The Vertebrate Retina: A Model for Neuronal Polarization *In Vivo*

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ABSTRACT: The vertebrate retina develops rapidly from a proliferative neuroepithelium into a highly ordered laminated structure, with five distinct neuronal cell types. Like all neurons, these cells need to polarize in appropriate orientations order integrate their neuritic connections efficiently into functional networks. Its relative simplicity, amenability to *in vivo* imaging and experimental manipulation, as well as the opportunity to study varied cell types within a single tissue, make the retina a powerful model to uncover how neurons polarize *in vivo*. Here we review the progress that has been made thus far in understanding how the different retinal neurons transform from neuroepithelial cells into mature neurons, and how the orientation of polarization may be specified by a combination of pre-established intrinsic cellular polarity set up within neuroepithelial cells, and extrinsic cues acting upon these differentiating neurons. Develop Neurobiol 71: 567–583, 2011

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INTRODUCTION

How neurons polarize, and orient their axis of polarity within a developing embryo is an essential question in developmental neurobiology. Most neurons are strikingly polarized. This polarity is obvious morphologically as a stereotypical neuron has a single long axon at one pole of the cell, and multiple shorter dendrites projecting from the cell body. The physiological function of neurons is also polarized. Electrical activity generally flows in one direction, from dendrite/soma through to the axon. Neurons are highly polarized intracellularly and molecular factors, such as presynaptic postsynaptic components, essential for axon and dendrite function respectively, are efficiently sorted and segregated to these domains. The study of neuronal polarization attempts to determine how axons and dendrites sprout in an appropriately oriented manner from the cell bodies of differentiating neurons, and how the distinct functions of these processes arise and mature.

Neuronal Polarization *In Vitro*

In the late 1980s it was discovered that isolated postmitotic rodent hippocampal neurons cultured on simple homogeneous substrates will polarize and extend a long axon and multiple dendrites after a few days (Dotti et al., 1988). This polarization can be described in five stages: at Stage 1 the spherical cell forms lamellopodia at its surface, Stage 2 is marked by the outgrowth of minor neurites from these lamellopodia, at Stage 3 one of the minor neurites starts growing

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faster than the others and becomes the axon, at Stage 4 dendritic growth starts and during Stage 5 the whole neuron matures. This polarization occurs in the absence of heterogeneous extracellular cues, demonstrating the remarkable intrinsic ability of neurons to break their initial morphological symmetry (Dotti et al., 1988; Craig and Banker, 1994).

The ability to form axons and dendrites *in vitro* has been demonstrated in other neuronal cell types such as cerebellar granule neurons and retinal ganglion cells (Powell et al., 1997; Zmuda and Rivas, 1998; Zolessi et al., 2006). This clearly demonstrates the existence of a strong cell autonomous polarization program in developing neurons, and a seemingly inherent requirement for neurons to achieve their *sina qua non* polarized morphology. *In vitro* the process of neuronal morphogenesis can go beyond simple polarization and isolated neurons may even achieve morphologies that approximate mature *in vivo* morphology. For example, cultured cerebellar granule neurons outgrow bifurcated axons (Powell et al., 1997; Zmuda and Rivas, 1998), cultured retinal photoreceptor cells form large outer segments and small axonal neurites (Adler, 1986), and cerebellar purkinje neurons grow a single axon and a densely branching dendritic tree (Dunn et al., 1998). This demonstrates that the autonomous polarization and morphogenesis programs are to some degree specialized for each cell type.

Research efforts on neuronal polarity over the past three decades have mainly concentrated on investigations using dissociated hippocampal neurons as a model system to study the early steps in the process of initial polarization and axon selection. This has led to the discovery of multiple factors required to for the neuron to form axons and dendrites, and then to ensure that only one axon is formed for each neuron (reviewed in Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Tahirovic and Bradke, 2009). These studies have pointed to a central role for cytoskeletal regulation and reorganization during polarization. Many cytoskeletal regulators such as CRMP-2, MARK2, Rho-family GTPases, and Kinesin motor proteins, have been implicated in axonogenesis. In brief, the current view is that actin destabilization and concurrent microtubule stabilization in preaxonal Stage 2 neurites allows for increased microtubule penetration into one of the growth cones, causing this process to grow faster and thereby forming the axon (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; Hoogenraad and Bradke, 2009; Tahirovic and Bradke, 2009). It is still unclear, however, what the first steps in the process that leads to this asymmetry in cytoskeletal growth are, although once initiated, the factors above may reinforce the decision.

Moving from the Culture Dish to the Brain

In contrast to the cell culture environment, neurons within the developing CNS are neither symmetrical prior to axon extension, nor are they in a homogenous environment. Thus it may be easier for neurons to establish their polarity, and the factors that are critical for axonogensis and dendritogenesis may be easier to discover *in vivo*. Many vertebrate CNS neurons, like hippocampal and retinal neurons, derive from the terminal divisions of neural progenitor cells within a polarized pseudostratified neuroepithelium. Neurons are born into environments with directionality in which a milieu of molecular gradients exist. If it is possible to determine which factors are critical for the initiation of polarity *in vivo*, then this information can be used to investigate how these factors are coupled to the intrinsic mechanisms that are being discovered *in vitro*.

Early anatomical observations of the CNS demonstrated that neurons of a given cell type are often oriented in parallel with each other, rather that at random orientations (Golgi, 1903; Cajal, 1906; Sotelo, 2003). This provided the first hint that cell type specific control over polarization exists. To achieve this remarkably reproducible orientation of thousands of cells, developing neurons might use common extracellular cues to fix their axis of polarization. This notion is supported by studies demonstrating that extrinsic factors such as extracellular matrix proteins and classical axon guidance factors can influence the orientation of vertebrate neuronal polarization *in vitro* (Gupta et al.; Esch et al., 1999; Menager et al., 2004; Shelly et al., 2007; Mai et al., 2009). Such factors then become good candidates for orienting neuronal polarization *in vivo* (Polleux et al., 1998; Zolessi et al., 2006; Lerman et al., 2007). The fact that many neurons have their axono/dendritic axis oriented in parallel with the apico/basal axis of the neuroepithelium (for example cerebellar purkinje neurons, retinal bipolar cells and cortical pyramidal neurons), suggests that pre-established polarity of the neuroepithelium may also be important for directing neuronal polarization. It is thus likely that developing neurons integrate information from their external environment and pre-established neuroepithelial polarity into intrinsic polarization programs, which direct cytoskeletal organization and molecular trafficking, so that they extend axons and in the correct cell-type specific orientation.

To understand fully the intricacies of how neurons polarize, investigations over the last decade have therefore begun to analyze cells within the context of their native environment using advanced microscopic techniques. Such studies have already indicated that not all neurons polarize by the same mechanisms as cultured hippocampal neurons. Furthermore, there are differences in mechanisms that guide polarization of the same types of cells when they develop *in vitro* versus *in vivo* (Rolls and Doe, 2004; Zolessi et al., 2006; Rusan et al., 2008). The fact that neurons often migrate over large distances from the position of birth adds to the complexity of the system. Live imaging of cortical neurons in slice cultures demonstrated that migration can occur in parallel with polarization (Noctor et al., 2004), but how neuronal polarization is coupled to cell migration *in vivo* is not understood.

