is a corresponding absence of neuronal differentiation.

We also included in this study an evaluation of the expression and function of the microglia-specific growth factor, Pgrn-a (Craig et al., 2008). At 24 hours post fertilization (hpf), pgrn-a is expressed throughout the forebrain, but beginning about 36hpf pgrn-a expression becomes limited to macrophages/microglial precursors in the yolk sac, brain and retina. Knockdown of Pgrn-a using morpholino oligonucleotides prevents microglial precursors from migrating into the retina. Compared to the genetic mutation or pharmacological treatment, which diminish the number of microglia in the retina, depletion of Pgrn-a results in more severe developmental defects, which likely reflect the sum of both the absence of retinal microglia and the depletion of Pgrn-a. Given that PGRN governs the cell cycle in peripheral tissues and transformed cells (Ong and Bateman, 2003), we evaluated cell cycle kinetics in retinal progenitors following Pgrn-a knockdown. Depleting Pgrn-a significantly increases the duration of the G2- and M-phases, and this results in an overall increase in the total length of the cell cycle. We also found that diminished Pgrn-a results in a
significant increase in the expression of genes that promote cell cycle progression, and a significant reduction in the expression of genes that promote cell cycle exit, demonstrating that Pgrn-a signaling regulates the duration of the cell cycle via governing the expression of genes that directly control cell cycle progression. From these data, we conclude that the physical presence of microglia in the embryonic retina and the presumptive regulatory molecules they secrete play fundamental roles in governing developmental neurogenesis. Further, we conclude that the microglia-specific molecule, Pgrn-a, functions both to recruit microglial precursors to the embryonic CNS and govern cell cycle kinetics and neuronal differentiation.

**MATERIALS AND METHODS**  
**Animals**  
Adult AB wild type (WT) and mutant zebrafish (*Danio rerio*; ZIRC, University of Oregon, Eugene, OR, USA) were maintained at 28.5°C on a 14/10-h light/dark cycle. Embryos were collected immediately following natural spawns, incubated at 28.5°C on a 14/10-h light/dark cycle and staged by hours post fertilization (hpf). Homozygous panther mutants (csf1ra^d01/^; AB background) were identified by early larval pigment phenotype (Parichy et al., 2000). The Institutional Animal Care and Use Committee at the University of Michigan approved all protocols and procedures.

**PLX5622 Treatment**  
PLX5622 is a sister compound of PLX3397, which is a selective small molecule inhibitor of colony-stimulating factor 1 receptor (CSF1R) kinase that, in adult mammals, promotes apoptosis of microglia (De et al., 2014; Elmore et al., 2014).
PLX5622 (1mg/mL stock in DMSO; Plexxikon Inc., www.plexxikon.com) was
diluted to a 1ug/mL working solution in embryo media (E3). Dechorionated WT
embryos were treated with 1ug/mL PLX5622 (experimental groups) or E3
containing 0.01% DMSO (control groups), from 20-72hpf at 28.5°C. At 48hpf,
72hpf or 8 days post fertilization (dpf), embryos were pulse-labeled with EdU
(see below), sacrificed and processed for EdU labeling (Click-it EdU Imaging Kit;
Invitrogen, Carlsbad, CA, USA) and immunohistochemistry.

**Immunohistochemistry (IHC)**
IHC was performed as previously described (Luo et al., 2012). Briefly, embryos
were fixed overnight in 4% paraformaldehyde (PFA) in 100mM phosphate buffer
at 4°C, cryoprotected with 20% sucrose in 100mM phosphate buffer, and
embedded in frozen Tissue-Tek optical cutting temperature (OCT; Sakura
Finetek USA Inc., Torrance, CA, USA) compound. Sections (10µm thick) were
mounted on slides, washed, incubated in heat inactivated normal sheep serum
(NSS), and incubated overnight at 4°C with primary antibodies. The following day,
sections were washed and incubated in secondary antibodies. Nuclei were
stained with DAPI. Antibodies are listed in Table 2.1.

**S-phase labels**
Cells in S-phase of the cell cycle were labeled with either 5-Bromo-
2’deoxyuridine (BrdU; Sigma-Aldrich Corp., St. Louis, MO, USA) or 5-ethynyl-
2’deoxyuridine (EdU; Invitrogen, Carlsbad, CA, USA) as previously described
(Ochocinska and Hitchcock, 2007; Luo et al., 2012). Briefly, dechorionated
embryos at 24 and 48hpf were incubated for 20 minutes in ice-cold 10mM BrdU
or 1.5mM EdU dissolved in E3 containing 15% DMSO (Westerfield, 2000). 72hpf embryos were incubated for 20 minutes in 10mM BrdU or 1.5mM EdU dissolved in E3 containing 15% DMSO at room temperature (RT). 8dpf larvae were incubated for 20 minutes in 2mM EdU dissolved in E3 at RT. Following the labeling step, embryos/larvae were returned to RT E3 for 10 minutes prior to fixation. For BrdU staining, sections were incubated in 100°C sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 minutes, cooled to RT for 60 minutes, and then immunolabeled using standard protocols described above.

**In situ hybridization**

Double in situ hybridization (ISH) on retinal sections (Hitchcock and Kakuk-Atkins, 2004) and whole mount in situ hybridization (wmISH) were performed as previously described (Hitchcock and Kakuk-Atkins, 2004; Ochocinska and Hitchcock, 2007; Craig et al., 2008; Luo et al., 2012). Sense and antisense digoxigenin (DIG)- or fluorescein-labeled riboprobes (Table 2.2) were synthesized from full-length cDNA clones for pgrn-a (NM001001949; a gift from Dr. Hugh Bennett), fms (NM131672; a gift from Dr. Philippe Herbomel), and atoh7 (NM131632; a gift from Dr. Deborah Stenkamp) using in vitro transcription (Roche Diagnostic Corp., Indianapolis, IN, USA). Embryos were incubated with hybridization solution containing approximately 100ng of probe. For double fluorescent in situ hybridization, the fms and pgrn-a probes were hybridized simultaneously.

