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Generation and circuit development of zebrafish retinal horizontal cells

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GENERATION AND CIRCUIT DEVELOPMENT OF ZEBRAFISH RETINAL HORIZONTAL CELLS

by

Philip Raymond Williams

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of Washington University in
partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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ABSTRACT

The construction of functional neuronal circuits requires proper coordination of many developmental processes. Neurons must be generated in the proper number and migrate to their mature locations within the nervous system. Upon completion and sometimes during neuronal migration, neurons must elaborate complex dendritic and axonal arbors and then form precise synaptic connections in the background of a plethora of possible synaptic partners. Glial cells must also establish specialized associations with a number of neuronal structures including synapses and axons. In order to create a detailed understanding of how a neuron progresses through all of these stages of development, I have studied a single class of neuron, the retinal horizontal cell (HC), from the stage of cell generation to the establishment of its synaptic circuits and associations with astroglia.

HCs are a key component of the circuitry of the outer retina of vertebrates and they function to modulate information transfer from photoreceptors. I examined the genesis of HCs using in vivo multiphoton time-lapse microscopy in the zebrafish retina and discovered a novel precursor cell that is dedicated to the generation of HCs. In contrast to the classical germinal cell, the horizontal precursor cell is unattached to the epithelial surfaces, and instead migrates freely and divides near the final laminar location of mature HCs.

I then determined what cellular changes take place in order for postmitotic HCs to establish their specific connectivity with rod and cone photoreceptors. In the zebrafish retina, HCs form well-defined circuits with specific subsets of photoreceptors. I determined how HC dendritic contacts were formed with only a specific subset of...
photoreceptors by performing in vivo time-lapse microscopy experiments in which a single HC was labeled in the background of all of its proper presynaptic partners. During the course of development, HC dendritic tips formed contacts with both proper and improper photoreceptor presynaptic terminals. However, improper contacts were removed while tips contacting the preferred photoreceptors were preferentially maintained. Thus, the final patterns of connections between HCs and photoreceptors are shaped by a process of refinement.

After establishing the events that take place during HC - photoreceptor circuit development, I examined whether Müller glia (MG) influence the development of these circuits. I found that synaptic contacts between HCs and photoreceptors are present before MG processes ensheath the nascent synapses. I also demonstrated that contact with MG did not influence the stability of newly formed HC dendritic tips. Furthermore, preventing the interaction of MG processes with photoreceptor pedicles and HC dendritic tips by targeted MG ablation did not cause the photoreceptor contact with HCs to disassemble. Thus, unlike in other parts of the nervous system, glial contact is not necessary to ensure the stability of newly formed synapses.

Together, my findings provide an in vivo view of the cellular mechanisms utilized to generate, localize and establish the circuitry of a single class of interneuron in the nervous system. My experiments also reveal a novel mechanism of cell generation and a contrasting view of the role of glial cells in stabilizing newly formed circuits.
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Summary
Identifying HC precursor cell progeny
Characterizing strategies of HC lateral migration
Determining the impact of reduced presynaptic input on HC dendritic arbor development
Examining the role of MG in the maintenance of immature retinal synapses

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE
The generation of a functional nervous system requires many fundamental steps. Neuronal and glial cells that will serve as the basic building blocks of the nervous tissue must be generated. These cells need to be positioned correctly such that they can form into structures observed at maturity. Countless precise connections must be made between neurons often involving the extension of long axons and the growth of complex dendritic arbors. Associations between these synaptic elements and supporting astroglia must be established, in addition myelinating contacts between axons and oligodendrocytes centrally and Schwann cells in the periphery must be established to ensure proper function of neuronal conductivity. Although much is known about each of these processes in isolation, a unified understanding of how a single cell progresses through these phases of cell genesis, migration, circuit integration and glial association is currently nonexistent.

The zebrafish retina is an excellent model system in which to study the development of a single cell type from genesis until mature synaptic and glial associations are formed. Zebrafish retinas can be maintained transparent and are amenable to repeated in vivo imaging until nearly two weeks of age, well after circuit maturity is reached. And since zebrafish develop en ovo embryos are accessible from the earliest stages of development. The laminated structure of the retina with stereotypical placements of cell somas and their synaptic contacts facilitates the assessment of cellular and circuit development. This thesis uses these advantages of the zebrafish retina in order to study how a single class of neuron develops from the point of cell genesis to the formation of mature circuits. I focus specifically on the development of a class of retinal interneurons, the horizontal cells (HCs), examining three key aspects of their
development. I will first describe how HCs are generated from a novel dedicated progenitor cell that divides at the location of mature HCs (Chapter 2). I will then examine the process by which HC dendrites establish contacts with their proper presynaptic partners, the photoreceptors (Chapter 3). Finally, I will examine the development of HC circuitry relative to Müller Glia (MG), the resident glial cells of the retina, and assess what role, if any, MG contribute to the formation and early maintenance of HC-photoreceptor circuits (Chapter 4).

**Non-classical modes of neurogenesis in the vertebrate central nervous system**

The first step in creating any tissue is generation of the cellular components. In the classical paradigm of neurogenesis during development, radial neuroepithelial progenitor cells undergo mitosis with their somas near the ventricle eventually leading to the production of most neurons that populate the CNS (Fig 1). These cell divisions can either generate a larger progenitor pool by creating two progenitor cells, generate a daughter neuron while self-renewing the progenitor cell, or terminate the progenitor cell by producing two daughter neurons. Such divisions occur during development throughout the length of the neural tube and in the retina. Neurons produced from this mode of neurogenesis typically migrate outward from the ventricular zone, often along the processes of radial glia, and cease migration in this local vicinity with little to no tangential migration taking place (Nowakowski and Rakic, 1979; Hallonet et al., 1990; Reese et al., 1995; Rakic et al., 2009). Such a strategy can ensure an even distribution of neurons provided that progenitor cell daughter genesis is regulated similarly from one
progenitor to the next within a local environment. However, many important exceptions to this simplified model of developmental neurogenesis exist.

For example, many neuronal subtypes are not generated by neuroepithelial progenitors of their local ventricular zone and instead must migrate long distances to their final destination. The majority of inhibitory cortical interneurons are generated in the medial ganglionic eminences where pallidal neurons are generated (Olsson et al., 1998). These immature neurons migrate tangentially into the neocortex and take up residence in multiple cortical layers (Lavdas et al., 1999). In other cases, the progenitor neurons themselves migrate, sometimes over a great distance, to form a secondary zone of neurogenesis. Progenitor cells from the lateral ganglionic eminence migrate to the subventricular zone of the lateral ventricle postnatally (Wichterle et al., 2003), where they establish a zone of neurogenesis that exists into adulthood (Lois and Alvarez-Buylla, 1993). A more extreme example of progenitor cell migration is the external granule layer of the cerebellum, which is formed by the mass migration of neural progenitors rostrally from the rhombic lip to cover the entire surface of the nascent cerebellum (Hallonet et al., 1990). The external granule layer is an important zone of neurogenesis that likely accounts for the production of all cerebellar granular neurons which migrate inward through the molecular layer after genesis (Hallonet et al., 1990; Gao and Hatten, 1994). Retroviral tracing, and transcription factor immunostaining also suggest that a portion of deep brainstem neurons might also arise from the external granule layer (Lin et al., 2001). A subtle specialized zone of neurogenesis is the subventricular zone, which is populated by progenitor cells generated in the directly adjacent ventricular zone. This layer is basal to a classical region of neurogenesis in the ventricular zone, and progenitor cells here do
not have the traditional radial morphology of ventricular zone neuroepithelial progenitors. Through careful time-lapse *ex vivo* imaging of cortical slices it is clear that progenitors in the subventricular zone always undergo symmetric divisions (that is producing two cells of the same “type”) that are usually terminal producing two daughter neurons (Haubensak et al., 2004; Noctor et al., 2004). Neuronal production from subventricular zone progenitor cells is particularly important for the production of early born neurons, when neuroepithelial progenitors cells in the ventricular zone primarily only produce more progenitor cells (Haubensak et al., 2004; Miyata et al., 2004). It has been indirectly suggested that subventricular zone divisions may account for the production of the vast majority of cortical subplate neurons (Takahashi et al., 1996), although a clear demonstration of the neuronal subtypes generated by this progenitor pool has not been carried out.

Another less studied zone of neurogenesis exists in the retina at relatively later stages of development. In the retina the apical surface adjacent to the pigmented epithelium is analogous to the ventricular zone. Radial neuroepithelial progenitor cells span the retina and undergo mitosis with their somas positioned at the apical surface. The vast majority of retinal cells then migrate basally displaying no tangential displacement, with the sole exception of horizontal cells (Reese et al., 1995). Curiously, after the formation of most, if not all, retinal ganglion cells and amacrine cells, mitotic figures can be observed in the inner nuclear layer of the retina of rats, cats and humans (Robinson et al., 1985; Rapaport and Vietri, 1991; Smirnov and Puchkov, 2004). Labeling dividing cells with tridiated thymidine at later stages, where this layer is the primary zone of neurogenesis, leads to labeled daughter neurons of multiple neuronal types suggesting
that these progenitor cells may be multipotent (Rapaport and Vietri, 1991). However, careful identification of the cells generated by this progenitor pool has not been undertaken.

The retina has the only reported neuronal progenitor population that is intermingled with differentiated neurons during development. A mixed population of dividing progenitor cells and differentiated neurons suggests a possible unique function for this population of neurogenic precursor cells, which may provide new insights into the purpose of secondary zones of progenitor cells in establishing neuronal networks in a precise manner, both spatially and temporally. Also it remains possible that divisions distinct from the apical surface generate specific populations of cells perhaps involving precursors dedicated to the generation of only a single class of neuron. In chapter 2, I describe a resident precursor cell of the inner retina that is dedicated to the production of a single retinal subtype, the horizontal cell, and that divides near the layer in which mature HCs reside. I demonstrate that this precursor cell is critical for the generation of the complete population of HCs since most, if not all, HC are generated from these precursor divisions.

**Circuit formation involving interneurons**

Although there are numerous studies that demonstrate the generation of both interneurons and projection neurons, few have directly addressed how these neuronal classes initially wire up with their presynaptic partners after they have migrated into position. In particular, only recently have there been studies examining how interneurons develop their synaptic circuits. The contributions of interneurons are critical for proper
neural processing in all CNS circuits. Interneurons are one of the only sources of synaptic inhibition in the CNS, yet their development has been studied very little, partially due to their complex arbors and dense patterns of connectivity.

(i) Axonal targeting of interneurons

Recent advances have increased our understanding of how interneurons of the CNS form their axonal outputs. Strategies of axon development in interneurons vary greatly across subtypes, although adhesion molecules have been demonstrated to play an important role. Stellate cells in the cerebellar cortex grow their axonal branches along the apical processes of Bergmann glia until they intersect with Purkinje neuron dendrites, the synaptic targets of stellate cells (Ango et al., 2008). An immunoglobulin family adhesion molecule, CHL1, was found to be expressed by both Bergmann glia and stellate cells. When this adhesion molecule was knocked out, stellate cells demonstrated deformed axonal arbors not following Bergmann glial processes, synaptic contacts with Purkinje cells were reduced in number, and eventually the stellate cell axons fully retracted (Ango et al., 2008). Cell adhesion interactions with target neurons have also been demonstrated. Cerebellar basket cells confine their specialized axonal terminals to the axon initial segment of Purkinje neurons by following a gradient of neurofasciculin186 established by ankyrinG, which is restricted to the axon initial segment. Removing expression of either neurofasciculin186 or ankyrinG leads to a lack of subcellular targeting of basket cell axons (Ango et al., 2004). Thus, interactions mediated by adhesion molecules of both neuronal and glial elements can be important for establishing proper interneuron axonal connections.
Synaptic activity is also important in regulating the number and density of postsynaptic targets by axons of interneurons. Basket interneurons of the cerebral cortex innervate multiple pyramidal neuron somas through an activity dependent mechanism. In slice cultures of postnatal cortex, the formation of new synaptic contacts between basket cell dendrites and pyramidal cell somas can be observed with time-lapse imaging. However, if the slice is bathed in tetrodotoxin, blocking spiking activity of all neurons in the culture, the formation of new associations is blocked (Chattopadhyaya et al., 2004). It should be noted that with these conditions it is impossible to determine if blockade of activity of the basket interneurons themselves was responsible for the prevention of axonal terminal differentiation. The importance of interneuron synaptic signaling in basket cell axonal terminal maturation was clearly demonstrated in GAD67 knockout mice where GABA synthesis is blocked. In these knockout mice, basket cells contact fewer pyramidal neurons and elaborate less extensively on their targets (Chattopadhyaya et al., 2007) an end result similar to global blockade with tetrodotoxin. These experiments demonstrate a clear role for synaptic transmissin from interneuron axons in establishing mature axonal contacts.

Proper interneuron axonal targeting is not always dependent on postsynaptic targets. Zebrafish amacrine cells, interneurons of the retina, stratify their neurites independently of their postsynaptic targets. Amacrine cell neurites project to the proper synaptic strata within the inner plexiform layer and appear to laminate normally even when their postsynaptic partners, the retinal ganglion cells, fail to be generated (Kay et al., 2004). In addition, multistratified retinal ganglion cells that laminate with specific amacrine cell strata do so after amacrine cell neurites are already present in the inner
plexiform layer (Mumm et al., 2006) suggesting that amacrine neurites may be important for organizing the retinal neuropil. Taken together these experiments demonstrate that the strategies by which interneuron circuits are formed may be as diverse as the functions of these cells in the mature CNS.

(ii) Dendritic development of interneurons

Although some insights have been made into the development of interneuron axons, very little is known about how interneurons form their dendrites. It is known that neurotrophic factors can promote dendritic growth and development. Cultured cortical interneuron dendrites are responsive to BDNF and NT4/5 during development. Biolistic transfection leading to the expression of these neurotrophic factors in cultured cortical interneurons causes more rapid dendritic maturation (Wirth et al., 2003). GAD67 positive cortical interneurons in slice culture also respond to BDNF with an increase in dendritic length (Jin et al., 2003). Sources of neurotrophic factors in the developing CNS are numerous indicating, presynaptic partners and surrounding glial cells which could have an impact on the development of interneuron dendritic arbors by a neurotrophic factor dependent mechanism.

The dendritic arbors of developing interneurons are highly motile. Wu & Cline (2003) studied the development of *Xenopus* tectal interneuron dendrites *in vivo*. Similar to zebrafish retinal ganglion cell dendritic arbors (Mumm et al., 2006), tectal interneuron dendritic arbors remodeled extensively over the first 24-48 hr post-cell genesis. After this time period, primary and secondary branches were stabilized while the arbor continued to grow. Large axon-like processes were removed in a subset of interneurons (Wu and Cline, 2003). Retinal ganglion cell axons that project to tectal interneurons, reach the tectum
and begin forming stable arbors slightly ahead of tectal interneuron dendritic stability (Harris et al., 1987), indicating a possibility for dendrites in this system to actively determine ultimate circuit connections.

An important aspect of dendritic arbor development is formation of correct synaptic partner choices. Whether interneuron dendrites actively seek out their proper presynaptic targets or are passively innervated by presynaptic axons remains unknown. These experiments require the visualization of dendritic development in the background of presynaptic inputs. Thus, using HCs as a model interneuron, I will examine the role of dendrites in actively selecting proper synaptic contacts during synaptogenesis in vivo (Chapter 3).

The importance of astroglia in the developing nervous system

At synapses, astroglia clear transmitter from the synapse (Schousboe et al., 1977; Schousboe and Waagepetersen, 2006), recycle neurotransmitter (Pow and Robinson, 1994; Lebon et al., 2002), physically separate synapses (Sykova, 2001), and secrete neurotransmitter receptor coagonists (Scolari and Acosta, 2007). Astrocytes have long been viewed as support cells of the CNS, whose main function was the maintenance of optimal conditions for neuronal function. Indeed, astrocytes are important for homeostatic functions like regulating levels of glucose (Escartin et al., 2006) and oxygen (Jakovcevic and Harder, 2007), while removing harmful molecules from the CNS milieu (Srebro and Dziobek, 2001). Studies over the last few decades have also demonstrated clear and critical roles for astrocytes in the development of the neuronal circuits in the nervous system.
(i) Role of astroglia in plasticity and refinement of synaptic circuits

The first known role for astrocytes in neuronal circuit development was in regulation of plasticity. The visual cortex contains ocular dominance columns that are primarily dedicated to the processing of visual information from a single eye in the cat. In the normal visual cortex the territory allotted to each eye is evenly distributed, but if visual stimulation is deprived to one eye during development, the unperturbed eye will take over the ocular dominance columns of the deprived eye. This redistribution of territories is only possible during a critical period of development, and can normally not occur in adult cats (Stryker, 1978). However, when astrocytes taken from kittens, which were still within the critical period, were transplanted into monocularly deprived adult cat visual cortex, they allowed for the redistribution of ocular dominance columns beyond the critical period (Muller and Best, 1989). Thus it appears as though activity from immature astrocytes is responsible for maintaining the plasticity seen during visual cortex development. Indeed, later studies demonstrated that the enzymatic degradation of chondroitin sulfate proteoglycans, an extracellular matrix molecule produced by mature astrocytes, could mimic the effects of immature astrocyte transplantation and reestablish the critical period in adult cats (Pizzorusso et al., 2002), suggesting astrocytic production of chondroitin sulfate proteoglycans brings about the end of the critical period.

A hallmark of neuronal circuit development in many regions of the CNS, is an overproduction of synapses that are later removed and refined. Astroglia have been demonstrated to be important for regulating the density of synapses during development, particularly in the cerebellum, where Bergmann glia are the resident astroglia. For example, mature Purkinje neurons receive input from single climbing fibers; however,
during development, multiple climbing fibers contact a single Purkinje neuron. In
dissociated cerebellar cultures, Purkinje neurons that are not contacted by Bergmann glia
maintain the immature multiply innervated pattern and display immature
electrophysiological characteristics (Drake-Baumann and Seil, 1999). Similar events take
place in vivo when Bergmann glia processes do not physically associate with synapses
due to misexpression of GluR2. Lack of Bergmann glia process association with synapses
in vivo also leads to maintenance of multiple climbing fiber inputs onto Purkinje neurons
as evidenced by electrophysiological recordings (Iino et al., 2001). Blocking Bergmann
primary text here...
postnatal rat hippocampal neurons form more inhibitory synapses and demonstrate more frequent IPSPs when exposed to astrocyte conditioned medium (Elmariah et al., 2005). These observations indicate that astrocytes produce secreted factors that promote synapse formation. Indeed, addition of thrombospondins can mimic the effects of astrocyte conditioned medium on retinal ganglion cell cultures (Christopherson et al., 2005). Furthermore, thrombospondins are expressed in immature astrocytes but not mature astrocytes in vivo, demonstrating spatial and temporal consistency with a role for thrombospondins in mediating synapse promotion by astrocytes. Cytokines and growth factors are also secreted by astrocytes and can promote synapse formation. Tumor necrosis factor-alpha and astrocyte conditioned media both strengthen synapses in cultured hippocampal neurons by increasing the number of AMPA receptors at the synapse (Beattie et al., 2002). Astrocytes can also produce astrocyte derived neurotrophic factor which promotes excitatory synapse formation via stimulation of NT-3 secretion in nearby neurons (Blondel et al., 2000). Thus secreted factors from astrocytes can influence synaptogenesis during development.

Astrocytes can also promote synapse formation through contact mediated mechanisms. Time-lapse imaging of astrocytes and neurons labeled in hippocampal slice cultures has demonstrated that astrocytic and dendritic filopodial rearrangement frequently occur in a coordinated fashion such that contact is observed more often than if rearrangements were random (Haber et al., 2006). Numerous experiments have now shown that these contacts are supportive of synapse formation. In dissociated hippocampal cultures astrocytic contact leads directly to increases in both excitatory (Hama et al., 2004) and inhibitory (Liu et al., 1996) synaptic drive and density. Careful ex
vivo time-lapse imaging of hippocampal slices has demonstrated that astrocytic contacts are important for the maturation of dendritic filopodia into spines. Dendritic filopodia contacted by astrocyte processes have longer lifetimes and are more likely to develop into mature spines (Nishida and Okabe, 2007). Inhibiting astrocytic process motility by expressing dominant negative Rac1 leads to a decrease in the lifetime of dendritic filopodia, which are morphologically much longer than normal. However, the lengthened filopodia that were able to contact immobilized astrocytic process were just as stable as those that contact normal astrocytic processes, indicating that decreased incidence of neuronal filopodial and astrocytic process contact led to the decrease in dendritic filopodial lifetime (Nishida and Okabe, 2007). Therefore, contact mediated mechanisms between astrocytes and synaptic elements can also promote synaptogenesis.

The signaling pathways that mediate contact induced stabilization are partially understood. In dissociated hippocampal cultures, contact mediated increases in excitatory synaptic drive and density are mediated by protein kinase C signaling cascades within the neuron (Hama et al., 2004). In these experiments protein kinase C was triggered by activation of neuronal integrin receptors by an unknown astrocytic source. Inhibition of protein kinase C signaling diminished the synaptogenesis seen following astrocytic contact (Hama et al., 2004). Ephrin signaling is also known to be critical for mediating the interactions of forming dendritic spines and astrocytic processes. EphA4 receptor is present on dendritic spines while one of its ligands, ephrinA3 is concentrated on astrocytic filopodia near synapses (Murai et al., 2003). Astrocytes in cultured hippocampal slices respond to addition of ephrinA3 ligands by increasing the length and number of processes (Nestor et al., 2007), suggesting astrocytic processes associate with
ephrin A3 ligands. Indeed, disrupting neuron-astrocyte Eph receptor-ephrin binding disturbs dendritic spine maturation (Murai et al., 2003). Addition of either exogenous EphA4 or ephrinA3 led to similar decreases in dendritic filopidal lifetime and also duration of dendritic filopidia-astrocytic process contact, suggesting that ephrin perturbation destabilized astrocyte-neuron associations which then lead to retraction of the dendritic filopodia (Nishida and Okabe, 2007).

Thus far astroglia have been shown to be important for the promotion of synapse formation in the CNS. Likewise, at the neuromuscular junction, in some instances perisynaptic Schwann cells are required for formation of synapses of the motor neuron nerve terminal onto muscle tissue. In developing *Xenopus* embryos, perisynaptic Schwann cells can be specifically targeted for ablation *in vivo* by treatment with antibodies that only recognize antigens on perisynaptic Schwann cell membranes, followed by complement serum which leads to cell lysis. When these ablations are carried out during formation of the neuromuscular junction, normal nerve terminal growth that occurs during this stage of development ceases, and in many cases the nerve terminal retracts completely leading to a loss of the neuromuscular junction entirely (Reddy et al., 2003). Thus, at some synapses, glial cells not only promote synaptogenesis but their contribution can be requisite.

