Topographic Wiring of the Retinotectal Connection in Zebrafish

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ABSTRACT: The zebrafish retinotectal projection provides an attractive model system for studying many aspects of topographic map formation and maintenance. Visual connections initially start to form between 3 and 5 days postfertilization, and remain plastic throughout the life of the fish. Zebrafish are easily manipulated surgically, genetically, and chemically, and a variety of molecular tools exist to enable visualization and control of various aspects of map development. Here, we review zebrafish retinotectal map formation, focusing particularly on the detailed structure and dynamics of the connections, the molecules that are important in map creation, and how activity regulates the maintenance of the map. © 2015 Wiley Periodicals, Inc. Develop Neurobiol 75: 542–556, 2015

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INTRODUCTION

Precise connections are formed between brain regions in the developing nervous system. In particular, topographic maps maintain the spatial organization between areas and are a frequent characteristic of systems that transduce sensory information. These maps are generated from a combination of mechanisms that include chemical guidance cues, competitive or cooperative interactions, and electrical activity. However, there are still many unanswered questions regarding how these processes interact in brain wiring. The development of the zebrafish retinotectal map provides a platform for studying the contributions of guidance, target recognition and plasticity in the formation, and maintenance of topographic maps, and offers a number of advantages over mammalian models. Here, we review zebrafish as a model system, including the development of map structure, the molecules that guide map creation, and the contributions of neural activity.

Zebrafish as a Vertebrate Model System

Zebrafish were introduced as a model for developmental biology in the late 1960s by Streisinger (reviewed in Grunwald and Eisen, 2002). Adult zebrafish have high fecundity and can lay hundreds of eggs in a single clutch, with an adult pair breeding every 1–2 weeks. The externally fertilized eggs can be kept in Petri dishes, and embryos and larvae can be screened at any time for developmental phenotypes or behaviors. As most fundamental developmental processes are conserved across vertebrates, observations made in this highly accessible model system are often applicable to the developmental processes found in higher vertebrates, including humans.

Current techniques in zebrafish allow for detailed anatomical studies, genetic manipulations, activity manipulations, and long-term noninvasive imaging. The combination of these methods holds significant

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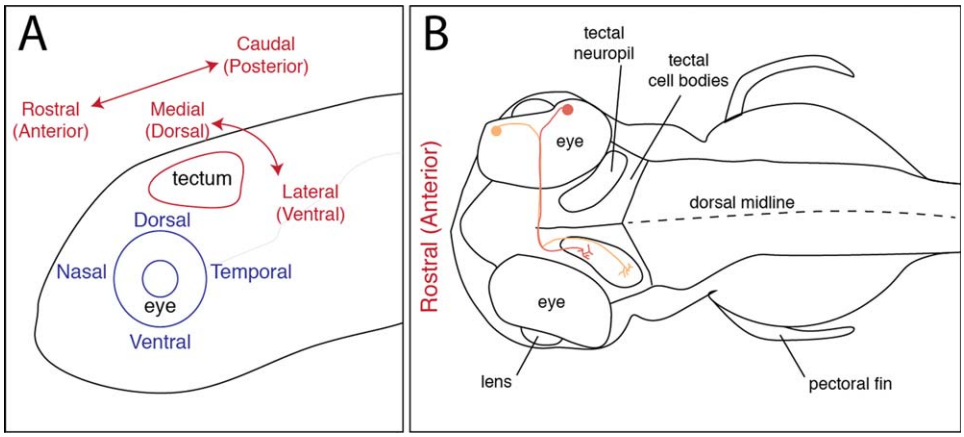

Figure 1 The retinotectal connection in zebrafish. The lateral view (A) displays the directional anatomical terms and the dorsal view (B) gives a schematic of the topographic map. Nasal axons (yellow) connect caudally, and temporal axons (red) target the rostral optic tectum. Similarly, dorsal RGC bodies have axons that travel to the lateral tectum, and ventral RGC axons arborize in the medial tectum, forming a smooth map.

potential for future studies of the precise anatomy of retinotectal circuits, how they change during ontogeny, and the role of neural activity in these processes.

ZEBRAFISH RETINOTECTAL MAP FORMATION: OVERVIEW

In zebrafish, the first retinal ganglion cells (RGCs), the output cells of the retina, differentiate between 29 and 34 h postfertilization (hpf) (Burrill and Easter, 1994; Xiao et al., 2011). Their main target is the optic tectum, although they also project to nine other arborization fields (Burrill and Easter, 1994). RGC pioneer axons leave the retina by 34–36 hpf (Stuermer, 1988; Burrill and Easter, 1994). The axons form the optic nerve until they reach the optic chiasm at the midline of the brain. Once there, all RGCs cross the midline. The axons then travel through the optic tract, where they are sorted into smaller brachial streams based on the location of their cell body within the retina (Stuermer, 1988; Poulain and Chien, 2013). The first axons reach the tectum at 46–48 hpf (Stuermer, 1988). There, they innervate the tectal neuropil, form a retinotopic map (Fig. 1), and create the first functional connections by 72 hpf (Stuermer, 1988; Easter and Nicola, 1996; Niell and Smith, 2005; Zhang et al., 2011). The initial connections are crucial for early survival, allowing zebrafish larvae to visually locate and evade predators and hunt prey soon after hatching (Roeser and Baier, 2003; Gahtan et al., 2005). Growth in the zebrafish visual system continues long after these early connections are made, although it is unevenly matched across the system. The retina adds cells in concentric peripheral rings, and the tectum expands caudally (Marcus et al., 1999). To maintain the retinotopic order, the connections are continuously rearranged. This plasticity ensures stability of the retinotopic map despite unequal growth between the tectum and the retina.