**Pgrn-a Knockdown with Morpholino Oligonucleotides (MOs)**
For Pgrn-a knockdown, two independent antisense MOs (Gene Tools, LLC, Philomath, OR, USA), targeting either the 5’UTR (0.25ng/embryo) or the splice site between exon 3 and intron 3 (SS, 1ng/embryo) of zebrafish pgrn-a, were diluted in 1X Danieau buffer (Nasevicius and Ekker, 2000) containing 2.5% phenol red and injected into the yolk of AB WT embryos at the 1-2 cell stage. 5-base pair mismatch (MM) and standard control (SC) MOs (Gene Tools, LLC, Philomath, OR, USA) were used as negative controls. To suppress potential off target effects induced by experimental MOs, p53 MO (Gene Tools, LLC, Philomath, OR, USA) was co-injected (1.5 fold) to block nonspecific cell death (Robu et al., 2007). The MO sequences are listed in Table 2.3.

mRNA Rescue
The coding sequence of zebrafish (zf) pgrn-a (NM_001001949.2; in pSPORT1 Vector from H. Bennett Lab) and the pgrn-a paralog, zf pgrn-b (NM_212738.1; in pBK-CMV Image Clone from Open Biosystems, GE Dharmacon, Lafayette, CO, USA), were PCR amplified starting from the ATG (primer sequences listed in Table 2.4), therefore removing the 5’UTR MO recognition sequence, using Platinum Taq DNA Polymerase HF (Invitrogen, Carlsbad, CA, USA). cDNAs were subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) using standard protocols, linearized with SaceI-HF (New England Biolabs, Ipswich, MA, USA), and capped RNAs were transcribed using the mMESSAGE mMACHINE T7 kit (Ambion, Thermo Fisher Scientific, Halethorp, MD, USA). Human progranulin (hGRN; GE Healthcare clone ID 3457813 in pCMV-SPORT6) and eGFP (pCS2+-EGFP) were linearized with NotI-HF and transcribed with SP6.
For rescue experiments, zf \textit{pgrn-a} (25pg/embryo), zf \textit{pgrn-b} (25pg/embryo) or human progranulin (\textit{hGRN}; 400pg/embryo) was co-injected with 5'UTR MO (.25ng/embryo) at the one cell stage. As a negative control, \textit{eGFP} mRNA (25pg/embryo) was co-injected with the 5'UTR MO (.25ng/embryo). At 72hpf Embryos were treated with EdU, sacrificed, and processed for IHC.

**Western Blot**
To confirm knockdown of Pgrn-a, Western blotting was performed as previously described (Gramage et al., 2015). Briefly, for each condition, protein was isolated from 50 embryo heads and separated by gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GenHunter Corp., Nashville, TN, USA) and incubated with rabbit anti-Pgrn antibodies (1:2000, a gift from Jen Leih Wu, see Li et al., 2010). Immunolabeled proteins were detected using enhanced chemiluminescence assay (ECL detection system, Amersham Biosciences, Arlington Heights, IL, USA). Anti-actin (1:1000, Calbiochem, EMD Millipore, Billerica, MA, USA) was used as a loading control.

**PCR**
To verify SS-targeting MOs altered processing of \textit{pgrn-a} pre-mRNA, PCR was performed on RNA isolated from SS morphant and uninjected control embryos at 48hpf. cDNA was reverse transcribed using the SuperScript kit (Invitrogen, Carlsbad, CA, USA). Primers flanking the SS MO target sequence (F: AGAATGTGTGAGGACCATC and R: CTGTGCTACTGGACAGCAG) were used to amplify the \textit{pgrn-a} locus. PCR products were separated by gel electrophoresis, purified and sequenced.
Cell Cycle Kinetics

Mitotic Index
The proportion of cells in the M-phase of the cell cycle was determined for embryos at 28hpf. Retinal sections were immunolabeled with anti-phosphorylated histone H3 (pH3), marking cells in the M-phase, and the number of M-phase cells as a function of retinal area (um²) was calculated. The spatial pattern of pH3 labeled cells was also assessed.

Percent Labeled Mitosis
The length of the G2-phase of the cell cycle was determined using the percent labeled mitosis technique (Quastler and Sherman, 1959; Luo et al., 2012). Embryos at 28hpf were incubated in BrdU for 20 minutes to label all cells in the S-phase of the cell cycle. Starting 10 minutes after BrdU exposure, embryos were collected at 30 minutes intervals through 35hpf, sectioned and immunolabeled with anti-pH3 and anti-BrdU antibodies. The proportion of double-labeled retinal cells ((pH3⁺BrdU⁺)/(pH3⁺)) was plotted as a function of time following BrdU exposure.

Pulse-Chase-Pulse
To determine the relative length of S-phase (Tₛ) and estimate the total cell cycle length (T_c), a pulse chase pulse method was used as previously described (Rachel et al., 2002). At 26hpf, embryos were incubated in 10mM BrdU dissolved in E3 containing 15% DMSO for 20 minutes on ice, rinsed in E3 at RT, incubated in 10mM thymidine at 28.5°C for 20 minutes (Otteson et al., 2001), washed in E3 for 1hr 40 minutes at 28.5°C and incubated in 1.5mM EdU dissolved in E3 containing 15% DMSO for 20 minutes on ice. Embryos were then rinsed for an
additional 10 minutes, sacrificed and processed sequentially to label BrdU and EdU. Nuclei were counterstained with DAPI. For each embryo, an optical section from the middle of a z-stack taken of one central retina section was selected and cells labeled with BrdU (nBrdU), EdU (nEdU) and DAPI (nDAPI) were counted. The length of the S-phase and total length of the cell cycle were determined using the following formulae, $T_s = 2/(n_{\text{BrdU+}}/n_{\text{total EdU+}})$ and $T_c = T_s/(n_{\text{EdU+}}/n_{\text{DAPI+}})$, respectively.

**Reverse transcriptase quantitative real-time PCR (qRT-PCR) for cyclins (B, D, and E) and cyclin-dependent kinase inhibitors (p27kip and p57kip)**

To determine the expression levels of genes that regulate cell cycle, qRT-PCR was performed as previously described (Taylor et al., 2015). For each condition, three biological replicates of 50 embryo heads were collected. Total RNA was isolated from pooled heads using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Three technical replicates of each biological replicate were run in a Bio-Rad CFX384 Touch Real Time PCR Detection System using 6ng cDNA and Bio-Rad IQ SYBR Green Supermix. Primers are listed in Table 2.5. Gene expression was calculated using Bio-Rad CFX Manager Software and normalized to $\beta$-actin.