Although there is now much evidence for a role of astroglia in normal circuit development and a simultaneous maturation of synapses and astrocytes has been clearly demonstrated in spinal circuits (Ullian et al., 2001), there are instances where synapse development precedes astroglial birth or differentiation. For instance, the subplate of the cortex is a very early developing cortical layer that receives input from thalamic axons as
the upper cortical layers are generated and undergo differentiation (Kanold, 2004). While
the upper cortical layers are developing, subplate neurons relay signals from thalamic
projections (Friauf et al., 1990). After maturation of the cortical layers, the thalamic
projections remove their synapses from subplate neurons and form mature circuits onto
layer 4 pyramidal neurons (Friauf and Shatz, 1991), followed by a degeneration of the
subplate neurons. In the somatosensory cortex of rats, thalamic projections onto subplate
neurons are functional by embryonic day 18 (Higashi et al., 2002). However, a large
number of cortical astrocytes are still being generated at later stages of development
(Levison et al., 1993). Therefore functional circuits are present at ages where large
numbers of regional astrocytes have yet to be generated. If differentiation of astroglia is
required for these cells to partake in promoting synaptogenesis, then a number of circuits
may form without the activity of astroglia. For example, the subplate circuit in cats is
developed by at least embryonic day 47 (Friauf et al., 1990). However, cat cortical
astrocytes do not express mature markers like glial fibrillary acidic protein, or connexins
uniformly until after postnatal day 60 (Muller, 1990; Rochefort et al., 2005). Thus
astrocytes are not fully mature for months after subplate circuits are functional.

Like the subplate, Müller Glia (MG) in the retina do not express glutamine
synthetase, a marker of their differentiation, until after synaptogenesis is initiated
(Johnson et al., 2003) and ribbon synapses are present in the synaptic layers (Prada et al.,
1998).

We therefore hypothesize that some synaptic circuits may be able to form independently
of contributions by astroglial cells. I will test this hypothesis using the zebrafish retina to
first determine MG development relative to retinal neurons and their synaptic circuits
using *in vivo* microscopy approaches. I will then examine how specific circuits in intact regions of the *in vivo* retina develop following focal ablation of MG (Chapter 4).

**Zebrafish retina as a model system**

We will use the zebrafish retina to determine:

(i) how the population of HC interneurons is generated

(ii) how HC interneurons establish specific synaptic connections with photoreceptors

(iii) whether synapse formation between HCs and photoreceptors is influenced by MG

The zebrafish retina has many advantages as a model system for such a set of experiments. The retina, across species, is a highly ordered structure. It is laminated into five well defined layers, three of which contain neuronal cell bodies separated by two layers in which all synaptic contacts are made. Since the cellular layers are separated by neuropil, even at very early stages of development (Godinho et al., 2005), vertical placement of cells within the retina can be easily determined *in vivo* with common membrane staining techniques (Das et al., 2003) or by using transgenic lines (Q01, Godinho et al., 2005). The synaptic layers are also highly organized. Specialized triad synapses between photoreceptors, and horizontal cell and bipolar cell dendrites are contained within the outer plexiform layer (OPL), whereas contacts between bipolar cell axons, amacrine cell neurites and retinal ganglion cell dendrites are present in the inner plexiform layer (IPL). Therefore, contacts between known neuronal subtypes occur in a discrete location and in a highly organized fashion, allowing for the easy tracking of the development of cellular arbors (Kay et al., 2004; Godinho et al., 2005; Mumm et al.,
2006; Schroeter et al., 2006) and their associations with synaptic partners (Mumm et al., 2006) during development. The outer retina is particularly amenable to the study of circuit formation because photoreceptors form a single large synaptic terminal that stratifies within a thin sheet of neuropil. These terminals are clearly invaginated by enlarged dendritic tips of HCs, making this synaptic contact easily identifiable by light microscopy. I will take advantage of these traits of outer retinal circuits in order to determine the time and course of development of HC-photoreceptor circuits (Chapter 3) and to examine HC-photoreceptor connectivity relative to MG maturation (Chapter 4).

My proposed studies require extensive in vivo time-lapse imaging approaches. Zebrafish are highly amenable to such time lapse imaging studies because they can be maintained transparent and healthy for up to two weeks, allowing for repeated imaging across many stages of development. Their embryos develop rapidly, such that the retina can process light stimulation by 3-4 days post fertilization (dpf) and visual hunting behavior is present by 5 dpf (Easter and Nicola, 1996). Therefore, combining the long duration over which experiments can be carried out in the zebrafish retina, with the rapid time course of development, experiments in which cells are tracked from the point of genesis to the elaboration of mature circuits are certainly feasible (Godinho et al., 2005, Mumm et al., 2006; Schroeter et al., 2006). This contrasts with ex vivo imaging of mouse retinal whole mounts which can only be maintained for approximately two days in culture, and where development is much slower. For example observations of HC migration in mouse retinal cultures was not able to extend for the entire course of HC vertical migration through the inner nuclear layer (Huckfeldt et al., 2009). In contrast, it is possible to track the migration and differentiation of zebrafish amacrine cells until their
neurites stratify at the appropriate mature depth within the IPL (Godinho et al., 2005). Therefore, I am confident that I can observe all stages of cell genesis and circuit development for zebrafish HCs.

With the growing popularity of zebrafish as a model system, tools are becoming more and more available for their study. For example, mutagenesis screens are commonly carried out in zebrafish generating useful new mutants that can be applied to the study of CNS development (Mullins et al., 1994; Brockerhoff et al., 1995; Gaiano et al., 1996). In addition, transgenic lines expressing fluorescent proteins in specific cellular subtypes are constantly being generated that are very useful for in vivo time-lapse imaging experiments. I will use transgenic lines generated by other groups for critical experiments throughout this thesis (Table 1). Furthermore, recent advances in transposase technology have made the generation of new transgenic lines a relatively simple task when compared to traditional methods. This technique has been demonstrated to lead to rates of transgenesis as high as 50% in zebrafish (Kawakami, 2004). In my hands new transgenic lines are generated at a rate of approximately 20% of F0 fish that are screened. I will use a transgenic line generated with this technique in Chapter 4. I will also use the Gal4/UAS system widely applied to Drosophila in which Gal4 transcription factor expression is driven by a promoter of interest (Koster and Fraser, 2001). The Gal4 transcription factor then activates transcription of genes downstream of the UAS, or Upstream Activating Sequence, promoter elements. The advantage of the Gal4/UAS system is that it is modular, in that, once a Gal4 transgenic zebrafish is generated it can be crossed with any available UAS transgenic zebrafish line in order to express the desired gene product.
Another important advantage of the zebrafish retinal system, is the ease with which individual cells of a desired subtype can be labeled. Consistent labeling of individual cells in a system conducive to extended time-lapse imaging is a powerful tool for studying the dynamic events that take place during development but is rare outside of organotypic slice culture. Immunofluorescent labeling of cells is hampered by its static nature, and difficulty in assessing the morphology of complete yet individual cells because entire populations are generally labeled. Stable transgenic lines are very useful for examining entire cohorts of cells, and can be used for time-lapse studies, but determining discrete changes in cellular morphology and connectivity is often difficult when the entire population is labeled. With zebrafish, mosaic fish where individual cells express a desired fluorescent protein can easily be generated by injecting plasmid DNA into one cell stage embryos. Currently promoter elements exist to drive expression of desired proteins in every cell type of the retina (Kennedy et al., 1994; Fadool, 2003; Takechi et al., 2003; Godinho et al., 2005; Bernardos and Raymond, 2006; Mumm et al., 2006; Schroeter et al., 2006; Shields et al., 2007). These techniques have been used to determine many unforeseen developmental events in the inner retina, and in my experiments I will use these techniques to examine the development of individual HCs (Chapters 2, 3 & 4), photoreceptors (Chapters 3 & 4) and MG (Chapter 4).

**Development of the zebrafish retina**

Neurogenesis in the zebrafish retina is a rapid process, commencing at 28 hpf and concluding by about 60 hpf (Hu and Easter, 1999). As in other species (Rapaport et al., 2004), retinal ganglion cells are the first neurons to be generated, presumably followed
by amacrine cells, horizontal cells and cone photoreceptors (Hu & Easter, 1999). Bipolar cells and MG are thought to be the last cells generated (Schmitt and Dowling, 1999). Unlike other cell types, rod photoreceptors are produced over an extended period, and their mature cohort is not present until 7 dpf (Fadool, 2003). For a summary of zebrafish retinal development in comparison to mouse retinal development see Figure 2.

Conventional synapses between retinal ganglion cells and amacrine cells are first observed at 60 hpf, while ribbon triads representing the contacts of photoreceptor pedicles onto horizontal cell and bipolar cell dendrites are first apparent at 65 hpf (Schmitt and Dowling, 1999). Bipolar cell ribbons in the inner plexiform layer are the final synaptic element to develop at approximately 70 hpf (Schmitt and Dowling, 1999), a time coinciding with the first observed light driven responses in the zebrafish retina (Easter and Nicola, 1996). The development of MG associations with retinal cells has not been carefully examined in the zebrafish, but it is known that associations between MG processes at the outer limiting membrane and photoreceptor inner segments are apparent very early in development (50 hpf, Schmitt and Dowling, 1999).

The dynamics of circuit development have not been examined in the zebrafish outer retina. Studies of developing inner retinal circuits show that neuronal processes are highly targeted. Zebrafish amacrine cells elaborate their processes in specific sublaminae of the IPL with almost no contributions straying into other strata (Godinho et al., 2005). Similarly, multistratified retinal ganglion cells build their dendritic arbors sequentially with new layers being precisely targeted to a specific sublaminae (Mumm et al., 2006). In this thesis, I will use in vivo time lapse imaging in order to determine how HCs form...
contacts with their specific presynaptic partners, rod and cone photoreceptors, in the outer retina (Chapter 3).

**Horizontal cells of the zebrafish retina**

HCs are the lateral interneurons of the OPL where they are thought important for mediating the center surround characteristics of bipolar cells and color opponency. Their activity is thought to be mediated through feedback mechanisms to photoreceptors (Kamermans and Spekreijse, 1999). Thus when HCs are stimulated, they are postulated to feed back inhibitory signals onto cone photoreceptors of similar and in some instances different subtypes. This modulation of cone photoreceptors is likely performed not performed via conventional transmitter release from HCs, although the exact mechanisms are still disputed (Kamermans and Spekreisje, 1999).

(i) **Horizontal cell connectivity**

In the zebrafish retina, there are four subtypes of HCs, three of which (H1, H2 and H3) connect with only cone photoreceptors, and one that is specific to rod photoreceptors, (rod HC). Each cone HC connects with different subtypes of photoreceptors. Zebrafish possess four subtypes of cone photoreceptors that are maximally sensitive to different wavelengths of light. They are termed UV cone, blue cone, green cone and red cone photoreceptors. The photoreceptor mosaic is highly regular in zebrafish to the point that it is termed near crystalline (Raymond et al., 1995). In this mosaic red-green double cones are present in rows with alternating orientations between rows of alternating UV and blue cone photoreceptors such that green cone photoreceptors always flank UV cone photoreceptors and red cone photoreceptors always flank blue cone photoreceptors (Fig
By labeling HCs with DiI in the SWS1::GFP transgenic background, in which all UV-cones express GFP (Takechi et al., 2003), the connectivity of each subset of cone connecting HC has been inferred (Li et al., 2009). H1 HCs (H1s) form contacts with red, green and blue subtypes of cone photoreceptor in the zebrafish retina (Li et al., 2009). H2s are morphologically very similar to H1s and in previous zebrafish studies have been morphologically inseparable (Song et al., 2008). H2s connect with green, blue and UV cone photoreceptors (Li et al., 2009). H3s have been reported to only connect with UV-cone photoreceptors (Song et al., 2008), or to contact both UV and blue cone photoreceptors (Li et al., 2009). A schematic of zebrafish HC-photoreceptor connectivity is provided in Figure 3.

It is important to note that Li et al. (2009) labeled HCs in the background of only UV cones, and used the extremely regular nature of zebrafish photoreceptor mosaics (Raymond et al., 1995) to extrapolate the remaining photoreceptors contacts. Therefore, the only partners that have been completely confirmed are either the presence (H2 & H3) or absence (H1) of UV-cone photoreceptor connections.

(ii) Horizontal cell electrophysiology

In the goldfish there are three electrophysiological subtypes of cone HCs (L1, C1 and C2) that have been matched to the above morphological subtypes (H1, H2 and H3 respectively, Stell et al., 1982). Photoreceptors are tonically active in the dark, and light stimulation cause hyperpolarization. HC dendritic tips have ionic glutamate receptors, which are sign conserving and, as such, light stimulation and hyperpolarization in photoreceptors causes subsequent hyperpolarization of invaginating HCs. H1s hyperpolarize to all wavelengths of light, but preferentially to longer wavelengths and as
such, are thought of as luminance detectors (Stell et al., 1982). H2s are biphasic in that they hyperpolarize to shorter wavelengths of light and depolarize to longer light wavelengths, thus inverting the sign of red photoreceptor stimulation (Stell et al., 1982). H3s are triphasic, hyperpolarizing to short and long wavelengths of light and depolarizing to middle wavelengths. It is somewhat perplexing that biphasic H2 would have a differential response to red stimulation since they do not connect to this subtype of photoreceptor, and even more intriguing that H3s, which connect to only shorter wavelength cones, display differential responses across a spectrum of light for which they connect to no representative cone photoreceptors. The mechanism by which these multiphasic responses are formed likely involves feedback onto photoreceptors through cone HC s. For example, it is thought that the monophasic H1 likely receives input primarily from long red cone photoreceptors while inhibiting the activity of medium and short wavelength photoreceptors to which its connects. This would result in red light inhibiting green cone hyperpolarizing responses via connections with H1s. This inhibition would lead to a depolarization of green cones, which provide input onto biphasic H2s leading to a depolarization response of H2s following a red light stimulus.

A recent conundrum facing zebrafish HC research is that there appears to be more electrophysiological subtypes of zebrafish HCs than there are morphological subtypes. Six unique electrophysiological responses of HCs across spectral stimuli have recently been reported (Nelson and Connaughton, 2008), whereas only three cone HC morphologies and wiring patterns have been described (Li et al., 2009). Two unique monophasic cells with varying peak intensities, a biphasic cell described above, two triphasic cells hyperpolarizing preferentially to blue or UV stimuli, and a tetraphasic cell
depolarizing to blue and red light were reported (Nelson & Connaughton, 2008). Li et al. (2009) reasoned that the two monophasic cells could simply be variants of the H1 cell type, and that the two triphasic and one tetraphasic cells could be explained varying the directionality of photoreceptor and HC communication while maintaining the H3 wiring pattern they observed (H3s connected to UV- and blue-cone photoreceptors). However, it is also possible that the H3 has different subclasses that make slightly differential connections between blue and UV cones but possess a similar morphology. In fact a UV specific H3 was previously reported by the same group that identified the H3 as a UV and blue contacting cell (Song et al., 2008).

Studies of mature HC-photoreceptor circuits have indicated subtype specific contacts between rod photoreceptors and rod HCs, and possibly UV-cone photoreceptors and H3s. In Chapter 3, I will label individual HCs in transgenic zebrafish embryos in which all rod photoreceptors or UV-cone photoreceptors express GFP. I will then examine the connectivity patterns of H3s and rod HCs in the developing retina, and determine how these arbors develop over time relative to their presynaptic partners. Specifically, I will ask whether specificity in the wiring pattern of rod and cone HCs arises by targeted dendritic outgrowth or by refinement of initial imprecise patterns of connectivity.

**The role of Müller glia in the retina**

(i) Morphology of Müller glia

In addition to the neuronal elements of the retina, activity from glial cells is critical for normal structure and function (Magalhaes and Coimbra, 1972; Newman et al., 1984; Pow and Robinson, 1994; Rich et al., 1995; Haberecht and Redburn, 1996;
Bernardos et al., 2005; Wang et al., 2009). Müller Glia (MG) are the only glial cells that contribute processes to the synaptic layers of the retina. MG also form the apical limiting membrane where their processes connect via adherent junctions, and the basal limiting membrane where they wrap retinal ganglion cell axons. MG have a similar morphology in all species that have been examined and are the only cells to span the retina at maturity. At the basal surface of the retina MG end feet form dense yet intricate processes that intercelate between retinal ganglion cell axons. MG contributions to the IPL are bushy and often contact synapses (Pedler, 1963; Rasmussen, 1974), similar to astrocytic processes in brain neuropil (Bushong et al., 2002). The INL receives a minimal contribution from MG; in most species very few MG processes are seen making contact with nearby neuronal somas. In the OPL, processes of MG are morphologically specialized to wrap each photoreceptor pedicle or spherule individually (Stell, 1967). MG contributions in the outer nuclear layer vary somewhat across species. In some cases photoreceptor somas are virtually wrapped by MG (Robinson and Dreher, 1990), and in other species MG have processes that course through the ONL contacting but not wrapping each photoreceptor (Bernardos and Raymond, 2006). This difference between the amount of MG contact on neuronal soma in the ONL versus INL is likely due to the higher metabolic demands of photoreceptors and specialized processes that MG carry out to support photoreceptor activity (Wang et al., 2009). At the outer limiting membrane, MG form a discontinuous apical barrier interrupted by photoreceptor outer segments. Specialized junctions between the processes of neighboring MG, and between MG processes and photoreceptor inner segments seal the apical barrier of the retina (Rentsch, 1973).
The population of MG has been investigated in numerous species using MG specific antibodies (Dreher et al., 1992). In most species MG soma occupy a single strata of the inner nuclear layer. As with almost all retinal cell types, MG are more densely packed in the central retina than in the periphery, while their peripheral territories are larger than those in the central retina. HRP labeling of neighboring MG in rabbit retina suggest that MG have overlapping territories (Robinson and Dreher, 1990). In contrast, astrocytes labeled variably with different color lipophilic dyes has demonstrated exclusive mosaics of protoplasmic brain astrocytes (Bushong et al., 2002).

(ii) Function of MG in the mature retina

MG play an important role as support cells of the retina. Trophic support is provided by MG. CNTF is produced by MG in vivo (Kirsch et al., 1997) as well as bFGF, NGF, BDNF, NT-3 and NT-4 in culture (Wexler et al., 1998; Oku et al., 2002; Taylor et al., 2003). bFGF produced by MG promotes survival of cultured rod bipolar cells (Wexler et al., 1998), and the addition of neurotrophic factors that stimulate bFGF production in MG through TrkC receptors is neuroprotective towards photoreceptors in models of experimental photoreceptor degeneration (Harada et al., 2000). MG actively support retinal neurons by supplying glucose through breakdown of glycogen particles stored within MG. In the rabbit retina, which is primarily avascular, MG are important for supplying glucose to the inner retina. This is evident in the high density of glycogen particles (Magalhaes and Coimbra, 1972) and the high rate of glycogen synthesis (Magalhaes and Coimbra, 1970) and breakdown (Magalhes and Coimbra, 1972) in the basal regions of MG.
MG are critical for normal visual processing in the retina. Synapses and neuronal excitability are directly effected by levels of free neurotransmitter and ions in the extracellular space. MG regulate glutamate by uptaking it at the synapse through glutamate transporters. Glutamate is then converted to glutamine by glutamine synthetase for transport back into neurons, which recycle it back into glutamate (Pow and Robinson, 1994). The effects of extracellular uptake of glutamate by MG are clear in the developing retina. During early stages of synaptogenesis levels of free glutamate in the extracellular space are high. After the onset of glutamate transporter expression in MG levels of extracellular glutamate quickly decline to their mature levels (Haberecht and Redburn, 1996). The induction of glutamate regulation by MG is likely induced by the release of glutamate from neurons since cultured MG isolated from photoreceptors will not induce glutamine synthetase expression, however, co-cultures will induce glutamine synthetase expression (Morris and Moscona, 1970; Prada et al., 1998). MG may also be responsive to local neuronal signaling via other neurotransmitters since they express a number of neurotransmitter receptors, including glutamatergic (Uchihori and Puro, 1993), purinergic (Bringmann et al., 2002), dopaminergic (Muresan and Besharse, 1993), glycinerergic (Cunningham and Miller, 1980) and GABAergic (Reichelt et al., 1997; Biedermann et al., 2004) receptors, and have been shown to respond to GABA and ATP application by increased intracellular Ca\(^{2+}\) levels (Bringmann et al., 2002; Biedermann et al., 2004).

Careful regulation of ionic concentrations is critical to optimal neuronal function. K\(^{+}\) concentrations in the retina are buffered by MG via siphoning, a process in which MG uptake K\(^{+}\) in the plexiform layers and then dump it into the perivascular space (Newman et al., 1984). Uptake of K\(^{+}\) is mediated by Kir2.1 channels on fine MG processes.
associated with synapses, while the outflow of \( \text{K}^+ \) into the perivascular space at MG endfeet is mediated by Kir4.1 channels (Kofuji et al., 2002).

MG also support photopigment recycling in cone photoreceptors. Salamander eyecups isolated from the retinal pigment epithelium demonstrate photopigment recovery in cone photoreceptors, albeit at a decreased rate, demonstrating that an activity present in the retina is capable of recycling photopigment in cones. Furthermore, the ablation of MG in these preparations using glial specific toxins leads to a complete loss of photopigment recycling in cone photoreceptors (Wang et al., 2009), indicating that the photopigment recycling properties of the retina were carried out specifically by MG. It is thought that in order to carry out photopigment recycling, MG uptake all-trans retinol from photoreceptors and convert it into 11-cis retinol. Cone photoreceptors, but not rod photoreceptors, can convert 11-cis retinol back into 11-cis retinal (Wang et al., 2009) explaining why MG can only aid cone photoreceptors in photopigment recycling.

(ii) Function of MG in the developing retina

During development of the retina, MG are important for cellular organization. Dissociated retinal cultures will only form normal laminae in the presence of MG (Germer et al., 1997). Disturbance of MG in vivo during retinal development by either administering the gliotoxin DL-\( \alpha \)-aminoadipic acid (Rich et al., 1995) or through transgenic overexpression of hBcl-2 in MG leading to their eventual death (Dubois-Dauphin et al., 2000), causes translocation of photoreceptors across the outer limiting membrane. These translocations, termed rosettes, occur variably across the retina and appear more frequently in regions with greater MG disturbances, particularly where adherens junctions between MG process in the OLM are disturbed (Rich et al., 1995).
Neuronal misplacements in the inner retina occur in the *mind bomb* mutant where MG fail to generate due to a loss of proper notch-delta signaling. In these mutants, retinal ganglion cells and amacrine cells are misplaced vertically, and the photoreceptor mosaic is disturbed with like photoreceptors clustering near one another (Bernardos et al., 2005). It should be noted however, that notch signaling and not MG may be an important factor in causing the observed defects in *mind bomb* mutants. Nonetheless, several experiments demonstrate that MG play an important role in organizing the retina early on in development. MG may also play a role in orienting retinal neuron lamination as their end feet promote retinal ganglion cell axons in culture (Bauch et al., 1998; Stier and Schlosshauer, 1998), while their soma inhibit axonal growth and promote dendrite development (Bauch et al., 1998). This is consistent with the orientation of retinal ganglion cells relative to the soma and end feet of MG. Overall, MG have many important functions for retinal organization during development.