The Vertebrate Retina as a Model to Study Neuronal Polarization *In Vivo*

The vertebrate retina, and especially the optically transparent zebrafish retina, is an outstanding model system for imaging cell behaviors *in vivo*, as has been demonstrated by pioneering studies (Das et al., 2003; Kay et al., 2004; Godinho et al., 2005; Poggi et al., 2005). As an outpocketing of the CNS on the surface of the embryo, the retina provides a system accessible to experimental manipulation, and is superficial enough to allow for high-resolution *in embryo* microscopy. This is in contrast to the study of other neurons in the CNS, such as cortical or cerebellar neurons, which, due to their occurrence in deeper tissue layers, rely on organotypic slice culture systems, in which aspects of the native environment, such as diffusible factors and axons projecting out of the slice, may be lost. Further, the retina contains five distinct neuronal cell types, which are unambiguously identifiable based on their individual morphology, immunohistochemical markers, and the expression of cell type specific transcription factors. Therefore, the retina provides a simple, yet powerful model to study common factors that govern the unique polarized morphology of different neuronal cell types.

Retinal neurons are born by terminal divisions of neuroepithelial progenitor cells at the apical surface of the neuroepithelium, and differentiate into one of the five neuronal cell types (see Fig. 1). Photoreceptors line the apical surface of the mature retina, with light sensitive outer segments pointing apically, and project axons to form synapses in the outer plexiform layer (OPL) basal to their cell bodies. Photoreceptors synapse onto bipolar cells (BCs), which have their dendrites pointing apically into the OPL, and have axons projecting into the inner plexiform layer (IPL). Retinal ganglion cells (RGCs) project dendrites into the IPL, synapsing with BCs, and extend axons along the basal surface of the retina, which collect at the optic disk and leave the eye to find partner neurons in the optic tectum. These three cell types form the excitatory pathway of the retina, and are all polarized in the same orientation (apical = dendrite, basal = axon). The two inhibitory retinal neurons: amacrine cells (ACs) and horizontal cells (HCs), modulate the visual information that flows through the excitatory pathways. In contrast to the other neuronal types, the inhibitory neurons have a unipolar morphology, and generally extend processes from one pole of the cell body into the plexiform layers, and are thus oriented towards them. The retina also has a single type of glial cell, the Muller glia, which spans the width or the retina from the outer limiting membrane (OLM) to the inner limiting membrane (ILM), and elaborates processes into the plexiform layers.

We will discuss progress that has been made so far in understanding how the different polarized morphologies of each retinal cell type arise from morphologically indistinguishable neuroepithelial progenitor cells, and how the extrinsic environment, the pre-established neuroepithelial polarization, and the cell-intrinsic polarization programs may be cooperating to direct neuronal polarization. However, before we discuss the specific types of retinal cells, we want to review the polarization of the retinal neuroepithelium.

The Pseudostratified Retinal Neuroepithelium

The progenitor cells of the retina are organized into a pseudostratified neuroepithelium, characteristic of the embryonic vertebrate CNS. The term pseudostratisfied means that despite the wide range of nuclear positions and multilayered appearance, all cells remain attached to the basal and apical surface of the epithelium. The development of pseudostratisfied epithelia can be described in three steps: first progenitors are anchored to the apical surface of the epithelium via adherens junctions, then an elongation of cells along the apical basal axis takes place leading to long apical and basal processes and finally cells begin to undergo interkinetic nuclear migration (IKNM) (Miyata, 2008). Intracellularly, neuroepithelial cells share characteristics with epithelial cells elsewhere in the body. They

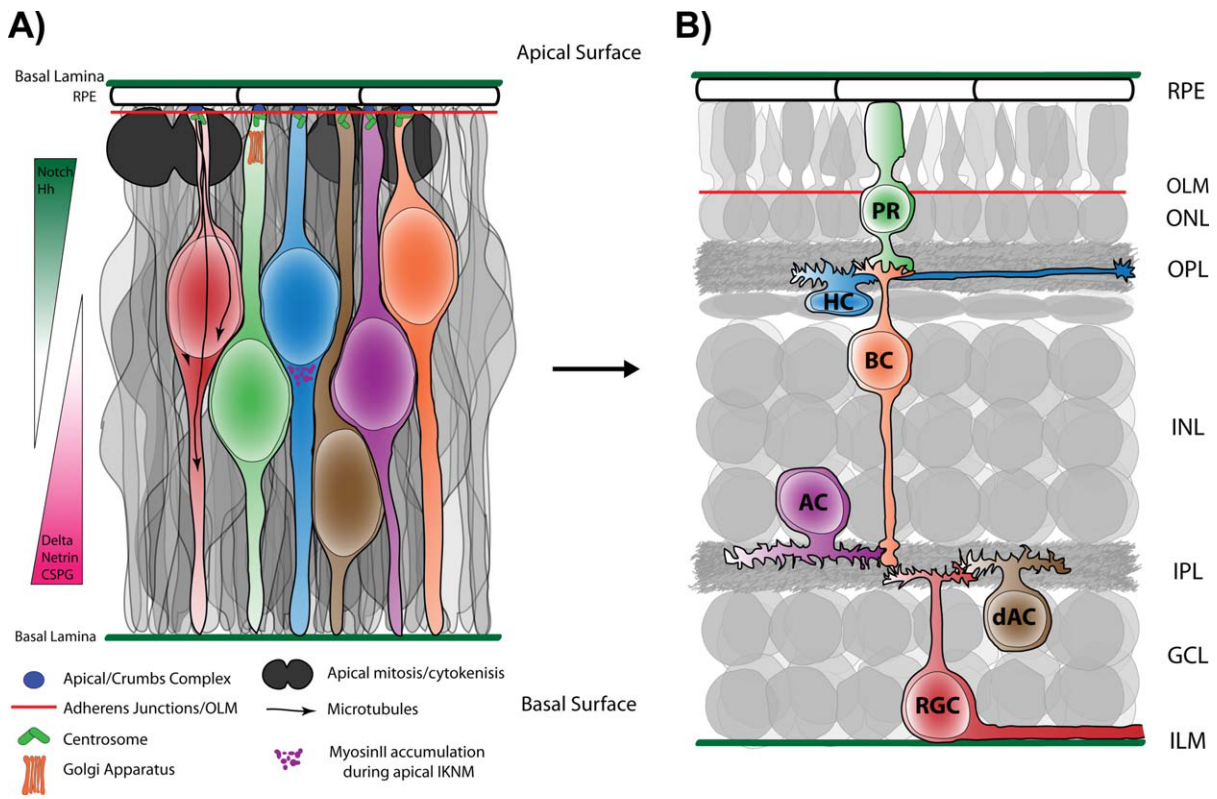

Figure 1 Organization of the vertebrate retina. A: Multipotant retinal progenitor cells in the pseudostratified neuroepithelium prior to neurogenesis. Apical processes extend to the apical surface of the neuroepithelium and are opposed to the retinal pigment epithelium. Apical and Crumbs complexes localize to the tip of the apical process (dark blue). Basal to these are adherens junctions, which attach to neighboring cells and form the OLM (red line). Basal to the OLM are the centrosome (green) and the golgi apparatus (orange), followed by the nucleus. Microtubules are nucleated at the centrosome, resulting in a polarized microtubule array with plus ends (arrowheads) pointing basally. During the apical movements of IKNM, MyosinII accumulates basal to the nucleus (purple), and is involved in propelling the nucleus towards the apical surface, where all mitosis and cytokinesis events take place. The basal process extends to the basal surface of the retina and attaches to the basal lamina through Integrin-based focal adhesions. Extrinsic factors are distributed in the retinal neuroepithelium, which may form gradients that serve to instruct the behavior of neuroepithelial cells. These include chondroitin sulfate proteoglycans (CSPGs) at the basal surface, Netrin-1 emanating from the optic nerve head, and perhaps high concentrations of Hedgehog in apical regions of the retina. A gradient of Notch/Delta activity has also been reported described the retina, with high Notch activity apically. (Brittis et al., 1994; Brittis and Silver, 1994; Capello et al., 2006; Costa et al., 2008; Del Bene et al., 2008; Hinds and Hinds, 1974; Norden et al., 2009; Perron et al., 2003; Stuermer and Bastmeyer, 2000; Wallace, 2008) **B)** The five neuronal cell types of the mature retina. Photoreceptors (PR, green) have cell bodies in the outer nuclear layer. Horizontal cells (HC, blue), bipolar cells (BCs, organge) and amacrine cells (ACs, purple) are in the inner nuclear layer. Retinal ganglion cells (RGCs, red) and displaced amacrine cells (dACs, brown) are situated in the ganglion cell layer. The outer plexiform layer (OPL) contains the synapses between photoreceptor axons, HCs and BC dendrites. The inner plexiform layer (IPL) contains the synapses of BC axons, dAC/AC neurites, and RGC dendrites. RPE - retinal pigment epithelium, OLM - outer limiting membrane, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer, ILM - inner limiting membrane.

feature a characteristic apico-basal polarity and are connected by adherens and tight junctions at the tip of the apical process.