**Statistical Analysis**

Quantitative data was represented by means and standard deviations. To calculate statistical significance, a one-way ANOVA or Student’s t-test was used (GraphPad Prism Software, La Jolla, CA, USA). A p-value $\leq 0.05$ was considered significant, unless otherwise noted.
Imaging
All sectioned material was sealed with glass coverslips and mounting media (Electron Microscopy Sciences, Hatfield, PA, USA). Fluorescence images were captured using a Leica TCS SP5 confocal microscope (Leica, Wetzler, Germany). Bright field images were captured using either a Leica DM6000 CFS or Leica M165FC microscope (Leica, Wetzler, Germany).

RESULTS
Absence of microglia in the retina alters retinal neurogenesis
Compared to warm blooded vertebrates, retinal morphogenesis in zebrafish is very rapid. By 24hpf, eyecups have formed from the anterior neural plate, and the retina is a layer of proliferating neuroepithelial cells (Schmitt and Dowling, 1994; 1996; Li et al., 2000). At approximately 28hpf, neuronal differentiation begins and ganglion cells are the first to exit the cell cycle in a small ventronasal patch (Hu and Easter, 1999). Neuronal differentiation and lamination progress sequentially from the ventronasal patch in circumferential waves that move dorsally, then temporally (Schmitt and Dowling, 1996; 1999; Hitchcock and Raymond, 2004). By 72hpf, the initial phase of neurogenesis is largely complete, and the retina is fully laminated and functional (Easter and Nicola, 1996). Beyond 72hpf and into adulthood, the retina continues to grow by expansion and the addition of new neurons from the ciliary marginal zone (CMZ; Hitchcock et al., 2004; Raymond et al., 2006).

In zebrafish, macrophages/microglial precursors migrate from the yolk sac into head mesenchyme between 22-40hpf, colonize neuroepithelial tissues, including the retina, between 30-48hpf, then undergo a phenotypic
transformation around 60hpf to adopt their final microglial state (Herbomel et al., 1999; Herbomel, 2001). The colony-stimulating factor 1 receptor (Csf1r) is a tyrosine kinase transmembrane receptor expressed by the macrophage lineage, and therefore microglia. Migration of microglial precursors to the brain and retina is dependent on Csf1r (Pixley, 2012). Panther mutants lack a functional fms gene, which encodes the Csf1r (Parichy et al., 2000), and in panther embryos, macrophages fail to migrate from the yolk sac into the embryonic brain and retina (Herbomel et al., 2001; Figure 2.1 A).

To determine if the absence of microglia in panther mutants alters retinal neurogenesis, qualitative and quantitative measures of cell proliferation, neuronal differentiation, and retinal size were compared in retinas of WT and panther embryos at 48 and 72hpf (Figure 2.2). In WT embryos at 48hpf, EdU-positive retinal progenitors are largely confined to the retinal periphery and the nascent outer nuclear layer (onl; Figure 2.2 A-C). In the central retina, the absence of EdU reflects exit from the cell cycle prior to EdU exposure by neurons in the inner nuclear and ganglion cell layers (inl and gcl, respectively) (Figure 2.2 A-C). Nuclear staining with DAPI reveals the presence of immature laminae, and markers of mature neurons label a small number of differentiated ganglion cells (Figure 2.2 A), amacrine cells (Figure 2.2 B), and photoreceptors (confined to the ventral patch; Figure 2.2 C). At 48hpf, the retinas of panther embryos are significantly smaller than WT retinas (Figure 2.2 M). In marked contrast to WT embryos, EdU-positive progenitors are present throughout the panther retinas, and there is an absence of both lamination and neuronal differentiation (Figure
2.2 D-F, N). In WT retinas at 72hpf, EdU-positive progenitors are few in number (Figure 2.2 N) and restricted to the CMZ (brackets, Figure 2.2 G-I). Further, the retina is fully laminated, and, reflecting its functional state, mature neurons are present throughout each of the retinal layers (Figure 2.2 G-I). In panther retinas at 72hpf, EdU-labeled progenitors are much more numerous than in WT retinas and are scattered throughout both the inl and onl (Figure 2.2 J-L, N). In addition, lamination is incomplete, and there is an overall paucity of differentiated neurons (Figure 2.2 J-L). Despite the overall lack of cell cycle exit and neuronal differentiation, at 72hpf retinal size is similar in WT and panther larvae (Figure 2.2 M). Collectively, these data show that the absence of retinal microglia results in a significant temporal delay in retinal neurogenesis. Progenitors fail to exit the cell cycle at the appropriate developmental time, and among postmitotic neurons there is diminished differentiation.

In panther mutants, the temporal progression of retinal development from 48 to 72hpf is delayed, but the spatial pattern of neurogenesis appears largely normal. This suggests that beyond 72hpf retinal development in panther mutants might eventually approximate WT. To determine if panther retinas recover from the initial developmental delay, retinal size, neuronal differentiation and the pattern of cell proliferation were compared in WT and panther larvae at 8 days post fertilization (dpf; Figure 2.3 A-C). At this time point, both the laminar structure and markers of differentiated photoreceptors and amacrine cells appear qualitatively equivalent, and there is no difference in the size of the retinas (Figure 2.3 B). Qualitative observations suggest that, compared to WT, the
retinas of *panther* mutants have fewer EdU-positive cells in the CMZ (Figure 2.3 A), because exposure to EdU for the same duration as WT did not label cells in the CMZ (Figure 2.3 A). However, subsequent staining with antibodies against PCNA revealed mitotically active cells in the CMZ (Figure 2.3 A). Notably, the paucity of microglia in *panther* retinas persists (Figure 2.3 C).

**Pharmacologically blocking macrophage migration into the CNS phenocopies *panther* mutants**

As a second test of our hypothesis, a pharmacologic method was used to block the migration of macrophages/microglial precursors into the brain and retina. WT embryos were treated with PLX5622, a selective small molecule inhibitor of CSF1R, from 20hpf, prior to the onset of macrophage migration from the yolk sac, until 72hpf. Control embryos were treated with vehicle solution only. Though not as complete as the genetic lesion, treatment with PLX5622 significantly arrests the migration of macrophages/microglial precursors into the retina (Figure 2.1 B), and this recapitulates the results observed for the *panther* mutants. At 48 and 72hpf, control embryos (Figure 2.4 A-C and G-I, respectively) closely approximate WT retinas at these ages (see Figure 2.2 A-C and G-I, respectively). Although, treatment with E3 and DMSO (vehicle solution) alone does not alter the normal developmental patterns, it does cause a slight temporal delay, as evidenced by the lack of HPC1 labeling at 48hpf (Figure 2.4 B) and the slight expansion of the CMZ at 72hpf (Figure 2.4 G-I). Nonetheless, in embryos treated with PLX5622, at both 48 and 72hpf, there is an increase in the number of EdU-labeled progenitors (Figure 2.4 D-F, J-L, M, N), laminae are either absent or immature, and there is a paucity of mature neurons. Similar to *panther*
mutants, at 48hpf PLX5622-treated retinas are significantly smaller than control retinas, and this, too recovers by 72hpf (Figure 2.4 M).