Although MG are involved in the organization of retinal layers during development, it is currently unknown whether MG are actively involved in the formation of proper synaptic circuits as has been demonstrated for astrocytes and Bergmann glia in the brain (Muller and Best, 1989; Iino et al., 2001; Nishida and Okabe, 2007; Lippman et al., 2008). Furthermore, a careful study of the timing of MG morphological development relative to synaptogenesis has not been carried out. The onset of immunoreactivity for glutamine synthetase in MG appears around the time that ribbons can be observed in the OPL (Prada et al., 1998), suggesting that MG differentiation at least lags synaptic development. I therefore propose to characterize the developmental time course of MG relative to the formation of synapses in the outer retina.
Previous experiments examining early MG morphology have been plagued by difficulties in unambiguously labeling these cells. Golgi staining of the retina early in development leads to many cells that appear similar to MG with a bipolar morphology that spans the entire width of the retina since progenitor cells and immature neurons take this shape (Ramon y Cajal, 1960; Morest, 1970). Similarly, immunolabeling techniques that unambiguously label MG, are dependent on cellular maturation, and all known markers of MG that label the entirety of the cellular components are not expressed until after synaptogenesis begins (Prada et al., 1998). In contrast, MG morphology is observed beginning at very early time points using the \textit{gfap}:GFP transgenic zebrafish line (Bernados and Raymond 2006). Using this transgenic background to unambiguously identify MG from early stages of development in conjunction with \textit{in vivo} time lapse imaging the formation of neuronal arbors in the outer retina, I will be able to determine the spatial interactions between retinal glia and neurons while circuits are being established. Furthermore, by applying recent advances in single cell ablation techniques (Huckfeldt et al., 2009) I can directly assess the effects of locally removing MG from the retina while circuits are being formed (Chapter 4). Targeted cell ablation will help separate the effects of MG on general retinal organization versus positional effects on local synapse formation and maintenance.

**Significance of this work**

A detailed description of how a single neuronal cell type progresses from genesis to integration within a mature circuit is currently unavailable. Such knowledge could be important for understanding the continuity of developmental events in neuronal
development and how early events impact later events in maturation. In my thesis I will focus on horizontal cells, specifically: (i) How they are generated; (ii) How they form specific circuits; and (iii) How interactions with glial cells may influence the formation and stabilization of their newly formed circuits.

In Chapter 2, I will characterize a unique population of neural progenitor cells that divides in regions intermixed with differentiating neurons. Using *in vivo* time-lapse imaging of transgenic zebrafish, I will be able to unequivocally identify the progeny of this unique progenitor pool, and determine the importance of this secondary zone of neurogenesis in the development of the retina. Results from these experiments could have implications for other secondary zones of neurogenesis throughout the nervous system.

In Chapter 3, I will determine how HC s form contacts with their presynaptic partners. While circuit refinement during development has been observed by electrophysiology, and dynamic rearrangements of the dendritic arbor occur during neuronal circuit development, the outcome of these rearrangements upon mature circuit composition is currently unknown. These experiments will be the first to examine the role of dendritic arbor rearrangements in circuit development, by observing dendritic morphological development in conjunction with the entire cohort of presynaptic inputs.

In Chapter 4, I will first determine the developmental time course of MG maturation relative to synapse formation in the outer retina. Such a comprehensive study has yet to be conducted, even though numerous examples of glial cells impacting synaptic development have been described. Furthermore, I will remove MG from regions of the retina where synapse formation is underway and examine how local deletion of glial cells impacts the formation of outer retinal circuits.
In summary, using *in vivo* imaging approaches, transgenic methods and laser ablation techniques I will examine many aspects of the formation of a single circuit during development in order to create a unified understanding of how a class of neuron progresses from a progenitor cell to a neuron integrated into a functional circuit, and forms intimate associations with nearby glial cells. The results of this study will contribute to the understanding of the roles of alternative modes of neurogenesis in the formation of neuronal populations, how precise connections arise in a well defined neuronal circuit, and whether astroglial cells are critical for the development and/or maintenance of the newly formed circuitry.
Ventricular (apical surface) of epithelium

DNA synthesis

<table>
<thead>
<tr>
<th>G1</th>
<th>S</th>
<th>G2</th>
<th>Mitosis</th>
</tr>
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Arrows indicate the progression through the cell cycle.
In the classical scheme of neurogenesis, neural progenitor cells cycle their soma from the apical to basal surface while maintaining processes connected to both the apical and basal limiting membranes. Cell cycle phases (indicated at the bottom) correspond to the location of the progenitor cell soma location along the apical-basal axis. In this example of asymmetric division, after mitosis the neuronal daughter cell loses its attachments to the apical and basal surface and migrates basally, while the progenitor daughter cell maintains its connections to out surfaces and will subsequently undergo another cycle of division.
<table>
<thead>
<tr>
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<th>Reporter</th>
<th>Cell Type</th>
<th>Chapter</th>
<th>Reference</th>
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<tr>
<td>ptfla:GFP</td>
<td>GFP</td>
<td>Amacrine and horizontal cells</td>
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<td>Godinho et al., 2005</td>
</tr>
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<td>pax6:MCFP Q01</td>
<td>MCFP</td>
<td>Ubiquitous</td>
<td>2</td>
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<td>3</td>
<td>Fadool, 2003</td>
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<td>GFP</td>
<td>UV Cone photoreceptors</td>
<td>3</td>
<td>Takeuchi et al., 2003</td>
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<td>MYFP</td>
<td>Reporter line</td>
<td>4</td>
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</table>


Table 1: List of transgenic zebrafish used in this thesis

ptfla is pancreas transcription factor 1a, Xops is *Xenopus* rhodopsin, *SWS1* is short wavelength selective 1, TαCP is cone promoter transducin α, gfap is glial fibrillary acidic protein, UAS is upstream activating sequence, GFP is green fluorescent protein, MYFP is membrane targeted yellow fluorescent protein.
In this comparison of zebrafish and mouse retinal development it is clear that development progresses rapidly in the zebrafish relative to the mouse. Progenitor cells are indicated in gray, photoreceptors in yellow, bipolar cells in green, horizontal cells in blue, amacrine cells in purple and retinal ganglion cells in red. The inner plexiform layer and outer plexiform layer are indicated in gray.
Figure 3: Schematic of horizontal cell-photoreceptor connectivity in the zebrafish retina

The connectivity patterns of the four zebrafish horizontal cell (brown) subtypes are depicted. H1s make contact with red, green and blue-cone photoreceptors. H2s make contact with green, blue and UV-cone photoreceptors. H3s make contact with blue and UV-cone photoreceptors. Rod HCs make contact with only rod photoreceptors. Connectivity patterns taken from Li et al., (2009).
References


CHAPTER 2

NONAPICL SYMMETRIC DIVISIONS UNDERLIE HORIZONTAL CELL LAYER FORMATION IN THE DEVELOPING RETINA IN VIVO
Abstract

Symmetric cell-divisions have been proposed to rapidly increase neuronal number late in neurogenesis, but how critical this mode of division is to establishing a specific neuronal layer is unknown. Using *in vivo* time-lapse imaging methods, we discovered that in the laminated zebrafish retina, the horizontal cell (HC) layer forms quickly during embryonic development upon division of a precursor cell population. The precursor cells morphologically resemble immature postmitotic, HCs and express HC markers such as ptf1a and Prox1, prior to division. These precursors undergo non-apical symmetric division at the laminar location where mature HCs contact photoreceptors. Strikingly, the precursor cell-type we observed exclusively generates HCs. We have thus identified a dedicated HC precursor, and our findings suggest a novel mechanism of neuronal layer formation whereby the location of mitosis could facilitate rapid contact between synaptic partners.
Introduction

In many regions of the central nervous system (CNS), neurons with similar structure and function are distributed into layers (Sanes and Yamagata, 1999; Wong and Ghosh, 2002). This is particularly evident in the neocortex, the hippocampus, cerebellum and retina. Because disruption of neuronal layers results in perturbed function, much work has focused on understanding the cellular and molecular mechanisms responsible for localizing neurons to their appropriate spatial configurations (D'Arcangelo et al., 1995; Chae et al., 1997; Gonzalez et al., 1997; Howell et al., 1997; Yoneshima et al., 1997).

The formation of neuronal cell layers is often considered to largely depend on mechanisms that regulate the migratory paths of cells after they exit the cell-cycle. This is because neurons are known to be generated in regions of cell proliferation distant to their final locations. For example, in the neocortex, progenitors undergo terminal mitosis either at the ventricular (apical) surface or at a short distance away, in the so-called subventricular zone (SVZ)(Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Newly generated post-mitotic cells then migrate significant distances to reach their final destination in one of the cortical layers. Thus, our current view of how neuronal cell layers are formed is that terminal mitosis occurs prior to cells arriving at their laminar locations. In contrast, we show here that a population of retinal cells undergo mitosis within their final laminar location, and in doing so, rapidly produce a layer of cells at their appropriate synaptic location.

The vertebrate retina is a laminated structure comprising five major neuronal classes (Fig 1A). Each of these major cell classes is generated from multipotent progenitor cells that undergo mitosis at the apical surface (Turner et al., 1990). Thereafter, like cortical neurons,
newly generated retinal cells migrate to laminar positions characteristic of their final phenotype (Fig 1A). While this sequence of cell generation and placement is true for some retinal neurons, the appearance of mitotic figures outside the apical surface of the retina (Rapaport et al., 1985; Robinson et al., 1985, Rapaport and Vietri, 1991; Smirnov and Puchkov, 2004) raised the possibility that retinal neurons may also be generated near their final destinations. Because static observations do not enable us to readily determine how the non-apical divisions contribute to the generation of a specific neuronal population or their layer, we examined the pattern of neurogenesis in the developing retina of zebrafish, a vertebrate whose rapid development and transparency has proven useful for in vivo time-lapse imaging studies. Using genetic-labeling and imaging approaches we focused on the development of horizontal cells (HCs), neurons that are born early but form their lamina in the outer retina relatively late in development (Hinds and Hinds, 1979; Schnitzer and Rusoff, 1984; Young, 1985). Taking advantage of genetic constructs that label HCs and their precursors during development in vivo, we obtained a global view of how the HC layer is generated, and discovered the presence of a precursor cell dedicated to generate this retinal subclass.

Materials and Methods

Immunocytochemistry

Embryonic zebrafish were fixed and processed for immunohistochemistry as previously described (Godinho et al., 2005). To visualize cells undergoing mitosis, cryosections were incubated overnight in rabbit anti-PH3 (1:1000, Upstate cell signaling solutions) rabbit anti-pax6 (1:200, Covance) and rabbit anti-prox1 (1:200, Chemicon) and then in goat anti-rabbit Alexafluor 568 (1:1000, Invitrogen) for 1 hour before being
cover-slipped in Vectashield (Vector Laboratories). To visualize general retinal architecture, Alexa Fluor 633 Phalloidin (1:50; Invitrogen) was included in the primary antibody incubation. Confocal image stacks were acquired on an Olympus FV1000 or FV500 with a 60X (NA 1.4, Olympus) oil objective. Counting of PH3 positive and GFP expressing cells was performed on 20 μm thick cryosections, in the dorsocentral retina using Amira 3D imaging software (Visage Imaging). Sample volume was estimated by multiplying the section thickness, by the width of the sampled image and the distance from the IPL to the apical surface of the retina.

**Transient expression of fluorescent reporters in HC precursors**

A Cx55.5:Gal4-VP16 driver (Shields et al., 2007) and UAS:MYFP reporter (Koster and Fraser, 2001) were co-injected into fertilized eggs at the one cell stage from crosses of either wild-type fish or one of two stable transgenic lines; pax6:MCFP Q01 as previously mentioned or pax6:MCFP Q02 where a subpopulation of amacrine cells are CFP-labeled (Godinho et al., 2005). To define the retinal location of FP-expressing HCs in a wild-type background, embryos were stained with the vital dye BODIPY Texas Red as previously described (Godinho et al., 2005).

**Live imaging and image analysis**

Embryos were prepared for *in vivo* imaging as previously described (Kay et al., 2004). Confocal or multiphoton image stacks were acquired on an Olympus FV500 confocal microscope, or a custom-built two-photon microscope respectively, using a 60X (NA 1.1, Olympus) long working distance water objective with correction collar. Image analysis, which included rotations and 3D reconstructions, was carried out using Metamorph (Medical Devices) and Amira software. Adobe Photoshop CS was used to
Tracking of individual HCs in the ptf1a:GFP transgenic background using 4D reconstructions was carried out with Amira software, where image stacks (51-67 optical planes, n=3 animals) could be freely rotated about in 3D space. Cells were tracked visually across time points relying on variability in GFP intensity to differentiate between neighboring cells. Labels and pseudocoloring were made using the paintbrush tool. The orthoslice tool was used to digitally remove the amacrine cell layer allowing for clearer visualization of the HC layer.

Distances over which HC daughter cells migrated apart were measured using Metamorph. First, maximum intensity projections were used to calculate the lateral distance (LD) between the daughter cells. Second, the distance in z-depth (ZD) between the cells was measured by taking into account the number of optical planes between the cells and the z-step size. Migration distance was then calculated as the square root of the sum of (LD)^2 and (ZD)^2.

**Results**

Mitosis occurs at distinct depths of the developing retina

To ascertain the location of mitoses during the period of neurogenesis in the developing zebrafish retina, we used antibodies directed against a phosphorylated form of histone H3 (PH3), a marker of cells in the M/late G2-phase of the cell-cycle (Hendzel et al., 1997). As expected from previous studies (Jensen et al., 2001; Das et al., 2003), early in the period of retinal neurogenesis, mitotic cells were located exclusively at the apical surface (data not shown). However, as retinal laminae become discernible, mitotic cells were detected both at the apical surface and in the inner nuclear layer, INL (Fig 1B; see also
Schmitt and Dowling, 1999). When PH3 immunolabeling was carried out on retinal sections from a transgenic fish line in which pancreas transcription factor 1a (ptf1a) regulatory elements drive GFP expression in amacrine cells and HCs (Godinho et al., 2005), it was evident that the INL mitotic profiles were localized primarily outside the amacrine cell layer (Fig 1B). Unexpectedly, we observed that some GFP expressing cells in the outer INL where HCs are labeled, were also PH3 positive (Fig 1B, arrow). This observation suggests that INL divisions may contribute to the generation of HCs.

In order to compare the extent and time-courses of INL and apical divisions, we quantified the density of PH3 positive cells located at the two division zones (Fig 1C). Apically-dividing cells declined steadily by the third day postfertilization (dpf), whereas INL divisions transiently peaked at 40-50 hpf. Prior to 50 hpf in the ptf1a:GFP line, a large fraction of PH3 positive cells in the INL were not GFP positive, but thereafter, GFP expressing cells contributed to the majority of the PH3 positive population.

Time-lapse imaging reveals non-apical divisions involve a HC precursor

In order to directly assess whether divisions in the INL produce HCs, we used Connexin 55.5 (Cx55.5) promoter elements to drive expression of membrane targeted yellow fluorescent protein (MYFP) specifically in HCs (Shields et al., 2007). Injection of the plasmid into fertilized zebrafish eggs resulted in sparse labeling of cells, enabling us to follow detailed changes in neurite morphology of individual cells over time. The laminar location of Cx55.5:MYFP-labeled cells in the outer margin of the INL and their mature morphology suggested that they were H1/2 HCs (Fig 2b) (Song et al., 2008; Li et al., 2009). To visualize the location of the YFP-expressing HCs, we either injected plasmids into fertilized eggs from a transgenic line in which all cell membranes are labeled by cyan
fluorescent protein (MCFP) pax6:MCFP Q01 or into embryos counterstained with BODIPY Texas Red methyl ester, a vital cell membrane marker (Godinho et al., 2005), prior to imaging.

We detected some Cx55.5:MYFP labeled cells which possessed a morphology reminiscent of immature HCs, at the inner margin of the INL (Schnitzer and Rusoff, 1984) (data not shown). Upon migrating towards the OPL, these YFP-labeled multipolar cells then assumed a rounded morphology with a concomitant loss of neurites, characteristic of cells undergoing mitosis (Fig 3, Movie 1). We followed the fate of isolated Cx55.5:MYFP labeled cells (n=20 cells; n=14 fish) by time-lapse imaging over the course of 50-75 hpf. Half (n=10 cells) of all the YFP-positive cells that were followed underwent mitosis. The remaining YFP-positive cells were present in pairs and located at the margin of the ONL and INL at the first time-point, suggesting that they too might have previously undergone a division prior to imaging. Following mitosis, daughter cells migrated apart laterally (between 19-31 µm, 5-6 hours following mitosis, n=3 pairs of HCs), but always maintained their laminar location at the outer INL where both cells later assumed the characteristic morphology of HCs (Fig 3A). Time-lapse at frequent intervals (every 10 min) demonstrated how rapidly (within minutes) division of Cx55.5:MYFP labeled cells can occur (Fig 3B). Whether each division produced daughter cells of the same subtype of HC could not be ascertained. Nevertheless, the high packing density of GFP-expressing HCs in the ptf1a:GFP line (Fig 1B; Fig 2B) suggests that it is likely that more than one subtype of HC is generated by INL divisions.

INL division of HC precursors gives rise to a complete layer of HCs

Although we observed many examples of dividing HC precursors using the Cx55.5 promoter, the few cells we could follow per eye did not provide a sense of how extensively
these divisions contributed to the formation of the HC population or layer. We thus performed
time-lapse confocal or multiphoton imaging of the ptf1a;GFP transgenic line from a stage
when there were few HCs at the OPL until a complete monolayer of HCs emerged. We
observed many GFP-positive cells in the inner margin of the INL migrating vertically towards
the OPL and the majority underwent division in the outer INL, at or near the OPL (Fig 4;
Movie 2). Mitoses in the outer INL occurred along random orientations, unlike divisions at the
apical surface (Movie 2; (Das et al., 2003). Within a field of view, mitosis could account for
approximately 87% of HCs (n=3 fish) that eventually populated that region. This was obtained
by dividing twice the number of mitotic profiles (n1=20, n2=27, n3=33 mitotic cells) observed
during the recording period by the total number of HCs in the field of view at the final time-
point (n1= 46; n2= 61; n3=78 HCs). This is a conservative estimate as mitotic figures were not
always present to mark a division because some cells divided more rapidly than the frequency
with which we collected images. Cell division may also have occurred in dense clusters of cells
where single cell bodies could not be readily resolved. By tracking individual precursors at
more frequent time intervals (every 25 minutes), we found that all 22 cells that could be
followed in one recording divided in the INL, at or near the OPL (Fig 4). The progeny of these
mitoses remained within the outer INL, suggesting that they became HCs (Movies 2 and 3).
Thus, mitosis within the INL is likely to be the primary mechanism producing the layer of HCs
in the zebrafish retina.

Our time-lapse experiments also showed daughter HCs, and in some instances HC
precursors, almost always migrated laterally at the interface of the OPL. The progeny of
many divisions at the outer INL migrated out of the field of view during time-lapse
recordings (Movie 3). HCs also migrated laterally into the field of view, presumably after
division outside (Movie 3). While we did not observe a daughter cell divide, this may have occurred outside the imaged area or at a much later time-point.

**HC precursors express progenitor cell markers**

To determine the phenotypic characteristics of the HC precursors, we immunostained retinas from 2-3 dpf *ptf1a*:GFP fish with antibodies against Pax6 and Prox1 that mark progenitor cells. In zebrafish, Pax6 is initially expressed in all neuroepithelial cells and at maturity is present in ganglion cells, amacrine cells and dividing Müller cells (Bernardos et al., 2007). We found that at 2-3 dpf, GFP expressing HC precursors were immunoreactive for Pax6 (Fig 5A). The absence of Pax6 immunoreactivity in some GFP positive cells in the outer INL at 57 hpf could reflect that these GFP positive cells are not HC precursors but rather post-mitotic HCs.

In the mouse retina, Prox1 expression is initiated in progenitor cells prior to the genesis of HCs and amacrine cells; it is upregulated in late S phase as cells enter the G2 phase and downregulated as cells exit the cell cycle (Dyer et al., 2003). Prox1 expression is then later upregulated in mature mouse HCs, some amacrine and bipolar cells. In the *ptf1a*:GFP retina, we found examples of GFP positive mitotic figures that were also Prox1 positive (Fig 5B). Some, but not all, GFP expressing cells that resembled HC precursors were immunolabeled for Prox1. Presumably, the Prox1 immunoreactive HC precursors may have been just entering the G2 phase of the cell cycle. Like mice, we also found that Prox1 is expressed by a large subset of HCs located at the OPL at later ages (63 hpf; data not shown). These patterns of Prox1 labeling match that described previously for the embryonic mouse retina (Dyer et al., 2003). Thus, HC precursors express at least two key progenitor cell markers.
Figure 1: Cell division occurs at two locations in the embryonic zebrafish retina

(A) Schematic showing typical morphology of progenitor cells and location of cell division in the vertebrate retina. A neuroepithelial cell attached to the apical and basal surfaces translocates to the apical surface where mitosis occurs. This form of division is thought to directly give rise to all major cell-classes of the retina. GC = ganglion cell, Am = amacrine cell, Bp = bipolar cell, HC = horizontal cell, ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer.

(B) PH3 immunoreactive cells are present at the apical surface (arrowheads) and INL of a 45 hpf retina. PH3 labeling in this retinal cross-section from a ptf1a:GFP transgenic fish reveals that some GFP expressing cells are PH3 positive (arrow), whereas others are not (asterisk). Cell membranes were labeled by phalloidin staining. Inset shows a higher magnification of the GFP-expressing mitotic figure.

(C) Density distributions of all PH3 positive cells within the INL or at the apical surface of the retina as a function of age. A plot of the densities of GFP expressing cells that were also PH3 positive is presented. PH3 positive cells were imaged within the dorsal-central retina across ages. The data was derived from 4-6 sections from 3-6 fish at each time point (33-63 hpf).
Figure 2: Labeling of zebrafish HCs in vivo

(A) Cells with HC morphology express GFP in the ptf1a:GFP transgenic zebrafish.
Frozen section of a 7dpf ptf1a:GFP zebrafish retina.

(B) Cells with HC morphology are transiently labeled by M-YFP driven by the Cx55.5 promoter. Cx55.5:Gal4VP16 and UAS:MYFP plasmids were co-injected into fertilized eggs from a transgenic line expressing CFP in a subset of amacrine cells pax6:MCFP Q02.
Figure 3: Cx55.5:MYFP labeled HC precursors produce HC-like daughter cells

(A) A single Cx55.5:MYFP-labeled cell divides near the OPL (pseudocolored in red for these 3D-reconstructions). The first frame shows the location of the cell in the background of BODIPY-Texas Red labeling.