The most apical plasma membrane of the retina and other neuroepithelial features an apical disc, comprised of an actomyosin based contractile system

interconnected with the lateral adherens junctions that, in connection with other cells, form the apical surface of the neuroepithelium. A focus of current research is to find out if the part of the plasma membrane surrounded by the apical disk can serve as a signaling platform in vertebrates as it has been proposed for invertebrate systems (reviewed in Sawyer et al., 2010). Additionally, most neuroepithelia feature a primary cilium, which also mediates signaling events, although this has not yet been characterized for retinal neuroepithelia. It is possible that the primary cilium in these cells is either rudimentary or transient, which would explain why it has been difficult to observe.

Components that mark the apical membrane domain of cells include the Crumbs complex and the polarity complex consisting of Cdc42, Par3, Par6, and aPKCs. Cdc42, Par3 and Par6, have all been shown to be important for neuroepithelial integrity in the mouse cortex. Upon knockdown or over-expression of any of these components, the epithelium looses polarity, dividing cells detach from the apical lamina, and many of such detached progenitors subsequently exhibit ectopic mitoses. Consequently, the correct spatiotemporal aspects of retinogenesis are perturbed (Cappello et al., 2006; Costa et al., 2008). The effects of Par and Crumbs complex mutants have been characterized in zebrafish retinal neuroepithelium. Common features of these mutants include an aberrant apico-basal polarity with the occurrence of basal mitoses and basally displaced centrosomes as well as delocalized apical complex markers. This eventually leads to severe disruptions of laminar organization (Pujic and Malicki, 2004).

Adherens junctions separate apical plasma membrane domains from basolateral domains. These belt-like structures are linked to the actin cytoskeleton. N-cadherin and β-catenin are crucial components of adherens junctions and a loss or mutation of N-cadherin leads to the collapse of adherens junctions, detachment of cells and basal mitoses in the zebrafish retina (Yamaguchi et al., 2010). The basolateral portion of the plasma membrane is characterized by the localization of Lethal giant larvae, Scribble, and Disks large (Li and Sakaguchi, 2002; Li and Sakaguchi, 2004). At the most basal end, cells are attached to the basal lamina lining the basal surface of the retina through integrin-based focal adhesion complexes.

Intracellularly, organelles and cytoskeletal elements are polarized in neuroepithelial cells. The centrosome is positioned apically, and serves as the basal body for the primary cilium in cortical neuroepithelia and probably also in the retina (see above). Additionally, the centrosome is the major microtubule-organizing center in neuroepithelial cells, resulting in most plus ends pointing basally (Norden et al., 2009). F-Actin distribution is not obviously polarized in progenitor cells but apical and basal accumulations can be observed where the endfeet of cells are connected to the laminae. Additionally a basal accumulation of MyosinII is seen during the apical movements in IKNM (discussed below), indicating that asymmetries exist in the contractile actomyosin system (Norden et al., 2009). The position of the Golgi adds to the overall intracellular polarity as it is always located apically between nucleus and centrosome (Hinds and Hinds, 1974; and our unpublished observations).

The extracellular environment of the neuroepithelial cells is also highly polarized. At the apical surface of the retina, neuroepithelial cells contact the RPE, which may contribute to neuroepithelial polarity and retinal organization (Rothermel et al., 1997; Layer et al., 1998; Zou et al., 2008). At the basal surface of the retina, cells contact a basal lamina, which features typical components like Laminin, Nidogen, Collagen IV, Agrin, Heparansulfate Proteoglycans and Chondroitin Sulfate [reviewed in (Stuermer and Bastmeyer, 2000)]. However, apart from serving as an attachment site for basal processes, not much is known about how these components influence the polarity of progenitor cells, although they may be critical for orienting neuronal polarization (see discussion of RGCs below).

Extracellular factors are also distributed asymmetrically in the retinal neuroepithelium, with high concentrations CSPGs basally (Brittis et al., 1992; Brittis and Silver, 1994). Recently an apico-basal gradient of Notch activity in the zebrafish retinal neuroeithelium has been described (Del Bene et al., 2008). It is also thought that other signaling pathways, such as Hedgehog are polarized, with Hh being released from the apically adjacent pigment epithelium and later from the basally localized differentiating RGCs (Perron et al., 2003; Wallace, 2008).

The polarized nature of the neuroepithelium results in the polarized behavior of neuropithelial cells. The most strikingly polarized behavior is that all cell divisions happen at the apical surface of the retina. This likely results from the apical position of the centrosome. Consequently, cells must translocate their nuclei in a polarized manner to ensure that they arrive at the apical surface for division, which is accomplished by interkinetic nuclear migration. IKNM was first described by Sauer, and serves to move the nucleus of progenitors away from their birthplace at the apical surface, and then back again for the next division (Sauer, 1935). IKNM has been

compared to an elevator movement; mitosis and cytokinesis happen at the apical side of the epithelium, during G1 nuclei exhibit a smooth transition towards the basal side of the cell, undergo S-phase there and during G2 migrate back towards the apical side (reviewed Miyata, 2008). However, this view has recently been challenged (Baye and Link, 2007; Norden et al., 2009). The latter report finds that IKNM in the zebrafish retina is actually best described by stochastic motions of nuclei that are punctuated with phases of directed movement around mitosis. It has been shown that the centrosomal components and its microtubule motors play a role in IKNM (Tsai et al., 2005; Xie et al., 2007; Del Bene et al., 2008). However, the microtubule cytoskeleton in the zebrafish retina can be completely disrupted without dramatically effecting IKNM. By contrast an actomyosin-based mechanism seems to be essential for both rapid directed as well as stochastic movements. One possible mechanism for rapid apical migration of the nucleus is actomyosin dependent cortex constriction through localized MyosinII activity basal of the nucleus (Norden et al., 2009). A role for actomyosin in basally directed IKNM has been proposed in the mouse cortex (Schenk et al., 2009).

Another polarized neuroepithelial progenitor behavior is oriented cell divisions. Oriented divisions that result in asymmetry can produce a progenitor cell and a neuron, as opposed to a symmetric division, which results in the production of two progenitor cells or two neurons.

Localized signaling in combination with IKNM has been implicated in the switch from proliferative to neurogenic divisions. This hypothesis was built on computational models and the observation that in zebrafish retinal neuroepithelia, cells with nuclei that migrate more basally are biased towards generating neurons (Murciano et al., 2002; Baye and Link, 2007). Consistent with this model, in the zebrafish retina cells with apical nuclei showed higher Her4 activity which is one component of the Notch pathway (Del Bene et al., 2008).

