Retinal development in *Drosophila*: specifying the first neuron

Frank Hsiung and Kevin Moses*

Department of Cell Biology, Emory University School of Medicine, 615 Michael Street, NE, Atlanta, GA 30322, USA

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In vertebrates, a proneural basic helix–loop–helix transcription factor (Ath5, Atonal homolog 5) plays a crucial role in the specification of the first retinal neuron: the retinal ganglion cell (RGC). *Math5* homozygous null mutant mice lack RGCs and have no optic nerve. Furthermore, the expression of the Ath5 protein is regulated to give a non-random dispersed pattern of RGCs. In *Drosophila*, retinal histogenesis is precisely coordinated and is associated with a progressive wave called the morphogenetic furrow. In the furrow, single precisely spaced cells are specified to become the first retinal neural cell type: the R8 photoreceptor cell. This *Drosophila* founder cell specification is coincident with and dependant upon the expression of the fly Ath5 ortholog: Atonal. Indeed, in both taxa, the process of founder cell specification may be viewed as the regulation of Atonal expression. It is now clear that, in flies, this regulation depends on the action of inductive and inhibitory signals. This review concentrates on the signaling mechanisms that produce this precise pattern of founder cells.

There has been recent and intense debate on whether vertebrate ciliary photoreceptor cells and insect rhabdomeric photoreceptor cells are homologous (1–9). Similarities in both taxa include the following: the eyes are specified by Pax6 and other genes (Table 1) (3,10,11), Hedgehog is the primary signal for patterning (12–16), TGF-α/EGF receptor signaling is involved in photoreceptor specification (17–22), Glass acts as a transcription factor for photoreceptor-specific gene expression (23–25), Crumbs/CRB1/RP12 functions to support the opsin-bearing membrane domain (26), and Atonal family basic helix–loop–helix (bHLH) transcription factors act to specify the first retinal neural cell type (27–36). On the other hand, there are some striking differences, such as the presence or absence of a microtubule-based ciliary body, the presence of the opsin photopigment on microvillar or disc membranes, the direction of the change in membrane potential in response to light (vertebrates hyperpolarize while insects depolarize), and the biochemistry of phototransduction (vertebrates signal via phosphodiesterase and invertebrates use phospholipase C; reviewed in 1,9,37,38). Whether or not these similarities reflect evolutionary history, *Drosophila* developmental genetics has often led to new insights into vertebrate eye development. Here we discuss our current understanding of Atonal-dependent retinal founder cell specification in the fly, because it may provide insights into the Ath5-dependent specification of the vertebrate retinal ganglion cell (RGC).

**COMPOUND EYE STRUCTURE**

The fly eye consists of hundreds of similar unit eye facets called ommatidia, each containing 20 cells (39,40). The eight photoreceptor neurons form the core of the ommatidium and they bear an array of microvillus extensions of their apical membrane called rhabdomeres, which carry the photosensitive opsin (41,42). These are functionally equivalent to the disc membranes of the rod and cone outer segments in vertebrates (26). Six rhabdomeres contain a blue-sensitive opsin called Rh1 and appear in sections to form a characteristic trapezoid, and these cells are called Retinula cell 1 through 6 (R1–R6; Fig. 1) (43). In the apical retina, there is also a central, smaller rhabdomere, associated with the R7 photoreceptor cell, which carries ultraviolet-sensitive opsin of the Rh3 or Rh4 type (44,45). Below the R7 rhabdomere lies the rhabdomere of the last photoreceptor cell: the R8 photoreceptor cell. The R8 expresses either Rh5 or Rh6 opsin, which are blue–green-sensitive (46,47). Above the photoreceptors lie four flattened cells that secrete the dioptic elements of the ommatidium: the lens and the crystalline cone. Surrounding the unit are a set of six pigment cells that optically isolate the ommatidium. There are also two cells that comprise a mechanosensory bristle. To function correctly, the compound eye depends on very precise cell number and shape: each ommatidium is focused on a point in space about 2° away from its neighbors (48). Any excess, missing or misshapen cells disrupt the crystalline regularity of the eye and degrade its resolution. This precision of final structure is based in very precise developmental processes, beginning with the initial spacing of the R8 cells, which are the first to be specified (49–51). The R8 ommatidial founder cells are specified in the morphogenetic furrow in the last larval phase of the life cycle (49,52).
THE MORPHOGENETIC FURROW