(B) Cell division is evident upon imaging at more frequent intervals. Shown here are a subset of time-frames of the recording.

All time points are in minutes.
Figure 4: Division of HC precursors generates the HC layer

Multiphoton time-lapse imaging of cell division in the mid-peripheral retina of a
ptf1a:GFP transgenic fish. Shown here are the 3D reconstructions of image stacks at
selected time-points (see movies for full recording). Sideview shown here is at 52 hpf;
the orthogonal rotation of the image stack, viewed from the top (with amacrine cell
profiles digitally removed; see Experimental Procedures) is shown below. Subsequent
images are top views of the HC layer as it forms over time. Cells are pseudocolored in
blue prior to division, in green during M-phase and red shows the daughter cells after
division. In the final time-point shown here (64 hpf), grey cells that populated the HC
layer arrived by lateral migration (Movies 2 and 3). A large proportion of daughter cells
that were generated eventually migrated laterally out of the field of view.
Figure 5: HC precursors express progenitor markers

(A) Immunostaining of a retinal cross-section from a ptf1a:GFP zebrafish for Pax6, a neuroepithelial cell marker. Example of a HC precursor-like GFP positive cell that expressed Pax6 (arrow).

(B) Immunolabeling for Prox1 demonstrates that it is expressed in GFP positive cells during M-phase (yellow arrow) of the cell cycle. Examples of HC precursor-like GFP positive cells that expressed Prox1 (white arrows, 49 hpf). INL, inner nuclear layer.
Figure 6: *ptf1a* mRNA is present in cells with similar morphology and location as HC precursor cells

Cross-section of the eye after *in situ* hybridization using a *ptf1a* antisense probe. Arrows, location of outer plexiform layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Asterisk: labeled cell in amacrine cell layer. (1,2) Labeled cells in outer half of INL. See (Lin et al., 2004) for Methods.
Discussion

Our *in vivo* time-lapse recordings have uncovered a retinal precursor whose terminal mitotic division occurs within the INL. Furthermore, this precursor cell-type exclusively generates HCs and contributes to the production of the vast majority, if not all, HCs that form a layer in the outer retina. Reports of unipotent neuronal precursors are rare. The only other retinal cell-type for which unipotent precursors have been detected in the developing rodent (Turner et al., 1990) and in the goldfish retina (Raymond and Rivlin, 1987) are rod photoreceptors.

We report here a novel mode of neurogenesis occurring during embryonic retinal development. It differs from the patterns of neurogenesis that occur in the mature retina of many species. In the retinae of fish, amphibians and to a lesser extent in birds, multipotent progenitor cells in the ciliary marginal zone contribute to persistent neurogenesis throughout the animal’s life (Wetts et al., 1989); (Fischer and Reh, 2000). In addition, the normal teleost retina is characterized by rod photoreceptor-committed progenitors in the mature INL and ONL (Johns and Fernald, 1981);(Johns, 1982);(Raymond and Rivlin, 1987; Julian et al., 1998; Otteson et al., 2001). The precursors we describe are committed to the generation of HCs. To date, HC specific progenitors have not been reported either during embryonic retinal development or at larval or adult stages.

INL divisions are not unique to fish, and in fact have been observed in the cat and human retina, late in the period of neurogenesis (Rapaport et al., 1985; Robinson et al., 1985; Rapaport and Vietri, 1991; Smirnov and Puchkov, 2004). The progeny resulting from these INL mitoses were found to be similar to the progeny of apical mitotic divisions (Rapaport and Vietri, 1991), suggesting that INL divisions produce many retinal cell types. Indeed, our
PH3 immunolabeling of the ptf1a:GFP retina suggests that INL divisions in zebrafish may generate cell-types other than HCs. Our preliminary time-lapse microscopy observations did not reveal INL division of GFP-labeled amacrine cells or cells resembling Müller glia (Bernardos and Raymond, 2006) at the ages we observed HC precursor divisions. Future experiments with specific markers of the precursors of these cells and to those giving rise to bipolar cells or photoreceptors will be highly informative.

The HC precursors described here may share a common lineage with other cell-types and represent the final precursors in a lineage tree. Indeed, at least some HCs in the zebrafish retina are derived from progenitors that also give rise to ganglion, amacrine and photoreceptor cells (Poggi et al., 2005). However, it is also plausible that HC-committed progenitors exist early in the period of neurogenesis but fail to undergo terminal mitosis until they reach the INL. In support of this model, a lineage study using retroviruses to mark embryonic mouse retinal progenitors reported HC-only clones (Turner et al., 1990). While some of these clones composed a single HC, it is conceivable that two HCs were originally generated and one of the daughters migrated away or died, thus evading inclusion in the clone.

Rather than resembling canonical retinal progenitors with neuroepithelial processes spanning the retina, HC precursors bear a strong morphological resemblance to HCs themselves, albeit at immature ages. Strikingly, HC precursors express Pax 6 and Prox1, markers of progenitor cells, and FP:s driven by the regulatory elements of genes [ptf1a (Fujitani et al., 2006; Nakhai et al., 2007) see also Fig 6 and Cx55.5 (Dermietzel et al., 2000)] normally found in postmitotic HCs. One interpretation of these findings is that HC precursors in fish may commit to a HC fate before terminal mitosis. Indeed, the bHLH transcription
factor ath5, normally expressed by retinal ganglion cells, is also expressed by ganglion cell precursors prior to their terminal mitosis (Masai et al., 2005). The expression of ptf1a in postmitotic horizontal cells in the mouse retina raises the possibility that the HC precursor divisions we observed in fish may not occur in the mouse retina, but this remains to be determined.

The INL could be regarded as the retinal correlate of the cortical SVZ. Like retinal HC divisions, mitoses in the SVZ occur away from the apical surface and are symmetric, with both daughter cells becoming neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Both precursor cell-types express neuronal markers of differentiation prematurely [Hu proteins (Miyata et al., 2004) and TUJ1 (Noctor et al., 2004)] and share a common morphology; neither have cytoplasmic attachments to the ventricular (apical) or pial (basal) surface. However, SVZ-derived neurons migrate radially from their birth-place to one of the upper layers in the cortical plate, HCs remain in the laminar location in which they are born. This unusual co-existence, in laminar position, of HC precursors and postmitotic HCs suggests an opportunity for local signaling between these two cell populations to regulate the number of cells produced, perhaps akin to the regulation of oligodendrocyte production recently reported in zebrafish (Kirby et al., 2006). Additionally, while cortical SVZ precursors arise from divisions of VZ precursors and migrate directly from the VZ to the SVZ, HC precursors take an indirect path, migrating initially to the inner retina before arriving at the outer INL to divide.

By dividing at the future synaptic location, HC precursors are able to rapidly form the HC layer while presynaptic photoreceptors are continuing to divide and elaborate their axonal processes (Hu and Easter, 1999; Schmitt and Dowling, 1999). Having the majority of
pre- and post-synaptic cells generated and differentiate simultaneously would greatly increase the rate at which their circuitry can form. This is especially pertinent as zebrafish develop rapidly and are visually responsive within less than a day after HC precursor division is first observed (Easter and Nicola, 1996). Moreover, although small clusters of rods derived from rod precursors in the INL can migrate across the OPL, HC precursors may divide in the INL rather than at the apical surface because movement of large numbers of HCs across the OPL could disrupt elaborating photoreceptor processes.

In summary, mitosis away from the ventricular surface appears to be a common occurrence in many parts of the developing nervous system at later stages of neurogenesis. In the cortex, because divisions in the SVZ are symmetrical and produce neurons, such divisions have been proposed to be a mechanism for “neuronal cell amplification” (Kriegstein et al., 2006; Martinez-Cerdeno et al., 2006). However, the percentage of cells forming the upper cortical layers that are derived from SVZ divisions is not known. Our in vivo observations in the zebrafish retina provide direct evidence for a critical role of non-apical divisions in generating an entire cell layer. In addition, the proximity of terminal mitotic division and final laminar destination we report here for HCs, demonstrates the existence of a novel mode of neuronal layer formation by which a specific cell-type is generated in a location that could facilitate rapid synaptogenesis with its afferents.
References


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CHAPTER 3

DEVELOPMENT OF HORIZONTAL CELL-PHOTORECEPTOR CIRCUITS IN THE ZEBRAFISH RETINA
Abstract

In order to form functional neuronal circuits during development, precise contacts must be formed between specific pre- and postsynaptic elements in the background of a vast quantity of inappropriate partners. Although great advances in our understanding of how axons properly target their postsynaptic partners have been made, comparatively little is known about the role of dendrites in creating specific circuits. Recent experiments have demonstrated that directed dendritic outgrowth can be important for establishing specific connectivity patterns. However, it is currently not known how dendritic rearrangements directly contribute to synapse formation or elimination. In order to address this, it is necessary to visualize the remodeling of dendritic arbors together with the axons of the complete population of presynaptic partners. Zebrafish horizontal cell (HC)-photoreceptor circuits are well suited for such a study because HCs form well-defined contacts with specific subsets of photoreceptors. Furthermore, photoreceptors in the zebrafish possess a single, large presynaptic terminal that is easily resolved with light microscopy. We thus used in vivo multiphoton time-lapse imaging to examine how specific contact between subsets of photoreceptors and HCs are established. Our results show that HC dendritic arbors initially contain a number of erroneously placed terminals or tips that are preferentially removed during the course of development, whereas correctly placed tips are stabilized. Thus our results show that dendritic rearrangement of HCs serves to refine its circuitry with photoreceptors. HC dendritic remodeling may be critical in the outer retina because the spatial constraint of photoreceptors into a mosaic pattern and their terminals to a thin lamina together limit axonal exploration.
Introduction

During development of the nervous system, individual neurons must make a multitude of highly precise pre- and postsynaptic contacts on their dendrites, soma and axon in order to create functional circuits. This selection process takes place in the background of a plethora of unwanted neurites from other neurons and has largely been thought to primarily involve axonal targeting. Many studies in the past have thus focused on the importance of adhesion molecules such as neural cell adhesion molecules (Schmid and Maness, 2008) and immunoglobulin family members (Thiery et al., 1982; Maness and Schachner, 2007), in addition to secreted guidance molecules like ephrin ligands (Cheng et al., 1995; Reber et al., 2007), netrins (Kennedy et al., 1994; Moore et al., 2007; Round and Stein, 2007), semaphorins (Kolodkin et al., 1992; Koncina et al., 2007) and neurotrophic factors (Lykissas et al., 2007) in axon guidance and refinement. Synaptic activity has also been shown to play a role in shaping axonal branching and thus their innervation patterns (Shatz and Stryker, 1988; Buffelli et al., 2004; Yu et al., 2004; Hua et al., 2005; Favero et al., 2007).

Although axonal targeting is clearly important for establishing the appropriate circuitry during development, recent studies have implicated dendrites also play an active. For example, in the Drosophila antennal lobe, each class of projection neuron targets its dendrites to specific presynaptic glomeruli through differential Dscam splice variant expression (Zhu et al., 2006). Also, retinal ganglion cells in the zebrafish grow their dendritic arbors toward specific sublaminae in the inner plexiform layer already occupied by presynaptic amacrine cells (Mumm et al., 2006). Despite the apparent role for dendritic targeting of afferents, it is not yet well understood how specificity in
connectivity patterns is achieved for circuits in which a single dendritic arbor contacts many presynaptic neurons, and how such specificity is shaped directly by dendritic outgrowth and refinement.

Numerous studies have examined dendritic remodeling of CNS neurons during circuit assembly, but it is still unclear how such remodeling shapes synaptic connectivity patterns at the level of individual pre- and postsynaptic cells. This is because live-imaging of dendritic remodeling has mostly been examined without knowledge of the identity of presynaptic partners that are contacted at each stage of development. This limitation is due to the complexity of most CNS circuits for which afferents comprise long-range axonal projections, arising from mixed populations of presynaptic cells outside the field of view of the postsynaptic cell. A suitable model system for determining how developing dendritic arbors gain specificity in their connectivity would thus be a circuit where a limited and local population of identifiable presynaptic cells contact the postsynaptic cell, and such contacts can be readily observed throughout the time course of circuit formation and maturation \textit{in vivo}. Such a system exists in the zebrafish retina where defined subsets of photoreceptors are the only presynaptic inputs onto horizontal cells (HCs). In addition, zebrafish HC dendritic fields are small enough to be observed in a single field of view with high optical resolution \textit{in vivo}.

HC dendrites stratify in a defined layer of retinal neuropil, the outer plexiform layer (OPL), where they invaginate axonal pedicles of cone photoreceptors (red, green, UV or blue) and rod photoreceptor spherules, depending on their subtype. In teleost retinas, photoreceptors contain a single pedicle or spherule within which all synaptic contacts are made (Brancheck and Bremiller, 1984). The size and stereotypic nature of HC
dendritic invaginations into photoreceptor synaptic endings in the zebrafish makes the inference of synaptic contacts by morphological criteria fairly reliable. Also, in the zebrafish retina, transgenic lines exist to drive GFP expression across the complete cohort of rod photoreceptors (Xops:GFP, Fadool, 2003) or UV-cone photoreceptors (SWS1:GFP, Takechi, 2003). Such transgenic lines therefore enable simultaneous visualization of presynaptic photoreceptor subtypes and individual postsynaptic HCs, the latter labeled by transient expression of a different color fluorescent protein.

Here, using in vivo multiphoton time-lapse imaging approaches, we determined how a type of HC that only contacts cones and a type that only contacts rods at maturity establish their stereotypic patterns of connectivity. We found that synaptic partner choice by HC dendrites initially involves numerous contacts with photoreceptor subtypes that are incorrect. Such incorrect contacts can persist for a significant duration (1 day), but are eventually eliminated as the dendritic tip retracts from the axon terminal of the inappropriate photoreceptor type. Thus, HC-photoreceptor synaptic patterns appear to be established by the active sampling of both correct and incorrect presynaptic partners by HC dendrites during synaptogenesis, and mature connectivity patterns arise upon elimination of the incorrect contacts.

Materials and Methods

Fish Husbandry

Zebrafish were maintained in accordance with University of Washington IACUC guidelines. The transgenic line Xops:GFP was used to identify the entire population of rod photoreceptors (Fadool, 2003). The transgenic line SWS1:GFP was used to identify the entire population of UV-cone photoreceptors (Takechi et al., 2003). The transgenic...
line TαCP:GFP was used to identify the entire population of cone photoreceptors (Kennedy et al., 2007).

**Transient Expression of Fluorescent Cell Labels**

Mosaic expression of fluorophores in retinal cells was performed by injecting plasmid cDNA into one-cell stage zebrafish embryos as previously described (Schroeter et al., 2006). Briefly, a pulled glass micropipette loaded with DNA was attached to a Picospritzer II (Parker) and anchored on a micromanipulator (Narshige) for small volume microinjections. Injection pressure was set to 10 psi and durations ranged from 100 to 1,000 ms. Zebrafish embryos were arrayed in custom built siliguard molds and oriented with their cells upwards to facilitate injections. Coinjection of Cx55.5:Gal4VP16 and UAS:MCFP was used to visualize HCs (Shields et al., 2007). DNA concentrations from 5-20 ng/μl diluted in 1X Danieau's solution (58 mM NaCl, 7 mM KCl, 0.6 mM Ca(NO₃)₂, 0.4 mM MgSO₄, 5 mM HEPES, pH 6.8) with 0.1% phenol red to aid injection bolus visualization were used for injections. At 10-12 hours post fertilization (hpf), embryos were placed in 0.2 mM PTU to prevent melanophore pigmentation.

**Multiphoton Live-cell imaging**

Embryos were prepared for *in vivo* imaging as previously described (Godinho et al., 2005). Briefly, transparent embryos were mounted in molten 40 °C, 0.5% low melting point agarose (Type VII, Sigma) with 0.02% tricaine anesthesia and 0.2 mM PTU in 60 mm organotypic culture dishes (Falcon). After the agarose set for 30 minutes samples were flooded with Danieau's solution containing 0.02% tricaine and 0.2 mM PTU. Multiphoton image stacks were acquired on a custom built two-photon microscope consisting of an FV300 scanhead (Olympus) and a Tai-Sapphire tunable infrared laser.
Laser intensity was measured as it entered the scanhead and ranged from 15-100 mW depending on the experiment. 850 nm laser was used for imaging CFP and GFP. A 1.1 NA 60X water dipping objective with a correction collar was used (Olympus). Zebrfish embryos were released from agarose and returned to a 28.5 °C incubator between 5-6 or 24 hr time points.

**HC Subtype Identification**

H1/2 HCs were identified by a lack of lemellopodial processes in the INL, presence of an axon and the presence of rosettes at some of their dendritic terminals. H3 HCs were identified by the presence of lemellopodial processes in the INL directly connected to their somata, the presence of an axon and simple dendritic terminals. Rod HCs were identified by the presence of lemellopodial processes in the INL primarily connected to the somata via neuritic process that developed over time from a forked neuritic terminal (Fig 5A), the absence or retraction of an axon and simple dendritic terminals. Most H3 and rod HCs (19 of 26) were also identified by their dendritic connectivity, or lack there of, with UV-cone photoreceptors or rod photoreceptors (Fig 3).

**Data Analysis**

Images were median filtered and levels were set manually using Metamorph software (Medical Devices). Amira software (Visage Imaging) was used for 3-dimensional reconstructions and to spatially align time-lapse series. Dendritic tips were labeled using the labelfield function in Amira. Dendritic tip areas were measured by fitting a convex polygonal to a 2-D projection of the dendritic tips using Metamorph software. Digital separations were carried out manually using the labelfield function in
Amira to trace the HC from the CFP channel. Correct or incorrect placement of dendritic tips was scored by examining the orthosliced images as well as by rotating the 3-D reconstructions.

**Results**

**Developing HCs demonstrate three unique morphologies**

Studies of zebrafish and goldfish retinas have identified four main subtypes of HCs, three dedicated to cone signaling pathways (H1, H2, H3) and one specific for rod signaling (Stell and Lightfoot, 1975; Stell et al., 1982; Nelson and Connaughton, 2008; Song et al., 2008; Li et al., 2009). Initial studies in the adult zebrafish could only distinguish three morphological subtypes (Song et al., 2008) since the H1 and H2 HCs are morphologically very similar and can only be differentiated by electrophysiological recordings (Stell et al., 1982; Nelson and Connaughton, 2008) or patterns of cone photoreceptor connectivity (Stell and Lightfoot, 1975; Li et al., 2009). In the developing zebrafish, we similarly identified three morphological subtypes of HCs (Fig 1) by labeling cells *via* transient expression of MCFP driven by Cx55.5 promoter elements (Shields et al., 2007; see Methods).

As early as 3-5 days post fertilization (dpf), we could identify a class of small, axon bearing HCs with dense dendritic tips similar to adult H1/2 HCs (H1/2s, Fig 1A&B, Song et al., 2008; Li et al., 2009). These H1/2s had processes that coursed along the edge of the OPL that were slightly larger than their dendritic fields. Also apparent in our sampling of labeled HCs were HCs with larger, less dense dendritic fields that morphologically resembled H3 HCs (H3s) previously described for the adult (Fig 1C&D, Song et al., 2008; Li et al., 2009). The H3s we observed had extensive neurite
contributions to the INL in the form of lamellopodial-like processes that were usually continuous with the soma and coursed just below neighboring HCs in the INL (Fig 1C&D, arrows). Finally, we observed a larger axonless HC subtype with a relatively low dendritic tip density in the OPL that was likely the rod HC subtype (Fig 1E; Song et al., 2008; Li et al., 2009). Like H3s, rod HCs also had lamellapodial contributions to the upper INL, but these were formed at the ends of forked dendrite like processes that were not continuous with the soma (Fig 5A, arrows). Our intracellular dye-filling of individual HCs in adult zebrafish HCs showed that the lamellopodial processes are not maintained in the adult H3 or rod HCs (data not shown, see also (Song et al., 2008; Li et al., 2009). In summary, we were able to label all identified HC morphological subtypes using Cx55.5 promoter elements to drive fluorescent protein expression transiently in HCs.

The relationship between dendritic arbor size and tip density is provided for all reconstructed HCs (n=37) at 4-5 dpf in Figure 1F. H1s were identified by the presence of an axon, and lack of lamellopodial-like processes in the upper INL. H3 and rod HCs were differentiated based on the presence or absence of an axon respectively, the presence of forked neuritic terminals in the upper INL in rod HCs (Fig 5A, arrows), and in most cases (19 of 26) by their connectivity with UV-cone photoreceptors or rod photoreceptors (see below). The three morphological subtypes of HCs we classified could be approximately separated into three clusters. H1/2s clustered into a clearly distinct subset primarily based on their high dendritic tip density, and because their arbor area was smaller than most H3s and all rod HCs. H3 and rod HCs formed two overlapping clusters, both with low dendritic tips densities. Although the average dendritic area was significantly larger for rod HCs compared to H3s (p=0.0005), there was one large H3 with an arbor more than
double the mean H3 dendritic area, as well as four small rod HCs that fell within the H3 cluster.

The sparse labeling of HCs in each eye precluded quantitative analysis of the spatial relationship between the dendritic territories of HCs of the same subtype. However, neighboring H1/2 pairs were labeled in three instances, and in all cases their dendritic arbors occupied exclusive territories and their processes at the OPL border did not overlap, but instead directly abutted one another (Fig 2A). If the members of each pair comprised only H1 or H2 subtypes, this would suggest that these subtypes tile. In the one instance where neighboring H3s were labeled, they exhibited overlapping dendritic territories suggesting non-exclusive territories in this HC subtype (Fig 2B). Rod HC pairs were never observed with adjacent territories; thus it was not possible to assess their territorial overlap.

**HC subsets contact specific photoreceptor subtypes**

Having classified HCs into distinct subtypes, we next examined the *in vivo* development of the dendritic arbors and their connectivity patterns using time-lapse multiphoton microscopy. It has been shown that mature goldfish rod HCs (Stell et al., 1982) and zebrafish H3s (Song et al., 2008) form connections with a single subtype of photoreceptor, rod and UV cone photoreceptors respectively. However, a recent report suggests that mature H3s likely contact blue-cone photoreceptors as well (Li et al., 2009). What morphological changes lead to rod HCs and H3s attaining specific connectivity with rods and UV (or blue) cone photoreceptors, respectively, is unknown.

By transiently expressing MCFP in rod HCs in the *Xops:GFP* transgenic background where all rod photoreceptors express GFP, we observed that by 5dpf nearly
all rod HC dendritic tips contacted GFP expressing rod photoreceptors (Fig 3A, n=6 cells). Separating the CFP and GFP signals digitally (see Methods) revealed obvious contacts between rod HCs and rod photoreceptors (Fig 3A, right). Rod HC dendrites contacted every Xops:GFP positive rod photoreceptor within their dendritic territory (n=9 rod HCs).

