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A Fish Eye View: Retinal Morphogenesis from Optic Cup to Neuronal Lamination

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Abstract

The neural retina, at the back of the eye, is a fascinating system to use to discover how cells form tissues in the context of the developing nervous system. The retina is the tissue responsible for perception and transmission of visual information from the environment. It consists of five types of neurons and one type of glia cells that are arranged in a highly organized, layered structure to assure visual information flow. To reach this highly ordered arrangement, intricate morphogenic movements are occurring at the cell and tissue levels. I here discuss recent advances made to understand retinal development, from optic cup formation to neuronal layering. It becomes clear that these complex morphogenetic processes must be studied by taking the cellular as well as the tissue-wide aspects into account. The loop has to be closed between exploring how cell behavior influences tissue development and how the surrounding tissue itself influences single cells. Furthermore, it was recently revealed that the retina is a great system to study neuronal migration phenomena, and more is yet to be discovered in this aspect. Constantly developing imaging and image analysis toolboxes as well as the use of machine learning and synthetic biology make the retina the perfect system to explore more of its exciting neurodevelopmental biology.

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INTRODUCTION

Vision is a fundamental sense for many species on earth. Visual systems allow organisms to move toward prey and away from predators, to sense the environment, and to adapt to day-night cycles. For humans, the perception of their visual environment has additional aesthetic components as vision allows for the appreciation of art, beauty, or nature. Visual systems have evolved multiple times, from the very primitive eye spots found today in ascidians and starfish (Arendt et al. 2002, Nilsson 2009) to the compound eyes found in arthropods (Harzsch & Hafner 2006) and the more sophisticated camera-type eyes found, for example, in cephalopods and vertebrates (Nilsson 2009).

Vision begins with the retina, the part of the nervous system responsible for the collection and transmission of visual information to the brain. In vertebrates the neural retina is located at the back of the eye and connected to the optic tectum by the optic nerve (**Figure 1*a***). The retina shows a hemispheric architecture with the lens in the middle (**Figure 1*b***). It hosts six different types of neurons and one type of glia cells, the Müller glia (**Figure 1*c***). Retinal ganglion cells (RGCs) occupy the most basal layer and their axons form the optic nerve. Photoreceptor cells, cones for color vision and rods for vision in darker environments, reside at the opposite side of the retina, the apical outer nuclear layer.

Bipolar cells reside in the inner nuclear layer (INL) and connect photoreceptors to the RGCs. The interneurons, horizonal cells, and amacrine cells are also found in the INL, although a small number of amacrine cells called displaced amacrine cells are also found in the RGC layer (Grimes et al. 2010, Masland 2012). Müller glia span the whole length of the tissue (MacDonald et al. 2015) (**Figure 1*c***). These general cell categories, in turn, each consist of a plethora of subtypes

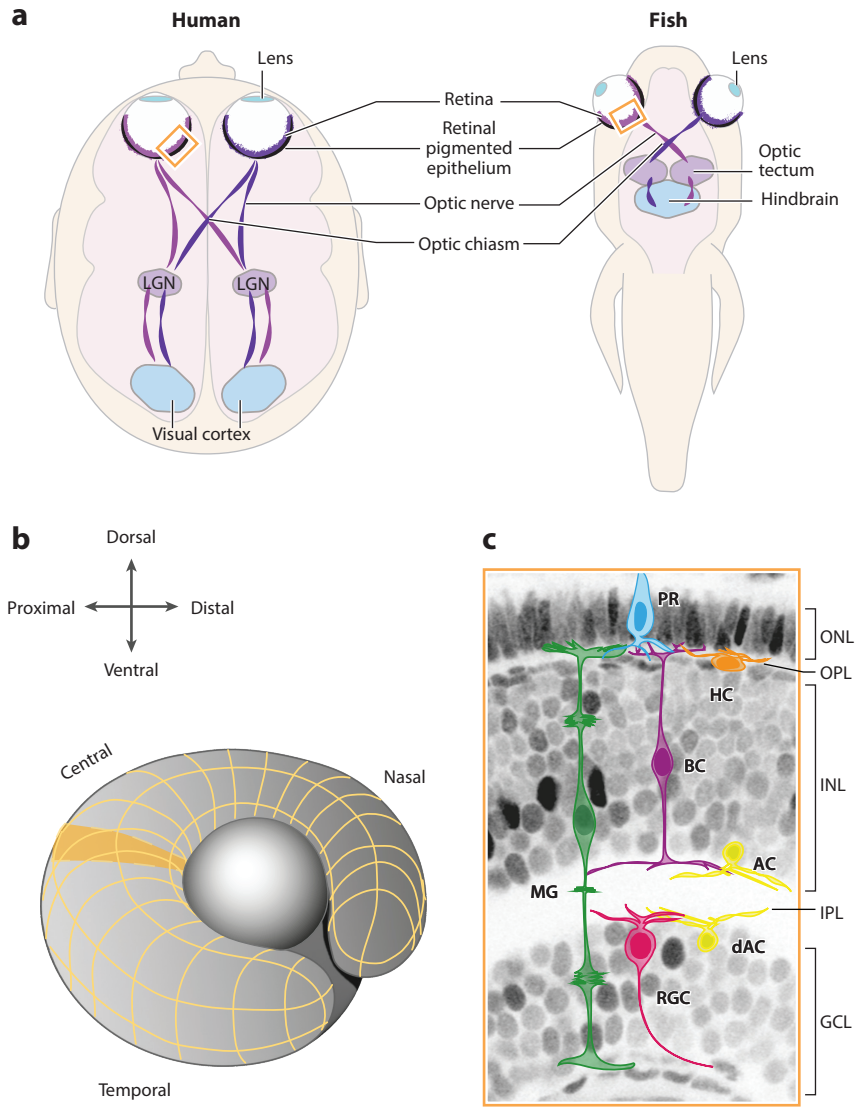

Figure 1

(*a*) Human (*left*) and teleost (*right*) visual systems. The orange boxed area is the retina at the back of the eye in both systems, whose composition is outlined in panel *c*. (*b*) The hemispheric optic cup with the lens in the center. The orange shaded area is the part of the retina shown in panel *c*. (*c*) Cell types and arrangements in the vertebrate retina. Abbreviations: AC, amacrine cell; BC, bipolar cell; dAC, displaced amacrine cell; GCL, ganglion cell layer; HC, horizontal cell; INL, inner nuclear layer; IPL, inner plexiform layer; LGN, lateral geniculate nucleus; MG, Müller glia cell; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptor; RGC, retinal ganglion cell.

that can be differentiated by a combination of molecular markers, position, morphology and electrophysiology.

Information flow via these cell types is slightly counterintuitive: Initially, light has to penetrate through the whole tissue as it is collected at the back of the retina by the photoreceptors. This information is then transmitted via the bipolar cells to the RGCs at most basal positions of the

retina. RGCs send the information to the brain via the optic nerve (**Figure 1*a***). Horizontal cells and amacrine cells help to integrate the visual message presented to the RGCs. It is thought that Müller glia, which span the complete width of the retina from the ganglion cell layer to photoreceptors, can serve as optical fibers to make the information flow more efficient (Franze et al. 2007). The neural retina is enveloped by the retinal pigment epithelium (RPE) cells. These cells have multiple functions, including nutrient transport and protection and phagocytosis of photoreceptor outer segments in the mature retina (Simó et al. 2010). Within this structure and enwrapped by it, the lens is found that captures the light that is then transferred to the neural retina. These three structures together build the vertebrate camera-type eye (Fernald 2000, Lamb et al. 2007) (**Figure 1*a***). The hemispheric structure of the retina with an embedded lens (**Figure 1*b***) is conserved in cephalopods even though their final retina has a very different developmental origin and neuronal composition (Kozmik et al. 2008, Napoli et al. 2022).