In early larval life, the presumptive eye is a monolayer columnar epithelium. Cells are undifferentiated and proliferate in no set pattern. By the end of the second instar, the eye field has been regionalized by Wingless signals into dorsal and ventral domains. The resulting midline is marked by elevated Notch signaling which is required to define the point at which the morphogenetic furrow will initiate (53–62). At about 186 hours post fertilization (at 18°C) (63), the morphogenetic furrow begins at the posterior edge of the eye field, where it joins the optic stalk (64,65). This depends on specific signals mediated by Hedgehog, Dpp, the epidermal growth factor (EGF) receptor homolog (Egfr) and Notch (63,66–68). The furrow was first described by August Weismann (69), but he did not notice that it moves. The furrow itself is the physical consequence of constriction of apical actin cytoskeletal rings, and is coincident with a band of cell cycle arrest at the G1 stage (49,70–72). The furrow sweeps across the eye epithelium over a period of about two days and lays down a new column of precisely spaced ommatidial founder cells roughly every two hours (49–51,73). The founder cells in each column are not specified simultaneously – instead, the first is specified at the midline, and then its dorsal and ventral neighbors are specified about 20 minutes later and so on to the ends of the column (74).
The establishment of ommatidial clusters was first studied morphologically (50). Cobalt and lead sulfide staining was used to highlight the apical profiles of cells in the furrow, and this revealed that the first distinguishable group of cells is core of four or five cells surrounded by a ring of about 15, called the ‘rosette’ (Fig. 2) (74,75). By the next column, the outer ring of cells opens and loses terminal cells to form a short arc of six to nine cells. By the third column, these arcs close up to form five cell preclusters, and the most posterior cell will become the R8 founder cell. These staining techniques depend on the deposition of very fine black precipitate onto the apical surface of living cells. Most likely, what is being revealed is active membrane flow, which sweeps the deposited material down onto the cell junctions, where it accumulates. Thus, cells that are more active in cycling their membrane (perhaps because they are more active in signaling) are more darkly highlighted.

Progression of the furrow depends on Hedgehog, which is secreted by the developing clusters and received by cells on the anterior side of the furrow (13,76–78). While it is clear that Dpp is required for furrow initiation and that Dpp is expressed just anterior to Hedgehog in the progressing furrow, there are contradictory data as to whether it actually functions in furrow progression (66,68,76,77,79–84). After the founder cells have been specified, the other 19 cells of each ommatidium are recruited by inductive signals from the Ras pathway (via the Egfr or Sevenless receptor tyrosine kinases, or both) and also by Hedgehog, and these inductive signals are opposed by inhibitory Notch signals (50,85–92).

In addition to the signals described above, ommatidial development depends on a complex and dynamic pattern of transcription factors (93–95). Far anterior to the furrow, the inhibitory factor Hairy is expressed, and its expression ramps up towards the furrow (96,97). The inhibitory activity of Hairy depends on a second factor: Esc (98). On the leading edge of the furrow, Hairy expression crashes abruptly (97). Most crucial for the formation of the R8 founder cells is the bHLH transcription factor Atonal, which functions together with a dimerization partner (Daughterless) that is uniformly expressed (27,28,98,99). Atonal is initially broadly expressed and its expression ramps up into the furrow (Fig. 3). In the furrow, Atonal becomes restricted first to groups of very roughly 20 cells (with about the same number in between ceasing to express it (28,100–102). These large groups have been called ‘intermediate groups’ and are also likely to be the same as the ‘rosettes’ described by Wolff and Ready (74). The intermediate-group stage is in the first column that can be distinguished by any criteria, and, more importantly, is the first time at which cluster spacing can be observed. A column later, Atonal is restricted to much smaller groups of two or three cells, which form an R8 equivalence group (100–102). By the third distinguishable column, Atonal is expressed in only one cell, now unambiguously the single founder cell, set at very precise spacing. This single-cell Atonal expression persists for three or four columns (28,100–102). As Atonal transcription becomes restricted, at least two other factors are upregulated in the surrounding cells that have lost Atonal: Rough and Glass (23,24,101,103–107). Following this, a number of other transcription factors act in specific cells as they are recruited into the growing ommatidia (93–95). In vertebrates, a similar pattern of bHLH transcription factor expression has been reported, as well as progressive restriction of Atonal homologs (31,108).

In summary: in the furrow, a sheet of...
At some stochastic frequency, founder cells might be specified if they express it. Cells are formed (27). However, Atonal expression is not alone sufficient to specify R8 fate, because anterior to the furrow all cells express it.