Unlike in panther mutants, pharmacological treatment can be temporally controlled. Therefore, for some larvae, PLX5622 treatment was stopped at 72hpf to determine if the retina recovers from the initial developmental delay and whether or not microglia recolonize the retina. At 8dpf, 5 days after PLX5622 treatment ended, the retinal size, laminar organization, neuronal differentiation and pattern of cell proliferation in experimental retinas were restored to control levels. (Figure 2.3 D+E). Interestingly, unlike panther mutants, at 8dpf, there was no significant difference in the number of retinal microglia in control and PLX5622-treated larvae (Figure 2.3 F).

**Developmental expression of pgrn-a**

We identified pgrn-a in the zebrafish retina through an unbiased screen for genes modulated during neuronal regeneration (see [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13999](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13999)), and determined that in the adult retina pgrn-a is expressed exclusively by microglia (Craig et al., 2008). Based on these data and the observation that, in vitro, Pgrn is a chemoattractant to microglia (Pickford et al., 2011), we hypothesized that Pgrn-a plays a fundamental role in developmental neurogenesis, and may also regulate the migration of microglial precursors into neuroepithelial tissues. We first established the developmental cellular pattern of pgrn-a expression using in situ hybridization (Figure 2.5 A-D, F-I). At 24hpf, pgrn-a is expressed throughout the forebrain and retina (Figure 2.5 A+F). Between 24 and 48hpf, pgrn-a expression
becomes restricted to putative macrophages/microglial precursors in the yolk sac, forebrain and retina (arrowheads, Figure 2.5 B, G-H), a pattern that persists through 72hpf (Figure 2.5 C-D, I). While at 72hpf it appears in retinal sections there is transcript expression in the CMZ (Figure 2.5 I), there is no evidence for this following whole mount ISH (Figure 2.5 C-D), and we infer that in sections this apparent labeling is spurious. There are no antibodies that detect zebrafish Pgrn-a in tissue sections, however, Western blot analysis showed that Pgrn-a is present at 24hpf (Figure 2.5 E), suggesting that the protein is reliably expressed when transcript is present. To confirm that pgrn-a expressing cells in the embryonic retina are microglia, pgrn-a in situ hybridizations were combined with immunostaining using the microglial markers, L-plastin (Herbomel and Levraud, 2005) and 4C4 (Raymond et al., 2006), and double in situ hybridizations using a fms probe, which encodes the CSF1R. In the retina, all cells that express pgrn-a (Figure 2.5 J+L) are co-labeled with antibodies to L-plastin (Figure 2.5 K) or 4C4 (Figure 2.5 M) and co-express fms (Figure 2.5 N-Q).

**Knockdown of Pgrn-a results in a paucity of microglia and diminished neuronal differentiation**

Two independent antisense MOs were used to block Pgrn-a synthesis. The ability to knockdown Pgrn-a with the 5’UTR-targeting MO was confirmed with Western blot analysis (Figure 2.6 A-B). Diagnostic PCR showed that the SS-targeting MO resulted in intron retention and a premature stop codon (Figure 2.7). There were no qualitative changes in the appearance of morphant retinas between 48 and 72hpf, so only data from only 72hpf embryos are described here. At 72hpf, the overall body size and shape of control embryos (Figure 2.6 D-F)
and \textit{pgrn}-a morphants (Figure 2.6 G-H) were comparable to uninjected WT embryos (Figure 2.6 C). Although, compared to controls, \textit{pgrn}-a morphants have markedly small forebrains and microphthalmia.

To determine the functional consequences of Pgrn-a knockdown, qualitative and quantitative measures of cell proliferation, neuronal differentiation and retinal size were compared between control and experimental groups. As for both the \textit{panther} mutants and PLX5622-treated embryos, the number of retinal microglia was significantly decreased in \textit{pgrn}-a morphants compared to controls (Figure 2.1 C). Regardless of the MO used, all control embryos (Figure 2.8 A-L) appear WT (Figure 2.2 G-I). In contrast, blocking the translation of Pgrn-a results in a retinal phenotype similar to, but more severe than that observed in either the \textit{panther} mutants or PLX5622-treated embryos. Compared to controls (Figure 2.8 A-L), retinas in morphants contain significantly more EdU-positive retinal progenitors and completely lack laminae and differentiated neurons (Figure 2.8 M-R). The microphthalmia observed in the whole mount embryos (Figure 2.8 A-F) was readily observable in retinal sections (Figure 2.8 S). As was also evident in the whole embryos, the alterations in the retinas of the 5'UTR morphants are slightly more severe than the SS morphants. We interpret this to be a dose-dependent effect resulting from the SS MO targeting zygotic transcripts only, whereas the 5'UTR MO targets both maternal and zygotic transcripts (Cadieux et al., 2005; Bill et al., 2009). All subsequent data presented here use the 5'UTR-targeting MO to knockdown Pgrn-a.
MO concentrations decrease with each cell division, and, as a result, MO-dependent translation inhibition lasts approximately 3-4 dpf (Nasevicius and Ekker, 2000). Therefore, we also evaluated the potential recovery of retinal development in pgrn-a morphants. When morphants are allowed to survive to 8 dpf, Pgrn-a synthesis recovers and cellular differentiation and the cytoarchitecture of the retina follow normal developmental patterns (Figure 2.9 A), however, the retinal size does not recover to control values (Figure 2.9 B). So too, the number of microglia remains significantly below control values (Figure 2.9 C).