In addition to patterns of IKNM, the length of G1 has been implicated in regulating the switch from proliferation to neurogenesis (Lange et al., 2009; Pilaz et al., 2009). The model is that the length of G1 phase is proportional to production of a yet to be unidentified cell fate determinant. Alternatively, G1 phase may be linked to the competence of the cell to respond such a factor and therefore as G1 kinetics vary so does the window of responsiveness to fate determinants (Calegari and Huttner, 2003).

In asymmetric neurogenic divisions, in which one progenitor and one neuron are produced, numerous factors have been proposed to play a role in influencing which cell becomes the neuron. Examples include the asymmetric distribution of Par3 (Bultje et al., 2009; Alexandre et al.), the inheritance of Numb protein (Cayouette and Raff, 2003), the inheritance of the more mature centrosome (Wang et al., 2009), the alignment of the spindle (Chenn and McConnell, 1995; Das et al., 2003) and the inheritance of the apical plasma membrane domain (Kosodo et al., 2004; Alexandre et al., 2010). Recently, the segregation of the thin elongated basal process upon division has been added to this list. The basal process splits upon cytokinesis but also this bifurcated basal process is usually inherited only by one of the two daughter cells (Kosodo et al., 2004). It was shown that in the zebrafish hindbrain the cell that inherits the basal process replenishes the progenitor pool (Alexandre et al., 2010).

So far it is not clear if all of these factors are needed for the switch from proliferative to neurogenic division in all neuronal cell types and which of these factors are upstream or downstream of each other.

Polarization of Retinal Neurons

Retinal Ganglion Cells. Mature RGCs have dendrites projecting into the IPL, and a long axon extending from the basal pole of the cell body. Despite being limited to the analysis of fixed tissue, early studies looking at RGC polarization painted a fairly clear picture of RGC morphogenesis through the analysis of multiple immature RGCs. These data indicated that the basal process transforms into a growth cone tipped axon, and dendrite formation happens at opposite pole of the cell body, perhaps by transforming the apical process into early dendritic structures (Hinds and Hinds, 1974; Holt, 1989). Time-lapse confocal imaging of differentiating RGCs in the living zebrafish retina confirmed this initial hypothesis for axon genesis, and demonstrated that shortly after their terminal division, axons begin to extend from the most basal part of the RGC—either the tip of the basal process, or the basal pole of the cell body (Fig. 2, Zolessi et al., 2006). Further, no evidence of a prolonged multipolar stage, similar to Stage 2 of polarizing hippocampal neurons, could be detected. Instead, protrusive activity was observed directly at the point where the growth cone sprouts. As the axon subsequently extends away, dendrites begin to develop from the cell body, which mature and laminate in the IPL located apical to RGC cell bodies (Mumm et al., 2006; Zolessi et al., 2006).

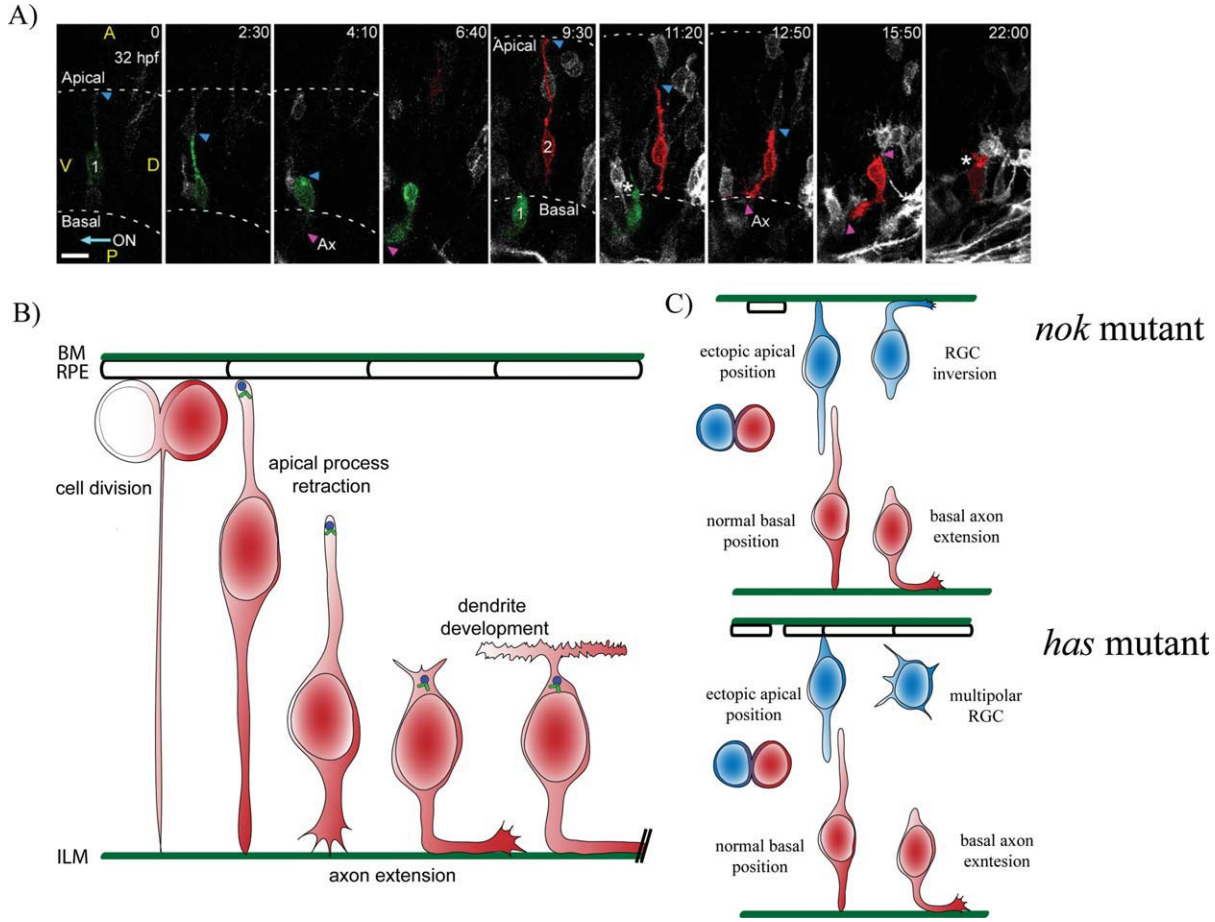

Figure 2 Extrinsic cues result in directed RGC axon extension. A: Timelapse imaging of polarizing RGCs labeled by *ath5:GAP-GFP* expression in the zebrafish retina. As the apical process retracts (blue arrowhead), axons project directly from the RGC (pink arrowhead), either from the basal surface of the cell body (cell 1), or from the tip of the basal process (cell 2). Dendrites subsequently project from the apical surface of the cell (asterix). Ax, axon; ON, optic nerve; A, anterior; D, dorsal; P, posterior; V, ventral (Zolessi et al., 2006). B: Schematic of RGC polarization. RGCs are born at the apical surface of the retina, and quickly reestablish a neuroepithelial-like morphology, with apical and basal processes. The apical complex and the centrosome remain apical in RGCs, and are observed in the retracting apical process as the axon emerges from the most basal point of the RGC, which is in contact with the basal lamina of the inner limiting membrane (ILM). C: RGC polarization is disrupted in disorganized retinas. Ectopically positioned RGCs in the *nok/pals1* mutant retina are exposed to the basal lamina of Bruch's membrane (BM) at the apical surface of the retina due to a discontinuous RPE. These RGCs are inverted, and extend axons along the apical surface of the retina. In *has/aPKCλ* mutant retinas apically mispositioned RGCs adopt a multipolar morphology, and axon extension is delayed. In both contexts normally positioned RGCs at the basal surface of the retina extend axons along the basal lamina of the ILM. Adapted from (Zolessi et al., 2006, 2009).