In the SWS1:GFP transgenic zebrafish all UV-cone photoreceptors express GFP (Takechi et al., 2003). Single cell-stage SWS1:GFP blastulas were coinjected with Cx55.5:G4VP16 and UAS:MCFP plasmids to drive MCFP expression in HCs. At 4dpf, H3s were seen to restrict their processes to UV-cone photoreceptors (Fig 3B, n=6 H3s). Separating the MCFP HC signal from the GFP UV-cone photoreceptor signal demonstrated clear contacts between H3 dendritic tips and the base of immature UV-cones (Fig 3B, arrow).

Thus, both rod HCs and H3s form exclusive contact with their appropriate photoreceptor type by 4-5 dpf.

Rod and H3 HCs gradually refine their arbors to contact the correct photoreceptor subtypes

Rearrangement of developing dendritic arbors has been reported previously in numerous neuronal types (Wong and Wong, 2000; Wu and Cline, 2003; Mumm et al., 2006). However, to date, it has not been shown how such remodeling directly affects contact with presynaptic cells in vivo. Observing specific HCs in the background of their presynaptic partners allowed us to assess the relevance of HC dendritic tip remodeling to achieving their mature synaptic connectivity patterns.
For rod HCs (n=7), some dendritic tips already appeared stable early in HC development; such tips were usually inserted into rods (Fig 4, green arrowheads). Tips that avoided rod photoreceptors were almost always removed (Fig 4, red arrowheads). In rarer instances, dendritic tips were seen to retract from prior contact with rods (Fig 4, violet arrowheads). Newly formed tips that projected directly towards presynaptic photoreceptor terminals were frequently observed (Fig 4, blue arrowheads). Similar dendritic rearrangements were seen to take place in time lapse recordings of H3s examined in the SW1:GFP transgenic background (data not shown, n=8).

Since the time course of HC dendritic development could be followed from the period of remodeling of HC dendritic tips (Fig 4; 3 dpf) to the formation of a stable, stereotypical dendritic arbor (Fig 3; 5 dpf), we performed long duration time lapse imaging studies in order to determine how HCs reorganize their dendritic arbors relative to their presynaptic partners in order to achieve proper connectivity. Rod HCs expressing MCFP in the Xops:GFP transgenic background showed a number of incorrectly placed tips just after 3dpf (Fig 5A&B). Gradually, most tips not contacting GFP positive rod photoreceptors (Fig 5B, orange dots) retracted, whereas tips contacting GFP positive cells were primarily maintained (Fig 5B, blue dots), indicating that contacts with proper presynaptic partners were preferentially stabilized. In this example, it was clear that during the period when incorrectly targeted tips were removed, even some tips that appeared to have contacted the correct photoreceptor subtype (rods) were also eliminated (Fig 5B, green dots). We verified that tips not contacting rod-photoreceptors were aberrantly contacting cone photoreceptors, and not unlabeled rod photoreceptors, by examining a rod HC in a transgenic background where all cone photoreceptors express
GFP (TacP:GFP, Kennedy et al., 2007). At early stages, dendritic tips contacted cone photoreceptors (Fig 5C, orange arrowheads) but eventually retracted. Stable tips avoided GFP positive cone photoreceptors at all time points (Fig 5C, blue arrowheads). This experiment demonstrated that misplaced rod HC tips do form contacts, albeit transiently, with cone photoreceptors.

We then performed similar long duration time-lapse experiments for the H3-UV cone circuit by observing MCFP expressing H3s in the background of all UV-cone photoreceptors expressing GFP in the SWS1:GFP line (Fig 6A). At the onset of GFP expression in UV-cone photoreceptors the majority of H3 dendritic tips contacted UV-cones; however, a number of incorrectly placed tips were observed (Fig 6B, orange dots). Misplaced tips gradually retracted over time and were seen to form transiently throughout the time course of imaging, suggesting that the formation of erroneous contacts was not limited to the earliest stages of dendritic rearrangement. Dendritic tips contacting UV-cones (Fig 6B, blue dots) were more stable over the time course. Such stable contacts were added to the dendritic arbor at time points throughout most the 22 hr observation period. Similar to experiments examining rod HC dendritic development, some correctly placed dendritic tips retracted during the observation period (Fig 6B, green dots).

Taken together, these examples suggest that H3 and rod HCs both begin with and elaborate a number of dendritic tips that are mistargeted early in development and are gradually removed. But, these results also demonstrate that even correctly targeted dendritic tips can be eliminated.

In order to determine if the remodeling of HC dendritic arbors was common across all HCs of the same subtype we quantified the change in dendritic tip error rates
(proportion of tips not in contact with the proper presynaptic photoreceptor) across the population of cells we imaged over time. To do so was, however, not trivial because retinal maturation can vary widely from animal to animal (by a few hours even within the same clutch of animals) as well as within an eye, from central to peripheral retina. Thus, we normalized HC dendritic development to the stereotyped morphological maturation of photoreceptors within their dendritic field.

To normalize our time-lapses of dendritic elaboration to the stages of photoreceptor development we first needed to characterize the morphological development of rod and UV-cone photoreceptors. Time lapse microscopy of rod photoreceptors in the \textit{Xops}:GFP transgenic line revealed a highly stereotypical time course where rod photoreceptors begin as simple columnar cells. Their apical processes elongate and become slimmer, meanwhile their somas expand laterally, biased towards the same direction across the retina, just above the OPL (Fig 7A). UV-cone photoreceptors also begin as simple columnar cells. They develop a pedicle connected to a squat soma in the center of the ONL by a thin stalk (Fig 7B). We thus normalized samples of developing rod HCs and H3s to the stages of photoreceptor morphological development described above.

When rod HC data were normalized to rod photoreceptor developmental stages, a common time course of dendritic refinement was evident (Fig 8A). At the onset of GFP expression in the \textit{Xops}:GFP transgenic background (approximately 75 hpf) a large fraction of dendritic tips in the OPL were misplaced and did not contact rod photoreceptors. Over the course of the next 40 hr, dendritic arbor refinement led to a near perfect connectivity where rod HCs almost exclusively contacted rod photoreceptors. In
some instances, single dendritic tips remained misplaced at late stages of development (n=3 cells). This may have been due to a delayed expression of GFP in rod photoreceptors because the onset of expression was highly variable across rod photoreceptors within the same field of view (Fig 5A).

Normalization of the H3 data showed a similar course of events for H3 dendritic development (Fig 8B). H3s had high error rates at the onset of GFP expression in the SWS1:GFP transgenic background (between 78 and 96 hpf). Over the course of 40hr, dendritic arbors remodeled to the point that all dendritic tips contacted exclusively UV-cone photoreceptors. It should be noted that while it appears from this data that H3s begin with a lower rate of misplaced tips compared to rod HCs, this was primarily due to differences in the onset of GFP expression between rod and UV-cone photoreceptor transgenic lines. Since rod photoreceptor expression of GFP in Xops:GFP transgenic retinas begins prior to the onset of GFP expression in UV-cone photoreceptors of the SWS1:GFP transgenic line, our analysis of misplaced dendritic tips may have commenced relatively earlier for rod HCs than for H3s.
Figure 1: Cx55.5 promoter elements drive expression of fluorescent proteins in all documented zebrafish HC subtypes

(A&B) Examples of H1/2s labeled by co-injecting Cx55.5:G4VP16 and UAS:MCFP plasmid DNA into single cell zebrafish embryos.

(C) An H3 (grayscale) expressing MCFP directly adjacent to an H1/2 (pseudocolored pink). A lemmelopodial process is indicated (arrow).

(D) Another example of an H3. A lemmelopodial process is indicated (arrow).

(E) A rod HC.

(F) Dendritic area versus dendritic tip density for all reconstructed HCs. H1/2s (red diamonds) were identified by their morphology (see Methods). H3s (green circles) and rod HCs (blue triangles) were identified by their morphology (See Methods) and in most instances photoreceptor connectivity. Solid black symbols represent the mean, and error bars represent the S.E.M.

Scale bars = 5 μm.
Figure 2: HC subtypes show differences in territory overlap

(A) A pair of directly adjacent H1/2s. One cell has been pseudocolored pink and the other is shown in grayscale. Upon orthogonal rotation of the image stack, it is evident that their territories are not overlapping (bottom panel).

(B) A pair of adjacent H3s. The cells can be differentiated by the intensity of MCFP expression. In the rotations (bottom panel), approximations of their dendritic tip territories are displayed as ellipses. Due to extensive overlap, it was impossible to digitally separate the cells.

Scale bar = 5 μm.
Figure 3: Some HC subtypes contact specific subsets of photoreceptors

(A) Maximum projection of a portion (approximately 15 μm) of a rod HC (grayscale left, yellow right) dendritic arbor at 5 dpf acquired in the Xops:GFP transgenic line where all rod photoreceptors (pink left, red right) express GFP. In the image on the left, the CFP and GFP signals have not been separated. In the image on the right, the rod HC has been manually traced using the label field tool in AMIRA and then pseudocolored yellow.

(B) Maximum projection of a portion (approximately 10 μm) of an H3 (grayscale left, yellow right) dendritic arbor at 4 dpf in the background of the SWS1:GFP transgenic line in which all UV-cone photoreceptors (pink left, red right) express GFP. In the image on the left, the CFP and GFP signals have not been separated. In the image on the right, the H3 has been manually traced using the label field tool in AMIRA and then pseudocolored yellow. The arrow indicates a UV-cone pedicle.

Scale bars = 5 μm.
**Figure 4: *In vivo* time-lapse imaging reveals dendritic rearrangements in a rod HC**

Time-lapse recording of a rod photoreceptor (grayscale) observed in the *Xops*:GFP transgenic background (pink). Tips contacting rod photoreceptors throughout the time course are labeled with green arrowheads. Transient tips that avoided rod photoreceptors are labeled with red arrowheads. Transient tips that contacted rod photoreceptors are indicated with violet arrowheads. Newly formed stable tips are indicated by blue arrowheads.

Scale bar = 5 μm.
Figure 5: Rod HCs make initial targeting errors and gradually refine their arbors

(A) Time-lapse recording of a rod HC (grayscale) expressing MCFP in the background of all rod photoreceptors (pink).

(B) Schematic showing dendritic tip placement at each time point above relative to rod photoreceptors generated from the images in (A). Dendritic tips that are contacting rod photoreceptors are shown in blue, and misplaced dendritic tips are shown in orange. Correctly placed tips that are transient are in green.

(C) Time-lapse recording of a rod HC (grayscale) labeled in the TaCP:GFP transgenic line in which all cone photoreceptors express GFP (pink). A portion of the dendritic field is shown in digital cross section. Tips that are contacting cone photoreceptors are indicated with orange arrowheads, whereas tips avoiding cone photoreceptors and thus presumably contacting rods are indicated with blue arrowheads.

Scale bars = 5 \( \mu \text{m} \).
Figure 6: H3s make initial targeting mistakes and gradually refine their arbors

(A) Time-lapse series of an H3 (grayscale) labeled in the SWSI:GFP transgenic background with all UV cone photoreceptors expressing GFP (pink). A cross section of the arbor is shown.

(B) Schematic showing the x-y view (orthogonal rotation of the image stack) of the locations of the dendritic tips of the time-lapse shown in (A). Stable dendritic tips contacting UV-cone photoreceptors are shown in blue, transient dendritic tips contacting UV-cone photoreceptors are shown in green, and misplaced dendritic tips are in orange. The dashed lines indicate the boundaries of z-planes of the image stack from which the maximum projection is shown in (A).

Scale bar = 5 μm.
Figure 7: Photoreceptors proceed through stereotypical morphological development

(A) Time-lapse of a rod photoreceptor in the Xops:GFP transgenic line. Asterisk indicates time 0 in Figure 8A. Arrow indicates rod spherule.

(B) Time-lapse of a UV-cone photoreceptor in the SWS1:GFP transgenic line. Asterisk indicates time 0 in Figure 8B.

Scale bar = 5 μm.
Figure 8: Rod and H3 HCs proceed through similar stages of dendritic refinement

(A) Scatterplot showing the development of accurate dendritic tip placement in rod HCs. The error rate indicates the fraction of dendritic tips that were not contacting a rod photoreceptor at the given time point. Each unique symbol represents a single rod HC sample. Samples were normalized to the morphology of rod photoreceptors within their dendritic field. Time 0 is shown in Figure 7A (asterisk).

(B) Scatterplot showing the development of accurate dendritic tip placement in H3s. The error rate indicates the fraction of dendritic tips that were not contacting a UV-cone photoreceptor at the given time point. Each unique symbol represents a single H3 sample. Samples were normalized to the morphology of UV-cone photoreceptors within their dendritic field. Time 0 is shown in Figure 7B (asterisk).
Discussion

HCs develop dendritic morphologies similar to their mature form at an early age

The HC subclasses reported here were morphologically similar to those described previously in adult zebrafish retina in regards to dendritic coverage area and tip density (Song et al., 2008) despite large differences in age (5 dpf versus adult). This contrasts with goldfish horizontal cell axonal arbors which increase nearly 20-fold in size during maturation (Raymond, 1990). In addition to dendritic arbor size and tip density, the fine structure of HC dendritic tips was also developed at early ages. Song et al. (2008) described rosette clusters at the terminals of adult H1/2s, which presumably contact multiple ribbons within the cone pedicle in a tight radial array of terminals. Rosette clusters are especially evident where HCs invaginate red and green-cone photoreceptors (Li et al., 2009). We observed rosette clusters at the terminals of many H1/2 dendrites, as early as 3 dpf (Fig 1A). Other HC terminals had spiral shaped endings reminiscent of some of the synaptic lamella described in electron microscopy studies of adult goldfish HC terminals (Stell, 1967). H3 and rod HCs in the adult zebrafish have small less elaborate dendritic terminals in the adult, and we observed a similar morphology for dendritic tips at 3-5 dpf for these HCs in our experiments. Mature ultrastructure within photoreceptor terminals is also apparent as early as 3 dpf as evidenced by the presence of synaptic triads opposed to photoreceptor ribbons (Chapter 4). Taken together our data suggest that overall, HC morphology matures rapidly in zebrafish, which is consistent with the early onset of visually dependent behavior observed in zebrafish embryos (Easter and Nicola, 1996).
A major difference between adult HCs and the embryonic HCs that we observed was the pattern of connectivity between H3s and subtypes of cone photoreceptors. Given that it has recently been reported that H3s likely wire to UV and blue-cone photoreceptors (Li et al., 2009), it is puzzling that all H3s we observed connected either exclusively to UV-cone photoreceptors (n=9) or entirely in avoidance of UV-cone photoreceptors (n=1, data not shown). Since the presence of two photoreceptor contacts was based on a repeated pattern of one H3 doublet tip contacting a \textit{SWS1}:GFP expressing cone photoreceptor terminal, and the other doublet tip terminating outside of the UV-cone pedicle (Li et al., 2009), it is possible that the external tip is not in fact contacting blue-cone photoreceptors but is instead extrasynaptic. However, in our experiments we should still observe this tip to be excluded from the \textit{SWS1}:GFP terminals. Perhaps a more likely explanation is UV-cone and blue-cone photoreceptor contacts with H3s develop at different stages of maturation according to the physiological properties of these contacts.

The idea that UV- and blue-cone contacts with adult H3 dendrites are of a different nature has been proposed previously. In order to explain the existence of three electrophysiological subtypes of HCs (blue triphasic, UV triphasic and tetraphasic, Nelson & Connaughton, 2008) with presumably identical H3 morphology and connectivity (Li et al., 2009), it was postulated that while H3s may all contact both blue and UV-cone photoreceptors, the directionality of contacts may vary (see Fig 3, Chapter 1). For example, UV triphasic H3s would receive hyperpolarizing input from UV-cone photoreceptors, but their connections to blue-cone photoreceptors would primarily serve a feedback purpose. This feedback would then mediate the depolarizing response to UV light seen in tetraphasic H3s. Our observations would suggest that these presumptive
feedback circuits develop later than the primary hyperpolarizing inputs onto H3s or vice versa, and thus H3s may form contacts with only UV-cone or blue-cone photoreceptors during developmental stages depending on their subclass.

We also observed differences in developing rod HC morphology compared to adult rod HCs in that some embryonic rod HCs clearly had an axon that retracted over the course of development (Fig 5A). Subsets of Xenopus tectal interneurons also develop long axon-like projections that retract during development (Wu & Cline, 2003). In some instances, portions of these projections from tectal interneurons are retained as part of a presumed dendritic arbor, which also occurred for rod HCs axon-like projections in our experiments. Why such axon-like processes would develop is unknown. It may be that the initial development of axons is maintained as some sort of vestigial sequence of development, or that cell subtype specification does not occur until some time after morphological development begins. Because we did not include the axon terminal in all our image fields, we could not determine if axon growth or retraction occurred in concert with rod photoreceptor targeting.

Aberrant synaptic contacts during development: implications for building retinal circuits

Across almost all species studied, HC circuits are discretely divided, processing rod photoreceptor signals independently of cone photoreceptor signals (Gallego, 1971; Boycott and Kolb, 1973; Kolb, 1974; Genis-Galvez et al., 1981; Stell et al., 1982). In the mouse for which there is only a single class of HC, HC dendrites are dedicated to cone photoreceptor circuits while the axons only contact rod photoreceptors. It is thought that the HC dendritic arbor and axon terminal are compartmentalized such that there is little crosstalk between HC dendrites and axons (Verweij et al., 1999). In contrast, in teleost
retina there is a dedicated rod HC, which processes rod photoreceptor signals separately from cone photoreceptor pathways.

Maintaining specificity in synaptic contacts of rod or cone specific HCs in the retina is critical for proper segregation of scotopic and photopic visual processing. However, there is evidence that abnormal crosstalk between these two pathways can occur in retinas with degenerating photoreceptors or in retinas where cone or rod photoreceptors fail to develop (Peng et al., 2000; Peng et al., 2003; Raven et al., 2007). For example in mouse, rat and pig rod photoreceptor degeneration mutants, rod bipolar cells, which normally only form synapses with rod photoreceptors, develop ectopic synapses with cone photoreceptors (Peng et al., 2000; Peng et al., 2003). However, in these instances, because rod photoreceptors die after rod bipolar cells have formed synaptic connections, it is possible that the potential for the ability to rewire with non-preferred photoreceptor types occurs after rod bipolar cells fully mature. Indeed widespread dendritic sprouting throughout the ONL is observed during outer retinal degeneration (Marc et al., 2003; Sullivan et al., 2007), indicating that the degenerative environment may potentiate the formation of ectopic synapses. The effects of the degenerative environment are avoided in Crxp-Nrl mice where cone photoreceptors are respecified into rod photoreceptors prior to circuit formation, creating a retina where only rod photoreceptors are present (Oh et al., 2007). HCs in the Crxp-Nrl transgenic retina still form synaptic terminals that associate with presynaptic markers in the OPL, which must represent rod terminals (Raven et al., 2007). HC dendritic contacts with rod photoreceptors indicate that when no proper presynaptic input is available, erroneous contacts may serve as a secondary input. While these experiments demonstrate that
remodeling can occur following manipulations or perturbations to photoreceptors, it was unknown if during normal retinal development improper contacts between rod and cone pathways occur. Furthermore, because a single mouse HC normally contacts both rod and cone photoreceptors it may not be surprising that the dendritic arbor of mouse HCs can contact rod HCs under abnormal conditions. In contrast, because zebrafish HC subtypes are normally dedicated only to rod or cone photoreceptor circuits, we further asked whether specificity in these wiring patterns is dependent on factors that determine the 'fate' of the postsynaptic cell (rod HC versus cone HC), or whether rod and cone HCs can contact cones and rods indiscriminately during synaptogenesis.

Using *in vivo* time-lapse imaging approaches we clearly demonstrated that rod HCs are capable of forming erroneous contacts with cone photoreceptors during the course of normal development. The fact that some of these improper contacts were maintained for long periods of time shows that some of these contacts may have differentiated into 'synapses'. Likewise, H3s which only target a single subtype of cone photoreceptor by 5 dpf, had erroneously placed dendritic tips during the course of dendritic arbor elaboration. Such observations reveal an inherent ability for improper contacts to form during circuit assembly in the outer retina, and underscore the importance of dendritic remodeling in refining these circuits.

Role of dendritic remodeling in circuit refinement

The existence of inappropriate connections during development has been inferred by loss of clusters of postsynaptic proteins such as PSD95 (postsynaptic protein scaffolding protein at glutamatergic synapses,(Marrs et al., 2001; Niell et al., 2004; Morgan et al., 2008), dendritic spines (Ziv and Smith, 1996) or loss of synaptophysin
clusters (Meyer and Smith, 2006) marking presynaptic terminals in time-lapse imaging experiments. Synapse elimination has also been inferred from numerous electrophysiological recordings of many circuits in vivo at various stages in development (Shatz and Stryker, 1988; Cramer and Sur, 1995; Kamiyama et al., 2006; Kano and Hashimoto, 2009). However, to our knowledge, experiments in this chapter are the first to examine the development of a dendritic arbor relative to its entire cohort of presynaptic inputs. Our in vivo experiments demonstrated that dendrites remodel not only to select from a pool of possible presynaptic cells, but also to eliminate aberrant contacts that should not exist in the mature circuit.

Dendritic rearrangements have previously been implicated in circuit refinement. For example, in the olfactory bulb the synaptic neuropil is organized into discrete glomeruli. Mature olfactory receptor neurons of the same type project to a single glomerulus where they form synaptic connections with mitral cell dendrites. Mitral cells also target a single glomerulus with their apical dendrite. However, during development, mitral cells have multiple apical dendrites that contribute to several glomeruli until postnatal day 10 in rats (Malun and Brunjes, 1996), but they lose all but one apical dendrite during the course of development. It may be that even at this early developmental stage when mitral cell apical dendrites contribute to neighboring glomeruli, they still contact their proper presynaptic partners. This is because individual axons of olfactory receptor neurons have been observed to target multiple, rather than individual, glomeruli as late at postnatal day 12 in rats (Klenoff and Greer, 1998; Royal and Key, 1999). However, labeling of the entire population of a single class of olfactory...
receptor neuron in conjunction with partnered mitral cell dendrites would be necessary to confirm whether dendritic remodeling of mitral cells serve to remove ectopic synapses.

Another example of dendritic refinement that could lead to specificity of connectivity during development is the structural reorganization of stellate cell dendrites in the rodent barrel cortex. Stellate cells receive synapses from thalamic projection axons within discrete "barrels" that are devoted primarily to the processing of sensory information form a single whisker, and in the mature circuits stellate cell dendrites and thalamic axons are confined to a single barrel (Welker and Woolsey, 1974; Woolsey et al., 1975). But during development, stellate cell dendrites straddle multiple barrels, and through an NMDA receptor dependent mechanism (Espinosa et al., 2009) remodel their arbors in order to occupy a single barrel by approximately postnatal day 6 (Espinosa et al., 2009) to postnatal day 10 (Greenough and Chang, 1988). However, as in the olfactory bulb, the presynaptic thalamic inputs are still observed to contribute to multiple barrels as late as postnatal day 7 (Senft and Woolsey, 1991), therefore while dendritic rearrangement clearly occurs during development of stellate cells it remains possible that this rearrangement occurs in response to redistributions of axonal targeting patterns.