This review mainly concentrates on the vertebrate neural retina. To understand the development and properties of the lens, the reader is referred to other excellent reviews (for example, Gunhaga 2011, Wride 2011, Ruan et al. 2020). This review focuses on morphogenesis events that form the neural retina: It covers shape acquisition as well as the proliferation, differentiation, and migration of neurons to their final position. Gene regulatory networks, signaling cascades, and the epigenetics that guide these events are only peripherally considered, as excellent reviews on these topics already exist (Neumann 2001, Agathocleous & Harris 2009, Corso-Díaz et al. 2018).

While many model species have contributed to our understanding of different stages of retinal morphogenesis, quantitative light imaging has allowed for numerous detailed and time-resolved studies in teleost systems, mainly zebrafish and medaka (**Figure 1*a***). Thus, teleost studies are a major focus of this review. In addition, I discuss ex vivo retinal organoids as more recently emerging systems to study shape formation, neurogenesis, and lamination of the vertebrate retinas.

THE BEGINNING: GETTING THE RETINA INTO SHAPE DURING OPTIC CUP FORMATION

The Optic Cup

The vertebrate retina is an outstanding biological example that demonstrates the concept of form following function. Its hemispheric shape, generated during optic cup formation (OCF), is important to ensure the most efficient use of the available light (**Figure 2*a*,*b***).

The final optic cup is a bilayered structure composed of two different epithelia: (*a*) The retinal neuroepithelium (RNE) consists of polarized elongated cells with apical centrosomes, cilia, and basal attachments to an extracellular matrix via integrins present in focal adhesions. Cells in the RNE will generate all retinal neurons later in development. (*b*) The RPE consists of, depending on the species, more or less columnar or flattened cells with the basal side pointing toward the brain ventricle (**Figure 2*b***). OCF in vertebrates generates its particular cup-shaped architecture (**Figure 1*b***) by a series of epithelial and overall tissue rearrangements as outlined below.

Early Steps: Toward the Optic Vesicle

The eye primordium, including the retina, arises from the diencephalon and contains a mixture of surface ectoderm and neuroectoderm. Eye-field specification starts at gastrulation when the eye field arises from the anterior neuroectoderm (Fuhrmann 2010, Graw 2010). As development proceeds, the eye field splits along the midline into two parts, each part giving rise to an optic vesicle. These events are driven by signaling pathways that are involved in midline and axis specification, such as sonic hedgehog and TGF-β/nodal (Fuhrmann 2010, Graw 2010). Further

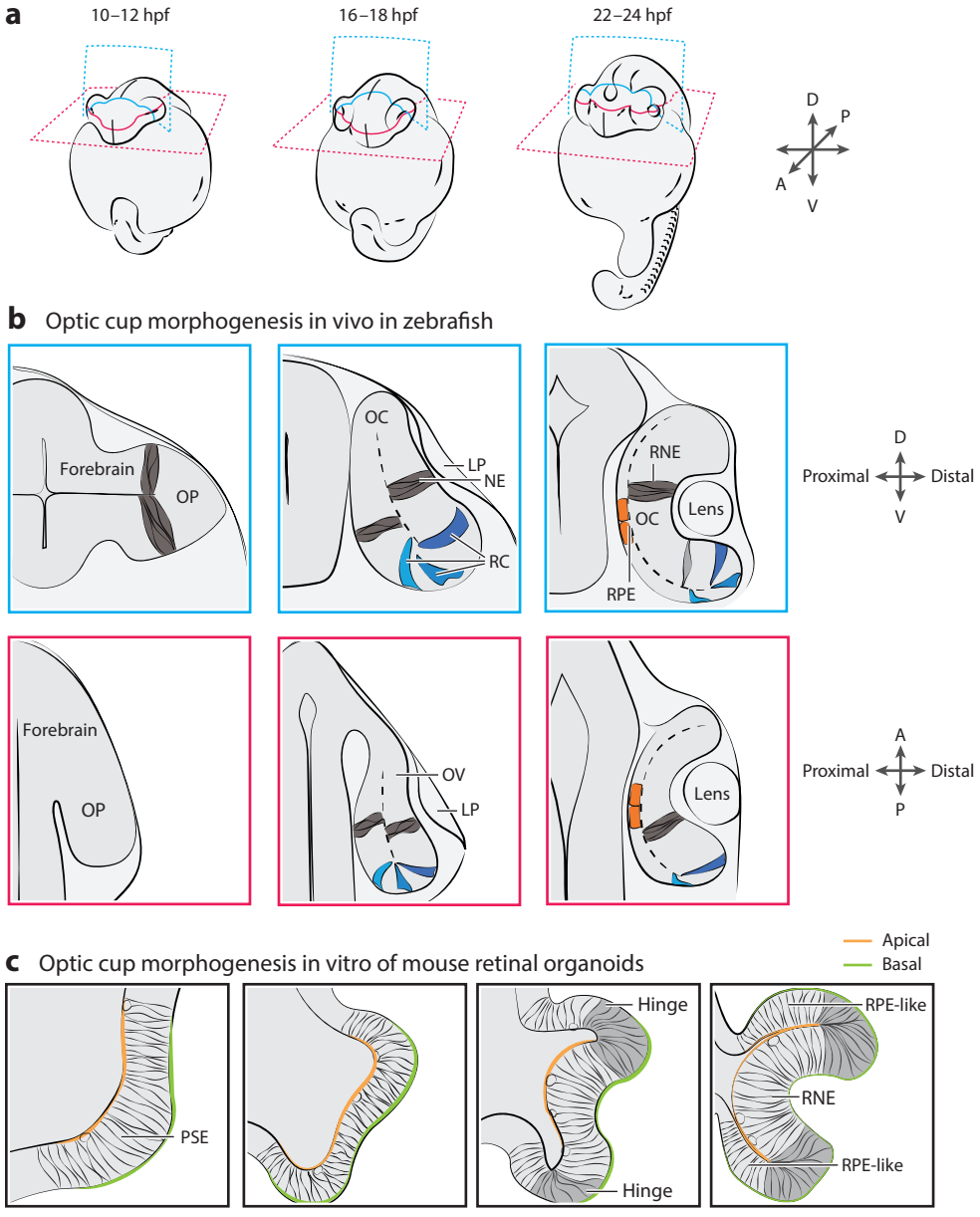

Figure 2

(*a*) Schematic of OC development in relation to the whole zebrafish embryo between 10 and 24 hpf. (*b*) Dorsoventral (*top*) and A-P (*bottom*) view of cell rearrangements during zebrafish OC development for the stages depicted in panel *a*. (*c*) Schematic of OC development in mouse organoids ex vitro. Abbreviations: A, anterior; D, dorsal; hpf, hours postfertilization; LP, lens placode; NE, neuroepithelium; OC, optic cup; OP, optic primordium; OV, optic vesicle; P, posterior; PSE, pseudostratified epithelium; RC, rim cells; RNE, retinal neuroepithelium; RPE, retinal pigment epithelium; V, ventral.

signaling cascades, in combination with transcription factor networks, are then translated into morphogenetic movements (England et al. 2006).