A study of cultured hippocampal neurons suggested that centrosome position is crucial for axon formation in that the axon emerges from the process closest to the centrosome, and, if the cell features more than one centrosome, more than one axon is formed (de Anda et al., 2005). Other studies suggest that members of the apical Par complex, including Par3, have been observed to localize to the axon. More directly, expression of Par3 dominant negative constructs was shown to disrupt neuronal polarization, suggesting that axonal localization of the apical complex is involved in specifying the axon (Shi et al., 2003; Nishimura et al., 2004). However, *in vivo* imaging of the centrosome and Par3 in RGCs using GFP fusion proteins demonstrated that these components remained at the tip of the apical process during

basal axon extension, demonstrating that they are as distal from the axon as is possible and that their localization does not direct axogenesis in the same manner as observed *in vitro* (Zolessi et al., 2006). Instead, it becomes clear that the intrinsic neuroepithelial polarity is what most strongly correlates with RGC polarization.

The polarity of the neuroepithelial environment and the associated extracellular cues are indeed critical for the normal sequence of RGC polarization (see Fig. 2). Zebrafish mutants, *has* (*aPKCλ*) and *nok* (*pals1*), feature dramatically disrupted retinal polarity; retinal lamination is grossly disrupted, different neuronal cell types are scattered throughout the retina and cell divisions occur at ectopic locations (Pujic and Malicki, 2004). Imaging of RGCs in *nok* mutants revealed that ectopically positioned RGCs near the apical retinal surface regularly invert their axono/dendritic axis, and extend axons along the apical surface of the retina. In the *has* mutant, similarly mispositioned RGCs were often seen in a dynamic multipolar phase, morphologically similar to Stage 2 hippocampal neurons and cultured RGCs, and seemed to have trouble extending axons (Zolessi et al., 2006).

The difference in polarization behavior of the RGCs in the *nok* and *has* mutants was reasoned to result from differences in the integrity of the RPE. In *has* mutants the RPE is not as severely disrupted, and overlies most of the apical surface of the retina, while in *nok* mutants large gaps occur in the RPE, which correlated to areas of RGC membrane accumulation. This presumably reflects axon extension in areas devoid of RPE (Zolessi et al., 2006). In support of this hypothesis, a recent study by Zou and colleagues demonstrated that RPE-specific expression of Nok results in RPE maintenance, and is sufficient to rescue many aspects of the *nok* mutant phenotype, including RGC patterning. This argues that RPE loss is the root cause of the inverted RGC phenotype (Zou et al., 2008). The inversion of RGC polarity in the *nok* mutants could result in a loss of RPE derived signals inhibitory towards RGC axon formation, or alternatively the novel presentation of positive signals. In support of the latter, the basal lamina of Bruch's membrane lies beyond the RPE, which has been shown to be a good substrate for RGC axon growth (Halfter, 1988), and it contains ECM components known to be able to polarize cultured neurons (Gupta et al.; Esch et al., 1999; Menager et al., 2004). Injection of collagenase into the vitreous of chick and quail retinas results in the disintegration of the basal lamina. This leads to a disorganization of RGC axons, which likely reflect polarization errors, and demonstrates the importance of the basal lamina in axonal polarization (Halfter, 1998). Differentiating RGCs in a wild type retina contact a single basal lamina lining the basal surface of the retina at the ILM, while in the *nok* mutant RGCs are also presented with an apical basal lamina. This double presentation likely explains why some RGCs polarize correctly, while others completely invert in this mutant.

The observation that apically positioned RGCs in the *has* mutant often fail to extend an axon likely reflects the presence of molecules inhibitory towards RGC axon extension in apical regions of the retina. In support of this, chick RGCs cultured on cryosections of apical retina extended fewer axons than those cultured on cryosections of basal retina (Bauch et al., 1998). Therefore, molecules outside of the basal lamina also appear to be able to influence polarization, although RGCs *in vivo* may not normally have access to them.

Chondroitin sulfate proteoglycans (CSPGs), which are asymmetrically distributed in the vertebrate retina, with high concentrations at the basal surface where axons form, are thought to mediate repulsive guidance of RGC axons towards the optic nerve head (Brittis et al., 1992). Incubation of cultured rat retinas with free chondroitin sulfate, which results in ectopic chondroitin sulfate in apical regions of the retina, caused RGC inversion and axon extension along the apical surface. These data, indicate that, in contrast to their proposed role in repulsive RGC axon guidance, CSPGs may be attractive during polarization, and act to direct RGC polarization basally (Brittis and Silver, 1994). Another candidate family of molecules that may mediate RGC polarization are axon guidance factors such as Semaphorin, Slit, Netrin-1, BDNF and Sonic Hedgehog, which are known to be able to affect RGC axon growth (Erskine and Herrera, 2007). BDNF and Netrin have been demonstrated to bias the polarization of cultured hippocampal neurons *in vitro* (Shelly et al., 2007; Mai et al., 2009), Slit can repolarize migrating SVZa neurons cultured in matrigel (Higginbotham et al., 2006), and Unc-6/Netrin is required for oriented polarization of *C. elegnas* HSN neurons *in vivo* (Adler et al., 2006). Furthermore, in Slit2 knockout mice a subset of RGCs in the dorsal retina extended axons towards the apical surface, indicating that Slit2 may be a repulsive cue preventing RGC axon extension in apical regions of the retina (Thompson et al., 2006). The relatively mild nature of the Slit2 knockout phenotype reinforces the idea that additional factors, both positive and restrictive, are likely collaborating to direct RGC polarization.

Bipolar Cells. Through the analysis of Golgi stained rat retinas, Morrest hypothesized that the axons of

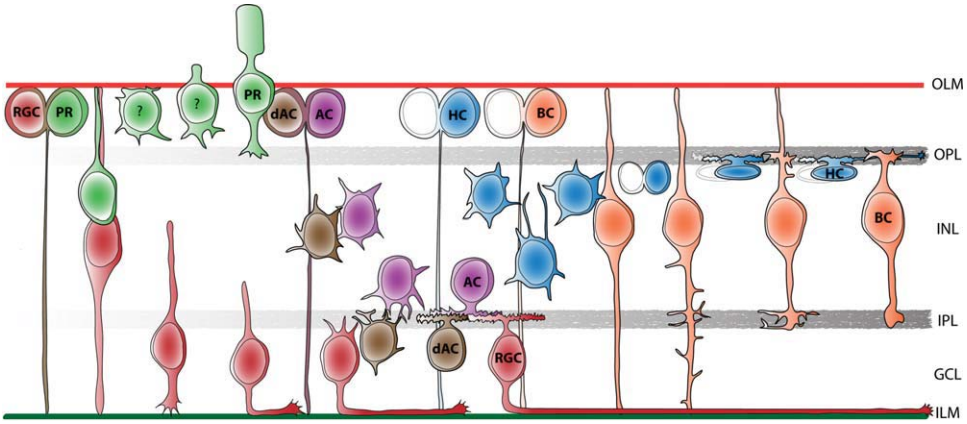

Figure 3 Morphogenesis and polarization sequence of retinal neurons. Neurons are born through terminal divisions of retinal progenitor cells at the apical surface of the retina. Retinal ganglion cells (RGCs, red) extend an axon from the basal surface of the cell while they retract their apical process. Dendrites then sprout from the apical surface of the cell and laminate into the inner plexiform layer (IPL). Phtoreceptors (PR, green) initially translocate their cell bodies basally, but then move back to the apical surface and form inner segments, followed by axons and outer segments. Amacrine cells (AC, purple) and displaced amacrine cells (dAC, brown) lose apical and basal attachments and adopt a multipolar morphology, extending dynamic processes during migration towards the IPL. After migration neurites become restricted to the IPL, where they stratify and mature into AC neurites. Horizontal cells (HC, blue) also undergo a basally directed multipolar migration, but upon reaching the IPL reverse direction and travel apically towards the outer plexiform layer (OPL). These cells can then undergo a second division, giving rise to a pair of horizontal cells, which extend neurites into the OPL. A subset of HCs then extends within the OPL. Bipolar cells (BC, orange) axon sprout from the basal process, where short processes are seen throughout the length of the basal process, and then become restricted to the IPL and the basal process retracts. Dendrites sprout from the apical process, where processes extend into the OPL, and the apical process subsequently retracts. (Prada et al., 1987; Schmitt and Dowling, 1999; Edqvist and Hallbook, 2004; Godinho et al., 2005; Morgan et al., 2006; Mumm et al., 2006; Zolessi et al., 2006; Godinho et al., 2007).