A third example of dendritic remodeling that correlates with circuit refinement occurs during the development of monostratified retinal ganglion cells. Mature monostratified retinal ganglion cell dendrites stratify within a sublayer of the IPL and have characteristic responses to light directly dependent on which strata their dendrites ramify. Early in development, individual retinal ganglion cell arbors have been observed to occupy the entire depth of the IPL, and electrophysiological recordings suggest that prior to dendritic stratification, retinal ganglion cells form synaptic contacts with
incorrect presynaptic bipolar cells (Wang et al., 2001). This suggests that refinement of the retinal ganglion cell dendritic arbors leading to a monostratified arbor in the IPL may be necessary to attain the mature light responses of the ganglion cells.

Despite these examples for a role of dendritic remodeling in circuit refinement, it remained unclear whether structural alterations in dendrites directly lead to circuit refinement or whether presynaptic (axonal) remodeling caused the restructuring of the dendritic arbor. In order to unequivocally demonstrate that dendritic, rather than axonal, remodeling is responsible for removing erroneous contacts formed during development, both the postsynaptic cells and all its presynaptic partners need to be visualized. In most systems this is an extremely difficult task. However, by taking advantage of the highly stereotypical and organized inputs onto zebrafish HCs, we were able to clearly demonstrate how dendritic remodeling relates to formation of a specific dendritic arbor with patterns of synaptic contacts observed at maturity. Our experiments clearly demonstrate that dendritic rearrangement of the postsynaptic cell can solely account for remodeling of connectivity required to form mature circuits between photoreceptors and HCs.

While we observed elimination of erroneous contacts, it was also interesting that we observed apparent targeting of forming HC dendritic tips towards their presynaptic partners as evidenced by the rapid transition from non-directed arbors to dendritic fields that were mainly contacting proper presynaptic partners. It should be noted however, that intervals between image acquisition in our experiments were rather large, and as such we likely did not observe many transient contacts. We attempted to acquire image stacks more frequently, but more frequent image acquisition over the long time course of
observation necessary to observe complete HC-photoreceptor circuit formation often lead to degeneration of photoreceptors most likely due to exposure to the infrared laser. However, our data clearly demonstrate that during development HCs mature from a phase of dendritic arbor growth characterized by many misplaced and transient contacts, to a later phase where the majority of dendritic tips are stable and are opposed to proper presynaptic partners.
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CHAPTER 4

DEVELOPMENT OF OUTER RETINAL SYNAPSES
IN THE ABSENCE OF MÜLLER GLIA
Abstract

Recent studies in the central nervous system (CNS) suggest an important role for astroglial cells not only in the maintenance, but also in the formation of synapses. However, there are regions of the CNS where synapses appear to form in the absence of differentiated glial cells, or without astroglia altogether. To date there are no studies that have directly visualized in vivo, the differentiation of glial cells in relationship to synaptogenesis between the neurons they contact. Thus, to further ascertain the potential role of glial contact in synapse formation and/or maintenance of nascent synapses in vivo, it is necessary to simultaneously image synaptogenesis and neighboring glial differentiation in the living animal. Outer retinal circuits are particularly amenable to such studies because of the relatively simple and highly organized connectivity between photoreceptors and their target neurons, horizontal cells (HCs) and bipolar cells, and because photoreceptor synapses are ensheathed by the processes of one type of glia, the Müller glial (MG) cell. All of these connections are contained within a thin synaptic lamina called the outer plexiform layer (OPL). Because zebrafish develop rapidly and they are relatively transparent, we conducted time-lapse in vivo imaging experiments of the zebrafish retina to: (i) Determine the time course of MG morphological development relative to the development of OPL synapses, and (ii) Performed in vivo MG ablations in order to elucidate how the lack of MG affects the formation and early maintenance of synaptic contact in the OPL. We focused on the emergence of the cone-HC synapse because this occurs prior to BC differentiation. Our findings clearly show that cone-HC synapses form before the elaboration of MG processes in the OPL. Furthermore, local
removal of MG had no appreciable effect on the stability of cone axonal pedicles or HC dendritic tips. Thus, contact from MG is not necessary for the formation and stabilization of early OPL synapses.
Introduction

Functional synapses involve not only pre- and post-synaptic neurons, but also surrounding glial cells. Although it has long been accepted that glial cells are involved in synapse maintenance (Ransom et al., 2003), recent studies have also implicated a role for glia in promoting synapse development and plasticity (Stevens, 2008). Coordinated motility of astrocytic processes and dendritic filopodia (Haber et al., 2006) has been observed to facilitate contact between glial cells and neurons in hippocampal slice cultures (Nishida and Okabe, 2007). Astrocytic contact was found to increase the lifetime of dendritic filopodia and increase the likelihood of filopodia maturing into spines (Nishida and Okabe, 2007). In the peripheral nervous system, ablation of perisynaptic Schwann cells led to a cessation of development of the neuromuscular junction, and in many cases, complete retraction of the nerve terminal at the synapse (Reddy et al., 2003). Several signaling pathways have been identified that are involved in the promotion of synaptogenesis by glial cells, including ephrin/Eph receptor signaling (Murai et al., 2003; Nishida and Okabe, 2007). These studies, across diverse circuits, have led to the current view that synapse formation and maturation is regulated by glia.

In contrast, in some circuits in vivo, the differentiation of glial cells appears to occur only after synaptogenesis has commenced. In particular, in the sensory cortex of mammals, thalamic projections innervate subplate neurons and make functional synapses during embryogenesis (Friauf et al., 1990). In the rat somatosensory cortex, subplate circuits are functional by embryonic day 18 (Higashi et al., 2002), a day or so before large numbers of cortical astrocytes are even generated (Levison and Goldman, 1993). Although there is overlap in the timing of astrocyte genesis and formation of thalamo-
subplate circuits, astrocyte differentiation occurs much later in development. In cat, astrocytes do not demonstrate many markers of cellular differentiation until more than two months after birth (Muller, 1990; Rochefort et al., 2005), long after subplate circuits are formed (Friauf et al., 1990). In addition, the expression of GFAP in astrocytes is delayed in dark reared kittens (Muller, 1990), suggesting a role for local synaptic activity in astrocyte maturation. Thus, it appears that at least in some early circuits, astrocyte differentiation occurs well after synapses are present and functional. But, subplate neurons eventually die as cortical circuits become established, raising the possibility that perhaps glial contact is only important for synapse formation and maintenance of persisting, rather than transient, connections in the nervous system.

Using the vertebrate retina as a model system, we re-examined the hypothesis in vivo that glial contact is not required for synaptogenesis or for the stability of newly formed synapses in some circuits that are maintained to adulthood. To do so, it is necessary to directly visualize synapse development and differentiation of neighboring glial cells in live animals. We focused on retinal Müller glial cells that have processes that ensheath synapses in the retinal synaptic layers, the inner and outer plexiform layers (IPL and OPL) because of the spatially confined and well-organized contacts formed between these glia and synapses.

MG are one of the last cell types generated in the retina (Rapaport et al., 2004), apparently just prior to the onset of circuit formation in the IPL (Johnson et al., 2003). Differentiation of MG is typically assessed by glutamine synthetase expression, which appears following synaptogenesis. Because extracellular glutamate is tightly regulated by mature MG, the relatively high levels of extracellular glutamate during the period of
synaptogenesis in the retina suggests that these glial cells are functionally mature only after synapses are present (Haberecht and Redburn, 1996). Indeed, onset of glutamine synthetase expression, a key enzyme in glutamate regulation, does not occur in MG until after the formation of ribbon synapses in the IPL and OPL (Prada et al., 1998). However, it is possible that other aspects of MG differentiation that are not detectable by the presence of marker proteins or require mature functions of these cells, affect synapse development. Thus, in order to determine the relationship between MG and retinal synaptogenesis, it is necessary to visualize these glial cells and the synapses they ensheath prior to the elaboration of glial processes into the future synaptic layers.

The complex circuitry of the retinal IPL makes it difficult to correlate the development of individual synapses with the differentiation of neighboring glia. However, in the OPL, synaptic connections between photoreceptors and their postsynaptic cells, bipolar and horizontal cells, are more readily identified and visualized. Thus, we determined the developmental relationship between process outgrowth of MG cells and synaptogenesis in the OPL. We determined this relationship by in vivo multiphoton time-lapse imaging of the fluorescently labeled cells in the developing zebrafish retina. Due to their transparent nature, zebrafish are ideal for such experiments because retinal cells can be observed for over a week in the intact animal, thus enabling the analysis of circuit formation throughout its entire time-course. To directly address the potential role of MG in synapse development in the OPL, we locally ablated MG in vivo before these cells extended processes into the forming OPL.

We found that the synaptic elements of cone photoreceptors and HCs develop prior to the arrival of MG processes in the OPL. Furthermore, ablation of a small number
of MG did not perturb the synapse formation and early maintenance of cone photoreceptor pedicles or HC dendritic tips in the OPL. These observations strongly suggest that outer retinal circuits involving these pre- and postsynaptic elements are established independent of glial cell contact.

Materials and Methods

Transgenic Zebrafish

Zebrafish were maintained in accordance with University of Washington IACUC guidelines. The transgenic line $Tg(gfap:GFP)^{mi2001}$ (referred to as $gfap:GFP$) in which all MG express GFP was used to visualize the full population of MG (Bernardos and Raymond, 2006). In order to visualize a complete or near-complete population of HCs, a $ptfla:Gal4VP16$ transgenic zebrafish (Parsons et al., 2009) in which most if not all HCs express the transcription factor Gal4VP16 was crossed to a pBleedingHeart $UAS:MYFP$ transgenic where cardiac myosin drives the expression of mCherry in the heart for ease of screening and all cells contain the Gal4 responsive promoter element $UAS$ driving the reporter MYFP. These zebrafish were created by coinjecting the pBleedingHeart $UAS:MYFP$ plasmid with Tol2 mRNA in order to increase the efficiency of cDNA integration (Kawakami, 2004). F0 founders were screened by expression of mCherry in the heart and $UAS:MYFP$ activity was confirmed by crossing F1 zebrafish with Gal4VP16 expressing transgenic lines. All transgenic lines were mated into the $roy orbison$ mutant background (Ren et al., 2002) to prevent iridophore formation allowing for in vivo imaging of the retina.

Plasmid Production and Cloning
$gfap$:cerulean was generated using standard cloning techniques. The GFP encoding region was excised from $gfap$:GFP (Bernardos and Raymond, 2006) using NotI and KpnI restriction enzymes. A DNA fragment encoding cerulean with NotI and KpnI restriction sites was obtained by restriction digest of a cerulean containing vector (Livet et al., 2007), and ligated with the backbone of $gfap$:GFP generated above.

To isolate the essential promoter elements of $tr\beta2$ a 1.8 kb upstream and a 2.0 kb downstream genomic fragment flanking exon1 of $tr\beta2$ were amplified from the zebrafish BAC clone, CH211-175H19 using the following primers:

-Upstream fragment:
5’-GGGGACAACTTTGTATAGAAAAGTTGGACTCTGGTGTTGAGGGGCTTT-3’
5’-GGGGACTGCTTTTTTGTACAAACTTGCACCGCATATCCGCCACTTACAC-3’

-Downstream fragment:
5’-AAAAATCGATAGGGCCACATAAGAAAGGTATTGC-3’
5’-AAAGGATCCACAGCTCATCCTTGTCCAGGTAAC-3’.

The fragments were combined with the tdTomato reporter using the Gateway based tol2kit (Kwan et al., 2007).

$SWS1$:tdTomato and $UAS$:MYFP were also generated by modifying the original constructs (Takechi et al., 2004; Schroeter et al., 2006) using the Gateway based tol2kit.

**Transient Expression of Fluorescent Cell Labels**

Mosaic expression of fluorophores in retinal cells was performed by injecting plasmid cDNA into one-cell stage zebrafish embryos as previously described (Schroeter et al., 2006). Briefly, a pulled glass micropipette loaded with DNA was attached to a Picospritzer II (Parker) and anchored on a micromanipulator (Narshige) for small volume
microinjections. Injection pressure was set to 10Psi and durations ranged from 100 to 1,000 ms. Zebrafish embryos were arrayed in custom built siliguard molds and oriented with their cells upwards to facilitate injections. \textit{gfap}:GFP and \textit{gfap}:cerulean were used to visualize MG (Bernardos and Raymond, 2006), \textit{SWS1}:tdTomato (Takechi et al., 2003) and tr2β:tdTomato were used to visualize cone photoreceptors, and coinjection of Cx55.5:Gal4VP16 and \textit{UAS}:MCFP was used to visualize HCs (Shields et al., 2007).

DNA concentrations from 5-20 ng/μl diluted in 1X Danieau's solution (58 mM NaCL, 7 mM KCl, 0.6 mM Ca(NO$_3$)$_2$, 0.4 mM MgSO$_4$, 5 mM HEPES, pH 6.8) with 0.1% phenol red to aid injection bolus visualization were used for injections. At 10-12 hpf, embryos were placed in 0.2mM propylthiouracil (PTU) to prevent melanophore pigmentation.

\textbf{Multiphoton Live-cell Imaging}

Embryos were prepared for \textit{in vivo} imaging as previously described (Godinho et al., 2005). Briefly, transparent embryos were mounted in molten 40 °C, 0.5% low melting point agarose (Type VII, Sigma) with 0.02% tricaine anesthesia and 0.2 mM PTU in 60 mm organotypic culture dishes (Falcon). After the agarose set for 30 minutes samples were flooded with Danieau's solution containing 0.02% tricaine and 0.2 mM PTU. Multiphoton image stacks were acquired on a custom built two-photon microscope consisting of an FV300 scanhead (Olympus) and a Tai-Sapphire tunable infrared laser (Spectraphysics). Laser intensity was measured as it entered the scanhead and ranged from 15-100 mW depending on the experiment. 845-860 nm laser was used for imaging cerulean/CFP and GFP, 880 nm laser was used for imaging GFP, 890nm laser was used for imaging GFP and YFP and 890-910 nm laser was used for imaging GFP and tdTomato. A 1.1 NA 60X water dipping objective with a correction collar was used.
Zebralsh fish embryos were released from agarose and returned to a 28.5 °C incubator between 6 or 24 hr time points.

**Photobleaching**

Individual MG were photobleached by scanning a small region of interest (<0.5 \( \mu \text{m}^2 \)) over the cell soma at twice normal laser acquisition intensity for 30-45 minutes as needed. Photobleaching was verified and if needed further scanning was allowed for increments of 10 min until fluorescence was appreciably diminished.

**Müller Glia Ablations**

MG were ablated using the multiphoton laser as previously described for mouse retinal HCs (Huckfeldt et al., 2009). The laser wavelength was adjusted to 750 nm to prevent photobleaching, the digital zoom was increased to the maximum and a small line scan across the apical process of the MG close to the soma was performed ten times. If the process was not severed, the line scan was repeated until changes in the signal were observed. Severed processes were indicated by either an increase in fluorescence intensity indicating damaged autofluorescent material, or loss of fluorescence indicating that the GFP containing process was no longer continuous within the scanned region. This resulted in the death of most MG observed. In instances where the MG survived apical process ablation, regeneration into the OPL was not seen. This procedure was repeated 6-8 times on adjacent MG in order to create a zone in the OPL free of MG processes.

**Immunofluorescence and Confocal Microscopy**

Transgenic zebrafish (78 hpf) were overdosed with 0.4% tricaine and fixed with 4% paraformaldehyde and 2% sucrose in phosphate buffered saline (PBS) for 20 minutes.
Samples were washed three times for 5 minutes in PBS, and then immersed in sucrose using a 5%, 10% and 15% series for 30 minutes each. Fish were then sunk overnight in 20% sucrose in PBS. Samples were frozen in OTC and 20 μm coronal sections were cut using a freezing microtome.

Immunofluorescence was then carried out. Slides were washed three times with PBS and blocked for one hour in blocking solution containing 0.3% Triton X-100 and 5% normal donkey serum in PBS. Primary antibodies against GluR2/3 (rabbit, 1:400, Upstate) and RIBEYE b (rabbit, 1:200; (Obholzer et al., 2008)) were diluted in blocking solution and incubated on slides overnight at room temperature. Following three washes with PBS, secondary antibody (goat-anti rabbit conjugated Alexa-Fluor 568, Invitrogen, 1:1000) diluted in blocking serum was applied to samples for 1 hr. Samples were washed three times and slides were coverslipped with VectaShield (Vector Labs).

Confocal image stacks were acquired on a FV1000 confocal microscope (Olympus) using a 1.35 NA 60X oil immersion objective. A multiline argon laser (488nm) and a krypton laser (568nm) were used for excitation.

Data Analysis

Images were median filtered and levels were set manually using Metamorph software (Medical Devices). Amira software (Visage Imaging) was used for 3-dimensional reconstructions and to spatially align time-lapse series. For retinal layer measurements, 2D projections of retinal layers were created in Amira by first properly aligning the 3D stacks and creating oblique slices above and below the regions of interest. Measurements were performed from these 2D projections using Metamorph software. Percent coverage was measured by manually thresholding the image and
dividing the number of pixels above threshold by the total number of pixels in the area. Photobleached and ablated areas were measured using the freeform region of interest tool. Centers of mass for MG arbors were calculated by assigning vector coordinates to each pixel in an image, and then measuring and averaging coordinates for all pixels within an arbor. Time lapse imaging of HC dendritic tips was analyzed using Amira 3D reconstructions of the cells for each time point. Dendritic tips present at each time point were first labeled using the label field tool. Then tips were determined to be present or absent in previous time points by comparing label fields across time. Dendritic tips were scored as contacting or not contacting MG processes in these 3D reconstructions. Photoreceptor invaginations were determined by both scrolling through the image stack plane by plane and by examining the 3D reconstruction. Photoreceptor – MG contacts were scored by scrolling through dual color image stacks and using 3D rotations in Amira. HC tips in the OPL were counted using the label field tool in Amira. Areas of the OPL devoid of MG processes in ptf1a:Gal4VP16; UAS:MYFP transgenic zebrafish were calculated by measuring the lateral distance lacking MG processes in each image stack and multiplying the sum by the z-plane thickness. Only areas of the OPL large enough to contain six or more HC dendritic tips (25 μm²) were analyzed.

Results

Before assessing the spatial and temporal relationships between MG cells and outer retinal synapses during development, we first characterized the morphology and distribution of MG processes in the zebrafish retina both at maturity and during development. We then asked how ingrowth of MG processes relates to synaptogenesis
and stability of newly formed synapses in the OPL. To directly assess the importance of MG in regulating synapse development in the OPL, we locally ablated small numbers of MG and performed time-lapse studies to follow the development of photoreceptor terminals and HC dendrites in the absence of the glial cells.

Processes of mature MG occupy exclusive territories within the synaptic layers

Like dendritic arbors of neurons, the processes of glial cells appear organized in their spatial distribution. For example, the processes of astroglial cells occupy distinct spatial territories that result in synapses being often contacted only by a single glial cell. In order to determine whether like astrocytes, the processes of MG in the zebrafish retina also tile, we examined the territories of MG just after they have completed arbor elaboration in all retinal layers at around 5 dpf. Single MG were visualized by injecting the gfap:GFP plasmid into one-cell stage embryos (Fig 1A). Isolated GFP-expressing MG were imaged in vivo and for each cell, the lateral extents occupied by their processes at each retinal depth (Fig 1A) were measured (see Methods).

As in all species studied to date (Ramon y Cajal, 1960; Uga and Smelser, 1973; Robinson and Dreher, 1990; Dreher et al., 1992; Prada et al., 1998), MG processes of the zebrafish retina terminate and stratify at the inner and outer limiting membranes (ILM, OLM) and the synaptic layers of the retina (Fig 1). Processes of zebrafish MG also course through the ONL, though no contributions to the INL were apparent. The lateral territories of MG were found to increase from the inner to outer retina: ILM (28.6± 3.6 μm²), IPL (62.6± 6.4 μm²), OPL (89.6± 9.1 μm²) and OLM (120.4± 8.5 μm²) (Fig 1A&C). Thus, larval zebrafish MG appear to take on a conical shape in contrast to the
cylindrical shapes of MG in other species (Ramon y Cajal, 1960; Robinson and Dreher, 1990; Dreher et al., 1992).

Having determined the spatial distribution of processes of individual MG, we next asked whether the arbors of neighboring cells overlap or formed distinct territories within each retinal layer. In the *gfap*:GFP transgenic line (Bernardos and Raymond, 2006) all MG are labeled by GFP expression (Fig 1B). By photobleaching single MG in the *gfap*:GFP transgenic line, territories occupied only by the bleached cell were unmasked by loss of GFP signal (Fig 1B). When images acquired before and after photobleaching were compared, it was evident that there were areas occupied solely by the bleached cell. These territories were similar in size to those of single MG labeled by transient fluorescent protein expression upon cDNA injection (Fig 1C). In the ILM, IPL and OPL, MG territories measured for single cells expressing GFP (n=10, 9 & 9 respectively) were similar to territories occupied by an individually photobleached MG (n=7, 6 & 4 respectively), indicating that MG territories at these retinal depths are unlikely to overlap. However, in the OLM, the territories occupied by the processes of individual GFP-expressing MG (n=11) were significantly larger (p<0.032) than those of a photobleached MG (p=7), indicating that processes of neighboring MG overlap somewhat in the OLM.

**MG tiling may be established by homotypic interactions**

In order to determine whether MG processes are intrinsically limited in their growth or establish their territories through MG-MG interactions, we ablated small patches (6-8 cells) of MG, one cell at a time using the multiphoton laser at 750 nm (See methods, Fig 2). A typical approach to unmask homotypic interactions is to perform ablations after territories are established (Gan and Macagno, 1995; Gao et al., 2000;
Grueber et al., 2003; Huckfeldt et al., 2009). However, when we attempted to ablate MG after their processes had fully elaborated in the OPL, the apical processes including arbors in the OPL and OLM remained intact for up to 3 days post ablation (dpa) despite the loss of the MG soma and removal of basal processes in the IPL and ILM. Thus, we attempted to investigate the role of homotypic interactions in the formation of mosaic MG territories by performing ablations prior to MG process outgrowth. If homotypic interactions are present, then ablation of a few MG would result in filling in by the processes of their remaining neighbors.

For most ablations performed at these early stages, surrounding cells were not obviously perturbed in their morphology (Fig 2A). However, in some instances MG processes in the OPL and OLM merged and photoreceptor somata crossed the OLM (Fig 2B); these gross perturbations were more likely to occur when a larger number of MG were ablated. Because the normal retinal architecture was significantly altered in these instances, ablations leading to merged OPL and OLM MG processes were not included for further analysis.