After eye-field specification and splitting, each of the two groups of cells grows outward from the anterior neural tube during evagination. Thereby, they form the optic vesicle from which the optic cup emerges (**Figure 2*a***).

Studies of teleost systems generated a detailed description of the evagination process of the optic cup. For example, live imaging and tracking of nuclei in medaka allowed researchers to follow individual cells and revealed that optic vesicle evagination is driven by the active migration of individual mesenchymal progenitor cells (Rembold et al. 2006). These cells adapt epithelial characteristics during migration in response to the underlying extracellular basal lamina. It was hence speculated that polarization of eye-field cells aids the efficient segregation of the eye fields and thereby facilitates the development of the optic vesicles (Rembold et al. 2006). Furthermore, at least in zebrafish, the eye field contains two distinct populations of cells, basally positioned epithelial cells that generate the pseudostratified epithelium (PSE) of the optic vesicle and apically positioned cells that undergo mesenchymal to epithelial transition to intercalate within the epithelium (Ivanovitch et al. 2013).

From Optic Vesicle to Optic Cup

After evagination, morphogenesis continues with the formation of the optic cup (**Figure 2*a*,*b***). This is a crucial step for later retinal formation, as it gives the tissue its typical hemispheric shape. The importance of successful OCF is shown by the fact that its impairment can lead to severe developmental defects. For example, a condition called coloboma is found in about 2.5 out of 10,000 human births (ALSomiry et al. 2019). In coloboma patients, the optic fissure in the optic cup does not close, which can lead to severe vision impairment.

To form and later close the optic cup, fascinating, complex epithelial rearrangements occur simultaneously, which are overall only beginning to be understood. Drastic cell shape changes accompany these events. While RPE cells flatten on the outside of the optic cup, RNE cells, which are already positioned in the neuroepithelium, constrict their basal process using a contractile basal actomyosin enrichment. This leads, at least partly, to the invagination of the overall structure. In addition, so-called rim cells move from the RPE layer into the future neuroepithelium (Martinez-Morales et al. 2009, Kwan et al. 2012, Heermann et al. 2015, Nicolás-Pérez et al. 2016, Sidhaye & Norden 2017) (**Figure 2*b***).

Basal constriction of RNE cells is an important component of efficient OCF. Medaka mutants for ojoplano (opo), a transmembrane protein involved in the formation of focal contacts between RNE cells and the underlying extracellular matrix, feature a flat eye phenotype (Martinez-Morales et al. 2009). Opo antagonizes Numb and inhibits integrin endocytosis, thereby leading to polarized basal integrin location (Bogdanović et al. 2012). This, in turn, is important to drive actomyosin contractions that basally constrict RNE cells and thereby facilitate the invagination of the optic cup (Nicolás-Pérez et al. 2016).

A study investigating zebrafish optic cup morphogenesis confirmed the actomyosin-dependent constriction of RNE cells (Sidhaye & Norden 2017). However, interference with basal constrictions by inhibiting actomyosin activity did not fully prevent invagination and OCF, indicating that invagination in the absence of actin-dependent constriction could be driven by a compensatory process. This process was found to depend on the rim cells that move from the future RPE into the future RNE during optic cup morphogenesis. Impairment of rim cell movement led to more severe problems with cup formation than interference with RNE basal constriction alone (Sidhaye & Norden 2017). The most severe phenotypes, however, were observed when both rim

cell movement and RNE basal constriction were compromised (Sidhaye & Norden 2017). Interestingly, cells that aberrantly reside outside the neuroepithelium nevertheless take on neurogenic fates (Bogdanović et al. 2012, Sidhaye & Norden 2017). Rim cell movement is not passive, as was suggested (Martinez-Morales et al. 2009, Kwan et al. 2012, Heermann et al. 2015), but active, driven by cryptic lamellipodia that are intricately linked to the underlying basal lamina and its topology (Sidhaye & Norden 2017, Soans et al. 2022).

Together, these studies show that invagination of the RNE and rim migration both play an important, active part in OCF. The role of RPE flattening in OCF has so far only scarcely been explored. A recent study showed that in zebrafish, where RPE cells flatten much more than, for example, in the mouse, this flattening depends on microtubules and actin. Impairment of either cytoskeletal component results in cells retaining a cuboidal morphology (Moreno-Mármol et al. 2021). Moreover, in zebrafish, cell proliferation plays only a minor role in RPE formation (Moreno-Mármol et al. 2021). This is different in chicken, mouse, and humans, where proliferation is more prominent. Here, as cells undergo less flattening, proliferation is an important factor for RPE spreading as it generates more surface to enfold the RPE (Moreno-Mármol et al. 2021). Overall, while progress has been made, more work is needed to explain the cross talk between the three epithelial rearrangements in the different species investigated.

Interestingly, in fish, chicken, and mouse, the process of optic cup morphogenesis is asymmetric, and generally invagination starts at the dorsal side, from which it progresses toward the ventral side. It is thought that this helps to enable the formation of the optic stalk, which connects retina and forebrain and will later host some of the developing axons (Bazin-Lopez et al. 2015). Current open questions are how this asymmetric morphogenesis event arises and how asymmetry is driven at the cellular and tissue scales. It was seen that along the nasal-temporal axis (spanning from the nose to the temporal lobe of the animal), rim cells mainly move from the temporal side, the side of the optic cup, into the RNE (Picker et al. 2009, Kwan et al. 2012, Sidhaye & Norden 2017, Soans et al. 2022). This might be due to the fact that more space is available in this part of the tissue as it is further away from the optic stalk. Upon inhibition of the FGF signaling pathway, the movement from the nasal/anterior side is impaired, but cells moving from the more temporal/posterior positions can compensate for this loss (Picker et al. 2009). This means that some plasticity exists within this in vivo system, most likely making the process more robust.

Comparison Between In Vivo and In Vitro Models of Optic Cup Formation

In vitro models of optic cup morphogenesis recently became popular with the advent of mouse and human retinal organoids. These models, derived from mouse and human induced pluripotent stem cells, are astonishing in the sense that they reveal emergent properties of the self-organization into structures resembling optic cups even without a developing lens (Eiraku et al. 2011, Nakano et al. 2012). In these systems, principles of some of the epithelial rearrangements outlined above occur. The timing of events varies between mouse and human organoids, as expected from their different in vivo development speeds. OCF takes about two days for the mouse systems and 26 days for human organoids (**Figure 2*c***) (Eiraku et al. 2011, Nakano et al. 2012). While the resulting optic cups look similar to in vivo tissues, differences are seen between ex vivo and in vivo cup formation: Cup invagination in mouse organoids depends on active proliferation of cells in the RNE (Eiraku et al. 2011), whereas this process is negligible in the zebrafish system, where the hemispheric shape is reached even when proliferation is blocked (Kwan et al. 2012, Sidhaye & Norden 2017). Furthermore, it was speculated that the so-called hinge region (**Figure 2*c***), the region that connects the RNE and the RPE, is responsible for cup invagination in mouse retinal organoids by being generally more rigid than the rest of the tissue and, in addition, driving the

process by apical constriction. A theoretical model hinted in a similar direction (Eiraku et al. 2011). Hinge rigidity seems to differ in teleosts: In these systems, particularly in this region, active rim migration occurs, meaning that there is epithelial flow (Kwan et al. 2012, Heermann et al. 2015, Sidhaye & Norden 2017). For human retinal organoids, a theoretical model proposes that, in addition to dynamics at the hinge, a reduction of apical actomyosin activity is involved in the invagination process (Okuda et al. 2018). This model does not seem to match the in vivo situation, at least in teleosts, where actin has been seen to be enriched at basal locations (Nicolás-Pérez et al. 2016, Sidhaye & Norden 2017). Such enrichment is also observed in human retinal organoids but not in mouse organoids (Eiraku et al. 2011, Nakano et al. 2012). However, whether this actin accumulation is actively involved in organoid morphogenesis is currently unclear.