**Neurogenic competence and cell death is not altered in morphants**

Morpholino oligonucleotides have the potential to create spurious results from off-target effects (Eisen and Smith, 2008). As a first of several approaches to assess this, we determined whether or not the retinal changes observed here were due to a non-specific developmental delay. atoh7 is required to initiate neurogenesis and serves as an indicator of the onset of neurogenic competence (Masai et al., 2000; Kay, 2005). Pgrn-a knockdown does not alter the timing or spatial restriction of the initial atoh7 expression (Figure 2.10 A-C), indicating that the changes in retinal development are not a consequence of a simple developmental delay. Cell death is another potential off target affect. In embryos at 24 and 48hpf, counts of pyknotic nuclei labeled with acridine orange and apoptotic cells marked by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) show no significant differences between control and experimental embryos (Figure 2.10 D), indicating the cell death cannot account for the changes in retinal development.
Co-injection of zf pgrn-a/b or hGRN mRNA rescues Pgrn-a knockdown

To validate the specificity of the Pgrn-a knockdown, mRNA encoding egfp, zf pgrn-a, zf pgrn-b, or hGRN was co-injected with the 5'UTR MO. To determine if exogenous mRNA rescued the knockdown phenotype, comparisons were made between embryos that were uninjected (Figure 2.11 A-C), injected with 5'UTR MO alone (Figure 2.11 D-F), and co-injected with 5'UTR MO and one of the mRNA constructs (Figure 2.11 G-O). As a control for the mRNA injections, the 5'UTR MO and egfp mRNA were co-injected, and this failed to rescue the developmental defects resulting from Pgrn-a knockdown (data not shown; Figure 2.11 P-R). In contrast, both zebrafish pgrn paralogs and the human pgrn mRNA rescued the morpholino-induced developmental defects in retinal growth, cell proliferation and neuronal maturation. In these larvae, EdU-labeled retinal progenitors were present only in the CMZ, retinal laminae were fully formed, and retinal size was equal to controls (Figure 2.11 G-O, P+Q). These data validate the specificity of the morpholinos targeted to pgrn-a, and demonstrate that Pgrn-a regulates both cell proliferation and neuronal differentiation. Finally, these experiments highlight the conserved function of progranulin protein between fish and humans. Interestingly, co-injecting mRNA only partially rescues the migration of macrophages/microglial precursors into the retina (Figure 2.11 R). In embryos co-injected with Pgrn mRNA, the total number of retinal microglia is significantly increased compared to morphants; however, there were still significantly fewer retinal microglia when compared to uninjected controls (Figure 2.11 R).

Pgrn-a knockdown alters cell cycle kinetics
In morphants, the persistence of retinal progenitors at a stage when these cells normally exit the cell cycle, suggests that Pgrn-a functions to promote progression through the cell cycle. Therefore, we determined the consequences of Pgrn-a knockdown on cell cycle kinetics using three approaches to measure the duration of individual components of the cell cycle and overall length of the cell cycle. First, the mitotic index was assayed at 28hpf. Sections through central retina were immunolabeled with anti-pH3, a marker of cells in M-phase and the proportion of pH3-positive cells was determined. In the retina, cells divide at the apical surface of the neuroepithelium (Das et al., 2003; Baye and Link, 2007). Following Pgrn-a knockdown, the location of pH3-positive cells was similar in morphants and controls (Figure 2.12 A), indicating that the fundamental polarization of the neuroepithelium and inter kinetic nuclear migration is unchanged by knockdown of Pgrn-a (see Baye and Link, 2007). In contrast, the number and proportion of M-phase cells was significantly less in the morphant retinas (Figure 2.12 B). Similarly, the mitotic index in both panther and PLX5622 treated retinas was also significantly less than in controls (Figure 2.13).

Second, the length of the G2-phase of the cell cycle was determined using the Percent Labeled Mitosis method (Quastler and Sherman, 1959). This assay measures the interval between the S-phase, when cells can be labeled with BrdU, and when BrdU-positive cells undergo mitosis and become positive for pH3. The resulting curve represents the percentage of BrdU-labeled cells that are co-labeled with pH3 at each of the time points sampled. In control embryos, the percentage of double-labeled nuclei increases sigmoidally over time (Figure 2.12
C; see Luo et al., 2012). For control groups, the curves plateau at 100% at about 2.5 hours, thereby defining the duration of the G2-phase. In contrast, in Pgrn-a morphants, the curve is shifted to the right. There were significantly fewer double-labeled cells at 60, 90, 120, 150, 300, and 330 minutes, indicating a G2-phase of about 3 hrs. Additionally, whereas the control curves begin to return to zero as BrdU-positive cells exit the M-phase and migrate away from the apical surface of the retina (Baye and Link, 2007), morphant retinal progenitors appear delayed in M. These cells eventually exit the M-phase and re-enter the cell cycle, because the number of progenitors does accumulate over time (cf. Figure 2.8 P-R, Figure 2.12 A). Therefore, these data show that knockdown of Pgrn-a results in a slower G2-phase and a prolongation of the M-phase.

Third, a pulse chase pulse paradigm was used to determine the relative length of S-phase ($T_s$) and estimate the total cell cycle length ($T_c$) (Rachel et al., 2002). This analysis showed the duration of the S-phase ($T_s$) is 6 hours (hrs) and is invariant in morphants and controls (Figure 2.12 D). The total length of the cell cycle ($T_c$) in uninjected controls is approximately 10hrs (Figure 2.12 E), which recapitulates previous findings in WT embryos (see Nawrocki, 1985; Li et al., 2000). However, the $T_c$ in morphant retinas is approximately 14hrs, which is significantly greater compared to controls (Figure 2.12 E). These and the aforementioned PLM data reveal that the G1-phase is also longer in morphants (~5hrs) than in controls (~1.5hrs).

To gain insight into the proximal molecular mechanisms that underlie Pgrn-a control of the cell cycle, qRT-PCR was performed at 30 and 72hpf to
measure the expression levels of genes that promote cell cycle exit, e.g., *p27kip* and *p57kip2* and genes that promote cell cycle progression, e.g., *cyclin B, D1* and *E*. Compared to controls, at both 30 and 72hpf the expression level of *p27kip* is significantly lower in morphants (Figure 2.12 F), whereas at 72hpf the expression levels of *cyclinB* and *cyclinD* are significantly higher (Figure 2.12 G).