However, in ablations for which the OPL and OLM were maintained, time-lapse imaging revealed that the OPL was eventually completely covered by the processes of MG surrounding the ablated region (Fig 3). Comparison of the number of MG somata in the field of view indicated that no new MG cells were generated by cell division during this period of process elaboration (n=35 retinas). In addition, this filling in of OPL space is unlikely to be due to local contraction of the tissue after cellular ablation because the arbors of MG cells bordering the ablation area expanded over time (Fig 4). MG directly adjacent to the ablation zone demonstrated enlarged, biased arbors atypical of normal
MG (Fig 4A, compare with Fig 1A). These remaining MG cells had significantly larger arbors in the OPL than cells in retinas without ablations of the same age (p=0.002, Fig 4B). Furthermore, MG at the border of the ablation zone appear to extend processes towards the space previously occupied by the ablated cells. To quantify this apparent biased outgrowth across cells, we calculated the distance between the apical stalk (blue dot, Figure 4A) and the center of mass of the OPL territory (yellow dot, Fig 4A) for each cell bordering the ablation zone, and compared these distances with those obtained from MG in non-ablated retinas. The distance between the position of the stalk and the OPL arbor center of mass was significantly increased in cells bordering the ablation (p<0.02; Fig 4C). These data suggest that MG territories are not inherently restricted in size, but are likely limited by interactions with adjacent MG.

**OPL process outgrowth is the last stage of MG morphological maturation**

Previous imaging studies showed that a laminated plexus separating the developing photoreceptor layer from the inner nuclear layer is present by 45 hpf (Chapter 2). At this stage, horizontal cells undergo their final division and their processes populate this outer lamina. Time-lapse imaging of individual MG showed that at this earliest stage of OPL establishment, MG processes have not yet elaborated within this layer (Fig 5A&B) although rudimentary processes were already present in the IPL. An arbor was established about 36 hr from the initiation of outgrowth and the final size of the arbor within the OPL varied between 60-140 µm² (Fig 5D). This late filling in of the OPL by MG process outgrowth was clearly evident from time-lapse recordings of the gflap:GFP transgenic line (Fig 5C). Interestingly, MG process outgrowth within the OPL appeared synchronized amongst cells in the field of view. The time taken for individual arbors to
reach a stable size matched the time it took for the population of MG arbors to occupy a maximal pixel density in the OPL (Fig 5E).

**Synapses are present in the OPL prior to MG process infiltration**

Ultrastructural analysis has previously shown that at maturity photoreceptor pedicles are ensheathed by MG processes in the OPL (Meller and Glees, 1965; Stell, 1967). Coinjecting zebrafish embryos at the single cell stage with *gfap*:GFP and *TαCP*:MCFP plasmids to drive GFP expression in MG and MCFP expression in cone photoreceptors (Kennedy et al., 2007), revealed the intimate associations of photoreceptors and MG (Fig 6A). Membrane-bound fluorophore expression in cone photoreceptors led to bright expression in the outer segments and the synaptic pedicle. All cone pedicles well within the territory of a labeled MG were wrapped by that MG. Since we observed that MG processes elaborated in the OPL relatively late in development, we wanted to assess whether or not synaptogenesis had already commenced prior to MG process outgrowth in the OPL. For this purpose we cloned promoter elements from *thyroid hormone receptor beta 2 (trβ2)*. Fluorophore expression under the control *trβ2* promoter elements enabled the visualization of cone photoreceptors as early as 24 hpf (Suzuki et al., in preparation). At 78 hpf, tdTomato expression in cone photoreceptors revealed invaginations in cone pedicles, evidenced by a dark spot in the pedicle, prior to the arrival of MG processes in the OPL (Fig 6B). These invaginations persisted throughout the timecourse of MG process elaboration (Fig 6B).

To verify that invaginations are sites of contact with postsynaptic cells, we visualized HCs in conjunction with cone photoreceptor pedicles. We chose to observe
HCs because in the vertebrate retina, the first synaptic contacts to form in the OPL are between cone photoreceptors and HCs (Maslim and Stone, 1986). Therefore, we determined that the invaginations we observed in cone photoreceptor pedicles contained postsynaptic processes of HCs. The transcription factor ptf1a is expressed by all HCs and amacrine cells in the zebrafish retina (Godinho et al., 2005). To visualize the fine processes of HCs, ptf1a:G4VP16 transgenic fish (Parsons et al., 2009) were crossed to UAS:MYFP transgenic fish we generated. ptf1a:G4VP16; UAS:MYFP double transgenic embryos were injected with the tm2:tdTomato plasmid. In every case, HC tips were seen inserted into photoreceptor pedicles (n=48). Performing these experiments in the gfap:GFP transgenic background demonstrated that invaginating HC-photoreceptor contacts were apparent in regions of the OPL where MG processes were not yet present (Fig 6C). Thus, prior to the arrival of MG processes in the OPL, at least one postsynaptic partner, the HC, is in contact with the cone pedicle.

To ascertain whether the cone photoreceptor-HC contacts were ‘synaptic,’ we performed immunolabeling for pre- and postsynaptic proteins in the OPL. Immunostaining with antibodies directed against glutamate receptor subunits 2 and 3 (GluR2/3) revealed that all observed HC dendritic tips in the ptf1a:G4VP16; UAS:MYFP double transgenic contained GluR2/3 positive puncta (n=198, Fig 7A). GluR2/3 puncta were observed in regions of the OPL where MG processes were not present (Fig 7C). Photoreceptor synaptic ribbons were labeled using antibodies directed against RIBEYEb (Obholzer et al., 2008). At 78 hpf, prior to MG infiltration of the OPL, abundant punctate labeling of the OPL was observed using this antibody (Fig 7D). Immunostaining for RIBEYEb in ptf1a:G4VP16; UAS:MYFP double transgenic fish also showed that HC tips
were directly apposed to ribbons (Fig 7B). The presence of cone-HC synapses in the OPL at these ages was confirmed by performing electron microscopy on age matched embryos (Fig 8). In 77 hpf samples, photoreceptor pedicles contained ribbons with vesicles (Fig 8B) were directly opposed to processes of apparent invaginating HCs (green in Fig 8). At this early stage, we also observed bipolar cell dendritic invaginations under EM (purple in Fig 8); thus classical triad structures were present postsynaptic to photoreceptor ribbons (Fig 8A). Importantly, cone pedicles were directly apposed to one another (Fig 8A&B, arrows) indicating a lack of ensheathing MG processes at these ages. At 5dpf, triad synapses were apparent in photoreceptor pedicles, and were clearly ensheathed by processes, presumably of MG (Fig 8C; pseudocolored orange).

HC dendritic tips and cone photoreceptor pedicles are not stabilized by MG processes upon contact

Although we have shown the presence of synapses in the OPL prior to the arrival of MG processes, these contacts may be nascent and unstable without contact with MG. In particular, HC dendritic tips continue to be motile for some time during the period when contact is made with cone photoreceptors and are not entirely stable until nearly two days later (Chapter 3). We thus examined whether the stability of developing postsynaptic HC dendritic tips was influenced by MG process contact.

Transient labeling of single HCs using Cx55.5 promoter elements to indirectly drive expression of membrane targeted CFP (Shields et al., 2007) revealed that HC dendritic rearrangement occurred during the time course of MG elaboration in the OPL (Fig 9A). Tips that persisted for at least 18hrs and were present at the final time point
were defined as "stable," whereas tips that were observed to retract during the 48 hr time lapse were defined as "transient."

The majority of transient HC dendritic tips were not associated with MG processes (Fig 9B). Transient tips were mostly present for only a single time point (Fig 9C), and possessed an average lifetime of 9.5±0.7 hr (n=65). In contrast, most stable HC dendritic tips were contacted by MG processes or vice versa (Fig 9B). However, the average stable tip received contact from MG processes 17.4±1.6 hr after its formation (Fig 9D, n=41). Furthermore, 12 stable tips did not receive contact from MG during the course of these experiments (total duration 26.5±1.2 hr). These results indicate that MG contact is unlikely to be necessary for the stabilization of newly formed HC dendritic tips. Figure 9E summarizes our findings of the interactions of HC dendritic tips with MG schematically.

In order to clearly demonstrate that components of the developing photoreceptor-HC circuit can form and be maintained in the absence of MG processes, we performed MG laser ablations prior to process elaboration in the OPL and observed cone photoreceptor and HC process stability in regions lacking MG processes. Areas lacking MG persisted for 1-5 days after ablations (Fig 2) and regions devoid of MG processes larger than 25 μm² at all time points were analyzed.

MG ablations were performed after crossing gfap:GFP transgenic zebrafish with ptf1a:G4VP16; UAS:MYFP double transgenics in order to observe the local effects of MG removal on HC dendritic tip stability. In most samples HC dendritic tips were clearly visible in large areas lacking MG processes (Fig 10A). HC dendritic tip densities in regions lacking MG processes were unchanged relative to directly adjacent regions in the
same sample where MG were maintained (Fig 10B, p<0.72). Therefore, removal of MG processes from the OPL had no apparent effect on the maintenance of HC dendritic tips. We then examined the effects of MG process removal from the OPL on the stability of cone contacts by observing invaginations following MG ablation. UV cone photoreceptors were visualized by injecting gfap:GFP embryos with SWS1:tdTomato plasmid DNA. In patches lacking MG processes, synaptic invaginations were clearly present in labeled photoreceptors (Fig 10C). There was no significant difference in the fraction of collapsed pedicles versus invaginated pedicles between regions in the OPL with or without MG processes (Fig 10D, p<0.43). Taken together, these results strongly indicate that MG structural contributions to the OPL and contacts with neuronal synaptic elements are not required for the early development and maintenance of OPL circuits.
Figure 1: MG territories do not overlap in retinal synaptic layers

(A) Maximum projection of a multiphoton image stack encompassing a single MG at 120 hpf labeled by transient expression of GFP. Orthogonal rotations of the image stack showing MG territories at the inner and outer limiting membranes (ILM, OLM) and within the synaptic layers, the inner and outer plexiform layers (IPL, OPL), are shown in the right column.

(B) A single MG (red dot) within the population expressing GFP in the *gfap*:GFP transgenic line was photobleached using an IR laser. In orthogonal rotations of the MG territories, the coverage of the photobleached cell is displayed by pseudocoloring the prebleaching image red, pseudocoloring the signal after photobleaching grayscale and overlaying the two images.

(C) Comparisons of the territories occupied by a MG cell obtained by transient GFP expression (SC) are compared to areas revealed upon photobleaching (PB). PB territories were similar to those attained for SC except for the OLM where PB territories were significantly larger (p<0.032). Open symbols indicate individual cells. Closed symbols indicate the mean. Error bars are S.E.M.

Scale bars = 5 μm.
Figure 2: Targeted ablations of MG using an infrared laser

(A) In the *gfap*:GFP transgenic line, a patch of MG (grayscale) were ablated one at a time (magenta dots) using the infrared laser. Image stacks acquired immediately after the ablations (15 minutes, center) and 1 day postablation (1 dpa, right) demonstrate no obvious gross perturbation of the tissue, and a continuous OPL. A patch of UV-cone photoreceptors transiently labeled by expression of tdTomato are pseudocolored red.

(B) In this example, MG ablation (magenta dots) led to a disturbance of the OPL and ONL. MG (grayscale) processes in the OPL merged with their processes in the OLM. UV-cone photoreceptors (red) translocated across the OLM and eventually retracted their pedicle and outer segments.

Scale bars = 5 μm.
Figure 3: MG processes eventually fill the territories of ablated cells

A patch of 6 MG were ablated at 4 dpf (indicated by red dots) prior to significant MG process elaboration into the OPL. Orthogonal rotations of the multiphoton image stacks demonstrate the time course over which neighboring MG processes infiltrate the region where MG were ablated. Scale bar = 5 μm.
Figure 4: Homotypic interactions likely establish MG territories

(A) An 8 dpf MG cell expressing cerulean (grayscale) in the gfap:GFP transgenic background (pink) five days after an adjacent patch of six MG were ablated. An enlarged arbor biased towards the ablated region (asterisk) is apparent. In orthogonal rotations, the apical and basal stalks are marked with a cyan dot and the centers of mass are marked with a yellow dot. Scale bar = 5 μm.

(B) Comparison of MG territory in the OPL between MG located adjacent to patches of ablated MG, and normal MG at 8 dpf. Territories next to ablated patches of MG were significantly larger than age matched controls (p=0.0002). Open symbols indicate individual cells. Closed symbols indicate the mean. Error bars are S.E.M.

(C) Comparison of the distance from the apical stalk to the OPL territory center of mass between MG adjacent to patches of ablated MG and normal MG at 8 dpf. Distances were significantly larger for MG adjacent to patches of ablated MG compared to age matched controls (p<0.02). Open symbols indicate individual cells. Closed symbols indicate the mean. Error bars are S.E.M.
Figure 5: MG processes in the OPL are the last to mature

(A&B) Time course of OPL territory development for two examples of MG, visualized by transient expression of GFP. Orthogonal rotations of the image stack showing the OPL territory in lateral view are provided below.

(C) Time course of MG process infiltration into the OPL for the complete population of MG imaged from the ventro-temporal retina. Orthogonal rotations show coverage of the OPL by elaborating MG processes.

(D) Graph of OPL territory growth across multiple MG. Each data point was normalized to the age at which the cell first demonstrated a stable and maximal OPL. Open symbols indicate individual cells. Closed symbols indicate the mean. Error bars are S.E.M.

(E) Time-course of OPL infiltration obtained by imaging MG in the *gfap*:GFP transgenic line. MG process infiltration was calculated by counting the fraction of pixels above a manually set threshold. Open symbols indicate individual retinas. Closed symbols indicate the mean. Error bars are S.E.M.

Scale bars = 5 μm.
Figure 6: Cone pedicle invagination precedes MG process arrival in the OPL

(A) A single MG expressing GFP (grayscale) associated with cone photoreceptors expressing MCFP (pink) at 5 dpf. At this age, cone photoreceptor pedicles are clearly ensheathed by MG processes. Cones were labeled by injecting TαCP:MCFP plasmid into single cell stage blastulas.

(B) A pair of cone photoreceptors (grayscale) transiently expressing tdTomato at 78hpf demonstrate synaptic invaginations in their pedicles (arrows) prior to the arrival of MG processes (red) in the OPL. Orthogonal rotations clearly show that cone pedicles were not contacted by MG processes at this stage. These invaginations are maintained throughout the time course of MG process elaboration in the OPL (shown here at 120 hpf, right). Cones labeled by injecting trβ2:tdTomato plasmid into the transgenic line gfap:GFP in which MG are labeled by expression of GFP.

(C) Insertion of a HC (green in merge) dendritic tip (arrowhead) into a cone photoreceptor pedicle (red) prior to MG (blue) process elaboration in the OPL. Scale bars = 5 μm.
Figure 7: Synaptic elements are present in the OPL prior to ingrowth of MG processes

HCs visualized in the ptf1a:Gal4VP16; UAS:MYFP double transgenic line and MG are visualized in the gfap:GFP transgenic line. Immunostaining was performed on 20 μm frozen sections.

(A) HC (red) dendritic tips in the OPL contain puncta that are immunoreactive for GluR2/3 (examples indicated by arrowheads).

(B) HC (red) dendritic tips are directly apposed to photoreceptor synaptic ribbons that are immunoreactive for RIBEYE b (grayscale).

(C) GluR2/3 immunoreactivity (cyan) in the OPL precedes the arrival of MG processes (yellow). Autofluorescent signal is apparent in the OPL of frozen sections as diffuse signal throughout the OPL in contrast to the localized signal from MG processes in the OPL (Fig 1).

(D) RIBEYE b immunoreactivity (cyan) in the OPL precedes the arrival of MG processes (yellow).

Scale bars = 5 μm.
Figure 8: Immature cone pedicles are not ensheathed by MG

(A&B). Electron micrographs of the OPL at 77 hpf show the presence of ribbons associated with vesicles (B) and triad invaginating synapses. Photoreceptor pedicle plasma membranes are directly adjacent to one another (red arrowheads) indicating a lack of MG processes in the OPL.

(C) Electron micrograph of the OPL at 5dpf. Processes of presumptive MG (pseudocolored orange) ensheath photoreceptor pedicles. HC dendritic tips are shaded green, bipolar cell dendritic tips are shaded purple.

Scale bar = 1 μm.
Figure 9: HC dendritic tips are not stabilized by MG contacts

(A) Time lapse imaging series of a single HC (grayscale) in the *gfap*:GFP transgenic background (pink) where images were acquired every 6 hr for 48 hr beginning at 73 hpf. Stable tips are labeled with green arrowheads and transient tips are labeled with red arrowheads. Newly formed tips are indicated by open arrows. Scale bar = 5 μm.

(B) The fractions of transient (red) and stable (green) HC dendritic tips that were either contacted (MG+) or not contacted (MG-) by MG processes during the 48 hr observation period are plotted here. In contrast to most stable tips, the majority of transient tips were not contacted by MG processes.

(C) Lifetime of transient HC dendritic tips.

(D) Time course of when stable HC dendritic tips become associated with MG processes. Plotted on the x-axis is the time taken for MG to contact the dendritic tip after the tip first appeared during the time-lapse. Less than 20% of all observed stable tips were contacted by MG <6 hr after they had formed, whereas for some tips, contact did not occur for more than 24 hr. Twelve stable dendritic tips did not make contact with MG during the duration of the recording (36 hr).

(E) Schematic diagram illustrating HC dendritic tip stability relative to MG process infiltration into the OPL. MG processes are colored 'tan'. Stable HC dendritic tips are colored green whereas transient tips are in red.
Figure 10: MG processes are not required for HC dendritic tip or cone photoreceptor pedicle stability in the OPL

(A) Six MG were ablated at 4dpf in a triple transgenic line labeling HCs (grayscale, ptfla::G4VP16; UAS:MYFP) and MG (pink, gfap::GFP). Areas lacking MG processes are indicated with arrowheads. Rotation at the plane of the OPL shows the extent of MG process loss in the OPL in the z-axis.

(B) HC dendritic tip densities were compared within the same sample for regions where MG were absent (red dots) and regions where they were present (green dots). Lines connect measurements from the same image stack. Measurements were recorded only in regions large enough to contain at least five dendritic tips, and were pooled across all time points after ablation (1-4 days postablation).

(C) Six MG were ablated at 4 dpf in retinas where UV-cones transiently expressed tdTomato (grayscale) in the gfap::GFP transgenic line (red). UV-cone photoreceptor invaginations (dark spot within the pedicles) are indicated with arrows. Rotation at the plane of the OPL shows the extent of MG process loss in the OPL in the z-plane. Arrows indicate the same cone photoreceptors in the image above.

(D) Fraction of UV-cone photoreceptor pedicles that collapsed or remained invaginated in areas lacking MG processes (red) or where MG processes were present (green) in the OPL.

Scale bars = 5 μm.
Discussion

Using an *in vivo* imaging approach, we found that MG occupy exclusive territories in synaptic layers of the retina. MG territories are likely established via homotypic interactions and develop asynchronously, with processes in the OPL being the last to elaborate. MG processes in the OPL arrive after the formation of photoreceptor and HC synaptic structures, but during the period when HC dendritic tips are still motile. HC dendritic tip stability is not influenced by contact with MG, and removal of MG from small patches of the developing retina had no effect on the maintenance of HC dendritic tip density or photoreceptor pedicle stability. Therefore, we conclude that glial contact does not play a critical role for the formation and early stability of synaptic circuits between photoreceptors and HCs.

Factors influencing the development of MG territories

Homotypic interactions are important for the formation of tiled mosaics in developing peripheral sensory neurons of zebrafish (Sagasti et al., 2005), leech (Gan and Macagno, 1995) and *Drosophila* (Grueber et al., 2003; Sugimura et al., 2003). Migrating HCs in the mouse retina display a transient stage with exclusive territories that are thought to underlie proper positioning of somata (Huckfeldt et al., 2009). Brain astrocytes also form similar non-overlapping territories (Bushong et al., 2002), but the mechanisms by which these territories develop are largely unknown. One expected observation for a system where contact mediated homotypic inhibition sets up a non-overlapping mosaic, is that during the development of the mosaic, processes of neighboring cells should come into contact and territories should transiently overlap. During stages of contact or overlap arbors that come into contact would repel one another, and over time occupy unique
territories. In fact, experiments examining the development of neighboring protoplasmic astrocytes demonstrated that their immature processes do in fact transiently overlap (Bushong et al., 2004) lending support to the idea that homotypic interactions are important for setting up exclusive glial domains. Furthermore, rapid time lapse imaging of zebrafish oligodendrocyte precursor cells in vivo revealed that when these glial cell processes came into contact, they retracted within minutes (Kirby et al., 2006). This mechanism was interpreted to be how oligodendrocyte precursor cells came to occupy an exclusive territory and how they migrated to the proper position within their mosaic, since migration patterns were random across cells. We have demonstrated that, like protoplasmic astrocytes, MG territories are exclusive or nearly exclusive throughout the retinal synaptic layers, in contrast to previous experiments in the rabbit that observed overlapping processes of neighboring HRP labeled MG (Robinson and Dreher, 1990). That we observed expansion of MG OPL territories towards regions where neighboring MG had been removed, further supports the claim that homotypic interactions, and not cell intrinsic programs, are important for establishing glial territories. Such a mechanism for elaboration, where processes fill nearby space until meeting their neighbors, would be an efficient way to establish full coverage across the retina where MG cell density varies along a centro-peripheral gradient (Dreher et al., 1992), while maintaining exclusive territories of individual cells.

Non-overlapping territories of MG in the synaptic layers would have similar implications for local neuronal domains as tiled territories of astrocytes does in the brain. Namely, that synapses, and in this case entire neurons, within a single glial territory may experience a unique microenvironment relative to their neighbors. Because MG regulate
extracellular glutamate (Pow and Robinson, 1994) and K+ ions (Newman et al., 1984), respond to extracellular neurotransmitters (Biedermann et al., 2004), and are critical for photopigment recycling in cone photoreceptors (Wang et al., 2009), individual MG could have a profound effect on the neurons within their territories. If MG regulate the above processes differentially, the activity of MG could prove important for processing of spatial information in the outer retina. However, because individual MG likely support different subtypes of cone photoreceptors as well as rod photoreceptors due to the near crystalline photoreceptor mosaic in the zebrafish ONL (Raymond et al., 1995), these glial microdomains would likely effect different color or scotopic circuits similarly.