bipolar cells may arise directly from a neuroepithelial-like basal process (Morest, 1970). Using a combination of fixed tissue and live confocal imaging in mouse, Morgan and colleagues confirmed that BCs, similar to RGCs, do not go through a multipolar phase. Instead, axons and dendrites sprout directly from apical and basal processes (Fig 3, Morgan et al., 2006). Similar to neuroepithelial cells, immature BCs have an apical process extending to the OLM, and a basal process extending to the ILM. Axons develop first, where dynamic neurites extend from the basal process into the IPL. Interestingly, this sprouting is initially not limited to the IPL, and neurites are seen extending from the basal process in the ganglion cell layer and inner nuclear layer. As the cell matures, the basal process retracts, axonal processes become restricted to the IPL, and subsequently to the On-sublamina. Dendrites develop from the apical process, and in contrast to the axons, seem to sprout more directly into the OPL, and no substantial sprouting is seen apical to the IPL (Morgan et al., 2006).

Perhaps due to their morphological similarity to neuroepithelial cells, BCs show the smoothest transition from neuroepithelial to neuronal morphology. However, as the marker used in the Morgan study did not label cells early enough to follow cells from their birth through polarization, it is not absolutely clear if true neuroepithelial basal and apical processes transform into axons and dendrites (Morgan et al., 2006). Alternatively, pre-axonal and dendritic processes could re-extend from the cell body to the retinal apical and basal surfaces prior to sprouting into the plexiform layers. Live imaging of *vsx1:GFP* and *vsx2:GFP* in transgenic zebrafish revealed GFP expression throughout the lifetime of BCs (Vitorino et al., 2009). Following these markers, no evidence of process re-extension prior to stratification was observed, supporting the conclusion that axons and dendrites sprout directly from neuroepithelial-like processes (Vitorino et al., 2009) and our unpublished observations.

Central questions that remain in BC polarization are how neuroepithelial processes transform into

axons and dendrites, what molecular factors are involved, and to what extent extracellular cues in the neuroepithelium influence this polarization. Obvious cues that could be involved are partner neuron processes in the plexiform layers, as bipolar cells are the last neurons to be born (Schmitt and Dowling, 1999; Rapaport et al., 2004). However, in terms of axonal development, it is clear that RGCs are not strictly required for IPL stratification, as zebrafish *lakritz*/*ath5* mutants which lack RGCs, as well as rats or ferrets whose RGCs were removed through optic nerve transection, showed relatively normal IPL stratification (Gunhan-Agar et al., 2000; Williams et al., 2001; Kay et al., 2004). However, a role for amacrine cells was suggested by careful analysis of the zebrafish *lakritz* mutants. In those specimens, amacrine neurite stratification was abnormal in patches of the IPL, at locations at which BC axon projections were also disrupted, suggesting that amacrine cells may be instructing the stratification of BC axons into the IPL (Kay et al., 2004).

It should be noted that plexiform layer stratification and neuronal polarization might be connected, but distinct processes. A lack of correctly placed terminals in a mature retina does not necessarily represent a polarization defect, but instead could reflect a problem in cellular response to cues directing sublaminar targeting and/or synaptic partner recognition (Huberman et al., 2010). Plexiform layer stratification likely results from a multi-step process, where neuroepithelial projections first polarize and become axons and dendrites, extending dynamic searching processes analogous to growth cones, and subsequently stratify within sublamina of the plexiform layers and synapse with partner neurons. This initial polarization may be completely intrinsic and autonomous based on neuroepithelial polarity, or alternatively may be directed by extracellular cues. The latter possibility is supported by the fact that BC axonal and dendritic process extension does not occur homogeneously throughout the apical and basal process, but instead neurite extension is concentrated near the plexiform layers. This suggests that factors present in the vicinity of the plexiform layers may be directing BC polarization (Morgan et al., 2006).

Photoreceptors. Photoreceptors are perhaps among the most polarized and compartmentalized cells in vertebrates. Both rod and cone photoreceptors line the apical surface of the retina. Outer segments, consisting of multiple layers of membranous folds containing the light sensitive pigments, point apically towards the RPE. Basal to this is the cilium and inner segment, followed by the cell body, and finally the axons projecting basally into the OPL. The inner segment can be further defined as a mitochondrium rich ellipsoid region and the golgi/endoplasmic reticulum dense myoid region. Although photoreceptor morphogenesis has been the subject of much attention, the majority of studies have focused on how the elaborate structure of the outer segments form, and how mature photoreceptor morphology is setup (Kennedy and Malicki, 2009). Relatively little is known about how photoreceptors change from neuroepithelial shaped cells into immature photoreceptors, and how initial neuronal polarity is established. EM and Golgi staining studies indicate that inner segments are the first to form, followed by the concurrent appearance of axons and outer segments (Morest, 1970; Hinds and Hinds, 1979; Schmitt and Dowling, 1999). Live imaging of presumed photoreceptors labeled by *ath5* promoter driven fluorescent protein constructs in zebrafish embryos suggests that after terminal division, the cell body of photoreceptors initially moves basally, but then translocates to the apical surface of the retina. At this point protrusive activity is obvious both basally and apically to the cell body, which is presumably involved in forming inner segments and axons (Fig 3, Poggi et al., 2005; and our unpublished observations).

Zebrafish mutants have demonstrated that PR morphogenesis depends on the minus end directed microtubule motor complex Dynein-1/Dynactin, as *dynactin1a* mutants show defects in PR nuclei positioning, and *cytoplasmic dynein heay chain 1* (*dync1h1*) mutants fail to form outer segments and intracellular organelle positioning is disorganized (Insinna et al.; Tsujikawa et al., 2007). Unlike in other retinal neurons, the apical adherens junctions of the OLM remain in photoreceptors, attaching them to neighboring photoreceptors, Muller glia and neuroepithelial cells. During morphogenesis, the inner and outer segments elaborate as a massive expansion of the apical domain of these cells, causing the OLM to lie within the photoreceptor cell body (Williams et al., 1990; Schmitt and Dowling, 1999). Remarkably, in dissociated retinal cultures, photoreceptors will mature from round, morphologically symmetrical cells, to adopt their complex elongated morphology. This occurs without the addition of heterogeneous cues or contact with other cells, demonstrating a high degree of autonomy in their morphogenesis (Adler, 1986; Madreperla and Adler, 1989). Perhaps the maintained contact to neighboring cells through the persistent OLM is sufficient to orient and position the autonomously polarizing photoreceptors within the retina.