Another interesting difference between mouse and human retinal organoids and in vivo teleost and other systems is symmetric versus asymmetric invagination of the optic cup. The optic cups in teleosts invaginate with different rates at the nasal versus temporal sides, leading to short-term asymmetries. In contrast, overall OCF in organoids seems to occur evenly from both sides, or at least, no prominent asymmetry has yet been reported (Eiraku et al. 2011, Nakano et al. 2012, Okuda et al. 2018). This difference between symmetric and asymmetric invagination most likely results from the fact that ex vivo systems develop in isolation without any instructive surrounding tissue. They also emerge as single cups, as opposed to the pairwise occurrence of optic cups in vivo. These different conditions might influence how morphogenetic processes take place and, thereby, could be at least partly responsible for the in vivo versus in vitro differences mentioned above. Understanding these differences will be important, and such understanding could improve the reproducibility of organoids and the efficiency of their production.

Furthermore, better understanding of differences and similarities would enable the development of organoids for understanding developmental programs in mouse and humans. In the long term, this could also facilitate the development of regenerative therapies, for which these systems have potential (Fathi et al. 2021). An additional advantage of comparative studies between in vivo and ex vivo models is that these comparisons can provide the possibility to generate fundamental insights about the role and influence of surrounding tissue and mechanical features in the in vivo system during OCF. To reach such insights, it will be crucial to investigate the regional differences and tissue constraints, including mechanical aspects during optic cup development in vivo, and compare them to in vitro systems. It will be important to explore which of the observed differences are dependent on the environment and which differences are species dependent.

To get to the core of these questions, it will be of advantage to involve theorists from different disciplines. Some cross-disciplinary studies have already improved our understanding of OCF in the in vivo versus in vitro systems. The theory involved could explain mechanical factors in the environment, as has already been done in some recent studies (Okuda et al. 2018, Soans et al. 2022). Theoretical efforts in addition and in parallel to experimental work need to be expanded, as otherwise the complexity of the in vivo systems will be hard to assess and explain. Theoretical models have the potential to generate more insights on the interplay and hierarchies between the different epithelial rearrangements. Ideally, theoretical approaches will then lead to more targeted experiments, creating a feedback loop between dry and wet science.

MOVING ON: FROM NEUROEPITHELIAL GROWTH TO NEURONAL DIFFERENTIATION ONSET

The Pseudostratified Retinal Neuroepithelium

Once the optic cup is formed, the RNE is inhabited by progenitor cells that will give rise to all retinal neurons later in development (**Figure 3*a***; 24–36 hours postfertilization). These

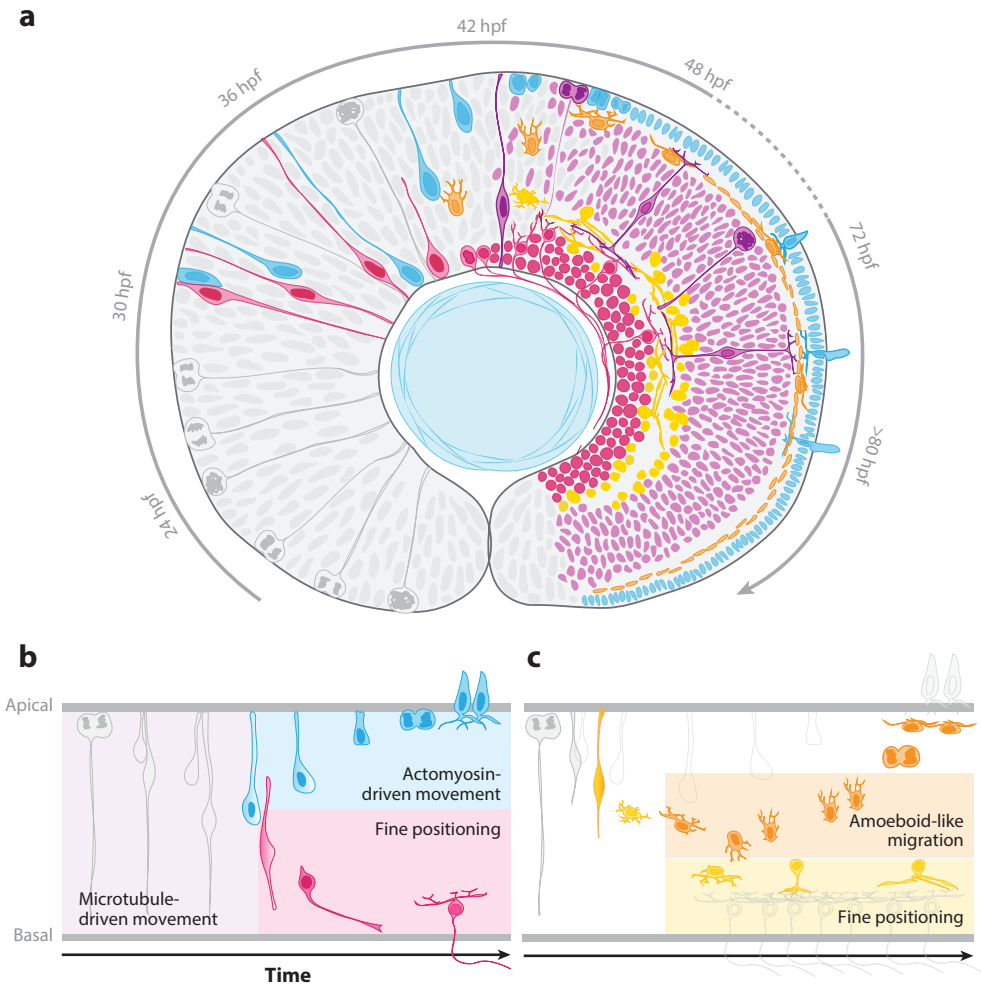

Figure 3

(*a*) Migration and lamination of the different cell types in the zebrafish retina between 24 and 80 hpf. (*b*) Somal translocation modes of retinal ganglion cells (*magenta*) and photoreceptors (*blue*). (*c*) Somal translocation followed by free migration of interneuron amacrine cells (*yellow*) and horizontal cells (*orange*). Horizontal cells undergo amoeboid-like migration when returning to the apical side. Abbreviation: hpf, hours postfertilization.

progenitors arrange in a so-called pseudostratified epithelium (PSE). PSE consist of elongated, spindle shaped cells that are apically and basally attached. Nuclei of these cells occupy positions along the apicobasal axis. Such pseudostratified epithelia are very common in development and form, for example, the lung, liver, and pancreatic buds, and otic and lens placodes (Strzyz et al. 2016). They also give rise to most vertebrate brain areas, including the neural tube, hindbrain, cortex, and retina (Norden 2017). It is thought that pseudostratified epithelia ensure high proliferation rates as they allow for dense nuclear packing (Smart 1972, Matejčić et al. 2018, Azizi et al. 2020). As packing increases and cell shapes change to accommodate more and more progenitors, nuclei become predominant in the proliferating PSE (Matejčić et al. 2018). Since nuclei are more viscous than the rest of the cells (Guilak et al. 2000), this dense packing of nuclei could result in

changes of the physical properties of the developing retina. So far, though, there are only a few 2D theoretical models that explore the effects of cell and nuclear packing (Kim et al. 2022), while a full 3D theory and analysis of this phenomenon in PSE is still lacking.