How can such precise spacing patterns be established in epithelia? Perhaps a trivial but simple model is that they are not. At some stochastic frequency, founder cells might be specified. They could then recruit surrounding cells into the cluster in a precise way, and when the clusters were complete, excess cells could be eliminated by apoptosis. Indeed, the developing clusters do both regulate the proliferation of their neighbors, and ultimately the excess cells do die (71,72,113,114). This stochastic model predicts that initially the founder cells would be randomly spaced and that order would only appear later. However, there are abundant data (discussed above) to dispose of this idea: founder cells are precisely spaced from the time of their inception (they are ‘overdispersed’), as can be seen by the pattern of Atonal expression (and other molecules such as dpErk and Scabrous, 115–118). Furthermore, there is no apoptosis in the furrow, so this pattern cannot be derived by any type of pruning. Also, the expression of the cell death inhibitor p35 does inhibit apoptosis in the eye but does not affect founder cell spacing (119). Thus, the initial precise pattern of founder cells must be controlled by some system of inductive signals.

An early model for this kind of patterning was suggested by Wigglesworth (120) in 1940, and called ‘lateral inhibition’, and has dominated thinking on this topic since then (121). Wigglesworth caused the abdomen of the blood-sucking bug Rhodnius prolixus to swell by rectal occlusion. He then observed that new bristles grew in on the animals’ backs at the points of greatest distance from old bristles. He suggested that this could be explained as a minimum point in the concentration field of some diffusible regulator secreted by the existing bristle cells. In this model, in the eye founder cells express an inhibitory and an inductive signal (Fig. 4). This idea was first made explicit for the developing retina in 1989, but there has been extensive debate over what the two signals are (122,123). Along with others (112), we suggest that the Notch ligand Delta is the inhibitor and Hedgehog is the inducer.

Three molecules have been proposed to act as positive signals: Hedgehog (136) and the Egfr ligands Spitz and Vein (134). We favor Hedgehog as the inductive signal because hedgehog loss of function does result in an immediate failure of intermediate-group specification (77,78), Hedgehog is expressed in the founder cells and does act on more-anterior cells (13,76,77,84,111,137), and hedgehog pathway signaling is thought to be a direct activator of atonal transcription (111,138). In vertebrates, Sonic hedgehog signaling is thought to play a similar role in driving progressive histogenesis in the retina (15,16). We do not favor Spitz as the positive signal, because our own data show no clear loss of function defect for Spitz in founder cell specification or in the spacing of the intermediate groups (139). Also, loss of function for the Spitz receptor (Egfr) and two downstream elements (Ras and Raf) has no discernable effects on intermediate-group spacing (118,135,136,140). Again (like Scabrous) there is evidence that Spitz/Egfr signaling may act at the next step(s) that select a single founder cell from the intermediate groups: in some Egfr pathway loss of function experiments, R8 ‘twinning’ is reported (135,136,140). Ectopic Spitz expression anterior to the furrow can produce ectopic photoreceptors – even without...
Atonal (131). We suggest that this is not a normal function, but rather an effect of ectopic expression away from sources of Delta/Notch inhibition. Finally, there are no clear loss of function data to support a role for Vein in founder cell specification. Indeed, as Vein is an Egfr ligand, it would be hard to envisage a role for Vein while Egfr has none.

It is important to note that while Egfr/Ras signaling does not appear to control intermediate-group spacing and probably founder cell specification, it is crucial for the differentiation of each neural cell type in the eye, including the transition from the founder cell type to the R8 cell type. When Egfr pathway function is withdrawn, the R8 cells regress at about the time that Atonal expression ceases (118). Also, ectopic Egfr/Ras signaling can have a dominant affect and lead to the specification of ectopic neurons (63,131). Thus, the regulation of MAP kinase activity is a central event in neural differentiation that must occur only at the right place and time. Spitz is expressed in the furrow (139), and Egfr functions there to activate the Rolled MAP kinase in the intermediate groups — yet no neurons are specified there (117,118,134). How is this Egfr/Ras pathway signaling blocked? We suggest that this is done in two different ways. Close to the founder cells, Rolled MAP kinase phosphorylation is opposed by the Delta-induced activation of Notch signaling (i.e. between the intermediate groups). In addition, there must be some mechanism by which the Hedgehog signal is blocked in these cells. We and others have shown that in the intermediate groups themselves, the Rolled MAP kinase is phosphorylated (117,118,134), but we have proposed that it cannot enter the nucleus through some form of cytoplasmic anchoring (118).

CONCLUDING REMARKS

As we have noted, there are astonishing similarities between fly and vertebrate eye development, and in particular in the specification and spacing of the first retinal neural cell type (the R8 photoreceptor in the fly and the RGC in ourselves). Current data can be explained in both taxa by a relatively simple model: a positive Hedgehog/Sonic hedgehog signal is opposed by a negative Notch signal to produce a precise and dispersed pattern of founder cells that express Atonal/Ath5 (Fig. 4). These mechanisms act in many other parts of the nervous system. Thus, an understanding of founder cell specification is likely to be of very broad relevance to neural development in many animals.

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