Classical epithelial polarity proteins of the Crumbs and Par complexes are localized apical and adjacent to the OLM. This pre-established neuroepithelial polarity could serve to direct the polarization of PRs with respect to the OLM, where inner/outer segments are defined by the apical complexes, and axons determined by basolateral polarity proteins. Mutations in Crumbs result in a disintergration of the OLM, and decreased PR apical domain size, although overall PR polarity is maintained (Mehalow et al., 2003; van de Pavert et al., 2004; Omori and Malicki, 2006). Zebrafish mutants of other members of the Crumbs complex, as well as the Par complex, also result in PR defects (Pujic and Malicki, 2004). However, in mutants and morphants of these genes, the general neuroepithelial polarity is dramatically disrupted prior to photoreceptor genesis (Kennedy and Malicki, 2009). Although many phenotypic aspects of the mutation of the Crumbs complex component *nok*/*pals1* in zebrafish were rescued by RPE specific expression of Nok, photoreceptor patterning as well as OLM formation remained disrupted. These retinas showed photoreceptors scattered throughout, which had not elongated properly, and failed to localize Crumbs to the inner segment region, (Zou et al., 2008). This provides some evidence that the apically localized Crumbs complex may be required within photoreceptors for their morphogenesis, perhaps through the maintenance of the OLM. Although it is clear that apical complex members are required for photoreceptor morphogenesis, it is not clear if they are required for polarization decisions, and much work remains to be done on whether the intrinsic neuroepithelial polarity and/or a persistent OLM could be instructive for PR polarization. Thus the extent to which polarization is regulated by extracellular cues in these cells is not yet known.

Amacrine and Horizontal Cells. Horizontal and amacrine cells are the only cells in the retina to undergo a phase of free migration, in which apical and basal processes detach and neurons migrate to their final positions (see Fig. 3). This was hypothesized from the analysis of fixed retinas in which young amacrine cells were seen to adopt a multipolar morphology, extending many short processes, which were presumed to be important for migration (Hinds and Hinds, 1978; Hinds and Hinds, 1983; Prada et al., 1987). Live imaging of differentiating ACs and HCs in zebrafish embryos confirmed that these cells undergo a multipolar migratory phase prior to polarization (Godinho et al., 2005; Godinho et al., 2007; Jusuf and Harris, 2009). Short dynamic processes, are extended from all poles of the cell body as the cell migrates from its birthplace at the apical surface to the vicinity of the IPL. ACs then extend neurites that stratify within the IPL. Their polarization appears to occur from biased stabilization of processes into the IPL, rather than selective sprouting, indicating that interactions between neurites and factors within the IPL may occur (Godinho et al., 2005).

Displaced amacrine cells (dACs) are a subpopulation of Sox2 expressing ACs that migrate further than other ACs, and come to lie basal to the IPL in the ganglion cell layer (Galvez et al., 1977; Taranova et al., 2006). dACs still have their neurites extending apically into the IPL, and therefore exhibit an inverted polarity with respect to the other AC subtypes. This inversion could result from intrinsic differences in this displaced population, causing them to not only to migrate further than other AC, but also to respond in an inverted manner to the same cues. Alternatively, there may be instructive signals within the IPL that cause all ACs to orient their neurites towards it, regardless of which side the AC happens to reside on. It is also possible that it is not the orientation of initial outgrowth with the apico/basal axis but selective stabilization of those processes that reach the IPL that is key to the reversed polarity of these cells.

How AC neurites become stabilized within the IPL is not known. Multiple cell adhesion molecules have been reported in the IPL, such as Cadherins, Sidekicks, Dscams, Contactin, Tenasin-c, NgCAM and NrCAM, (D'Alessandri et al., 1995; Honjo et al., 2000; Drenhaus et al., 2003; Yamagata and Sanes, 2008). ACs in zebrafish N-cadherin mutants showed exuberant outgrowth and extended misdirected processes into the INL, implicating cadherin interactions in mediating AC polarization, perhaps through process stabilization (Masai et al., 2003).

How could adhesion molecules such as cadherins localize to the IPL? An obvious model would be that relevant adhesion molecules are present on the dendrites of RGCs, since they are synaptic partners of ACs, and RGCs are born earlier than ACs (Schmitt and Dowling, 1999; Rapaport et al., 2004). However, live-imaging of RGC dendrites demonstrated that amacrine cells appear to stratify into the IPL before RGC dendrites, and in retinas lacking RGCs, ACs are still able to organize into an IPL (Gunhan-Agar et al., 2000; Williams et al., 2001; Kay et al., 2004; Mumm et al., 2006). Time-lapse analysis of ACs in the zebrafish *lakritz*/*ath5* mutant, which completely lack RGCs, demonstrated that an IPL still forms in the absence RGCs, although IPL formation was delayed,

fairly disorganized, and formed closer to the ILM (Kay et al., 2004). This suggests that RGCs may play an early, albeit non-essential role in polarizing ACs towards the IPL. The IPL subsequently becomes much more ordered in older *lak* mutant retinas, indicating that the AC are able to correct early aberrantly projecting neurites, and AC/AC interactions could be important for polarization, resulting in a self organizing system (Kay et al., 2004). Alternatively, other later born cells such as BCs or Muller glia could influence corrective behaviors and refine AC processes.

Careful timelapse imaging demonstrated that ACs and dACs extend neurites towards each other at approximately the same time during IPL formation (Godinho et al., 2005). This leads to the intriguing hypothesis that dACs may influence the stratification of normally positioned ACs (Godinho et al., 2005). It might be that RGCs simply act as a barrier to AC migration rather than playing a direct role in polarization (Kay et al., 2004). dACs migrate farthest, and come to lie at the AC/RGC border. dACs then polarize away from the RGCs, forming an immature IPL and physically forcing themselves into the ganglion cell layer (Godinho et al., 2005). dAC dependent polarization would provide an explanation for how the IPL always forms directly adjacent to the ganglion cell layer, but can form without RGCs, as well as why the IPL initially forms close to the ILM in *lak* mutants, as there are no RGCs to act as the migratory border in the mutant retinas (Kay et al., 2004). Although the exact mechanisms still need to be determined, it is apparent that extracellular cues on neurons in the vicinity of the IPL likely direct the polarization of ACs.

The early phase of HC morphogenesis is undistinguishable from ACs, where they undergo free migration towards the IPL. Amazingly, these neurons then reverse their migratory direction and travel back to the base of the OPL (Edqvist and Hallbook, 2004). The reason for this apparently inefficient migration is not known, but requires the expression of the transcription factor Lim1 (Poche et al., 2007). Live imaging in zebrafish demonstrated that HCs undergo a final mitosis once they reach their correct location, producing a pair of HCs that extend neurites and stratify into the OPL (Godinho et al., 2007). The cues directing HC stratification are not known, but they are likely to be similar to the cues directing AC polarization towards the IPL, as HCs that lack Lim1 expression not only fail to migrate towards the OPL, but also extend processes and stratify into the IPL (Poche et al., 2007). This does not seem to be a result of a cell fate switch to AC identity, as the Lim1 negative cells still express HC specific markers and are negative for AC markers (Poche et al., 2007).

A subset of HCs extend a long axon within the OPL. An analysis of Golgi stained chick retinas indicated that this axon may sprout from one of the dendrites (Genis-Galvez et al., 1981). However, this hypothesis awaits live imaging confirmation, and a study of immature HCs in mouse demonstrated that long axon-like processes can be seen before mature dendrites are formed, indicating that dendrites may not always precede axons (Huckfeldt et al., 2009). Nevertheless, axon-bearing HCs may go through a two step polarization process in which neurites are first directed into the OPL, and subsequently select and then extend their axon from one these neurites. It is tempting to speculate that a mechanism similar to dissociated hippocampal neuron polarization may occur, in which one neurite/dendrite is selected from many morphologically equivalent processes to become an axon.