While progenitor nuclei are distributed along the whole apicobasal axis for the majority of the cell cycle, mitosis always needs to occur at apical positions. When nonapical mitosis occurs, cells that do not inherit the apical process delaminate and occupy more basal positions in the epithelium (Nakajima et al. 2013, Strzyz et al. 2015). Interestingly, in the retina, these cells do not seem to enter differentiation programs, indicating that they get lost in time and space (Strzyz et al. 2015). Thus, ensuring apical mitosis is an important factor to facilitate tissue integrity and further maturation. To undergo apical mitosis, nuclei need to move to the apical side before division, a phenomenon called interkinetic nuclear migration (Lee & Norden 2013, Strzyz et al. 2016). Different ideas were proposed regarding which cytoskeletal element drives apical movement of nuclei (Del Bene et al. 2008, Norden et al. 2009). However, it now seems clear that, at least in the zebrafish retina, this process is actin dependent (Norden et al. 2009, Strzyz et al. 2015, Yanakieva et al. 2019, Azizi et al. 2020). Interestingly, in the zebrafish RNE, actin propels the nucleus via formin-dependent polymerization (Yanakieva et al. 2019), which is different from myosin actomyosin contractility–derived forces that move organelles in many other contexts (Chalut & Paluch 2016, Gilmour et al. 2017). It was speculated that this difference could be linked to the hemispherical architecture of the tissue. An initial toy model corroborates this view (Yanakieva et al. 2019). More theoretical and experimental work is, however, needed to fully understand how tissue and cell architecture influence the cytoskeletal machineries at play during proliferation and neurogenesis onset.

From Progenitor to Neurogenesis Onset

The multipotent progenitors that inhabit the pseudostratified RNE eventually give rise to all retinal neurons. The overlapping birth order of retinal neurons is the following: RGCs and cone photoreceptor cells are born first. Then, the interneurons, horizonal cells, and amacrine cells appear, which are followed by bipolar cells and rod photoreceptor cells. Müller glia are born at late developmental stages with bipolar cells (Rapaport et al. 2004) (**Figure 3*a***; 36–72 hours postfertilization).

In the fast-developing teleost, but also in mammals, retinal growth and differentiation occur concomitantly. This means that even as the progenitors still proliferate, some cells have already started entering neurogenesis programs. The first neurogenic progenitors to appear are Atoh7 positive progenitors (Kay et al. 2001, Poggi et al. 2005) (**Figure 4**). A long-standing question has been how and by what division mode these neurogenic progenitors arise (Poggi et al. 2005, Paolini et al. 2015). One hypothesis was that the onset of Atoh7 expression and, thereby, neurogenic progression could be linked to the depth of interkinetic nuclear migration (Baye & Link 2007, Del Bene et al. 2008). This hypothesis was based on the idea of a Notch gradient along the apicobasal axis, with higher Notch activity at apical than at basal positions. Following this logic, the depth of nuclear migration during interkinetic nuclear migration defined the nuclear exposure to this signaling molecule (Del Bene et al. 2008), and basal interkinetic nuclear migration could be linked to the onset of neurogenesis. Indeed, some correlative evidence has shown that this could be the case (Baye & Link 2007, Del Bene et al. 2008). Although a more recent study confirmed that Notch was involved in neurogenic decisions and that the more basal nuclear position of one sister cell correlated with the deeper movement of nuclei, it also showed that these two factors did not necessarily depend on each other. Even when nuclear positions of sister cells were equalized apically or basally, asymmetric divisions that give rise to one Atoh7 positive and one Atoh7 negative progenitor prevailed. The work concluded that Notch actually