Our in vivo imaging study also revealed that MG form each of their four distinct territories in different retinal layers independently with MG maturation in the OPL proceeding last, after contact between photoreceptors and HCs have formed. Previous experiments labeling developing MG with Golgi impregnation methods in chicken and mammalian retinas have observed that the basal stalk of MG at the ILM and in the IPL begins to develop fine processes prior to morphological maturation of the apical stalk (Ramon y Cajal, 1960; Morest, 1970). Conversely, we found that the OLM appeared to be the first territory to fully differentiate (Fig 5A&B), although early development of contacts between the apical processes of MG and photoreceptor inner segments (50 hpf) has been observed in the zebrafish by electron microscopy (Schmitt and Dowling, 1999). The late differentiation of MG processes in the OPL would imply that MG processes either target existing synapses in the OPL or passively grow throughout the layer and form associations with synapses. Slice cultures of mature brainstem showed that regions of rapid astrocytic filopodial remodeling occur exclusively around active synapses.
labeled by FM dyes (Hirrlinger et al., 2004), suggesting that astroglia can differentiate between synaptic and non-synaptic zones. Thus synaptic activity may play a role in stimulating MG process outgrowth in the OPL.

**MG contact is not critical for synapse formation in the OPL**

There is a large amount of evidence showing that astroglia play an important role in promoting synapse formation. Coordinated rearrangements of astrocytic processes and neuronal filopidia leading to contacts between these two structures have been observed in slice cultures (Haber et al., 2006), showing that these processes actively target one another during development. The contacts between astrocytes and neurons have functional implications since astrocytic contacts can promote synaptogenesis. In dissociated co-cultures of neurons and astrocytes, significant increases in the number of presynaptic sites, and the amplitude and number of EPSCs specifically in regions where postsynaptic cells are contacted by astrocytes were observed (Hama et al., 2004). Furthermore, in hippocampal slice cultures, astrocytic filopodia can stabilize developing dendritic spines through ephrin signaling (Nishida and Okabe, 2007). Blocking astrocytic process motility with dominant negative Rac lead to a decrease in the number of spines; however, dendritic filopodia that grew over longer distances than normal to contact astrocytes were just as likely to develop into spines as in normal controls. This synapse promoting activity is not limited to excitatory connections or projection neurons. The intensity of GABA signaling is significantly upregulated by astrocytic contact in dissociated hippocampal cultures (Liu et al., 1996). In some instances synapses require glial contributions during development. For example, targeted ablation of perisynaptic Schwann cells *in vivo* leads to a collapse of the neuromuscular junction (Reddy et al.,
2003). In contrast to these examples, our experiments showed that during normal development synapses are present prior to the arrival of MG processes within the neuropil. In addition, HC dendritic tips are stabilized independent of contact with MG processes. Finally, the removal of MG had no effects on the stability of presynaptic cone photoreceptor pedicles or postsynaptic HC dendritic tips.

While direct contact between MG and neuronal elements was not required for OPL synapse formation, MG may still provide support for synapse development via diffusible factors. Culture experiments using neurons from a number of nervous system regions have shown that multiple diffusible factors can positively regulate synapses including cholesterol (Mauch et al., 2001), thrombospondins (Christopherson et al., 2005) and activity dependent neurotrophic factor (Blondel et al., 2000) from astrocytes, as well as tumor necrosis factor α (Beattie et al., 2002) from Schwann cells. Astrocyte conditioned media has also been shown to increase the number of interneuronal inhibitory synapses on cultured rat hippocampal neurons (Elmariah et al., 2005). In these experiments astrocyte conditioned media indirectly upregulated BDNF/TrkB signaling from local neurons through an unidentified factor.

The role of astroglia in plasticity and circuit maturation

Even though contact with MG is not important for initial synapse differentiation in the OPL, MG may still be involved in reducing structural plasticity in developed circuits. Contrary to their role in promoting synaptogenesis during development, astrocytes are critical for preventing synapse formation during the later stages of development and limiting plasticity in mature circuits. One of the first experiments to show that astrocytes can regulate synaptic plasticity concerned the critical period of
ocular dominance columns in cats. Restriction of visual stimulation to a single eye during development leads to a shift in ocular dominance columns such that the deprived eye loses a large amount of cortical territory to the experienced eye. There is a well defined critical period past which rearrangement of ocular dominance columns cannot be altered by experience. However, if immature astrocytes are transplanted into the visual cortex after the critical period, ocular dominance columns can still rearrange suggesting that the plasticity observed before the end of the critical period is restored by activity from immature glia (Muller and Best, 1989).

The role of glia in synaptic pruning during development has been extensively studied in the cerebellum, where Bergmann glia are important for removing exuberant synapses made during early stages of development. Mature Purkinje neurons receive input from single climbing fibers; however, during development, multiple climbing fibers contact single Purkinje neurons. Experiments limiting Bergmann glial associations with Purkinje cell dendrites in culture (Grosche et al., 1999) and in vivo (Iino et al., 2001) have demonstrated that lack of contact with Bergmann glia leads to a maintenance of the immature pattern of climbing fiber innervation onto Purkinje cells. Furthermore, blocking Bergmann glial process motility in vivo also leads to significant increases in AMPA receptor density in the neuropil surrounding Purkinje cell dendrites (Lippman et al., 2008) demonstrating that maintenance of exuberant synapses is not limited to the climbing fiber circuits.

Astroglia can also regulate the remodeling of synapses in mature circuits. In the mouse supraoptic nucleus, the dendrites and somas of oxytocin producing neurons are normally highly associated with astrocytes. Maternity induces a retraction of associated
glial processes allowing for the formation of new contacts from nearby GABAergic, glutamatergic and noradrenergic neurons (Theodosis and Poulain, 2001). This mechanism is thought to be mediated by expression of normally development associated adhesion molecules, including NCAM and tenascin-C, by astrocytes. Taken together studies from multiple brain regions suggest that astroglia can be important for both maintaining synapses during development, and preventing the formation of synapses at many ages.

Given that MG may thus be important for the long term stability of neuronal circuitry, we actually attempted to use focal ablations to investigate the role of MG in stabilizing more mature OPL circuits; however, this proved difficult to achieve with laser ablations. Although MG could be killed at later ages, we found that their apical processes remained intact with normal morphology spanning the OPL to the OLM for 1-5 dpa (data not shown). While these processes were gradually removed from the OPL, neighboring MG arbors elaborated to take over the unoccupied area. Thus with laser ablations it was impossible to remove MG processes from the OPL or OLM once they had elaborated.

Finally, the overall stability of outer retinal circuits at the ages we examined is currently unknown. While photoreceptor pedicles appeared stable within the time frame we observed, some HCs were still adding new dendritic tips at the end of the recording period, albeit much less frequently than at the beginning of the experiments. It should also be noted that the cone photoreceptor mosaic is not yet regular over the ages we observed, thus rearrangements in the positioning of cone photoreceptors must occur over the course of further development in order to create the near perfect array of cone photoreceptors present in the adult zebrafish (Raymond et al., 1995). Furthermore, rod
photoreceptors are produced until 7 dpf in the zebrafish retina (Fadool, 2003). This ongoing rearrangement of cone photoreceptors and addition of rod photoreceptors indicates that some HC-photoreceptor circuit remodeling is required for attaining the adult circuits. It is therefore unlikely that MG at these ages restrict synaptic rearrangement. Our current study however, strongly suggests that the initial differentiation of photoreceptor-HC synapses in the OPL is not dependent on direct structural associations with MG.
References


CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS
Summary

In my thesis, I have examined the entire time course of horizontal cell (HC) development in the zebrafish retina. These are the first experiments to define how a neuron matures from the point of cell genesis to when it acquires a stable set of specific synaptic contacts, thus generating a detailed understanding of the development of a single neuronal subclass. My in vivo imaging study has led to the discovery of many novel aspects of neuronal development and provides new insights into the cellular mechanisms necessary for the assembly of neuronal circuits.

I first investigated the generation of HCs. In these experiments I identified a dedicated precursor cell that only produces HCs. Importantly, these divisions take place at the retinal layer in which mature HCs are located, rather than at the apical surface typical of mitotic divisions. To date these are the only vertebrate neuronal precursor cells to divide at the location of their mature progeny. Furthermore, I demonstrated that this form of cell division is required for the generation of the mature cohort of HCs in the zebrafish retina.

I next determined how HCs form their mature connection patterns with presynaptic photoreceptors. Since I was able to label the entire cohort of presynaptic inputs onto a single HC dendritic arbor, I could accurately characterize the rearrangement of dendritic tips relative to their available presynaptic partners. I demonstrated that although many dendritic tips appeared to target their proper presynaptic targets, a number of tips were misplaced. These misplaced dendritic tips were preferentially removed while properly placed dendritic tips were more likely to be stabilized. These experiments
directly demonstrated that reorganization of the dendritic arbor can be solely responsible for maturation of a neuronal circuit.

In my final set of experiments I examined the possible role of Müller glia (MG) in the formation of synapses in the outer retina. I first determined the time course of MG elaboration into the OPL relative to synaptogenesis between photoreceptors and HCs. I demonstrated that synaptic elements are present prior to the arrival of MG processes in the OPL using both immunofluorescent labeling of synaptic proteins and electron microscopy. Furthermore, with time-lapse in vivo microscopy I determined that HC dendritic tips are stabilized independent of contact with MG processes in the OPL. Ablating MG in small regions of the retina in vivo, I demonstrated that MG contact is not required for the stabilization of either photoreceptor pedicles or HC dendritic tips. These experiments are some of the first to carefully examine the onset of synaptogenesis relative to glial maturation on the vertebrate CNS, and they clearly demonstrate that some neuronal circuits can develop normally without contributions for resident astroglia.

**Identifying HC precursor cell progeny**

In Chapter 2, I identified a dedicated HC precursor cell that is important for the formation of most, if not all HCs in the zebrafish retina. However, there are four subtypes of HCs in the zebrafish retina and as such this precursor cell may generate all four types from asymmetric divisions, or the precursor cell may in fact represent four separate precursors themselves dedicated to the production of H1, H2, H3 and rod HCs separately. Recent experiments in chick retina using retroviruses to label progeny of retinal progenitor cells suggest that HCs of the same subtype may be generated by a dedicated
precursor. The chicken retina has three subtypes of HCs that can be morphologically discriminated (Genis-Galvez et al., 1981). When the progeny of retroviral clones were examined, HC subtypes that generated by the progenitor cell were almost always present in multiples of two suggesting that they could be the result of a terminal neurogenic division. Furthermore, a few clones consisted of either pairs or sets of pairs of HCs that comprised the same subtype, implicating a dedicated precursor cell generated these pairs (Rompani and Cepko, 2008). However, this data does not directly demonstrate that progenitors dedicated to subtypes of HCs exist and time lapse imaging studies following the progenitor cell division and progeny differentiation in vivo are needed to clearly demonstrate whether or not an individual HC precursor cell is dedicated to the production of daughter cells of the same subtype.

To conduct such experiments, single zebrafish HC precursor cells must be labeled and the progeny of this division must be unambiguously identified at the point that mature morphology is evident approximately two days after division. This can be done with two methods. The first is to label HCs using Cx55.5 promoter elements to drive the expression of a membrane tagged fluorophore. This is less than ideal because transient injections will often lead to multiple labeled HC precursors in close vicinity. Since immature HCs can migrate rapidly, frequent image acquisition over the course of two days is required to accurately detail the progeny of a precursor division. Besides being difficult experiments, the rigor of such frequent imaging on a cell expressing relatively high levels of a membrane targeted fluorescent protein can often lead to cell death, making it impossible to determine the result of the precursor division.
A better approach is to use a photoactivatable or photoconvertable fluorescent protein in order to selectively label a single precursor cell prior to completion of mitosis. To do this, I have generated UAS:kaede transgenic fish, that can be crossed to the ptf1a:Gal4VP16 transgenic line used in Chapter 4 to label the entire population of HCs. Kaede is a photoconvertable fluorescent protein that emits a green wavelength light under baseline conditions, but after conversion with a short wavelength laser (405 nm), becomes red fluorescent (Ando et al., 2002). By scanning a very small area at the center of a HC precursor cell expressing kaede in this double transgenic line I have been able to photoconvert a single precursor cell creating a single red precursor cell in the background of green HCs and precursors (Fig 1A). Reexamining this cell at a later time point, two daughter cells were evident (Fig 1B). To date, I have only tested kaede with these experiments using an inefficient conversion laser (440 nm) and as such, conversions I have observed have been suboptimal, and red progeny could not be detected at mature time points because the converted kaede signal was too dim. However, I will soon have access to a 405 nm laser line that will convert kaede much more efficiently, and as such this approach should be feasible for identifying whether or not HC precursors are dedicated to the production of a single subtype of HC daughter cell.

**Characterizing strategies of HC lateral migration**

It has been known for quite some time that HCs are the only retinal subtype to migrate laterally during development of the retina. Classic experiments with X-inactivation lineage tracing demonstrate that most cellular products of retinal progenitor cells are contained within a single discrete column with the exception of HCs (Reese et
al., 1995; Reese et al., 1999). However the strategies that HCs use to localize their somas during development are not well characterized. In mouse ex vivo experiments, it has been shown that homotypic interactions of the processes of immature HCs contribute to correct somal spacing. In these experiments immature vertically migrating HCs were ablated, and neighboring cells were seen to occupy the territories of ablated cells (Huckfeldt et al., 2009). It was postulated that the mosaic positioning of the HC somata is influenced by tiling of the early neuritic territories. However, due to the limits of ex vivo imaging and the slow development of mouse retina, direct observations of laterally migrating HCs were limited (Huckfeldt et al., 2009).

In my time-lapse recordings of HC precursor division, lateral migration of daughter cells just below the surface of the OPL was quite robust. I have collected extended time-lapse series of HC migration in the ptf1a:GFP at frequent intervals over a wide field view of the retina (Fig 2, Movie 1). In these time-lapse recordings, individual HCs could be resolved due to variable GFP expression across the HC population (Fig 3). In a single experiment many HCs can be tracked from the time point at which they are generated to when they cease migration and presumably establish their territory. At first glance cellular movements appear somewhat random with a slight bias towards the central retina where a complete layer of HCs expands towards the periphery during maturation. More detailed analysis of individual cell trajectories should shed important light onto the course individual HCs take in finding their ultimate destination.

**Determining the impact of reduced presynaptic input on HC dendritic arbor development**
My experiments in Chapter 3 demonstrated that HC dendrites make errors while establishing their synaptic contacts with photoreceptors. Previous experiments in mice have demonstrated an interesting ability for synaptic partners to be respecified when the normal presynaptic partners are lacking. Unlike zebrafish HCs, individual mouse HCs exclusively contact cone photoreceptors with their dendrites and rod photoreceptors with their axons. Interestingly, this wiring pattern can be altered in transgenic mice where cone photoreceptors are respecified into rod photoreceptors (Oh et al., 2007) or rod photoreceptors aberrantly differentiate into cone photoreceptors (Raven et al., 2007). In such transgenic mice, HC dendrites contact rod photoreceptors and presumably form synaptic contacts in the rodfull retina, while their axons contact cone photoreceptors in the coneful retinas (Raven et al., 2007). While this result is intriguing, it remains unknown exactly how drastic a loss of presynaptic input must occur in order to cause respecification, and whether or not single chimeric dendritic arbors could exist that connect with both correct and incorrect photoreceptor terminals.

I plan to address these questions using a recently identified zebrafish mutant lots of rods (lor). In lor mutant retinas the vast majority of UV-cone photoreceptors are fate switched into rod photoreceptors, thereby drastically reducing UV-cone numbers, but not completely removing these cells altogether (Fig 4A)(Alvarez-Delfín et al., 2009). Rod numbers are directly increased by the number of fate switched UV-cone photoreceptors, and the other subtypes of cone photoreceptor remain unaffected. Since I have demonstrated that H3s form specific contacts with UV-cone photoreceptors early in development, I will label H3s in the SWS1:GFP transgenic background as in Chapter 3; however, I will perform these experiments in the lor mutant background.
There are a number of possible outcomes from these experiments (diagrammed in Figure 4B). First H3s may maintain contact with UV-cone photoreceptors. If H3s continue to only contact UV-cone photoreceptors, as I observed in Chapter 3, then dendritic densities should decrease proportionally with the loss of UV-cones (Fig 4B left). The dendritic arbor may either be larger in order to contact the same average number of presynaptic cells seen in normal retina, or be smaller because of the diminished number of UV-cones within the dendritic territory. H3s might also contact UV-cone photoreceptors along with other photoreceptors because few UV-cone photoreceptors are insufficient to support a sizeable dendritic arbor (Fig 4B top). Likely, blue-cone photoreceptors will also contact H3s along with UV-cones because this has been observed as a normal pattern of connectivity in adult H3s (Li et al., 2009). Alternatively rod photoreceptors now contact H3s because these cells will be present at higher densities than normal in the lor retina. It is also possible that red or green photoreceptors could be contacted by H3s lacking UV-cone photoreceptor input, but this is less likely because H3s have not been observed to contact red- or green-cone photoreceptors and their numbers are normal in the lor mutant retina. If H3 dendritic arbors are observed that contact UV-cone photoreceptors and another photoreceptor subtype in the lor mutant, this would indicate that H3 dendritic arbors are not mutually exclusive in their connectivity if their normal presynaptic partners cannot provide enough presynaptic input. Results where UV-cone photoreceptors and rod photoreceptors are present on the same dendritic arbor would be extremely interesting because rod and UV-cone photoreceptor inputs would indicate mixing of photopic and scotopic pathways within a single HC.
A final possible result for these experiments is a complete respecificiation of H3 dendritic targeting (Fig 4B bottom). If such respecification occurs, then I expect H3s to connect with only blue-cone photoreceptors because these connections have been previously reported (Li et al., 2009), or for H3s to connect with rod photoreceptors because of their increased density in the lor mutant retina. Depending on the photoreceptor subtype contacted, complete respecification of the H3 dendritic arbor in the lor mutant (especially if GFP positive UV-cone photoreceptors are observed within the H3 dendritic arbor) would indicate that either blue- and UV-cone photoreceptor connectivity, or rod and cone photoreceptor connectivity in H3s is mutually exclusive.

**Examining the role of MG in the maintenance of immature retinal synapses**

In Chapter 4, I demonstrated that MG contact plays no role in the early development of synapses in the zebrafish OPL. However, astrocytes have been shown to partake in many aspects of circuit formation beyond the initial stages of synaptic development. For example, astrocytes in the visual cortex are known to be important for the regulation of the critical period of ocular dominance column development (Muller and Best, 1989). In addition, in the cerebellum Bergmann glia process associations appear to be important for limiting the density and distribution of synapses seen in the mature circuits (Grosche et al., 1999; Iino et al., 2001; Lippman et al., 2008). Unfortunately, with my ablation techniques, these later stages of MG contribution to the development of retinal circuits could not be assessed since MG apical processes remained for as long as 3 days after ablating the cell (data not shown).
It should be noted however, that MG soma and basal processes were removed by these later stage ablations. Thus, it is feasible to use focal laser ablations to remove MG processes from the inner retina and subsequently examine the stability of retinal processes in this layer. For such experiments, single amacrine cells can be labeled in the background of all MG expressing GFP by injecting UAS:MXFP into double transgenic ptf1a:Gal4VP16, gfap:GFP zebrafish embryos. Amacrine cells appear to have a mature stratified arbor as early as 60hpf (Godinho et al., 2005). Therefore, from 5 dpf, after MG processes have already elaborated in the IPL, MG ablations can be carried out in order to remove MG contributions to the neuropil. Although neighboring MG processes will invade the neuropil vacated by the ablated MG over the course of approximately three days, this should be ample time to assess the effects of MG removal from the IPL. Time lapse imaging will be carried out to compare the amacrine cell arbor before the ablation to its arbor after the ablation in order to assess any alterations in arbor area, stratification or motility. If MG are important for stabilizing and maintaining neuronal connections at later stages of development, retraction of amacrine cell neurites would be expected (Fig 5 top). On the other hand, if MG play a role in limiting synapse densities as Bergmann glia do in the cerebellum, amacrine neurite growth and mistargeting may be observed (Fig 5 bottom). Similar experiments can also be performed with retinal ganglion cells, although later time points would be desired since these cells have been observed to elaborate their dendrites until 6 dpf (Mumm et al., 2006). Bipolar cells are not a good candidate for such experiments because their apical dendrites often course adjacent to MG apical stalks (unpublished observations) and thus they may be directly affected by laser ablations. If there are concerns about alterations in the bipolar cell population due to laser ablations.
leading to remodeling of amacrine cell arbors because of bipolar cell-amacrine cell
circuitry, experiments can be carried out in double transgenic zebrafish lines in which
both MG and bipolar cells (Schroeter et al., 2006; Vitorino et al., 2009) are labeled. In
double transgenic experiments, if MG ablations leads to any alterations in the bipolar
cells within the ablated area it will be obvious and such samples can either discarded or
analyzed as a separate experimental condition.
Figure 1: Photoconversion of HC precursor cells expressing kaede

(A) Example of a mitotic HC precursor cell expressing kaede in the ptf1a:Gal4VP16; UAS:kaede double transgenic retina. The HC precursor cell has been successfully converted in vivo to the red fluorescent state (magenta) by confocal microscopy using a very small region of interest and the 440 nm laser for approximately 5 minutes. The surrounding HCs and amacrine cells remain unconverted (green). Four hours later, two daughter cells expressing red kaede can be observed.

(B) A second example of a HC precursor cell expressing kaede that was photoconverted (magenta) during migration. The surrounding HCs and amacrine cells remain unconverted (green). Five hours later, two daughter cells expressing red kaede can be observed.

Scale bar = 5 μm.
Figure 2: Formation of a continuous layer of HCs is achieved by lateral migration

An extended in vivo multiphoton time-lapse recording of HC layer formation observed in the ptf1a:GFP transgenic line. HC precursor cells divide towards the periphery (top). Two daughter cells of different precursor divisions are pseudocolored magenta and green (shown in Figure 3). Scale bar = 5 μm.
Figure 3: Migration of individual HCs tracked in the *ptf1a*:GFP transgenic background

The migration of two daughter cells from an *in vivo* time lapse of HC layer formation (shown in Figure 2) were tracked based on differential levels of expression of GFP. Scale bar = 5 μm.
(A) Schematic of the wild type (wt) photoreceptor mosaic and a single row of blue and UV-cone photoreceptors (white dashed line) compared to the same region of the mosaic in the lor mutant retina.

(B) Schematic of the different outcomes of H3-photoreceptor connectivity in the lor mutant retina relative to rod photoreceptors, and blue-and UV-cone photoreceptors. Photoreceptors not contacting the H3 dendritic arbor are made dimmer for clarity.

Green cone shown in green. Red cone shown in red. Blue cone shown in blue. UV cone shown in purple. Rod photoreceptor shown in charcoal. H3 shown in tan.
Figure 5: Schematic of expected changes in amacrine cell neuritic arbor following local MG ablation

Following ablation (red dots) of a patch of MG (green) amacrine cell (cyan) neurites may retract (top right), expand (bottom right) or remain unchanged (not shown).
References