Dendrite Formation

The events leading to properly oriented dendrites have been less explored than those for axon formation. Similar to neurons in culture, RGCs and BCs form axons before dendrites (Hinds and Hinds, 1974; Holt, 1989). However, PRs and axon bearing HCs seem to form dendritic structures before axons, indicating that dendrite formation need not always follow axon formation (Prada et al., 1987; Schmitt and Dowling, 1999). As with axon formation, two mechanisms could contribute to RGC and BC dendrite formation: dendrites may sprout cell autonomously, directed by intracellular asymmetries setup during axon formation and/or neuroepithelial polarity. Alternatively, dendrites may be influenced by extracellular cues, resulting in their directed sprouting.

BC dendrites seem to sprout directly into the OPL, rather than at the tip or throughout the apical process, indicating that this event may be directed by extracellular cues (Morgan et al., 2006). Interestingly, a subpopulation of RGCs in the chick retina are displaced, and do not migrate to the ganglion cell layer. These cells remain in the inner nuclear layer, and must form dendrites basal to their cell body to stratify in the IPL. The morphology of these neurons was analyzed by Golgi staining in chick embryos, and demonstrated that they can adopt a monopolar morphology extending dendrites from the axonal shaft, which travels through the IPL (Prada et al., 1992). These two observations, and the fact that HC and PR form dendrites before axons, indicate that dendrite polarization is not

completely passive and cell autonomous, but is likely to be as directed and complex as axon formation.

Conclusions and Perspectives

In vivo studies in the retina have shown that RGCs and BCs make a smooth transition from neuroepithelial to neuronal morphology and that axons and dendrites sprout directly from the cell (Morgan et al., 2006; Zolessi et al., 2006). This is in contrast to the multipolar Stage 2 behavior that is transiently seen in cultured hippocampal neurons. Moreover, in these cultured neurons, members of the apical complex including Par3, localize to the immature axonal growth cone and are important for neuronal polarization (Shi et al., 2003; Nishimura et al., 2004). Furthermore the position of the centrosome at the base of one neurite has been reported to correlate with this neurite forming the axon (de Anda et al., 2005) although the centrosome may be dispensable for axon extension in these cells (Stiess et al., 2010), and no correlation between centrosome localization and axon formation was found in embryonic chick forebrain neurons polarizing *in vitro* (Seetapun and Odde., 2010). In contrast, in zebrafish RGCs polarizing *in vivo*, the centrosome as well as the apical complex remain in the apical process (Fig. 2; (Zolessi et al., 2006) while axons emerge. These findings challenge the role of these factors in the normal polarization process. It is therefore important to look at studies in which the roles of the apical polarity complex and the centrosome are explored in different cell types *in vivo*. In *Drosophila* mushroom body neurons, Rolls and Doe demonstrated that neurons lacking apical complex members Par-3, Par-6 or aPKC, as well as ACC motorneurons overexpressing Par-3 or Par-6, have normal axon and dendrite morphology (Rolls and Doe, 2004). Furthermore, Drosophila DSas-4 mutants, which lack centrosomes, have a morphologically normal nervous system and properly oriented axons in eye disk neurons (Basto et al., 2006). These studies highlight the fact that results obtained from one type of neuron *in vitro* may not apply to all neuronal polarization events, especially those *in vivo*, and so it will be important in the future that results pertaining to neuronal polarization obtained *in vitro* need confirmation *in vivo* in order to demonstrate their general relevance.

In the case where neurons lose contact with the neuroepihelium, the situation may become more like that *in vitro*. For example, cortical interneurons have been observed in a multipolar state prior to the emergence of the trailing process that becomes the axon, and the leading process from which dendrites develop (Hatanaka and Murakami, 2002; Tabata and Nakajima, 2003; Noctor et al., 2004). This multipolar stage appears to be similar to the multipolar Stage 2 of polarizing hippocampal neurons *in vitro* (Barnes et al., 2008; Barnes and Polleux, 2009). This stage may be equivalent in some ways to the multipolar migration of AC/HCs. It will, therefore, be very interesting to determine how the multipolar migratory neurites of AC/HCs compare with maturing axono/dendritic neurites of cortical neurons, and to understand at which point the polarity of the neurons becomes manifest. Do these neurons "break symmetry" autonomously using mechanisms that hippocampal neurons *in vitro* use? Or do they rely on local extracellular cues using distinct mechanisms? An analysis of the dynamics and determinants of HC axon extension could be an avenue for research into how axons sprout from multipolar cells *in vivo*.

Extracellular cues are not required for RGC or PR polarization, as these neurons can polarize in dissociated culture (Adler, 1986; Zolessi et al., 2006). However, extracellular factors certainly influence the orientation polarization of RGCs in the retina (Brittis and Silver, 1994; Halfter, 1998; Zolessi et al., 2006). Moreover, the stratification of ACs, dACs, and mis-migrated Lim1 mutant HCs into the IPL strongly suggests that extracellular factors within the IPL direct neuronal polarization (Godinho et al., 2005; Poche et al., 2007). Evidence from cultured neurons shows that extracellular cues are capable of directing the orientation of polarization (Gupta et al.; Esch et al., 1999; Menager et al., 2004; Shelly et al., 2007; Mai et al., 2009). Furthermore, Slit2 knockout mice show problems in neuronal orientation, as do the dorsal root ganglia of Sema3A knockout mice, suggesting that these molecules may direct polarization in the mammalian nervous system *in vivo* (Thompson et al., 2006; Lerman et al., 2007).

Cell culture experiments have indicated that the RPE may be a source of important secreted factors. Dissociated cells from avian retinas grown in rotating culture conditions have a remarkable ability to reaggregate and form spherical structures containing all of the cell types of the retina. These reaggregates form rosettes, which are fairly disorganized, but grossly resemble an inverted retina—PRs on the inside, RGCs on the outside (Bauch et al., 1998). When reaggregates are assembled in the presence of RPE (or supernatant derived from RPE cells) these spheres become much more organized, and recognizable cell and plexiform layers form, which are not longer inverted (Rothermel et al., 1997). This organization could also be achieved by culturing the reaggregates in the presence of the anterior rim of the retina, Wnt-2b, or a monolayer of Muller glia cells (Willbold et al., 2000; Nakagawa et al., 2003).

Although clearly able to dramatically affect polarity, the mechanisms by which these factors influence retinal organization is not known and it is not clear if they have a direct influence on neuronal polarization.

How much of polarization is dictated by the environment, and how much results from pre-established intrinsic polarity may vary between cell types. Determining how these two sources of polarization information are integrated is a critical step in understanding how neurons orient within the brain. From the data available in the retina, it appears that for cells undergoing somal translocation, like RGCs and BCs, neuroepithelial polarity is inherited from intracellular asymmetries, which are set up in the proliferative neuroepithelium. This may bias the preferred axon/dendritic orientation of the neuron, but the final commitment to polarize may need reinforcement by extracellular cues acting on the neuron, resulting in directed axon/dendrite extension in the correct orientation.

Many of the unsolved questions about neuronal polarization in the retina are only beginning to emerge and we are still a long way from understanding the relevant molecular mechanisms. A more difficult, but very exciting challenge, will be to determine how the programs that lead to cell type specification and the dramatically different cellular architectures of these cell types is superimposed on the polarization process. For example, PRs and RGCs can arise from the division of a single progenitor cell, and thus are identical prior to their birth. However, during subsequent differentiation, RGCs extend a massive axon and a modest dendrite, while PRs extend a massive photosensitive dendrite and a modest axon. Similarly, HCs and ACs seem identical during the first phase of migration towards the IPL, but the majority of mature ACs are oriented basally into the IPL, while HCs are oriented apically towards the OPL. All polarization and morphogenesis events depend upon the cytoskeleton, whose rearrangements physically drive the cellular transformations. Determining how cell type identity differentially directs these rearrangements amid the same polarization cues will be an essential part of understanding how nervous systems develop and begin to form functional networks.

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