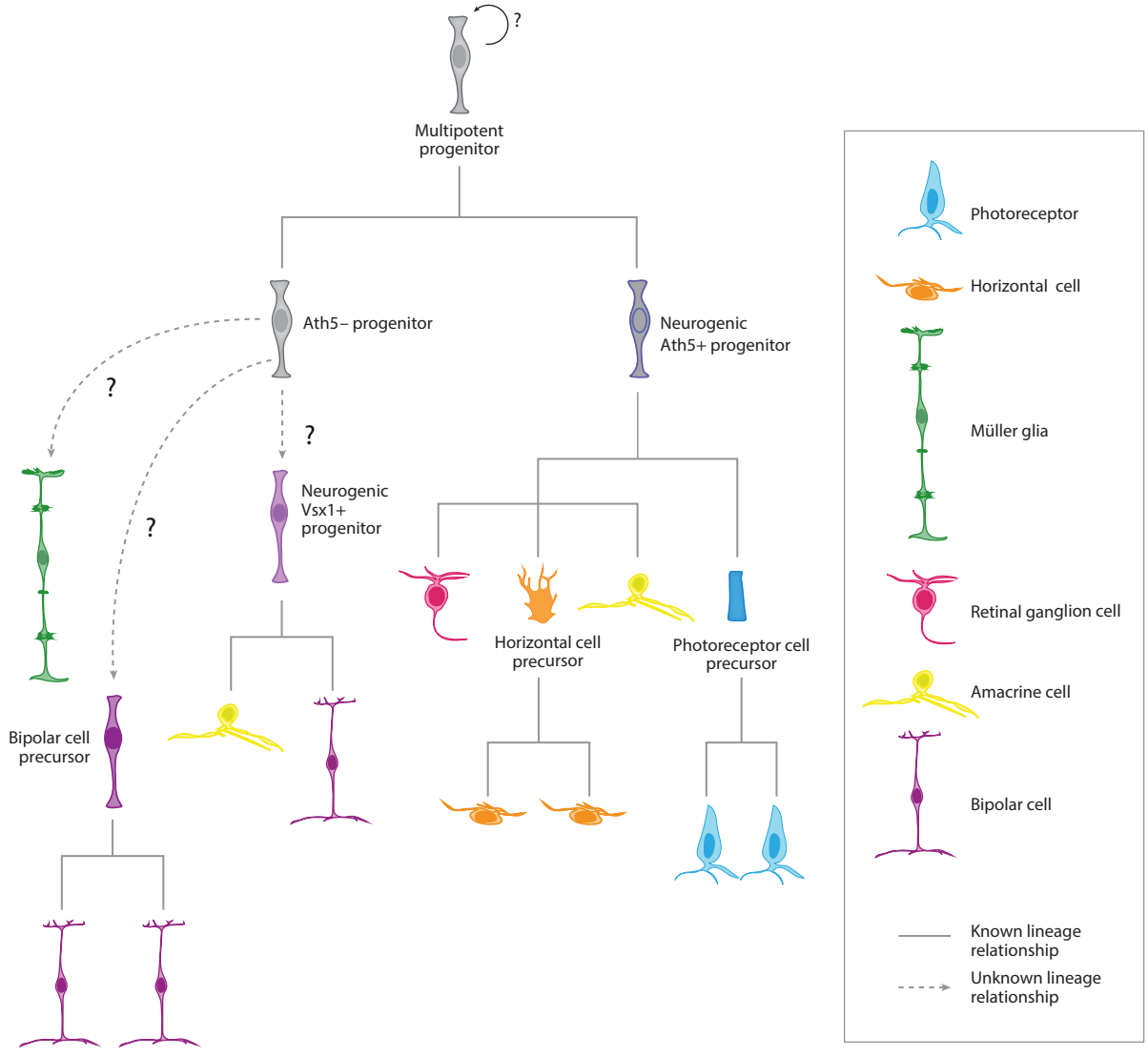

Figure 4

Current knowledge of the lineage topology in the zebrafish retina.

regulated neurogenesis onset via asymmetric inheritance of Sara-positive endosomes (Nerli et al. 2020) and not through an apical-basal gradient. This mechanism had previously also been seen in other examples of vertebrate neurogenesis (Kressmann et al. 2015). The more basal migration of Atoh7+ progenitors was explained by the facts that (*a*) these cells were more likely to inherit the basal process (Okamoto et al. 2013, Icha et al. 2016) and (*b*) a more stabilized apical microtubule cytoskeleton was present in the Atoh7+ cells (Nerli et al. 2020).

Further mechanisms suggested to be involved in neurogenesis onset of Atoh7-expressing progenitors are linked to the redox pathway that is downregulated upon differentiation onset (Albadri et al. 2019) as well as the inheritance of the apical domain of the progenitor depending on the actin regulator anillin (Paolini et al. 2015).

Atoh7+ progenitors divide once more, giving rise to neurons and committed precursors (see the section titled Committed Precursors) (Poggi et al. 2005). How these fates are distributed has been an area of intensive research. Some studies have shown that deterministic transcription factor programs are involved (Jusuf et al. 2011, 2012). However, work using rat retinal progenitor cells revealed that not all fate decisions are deterministic but that some amount of stochasticity can be observed in retinal fate decisions (Kechad et al. 2012). This was later also shown by in vivo studies in zebrafish (He et al. 2012, Boije et al. 2015). In both studies, simple stochastic models were able to recapitulate the experimental findings (Kechad et al. 2012, Boije et al. 2015). The potential coexistence of deterministic and stochastic fate decisions led to some confusion in the field. However, a recent study was able to at least partly reconcile these findings. By following hundreds of division outcomes over time in different conditions in zebrafish embryos, Nerli et al. (2023) showed that deterministic and stochastic fate branches can coexist. Furthermore, they found that interference with deterministic fate decisions was more detrimental than interference with more stochastic decisions, for which an unexpected degree of plasticity was observed.

Together, these findings imply that mechanisms to buffer fate plasticity exist and are activated in case of developmental problems. To further expand on these findings, it will be important to relate clonal ex vivo to in vivo studies and live imaging, in combination with modeling approaches to explain complex lineage outcomes in the retina.

While these findings were a leap forward for the field, a lot of further investigation across model systems is necessary to determine the exact nature of the fate buffering, whether it occurs at the transcriptional level or another level, and how processes are coordinated to always produce the correct proportions of neurons at the right position. All known fate outcomes are summarized in **Figure 4**.

Committed Precursors

One particularity of retinal neurogenesis in some vertebrates is the presence of so-called committed precursors. Committed precursors differ from the multipotent progenitors described above in the sense that these cells already exhibit a more neuron-like morphology but divide once more, and only once more, to give rise to two neurons of the same fate (although whether it is always the same subtype of neuron is currently unclear) (**Figures 3*a*** and **4**). Committed horizontal cell precursors were initially reported in the zebrafish retina. Here, cells that already appeared to be interneurons divided once more to give rise to two horizontal cells (Godinho et al. 2007). This type of committed precursor was also reported in chick retinogenesis (Boije et al. 2009). It was shown that horizontal cell precursors usually divide just beneath the photoreceptor cell layer where they will later reside (Godinho et al. 2007, Amini et al. 2019). However, in principle, they can also divide at other locations along the apicobasal axis of the tissue. In this case, both sister cells move to their final location after division (see the section titled The Main Act: Getting Cells to the Right Place at the Right Time, Neuronal Migration, and Lamination; Amini et al. 2019).

Other types of committed precursor were reported in zebrafish, namely photoreceptor cell precursors (Suzuki et al. 2013, Weber et al. 2014) and bipolar cell precursors (Weber et al. 2014, Engerer et al. 2017). Photoreceptor cell precursors already show columnar morphology (Weber et al. 2014) and divide once more in the photoreceptor cell layer (Suzuki et al. 2013, Weber et al. 2014, Nerli et al. 2023). Bipolar cell precursors divide either at apical locations, very much like multipotent progenitor cells, or, later in development, just beneath the photoreceptor cell layer (Weber et al. 2014, Engerer et al. 2017). The position of bipolar cell division, apical or subapical, is influenced by the maturity of the photoreceptor cell layer that acts as a steric hindrance for bipolar cells and overall differentiation state of the tissue (Weber et al. 2014, Engerer et al. 2017).

It should be noted that currently, photoreceptor and bipolar cell committed precursors have been reported only in the zebrafish system. However, mouse retinal progenitors that express the transcription factor Olig2 at late developmental stages show a strong bias to produce either two horizontal or two cone photoreceptor cells. Thus, similar concepts might be at play in the rodent retina (Hafler et al. 2012). Are committed retinal precursors prevalent in rodents and other species, or are they are a peculiarity of teleosts, possibly due to their fast development? This question needs to be further explored.

THE MAIN ACT: GETTING CELLS TO THE RIGHT PLACE AT THE RIGHT TIME, NEURONAL MIGRATION, AND LAMINATION

General Themes in Retinal Migration and Lamination

Upon neurogenesis onset, as cells adopt neuronal or committed precursor fates, they need to move to the correct position at which they will later function. This is similar to neurons in other areas of the central nervous system (example reviews include Marín & Rubenstein 2003, Ayala et al. 2007, Cooper 2013, Icha & Norden 2014, Rahimi-Balaei et al. 2018). In the retina, overall traveling distances are shorter and lie in the tens of micrometers range, compared with hundreds of micrometers to millimeters in other parts of the brain. However, while neuronal migration in other parts of the brain, especially the neocortex, is an area of intense and decades-old research (examples include Rakic 1972, Tan et al. 1998, Nadarajah & Parnavelas 2002, Sekine et al. 2011, Hatanaka et al. 2016), comparatively little is known about neuronal migration in the retina (Amini et al. 2018). The lack of knowledge is surprising, as correct lamination is key to correct neuronal connectivity and thereby information flow.