**DISCUSSION**

Historically, microglia were characterized as resident CNS macrophages, the primary responders to brain injury or disease that cleared cellular debris and released factors as part of the immune response (Peri and Nüsslein-Volhard, 2008; Saijo and Glass, 2011). However, this narrow view is widening as evidence of the neurogenic role microglia play in both the developing and adult/regenerating brain increases. *In vitro* studies have shown microglia regulate aspects of neural progenitor proliferation, differentiation and survival (Review by Su et al., 2014; Sato, 2015). *In vivo*, in birds, microglia are required for the injury-induced proliferative response of Muller glia-derived neural progenitor cells in the retina (Fischer et al., 2014). In mice, microglia regulate the size of the neural progenitor pool in the embryonic subventricular zone (SVZ) (Cunningham et al., 2013), and promote neural progenitor survival and migration in the adult SVZ (Ribeiro Xavier et al., 2015). The data reported here adds to this growing body of literature by illustrating the fundamental role microglia play in governing early retinal neurogenesis.
In this study we utilized independent experimental approaches that blocked macrophages/microglial precursors from colonizing neuroepithelial tissues to determine whether the presence of microglia within the parenchyma governs developmental neurogenesis in the zebrafish retina. The results showed that when microglia fail to colonize the retina, neurogenesis is significantly altered. Retinal progenitors do not exit the cell cycle at the appropriate developmental time, and both neuronal differentiation and early retinal growth is delayed. Further, either excluding microglia from colonizing the retina or knocking down the microglia-specific protein, Pgrn-a, results in a significant lengthening the cell cycle, and this serves as the mechanistic explanation for the delay in retinal development when microglia and the molecules they release are excluded from the retina. Collectively, these data demonstrate that the microglia that colonize the embryonic brain and retina play a fundamental role in governing early neurogenic events and serve as cellular components that regulate the brain’s intrinsic timing of the cell cycle.

Members of the zebrafish community have recently expressed concerns regarding the use of MOs as experimental tools (Schulte-Merker and Stainier, 2014), and some have declared data based on MOs untrustworthy (Kok et al., 2015). Nonetheless, we are confident in the data presented here that are based on MO-induced knockdown of Pgrn-a. First, we used two independent MOs that targeted different regions of pgrn-a, and each MO produced essentially identical results. Second, we confirmed specific knockdown of Pgrn-a protein by Western blot analysis. Third, we used low, minimum doses of MOs: 0.25ng and 1ng for
the 5'UTR and SS MO, respectively. Fourth, we co-injected each experimental MO with a p53-targeting MO to suppress p53 activity and minimize off target/non-specific effects, including ectopic cell death. Fifth, we characterized the retinal phenotype in morphants at 72hpf, a time at which the MO-induced knockdown and the concentration of MOs in individual cells is nearing its effective limit (unpublished observations; Bill et al., 2009). Sixth, co-injecting Pgrn mRNA uniformly rescued the morphant phenotype, which validates the specificity of the MOs and the relationship between Pgrn-a knockdown and the retinal phenotype. Finally, the morphant phenotype was largely recapitulated by simply excluding microglia, and the presumptive Pgrn-a they release, from the embryonic retina. Together, these observations support the data presented here and suggest that, when used at limiting doses and rigorously controlled, MOs have a place in the repertoire of zebrafish biologists.

Though the ontogeny of microglia was debated for many years (Reviewed in Cuadros and Navascués, 2001; Ginhoux and Prinz, 2015), it is now established in fish (Herbomel, 2001), birds (Cuadros et al., 1993), and rodents (Ginhoux et al., 2010) that embryonic microglia originate as primitive yolk sac macrophages. The migration of macrophages/microglial precursors into the developing CNS is also highly conserved across vertebrate species (fish: Herbomel, 2001; rodents: Swinnen et al., 2013; humans: Verney et al., 2010). After colonizing the parenchyma of the embryonic brain, early macrophages concurrently undergo a phenotypic transformation to become microglia (Herbomel, 2001). Although the mechanisms that regulate microglial migration
and maturation are not completely understood, these processes depend on multiple factors and are critical for normal CNS development and function (Reviewed in Nayak et al., 2014; Prinz and Priller, 2014).

In vertebrates, microglia colonize the embryonic CNS and govern early neurogenic events. When microglial precursors fail to colonize the CNS, developmental neurogenesis is aberrant. Csf1r knockout mice (Csf1r<sup>-/-</sup>) completely lack CNS microglia (Ginhoux et al., 2010; Erblich et al., 2011) and have multiple developmental brain abnormalities, including small brains, increased neuronal density and decreased cortical thickness (Nandi et al., 2012). Csf1r<sup>-/-</sup> mice do not live beyond one month of age (Dai et al., 2004). Further, in the neocortex of Csf1r<sup>-/-</sup> mice, the number of neural progenitors and overall cellular proliferation is increased, while neuronal differentiation is decreased (Nandi et al., 2012). Our data shows that, similar to Csf1r<sup>+/+</sup> mice, genetically or pharmacologically blocking Csf1r function during zebrafish embryogenesis causes neurodevelopmental defects within the retina. Both panther mutant and PLX5622-treated embryonic retinas lack microglia and have a significantly increased number of proliferating retinal progenitors, which corresponds to an absence of neuronal differentiation at the time when initial retinal development is normally complete. Therefore, the presence of microglia in the embryonic brain and retina is required for normal developmental neurogenesis. Neither the panther mutants nor Pgrn-a morphants recover WT levels of retinal microglia by 8dpf, though in both models, the retinas were fully differentiated at this time. These results suggest there is an early critical window of retinal neurogenesis
that depends on the presence of microglia and/or Pgrn-a, and outside this window other cellular and/or molecular mechanisms are sufficient to drive retinal neurogenesis to completion.

There are numerous factors, in addition to Csf1r, that regulate microglial migration into the developing CNS. In zebrafish, cell autonomous factors, such as the Leucine/Arginine transporter, slc7a7 (Rossi et al., 2015), phosphate exporter XPR1 orthologue, xpr1b (Meireles et al., 2014), and noncanonical NOD-like receptor, nlrc3-like (Shiau et al., 2013), are required for primitive macrophage migration to and colonization of embryonic neuroepithelial tissues. Zebrafish slc7a7, xpr1b, and nlrc3-like mutants all lack microglia (Shiau et al., 2013; Meireles et al., 2014; Rossi et al., 2015), though the embryonic retinal and brain phenotypes among these mutants remain to be determined. Non-cell autonomous factors produced by neuroepithelial tissues, such as cell death signals and chemokines, also promote macrophage chemotaxis during development of the brain and retina (Reviewed in Polazzi and Contestabile, 2002; Schwarz and Bilbo, 2013). Based on our findings, Pgrn-a can be added to the list of factors that originate in neural progenitors and are required for microglial colonization of the developing zebrafish CNS.