Due to the overlapping birth order of retinal neurons outlined above, translocation of different types of neurons occurs concomitantly. While the initial stages of retinal neuronal migration still appear relatively ordered and not very different from what is observed in other areas of the developing brain (Cooper 2008), very soon thereafter, different types of neurons move in different directions and employ diverse types of migration modes (see **Figure 3*a*,*b***). This is unlike what is seen in the neocortex, where radial glia-guided migration is a prevailing neuronal migration mode leading to an ordered, inside-out layering of this part of the brain (Cooper 2008). The lack of such ordered migration in the retina could partly be due to the fact that radial cells like bipolar cells or Müller glia, the only glia cell type in the retina, only emerge later in development (Amini et al. 2022).

Due to the fact that migration of different cells in different directions starts while proliferation is still ongoing, a phase of chaos arises from the previously very ordered pseudostratified epithelium, in which cells move by different modes in different directions (**Figure 3**). However, fascinatingly, this chaos is always resolved during development, leading to the highly ordered structure of the neural retina, with its nuclear and plexiform layers. So far, however, how this chaos is resolved is only starting to be understood.

Retinal Ganglion Cell Movements

The use of Golgi staining of rat and mouse retinas indicated in the 1970s that the early born RGCs mainly undergo somal translocation (Morest 1970, Hinds & Hinds 1974) (**Figure 3*a*,*b***). Somal translocation means that cells stay attached at either one side or both sides of the developing structure, and in this case, the only moving part is the cell's soma, including the nucleus. The idea that RGCs undergo somal translocation was later confirmed by live imaging in zebrafish (Poggi et al. 2005, Zolessi et al. 2006, Icha et al. 2016). Light sheet imaging of dozens of cells from many

embryos showed that RGC translocation can be divided into two stages: a rather rapid phase of directional translocation, as already suggested in the fixed analysis in rodents (Morest 1970, Hinds & Hinds 1974), followed by a slower and nondirectional fine positioning phase. At the end of these two phases, cells occupy their final location in the ganglion cell layer (Icha et al. 2016).

The mechanism of this translocation was already suspected to be microtubule dependent based on electron microscopy staining of mouse retinas (Hinds & Hinds 1974). This finding was confirmed using live imaging in the developing zebrafish retina (Icha et al. 2016). In addition, basal process reattachment was necessary for successful RGC translocation (Icha et al. 2016). Surprisingly, however, even when microtubules are destabilized or basal process reattachment is impaired, the majority of RGCs nevertheless find their way to their final position and undergo successful axonogenesis (Icha et al. 2016) (**Figure 3*a*,*b***). It was found that RGCs can employ a backup mode of migration that was shown to be multipolar and microtubule independent. This backup mode is almost as efficient as canonical somal translocation, and final positioning of neurons is similar for both modes. The exact molecular mechanisms of this backup mode are not yet understood, but preliminary data hint that it could depend more on the actin than on the microtubule cytoskeleton (Icha 2016).

However, when both modes of migration are impaired, RGCs are positioned in central retinal locations, unable to reach their functional locations, and further tissue maturation and neuronal lamination are affected. That two modes of migration exist suggests that RGC translocation is a robust process, most likely because it sets the stage for all following retinal migration phenomena (Icha et al. 2016). How cells sense impairment of somal translocation and consequently activate the multipolar migration mode, why movement is nevertheless directed (uncommon for multipolar migration events), and what drives this mode of migration are currently open questions.

Photoreceptor Cell Movements

RGCs emerge from Atoh7-expressing progenitors by asymmetric divisions, and their sister cell is very frequently a photoreceptor cell precursor (Poggi et al. 2005, Nerli et al. 2023) (**Figure 4**). Intuitively, one would think that the photoreceptor cell precursors do not need to translocate since they are born at the same position at which they later divide, mature, and undergo their function: forming the apical side of the developing retina. However, anecdotal reports mentioned photoreceptor cells distributed along the apicobasal axis at early lamination stages in fish and human retinal organoids (Kaewkhaw et al. 2015, Suzuki et al. 2013). A live imaging study in zebrafish revealed that photoreceptors undergo apical migration and that this migration depends on N-cadherin adhesions. When N-cadherin is knocked down, the bipolar morphology of moving photoreceptors is lost, and translocation becomes more random. Furthermore, photoreceptors were seen to move in apical directions in human retinal organoids (Rempel et al. 2022).

To understand the basis of these observations, a recent study compared photoreceptor distribution over development in zebrafish, human retinas, and human retinal organoids (Rocha-Martins et al. 2021). In all these systems, cells were found along the apicobasal axis before residing and maturing at the apical surface. In zebrafish and human organoids, live imaging revealed that photoreceptors undergo bidirectional translocation (**Figure 3*a*,*b***). Interestingly, in zebrafish, the two directions of movement are driven by different cytoskeletal components. Similar to RGCs, microtubules are involved in basal migration, while actomyosin drives the return of cells to the apical surface (Icha et al. 2016, Rocha-Martins et al. 2021). This counterintuitive migration pattern was explained by the fact that proliferation is still at a peak when photoreceptor cells are born (Matejčić et al. 2018). It was shown that when photoreceptor cells cannot leave the apical surface, progenitors undergo basal divisions, delaminate, and generate problems with overall tissue lamination

(Rocha-Martins et al. 2021). This study exemplifies the importance of taking overall tissue development into account when trying to understand the behavior of individual cells or groups of cells. It also shows that neuronal migration in the retina, in addition to its canonical role in translocating cells to the correct position, can be involved in the coordination of growth and morphogenesis. However, many open questions remain. For example, it is unclear what triggers the switch of direction from basal to apical in these cells, whether it occurs due to cues in the environment or an internal cellular timer, and what drives the cytoskeletal switch at the molecular scale.

Amacrine and Horizontal Cell Movements

Initial studies using a combination of Golgi staining and electron microscopy in diverse species revealed that the two types of interneurons, horizontal cells and amacrine cells, can exhibit both bipolar as well as multipolar morphology (Génis Gálvez et al. 1977; Hinds & Hinds 1979, 1983; Gallego 1986; Prada et al. 1987). This was validated by live imaging in zebrafish that showed that after birth, both cell types initially keep an apically attached process and translocate basally via somal translocation. This movement is directional and quite similar to what is seen for RGCs (Chow et al. 2015, Amini et al. 2019). However, this phase of movement is relatively short, and amacrine and horizontal cells afterwards fall into a multipolar migration mode that is less directed and shows more frequent direction changes (Chow et al. 2015, Amini et al. 2019) (**Figure 3*a*,*c***).

Amacrine cells lose their basal and apical contacts when reaching the INL and switch to the multipolar migration mode (Chow et al. 2015, Icha et al. 2016). This initial migration phase might serve to place these cells close to their final location. They then undergo multipolar migration, during which they move within the INL in 3D until they occupy their final positions. The distances along which these cells move are shorter compared with those in the bipolar migration phase (Chow et al. 2015). The zebrafish system thus refuted the hypothesis that amacrine cells merely stack on top of the inner plexiform layer (Rapaport 2006, Voinescu et al. 2009) and instead showed that they do undergo fine positioning. The general themes of amacrine cell migration seem to be conserved in mouse, as studies on organotypic slice culture showed that both bipolar and multipolar modes of migration exist (Krol et al. 2016). In mice, amacrine cell translocation depends on the atypical cadherin Fat3, which interacts with actin via the Ena/VASP complex (Deans et al. 2011, Krol et al. 2016). Overall, so far, the evidence presented indicates that amacrine cells initially use somal translocation followed by a fine-positioning free-migration mode. However, the exact molecular details and cytoskeletal machineries, as well as the attracting or repelling cues—biochemical, mechanical, or both—that guide the free migration mode still need further investigation.

The initial phases of horizontal cell migration are very similar to what is seen for amacrine cells, as these cells also undergo bipolar migration before switching to multipolar migration. Interestingly, they initially move to very similar depths within the INL as amacrine cells, despite the fact that they reside just beneath the photoreceptor cell layer in the mature retina. Thus, these cells undergo a U-turn to relocate to the apical side. Hints toward such bidirectional migration were already seen using fixed analysis in a variety of species including chicken (Prada et al. 1987), mouse (Liu et al. 2000), rabbit, cat, agouti, capybara, and macaque (Wässle et al. 2000). In chicken and zebrafish, bidirectional migration was confirmed by live imaging (Edqvist & Hallböök 2004, Godinho et al. 2007, Chow et al. 2015, Amini et al. 2019) (**Figure 3*a*,*c***). Work that more thoroughly investigated the multipolar migration mode of horizontal cells showed that the trajectories of these cells are heterogenous. Movement can last different amounts of time, varying by hours, and cover different depths. Furthermore, migration within the INL can be more confined or cover significant volumes in 3D. No common denominators to predict exact horizontal cell migration behavior were found (Amini et al. 2019). Follow-up work showed that horizontal cells fulfill

many criteria of amoeboid-like migration (Amini et al. 2022). Characteristics of amoeboid-like migration include cell direction changes, space adaptation at the cellular and nuclear scales, and protrusive activity not directly involved in migration (Lämmermann & Sixt 2009). All of these features were found for horizontal cells (Amini et al. 2022). So far, however, the cues sensed by the horizontal cell to always find the correct position beneath the photoreceptor cell layer despite the randomness of migration are not understood. It is likely that these cues are of chemical rather than of mechanical nature, as no obvious difference of INL stiffness was observed neither in location or over development (Amini et al. 2022). These cues need further investigation, as do the exact cytoskeletal arrangements that drive horizontal amoeboid-like migration.

Bipolar Cell Movements

Currently, final positioning kinetics and dynamics of bipolar cells are the least understood in the developing retina. This is astonishing, as these are the cells that connect photoreceptors and RGCs and thereby can be seen as the highways of information flow that are very important for visual functionality.

Once born, bipolar cells do not migrate extensively but rather reposition their nuclei and somas within the INL. To date, work done in zebrafish has concentrated more on the location of bipolar cell division than on their final positioning (Weber et al. 2014, Engerer et al. 2017). As mentioned above, divisions can occur at apical or subapical positions, depending on developmental stage and linked to the presence or absence of the photoreceptor cell layer. Subapical divisions can occur with any cleavage plane. When cells divide subapically, they lose their apical process during division but stay attached at the basal side (Weber et al. 2014). One cell inherits the basal process after division, while the other daughter cell regrows it. After division, nuclei reposition within the INL, and cells remodel their apical and basal processes into neurites reaching into the outer and inner plexiform layers (Weber et al. 2014, Engerer et al. 2017). It will be interesting to see whether and how apically and subapically dividing bipolar cells differ in migration behavior and final positioning.

Open Questions in Retinal Lamination

At least in zebrafish, the retina laminates with the correct number of nuclear as well as plexiform layers even when particular cell types are not produced (Randlett et al. 2013, Almeida et al. 2014, Nerli et al. 2023). Upon genetic depletion of RGCs or amacrine and horizontal cells, overall retinal size is almost unchanged, and only the different layers are expanded or shrunken. Even when all three cell types are lacking, retinal organization of photoreceptor cells and bipolar cells still maintains an overall organization of nuclear and plexiform layers, even though the tissue is smaller (Nerli et al. 2023). Interestingly, plexiform layers can form at more or less correct positions even when retinas consist merely of bipolar and photoreceptor cells (Randlett et al. 2013). However, whether and how this reflects on migration behavior of the different cell types has so far not been explored.

Overall, the combination of fixed analysis and the recent advances in quantitative live imaging has enhanced our understanding of neuronal migration and lamination in the retina. However, while the kinetics of migration modes have been elucidated for most cell types, the cellular and molecular factors involved are still unresolved. Even less understood is what interactions exist between different neurons that are moving in different directions. Future studies need to explore how cells interact and communicate with each other and the surrounding environment. It is unclear how cells move in different directions in the very crowded retinal tissue (Matejčić et al. 2018) without interfering with each other or the proliferating progenitors. It will be fascinating to probe

how, within all this chaos, cells nevertheless manage to always reach the correct position to ensure the establishment of the right synaptic connections.

To address these very complex issues, it will be important to involve theorists and physicists who can contribute with modeling approaches. These models can then be used as entry points to formulate and test future hypotheses. As much of our current knowledge is based on teleost systems, it will also be interesting to ask which phenomena and parameters are conserved across species, including those that exhibit different, usually longer, developmental timescales and at the same time develop thicker retinas in which cells have to move over longer distances.

Furthermore, the comparison of in vivo findings to organoid systems, including human organoids, will provide important insights. This has so far only been done for photoreceptor cell migration in zebrafish and human organoids, where many similarities between these otherwise very different systems were found (Rocha-Martins et al. 2021). Besides the species-relevant similarities and differences, the organoids also provide a system in which to probe how much ex vivo versus in vivo development influences the processes of retinal neuronal migration and lamination. This, in turn, has the potential to improve human organoids in particular and to make human developmental morphogenesis programs more accessible.

FUTURE CHALLENGES FOR INVESTIGATING RETINAL MORPHOGENESIS

I hope that this review has revealed that retinal morphogenesis is a fascinating, multilayered process involving different rearrangements of tissues as well as cells interacting within tissues at different stages of development. While massive progress has been made in recent years, we are very far from fully understanding any of these phenomena. As mentioned above, the next challenge will be to move from understanding the behavior of single cell types, such as rim or RNE cells during OCF or the migrating neurons during lamination, to an understanding of the interactions and hierarchies between cell types within the tissue. This will be a tremendous effort that requires advanced imaging, image analysis, and spatiotemporally targeted perturbations. For example, the role of tissue mechanics and other physical components involved in all processes discussed above still needs further investigation, as does the influence of chemical attractions and repulsions. It is clear that chemotaxis, together with durotaxis and topotaxis, is at the core of many morphogenetic phenomena, but how these different cues interact needs to be further explored. This, together with previous knowledge on transcriptional and signaling factors, which has been cut a bit short in this review but is of course of tremendous importance, will allow for a more complete understanding of the exact processes and their interactions.

Another important but so far understudied question is how retinogenesis occurs in such a robust manner and results in two eyes of the (supposedly) same size and composition with the symmetry observed. Whether and how much variability exists in the developmental processes at different stages of retinogenesis is only beginning to be explored (Randlett et al. 2013, Young et al. 2019). Dissecting the extent of variability in the system and how robustness across scales is achieved are exciting avenues to focus on in the future. Such studies, in addition to enhancing the understanding of organogenesis of one of the most beautiful and symmetric structures in the developing body, could also have therapeutic potential in the long term.

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