Progranulin is a soluble growth factor that has multiple functions in developing and adult vertebrates. In vitro, PGRN promotes migration and invasiveness of transformed cells (Tangkeangsirisin and Serrero, 2004; Monami et al., 2006; Swamydas et al., 2011; Dong et al., 2015), stimulates proliferation and migration of endothelial and fibroblast cells (Bateman and Bennett, 2009),
and acts as a chemoattractant to microglia (Pickford et al., 2011). *In vivo*, application of PGRN to a cutaneous wound results in an increase in macrophage, neutrophil, and blood vessel accumulation in the wound (He et al., 2003). In humans, macrophage infiltration into adipose tissues correlates with circulating PGRN, further suggesting PGRN acts as a macrophage chemotactic factor (Youn et al., 2009). Our data are the first to demonstrate Pgrn-a regulates the migration of microglial precursors into neuroepithelial tissues and therefore acts as a chemoattractant to microglia *in vivo*. We hypothesize that during early zebrafish embryogenesis, the developing forebrain serves as a source of Pgrn-a, thereby establishing a morphogenic gradient between the brain and yolk sac. Microglial precursors migrate up the Pgrn-a gradient. Consistent with our hypothesis, depleting Pgrn-a significantly decreases the number of microglia that invade the retina. In rescue experiments, exogenous *pgrn-a* (or *pgrn-b*) is ubiquitously expressed in every cell. While this restores the function of Pgrn-a during retinal neurogenesis, there is no discrete source of Pgrn-a protein that is sufficient to fully rescue the failure of microglia to invade the retina. Together, these data suggest that, similar to *in vitro* results (Pickford et al., 2011), *in vivo*, Pgrn-a can act as a chemoattractant and direct microglial migration into neuroepithelial tissues.

In zebrafish, Pgrn-a is a growth factor that governs both migration of microglial precursors into the embryonic brain and cell cycle kinetics among neural progenitors. Progranulin was first identified as a novel autocrine epithelial cell growth factor in peripheral tissues (Review by Bateman and Bennett, 1998).
Subsequent studies have shown that in humans Progranulin is involved in both neurodegeneration and glioblastoma (De Muynck and Van Damme, 2011). PGRN was first identified as a mitogen in the tumorigenic PC cell line (Zhou et al., 1993). Later studies established that the level of PGRN expression is directly proportional to proliferation rate of cells in vitro and tumorigenicity in vivo, such that increased PGRN levels accelerates cell division (He and Bateman, 1999) and decreased PGRN levels attenuates tumor growth (Zhang and Serrero, 1998). Acting as both a competence and progression factor, PGRN promotes mitosis in embryonic and adult epithelial cells by stimulating classic growth factor signal transduction cascades, such as mitogen-activated protein kinase (MAPK)/extracellular-signal-related kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) pathways (Reviewed by Ong and Bateman, 2003; Bateman and Bennett, 2009). Modulation of PGRN levels within the CNS appears to similarly effect cell cycle activity. Our data demonstrates Pgrn-a promotes mitosis in retinal progenitors in vivo. Pgrn-a knockdown results in a significant lengthening of the cell cycle in retinal progenitors. Further, when Pgrn-a is reduced, the number of cells in the retina undergoing mitosis and terminally differentiating is significantly decreased. From the mitotic index data determined for each of the three models studied here, we conclude that the changes in cell cycle kinetics observed among the retinal progenitors in morphants are likely present in panther mutants and embryos treated with PLX5622. In Pgrn-a morphants, mitotically active cells take longer to transit G1 and G2 and undergo mitosis. Overall, these data indicate that in retinal progenitors Pgrn-a governs the
duration of the G1-, G2- and M-phases of the cell cycle. Therefore, Pgrn-a
normally functions to promote cell cycle progression and neuronal differentiation
in the developing retina. This illustrates that Pgrn-a functions similarly in
peripheral tissues and the CNS by driving cells through the cell cycle. The cell
cycle data were analyzed at a stage when all neural progenitors express pgrn-a,
and suggest that among these cells Pgrn-a may function as either an autocrine
or paracrine growth factor. In contrast, as 72hpf, when Pgrn-a expression can be
ascribed solely to microglia, the changes in cell cycle kinetics likely reflects the
consequence of both the absence of Pgrn-a and microglia, including other
molecules released by microglia that may regulate cell cycle kinetics. Together,
our findings indicate that, although relatively few in number, microglia regulate
neurogenesis in the developing retina, and that among retinal progenitors, factors
expressed or secreted by microglia, including the growth factor Pgrn-a, function
to promote progression through the cell cycle and cell cycle exit.
Retinal Microglia

A

# of Retinal Microglia (72 hpf)

WT
Mutant

B

# of 4C4+ Cells

DMSO
PLX5622

C

# of 4C4+ Cells

UI
5'UTR MM
5'UTR MO
Figure 2.1: Quantification of retinal microglia at 72hpf. (A) Histogram showing the number of microglia (4C4* cells) in the retinas of wild type (WT; 63.8 ± 18.7 cells; n=6) and panther mutants (1.0 ± 1.0 cells; n=11) at 72hpf; ***p≤0.001. (B) Histogram showing the total number microglia in DMSO-treated control retinas (45.2 ± 7.7; n=6) and retinas treated with PLX5622 (10.3 ± 8.6; n=14) at 72hpf; ***p≤0.001. (C) Histogram showing the number of microglia in uninjected (UI; 55.4 ± 21.5 cells; n=20), 5'UTR mismatch morpholino injected (5'UTR MM; 43.4 ± 4.6 cells; n=8), and Pgrn-a morphant (5'UTR MO; 4.4 ± 3.4 cells; n=11) retinas at 72hpf; ***p≤0.001. Data are represented as mean; error bars reperesent the standard deviation.
**Figure 2.2: Developmental neurogenesis is altered in the retinas of *panther* mutant embryos.** (A-L) Transverse sections through central retina of wild type (WT; A-C; G-I) and homozygous *panther* mutant (D-F; J-L) embryos at 48 and 72hpf, respectively. Sections are immunolabeled with cell-specific antibody markers (cyan) for ganglion cells (Zn5, top row), amacrine cells (HCP1, middle row), and red-green double cone photoreceptor cells (Zpr1, bottom row), EdU (fusia), and DAPI (gray). (M) Histogram showing relative retinal area of WT (10938.2 ± 959.6 μm²; n=7) and *panther* mutant (5879.2 ± 983.0 μm²; n=15) retinas at 48hpf (**p≤0.001), and WT (13498.4 ± 2427.9 μm²; n=5) and *panther* mutant (11713.7 ± 2715.9 μm²; n=22) retinas at 72hpf. (N) Histogram showing the percent of the retina labeled with EdU in WT (43.2 ± 2.2%; n=15) and *panther* mutant (80.7 ± 5.5%; n=7) retinas at 48hpf, and WT (7.0 ± 2.0 %; n=5) and *panther* mutant (35.2 ± 11.1%; n=22) retinas at 72hpf; ***p≤0.001. Data are represented as mean; error bars represent the standard deviation. Outer nuclear layer (ONL); inner nuclear layer (INL); ganglion cell layer (GCL); ciliary marginal zone (CMZ, brackets). Scale bar equals 50μm.
A. AB WT Mutant

B. Relative Retinal Area

C. Retinal Microglia

D. DMSO PLX5622

E. Relative Retinal Area

F. Retinal Microglia
Figure 2.3: Retinal phenotype of 8dpf panther mutant and PLX5622-treated embryos. (A) Sections through central retina of WT (left column) and panther mutant (right column) larvae at 8dpf, immunolabeled (cyan) with markers of red-green cone photoreceptors (ZPR1, top row) and amacrine cells (HCP1, middle row), BrdU (fusia), PCNA (fusia, bottom row), and DAPI (gray). (B) Histogram showing relative retinal area between WT control (21533.4 ± 4112.8 µm²; n=15) and panther mutant (21333.4 ± 2365.8 µm²; n=10) larvae at 8dpf. (C) Histogram showing the number of retinal microglia in WT control (95.7 ± 25.7 cells; n=14) and panther mutant (5.9 ± 6.0 cells; n=15) larvae at 8dpf; ***p<0.001. (D) Sections through central retina of DMSO-treated control (left column) and PLX5622-treated (right column) larvae at 8dpf, immunolabeled (cyan) with markers of red-green cone photoreceptors (ZPR1, top row) and amacrine cells (HCP1, bottom row), BrdU (fusia), and DAPI (gray). (E) Histogram showing relative retinal area between DMSO-treated control (19503.8 ± 2177.6 µm²; n=10) and PLX5622-treated (18900.2 ± 2781.1 µm²; n=11) larvae at 8dpf. (F) Histogram showing the number of retinal microglia between DMSO-treated control (113 ± 43.8 cells; n=10) and PLX5622-treated (113.9 ± 57.8 cells; n=11) larvae at 8dpf. Quantitative data are represented as mean; error bars represent the standard deviation. Outer nuclear layer (ONL); inner nuclear layer (INL); ganglion cell layer (GCL); ciliary marginal zone (CMZ, brackets). Scale bar equals 100µm.
Figure 1: Loss of EdU+/DAPI+ double-labeled retinal cells after PLX5622 treatment.

(A-L) Representative images showing the effects of DMSO control (A-C) and PLX5622 treatment (D-L) on EdU+/DAPI+ double-labeled retinal cells at 48hpf and 72hpf.

(M) Relative Retinal Area:
- Bar graph showing the comparison of DAPI area (um²) between DMSO and PLX5622 at 48hpf and 72hpf.

(N) % Retina Labeled with EdU:
- Bar graph depicting the percentage of retina labeled with EdU at 48hpf and 72hpf for DMSO and PLX5622 conditions.
Figure 2.4: Treatment with PLX5622 results in altered neurogenesis in the developing zebrafish retina. (A-L) Transverse sections through central retina of DMSO-treated control (A-C; G-I) and PLX5622-treated (D-F; J-L) embryos at 48 and 72hpf, respectively. Sections are immunolabeled with cell-specific antibody markers (cyan) for ganglion cells (Zn5, top row), amacrine cells (HCP1, middle row), and red-green double cone photoreceptor cells (Zpr1, bottom row), EdU (fusia), and DAPI (gray). (M) Histogram showing relative retinal area of DMSO-treated control (14462 ± 1749.6 µm²; n=10) and PLX5622-treated (10007.5 ± 1673.2 µm²; n= 14) retinas at 48hpf (**p≤0.001), and DMSO-treated (13901.9 ± 712.2 µm²; n=6) and PLX5622-treated (12857.9 ± 1524.2 µm²; n=16) retinas at 72hpf. (N) Histogram showing the percent of the retina labeled with EdU in DMSO-treated control (41.6 ± 5.0%; n=10) and PLX5622-treated (73.4 ± 8.1%; n=14) embryos at 48hpf, and DMSO-treated control (13.3 ± 2.2%; n=6) and PLX5622-treated (35.6 ± 11.5%; n=16) embryos at 72hpf; ***p≤0.001. Data are represented as mean; error bars reperesent the standard deviation. Outer nuclear layer (ONL); inner nuclear layer (INL); and ganglion cell layer (GCL); ciliary marginal zone (CMZ, brackets). Scale bar equals 50µm.
Figure 2.5: Expression of pgrn-a in the developing retina and Western blot of Pgrn-a expressed at 24hpf. (A-D) Whole mount in situ hybridization showing pgrn-a expression in wild type (WT) at 24 (A), 48 (B), and 72hpf (arrowheads in C+D). Panel D is a higher magnification image of the eye in panel C. In each panel, the dashed line marks the boundary of the eye. Lens (L). (E) Western blot showing Pgrn-a and Actin expression in 24hpf AB WT embryos. (F-I) Cross-sections of the retina following whole mount in situ hybridizations showing pgrn-a expression in WT at 24 (A), 36 (B), 48 (C), and 72hpf (D). (J-Q) pgrn-a is microglia-specific by 72hpf. pgrn-a in situ hybridization (J+L) followed by immunohistochemistry of L-plastin (K) and 4C4 (M) showing co-localization of pgrn-a and microglial markers (arrowheads). Double in situ hybridization in the same section showing DAPI (N), pgrn-a (O), and fms (P) expression, and overlay (Q) showing co-localization of pgrn-a and fms in the retina (arrowheads). Outer nuclear layer (ONL); inner nuclear layer (INL); and ganglion cell layer (GCL); (L) lens. Scale bar equals 50µm.