The Tectum is an Intricate, Integrative Structure

The ingrowing RGCs are not limited to a single layer of the tectal neuropil, and horizontal laminae at different depths in the optic tectum add complexity to the system. RGC axons enter the tectum at one of four sublaminae and remain in those layers to find and refine their topographic connections (Robles et al., 2013). The lamina targeted by each RGC depends on the RGC subtype and intrinsic properties, although most RGCs innervate superficial laminae within the neuropil (Yamagata and Sanes, 1995; Xiao et al., 2005; Huberman et al., 2008, 2009; Gabriel et al., 2012; Nikolaou et al., 2012; Antinucci et al., 2013). The secreted protein Slit1, expressed in a high-superficial to low-deep gradient, signals to Robo2 receptors on the RGC axons to guide axons into the correct lamina (Xiao et al., 2011). Thus, several retinotopic maps are superimposed on the tectum.

The tectum acts as a center for integration of input from different sensory modalities. It coordinates tasks that involve spatial components such as phototaxis, prey capture, and predator avoidance (Nevin et al.,

2010). The visual system is the largest and most direct sensory input to the tectum. Visual information is relayed from superficial tectal layers and is filtered as it moves deeper into the tectum (Del Bene et al., 2010). Mechanosensory information from the lateral line in *Xenopus* also maps topographically onto the tectum (Lowe, 1986). Although lateral line input to the tectum has been anatomically described in zebrafish (Fame et al., 2006), it has not yet been determined to be topographic. Additionally, in gymnotiform fish, electrosensory information from the environment is relayed from other brain regions into the deep layers of the tectum, in register with the visual map on the more superficial layers (Carr et al., 1981; Northmore, 2011). Zebrafish have topographic tectal afferents connecting directly from the zebrafish cerebellum (Heap et al., 2013). The deep layers of the tectum have topographic outputs to the hindbrain (Sato et al., 2007) and can send output commands to motor centers (Scott and Baier, 2009), initiating swimming, hunting, and escape behaviors with respect to the location of the stimuli (Nevin et al., 2010). In this manner, the tectum combines sensory inputs and rapidly generates survival behaviors in response. Experiments comparing acoustic and visual maps after altering visual space with prismatic lenses on barn owls show that maps remain in register after one is shifted. Cells sprout new connections to the correct alignment and inhibit the original synapses (Knudsen, 2002). Although the tectum integrates a variety of sensory stimuli, the superficiality of RGC axons and the prominence of retinotopy have led most descriptions of topographic map formation to focus on RGC axons.

Live Imaging of Retinotectal Map Formation

In early descriptions of RGC axon pathfinding, fixed images suggested straight trajectories to target regions where the retinotopic connections formed (Stuermer, 1988; Stuermer et al., 1990). RGC axons from nasal cell bodies only arborized after passing through the rostral tectum and into the caudal tectum. Temporal axons arborized directly in the rostral tectum and remained there (Kaethner and Stuermer, 1992). Time-lapse imaging revealed the intermediary dynamics of axon movements across the tectum. The growth cones at the tip of the axon moved forward by alternating rapid advances and periods of rest, a pattern matching the behavior of axons passing through decision points (Kaethner and Stuermer, 1994). In these initial publications, branching was first observed only when the axon reached the target area. The branches extended and retracted, exploring the area where the final arbor was elaborated (Kaethner and Stuermer, 1992).

With imaging improvements, including the use of a genetically encoded membrane bound green fluorescent protein (mGFP) rather than injected dyes, and confocal imaging over 24 h of development, branching was observed contributing to pathfinding toward the target arborization zone rather than branches initiating only after the growth cone found the target (Simpson et al., 2013). During pathfinding, axons continuously add and retract branches, some of which are tipped with growth cones. The growth cones travel outward from their branches in straight trajectories rather than turning toward the target as suggested by the earlier work (Fig. 2; Kaethner and Stuermer, 1992). Instead, branches that do not decrease the distance to the target are often retracted, while those oriented toward the target are more often maintained. A continual bias can be seen in the directionality of branches, where more branches point toward the eventual target than away from it. The result, viewed at low temporal resolution, is an essentially direct trajectory to the target, explaining why the mechanism of selective branch stabilization has only recently been identified. Iterative rounds of selective branching provide a means of ever-closer approach (Simpson et al., 2013). The mechanisms that control this biased branching have not yet been conclusively determined, but this pathfinding behavior could potentially be informed by the same principles that govern branch-based pathfinding in other species (Triplett, 2014), synaptogenesis, which guides the branch stabilization and outgrowth in arborizing RGCs (Meyer and Smith, 2006) or guidance molecules.

Time-lapse imaging has established the dynamic methods used by RGC axons to find their targets, in a manner that still images could not. The rapid growth and optical transparency of zebrafish allows the development to be monitored closely and continuously, and by adding in different manipulations or perturbations, there is rich potential for new insights into classic results to be gained.

Guidance Strategies Differ Between Model Organisms

Even with the biased branching behavior, zebrafish and *Xenopus* axons travel rather directly to their final target locations on the optic tectum when compared with other species (McLaughlin and O'Leary, 2005). Both chick and mouse initially use an "overshoot and refine" strategy for axons to find their targets (Fig. 2). Axons extend into the caudal regions of the tectum (chick) or superior colliculus (mammals), regardless of the rostrocaudal location of their retinotopic target.

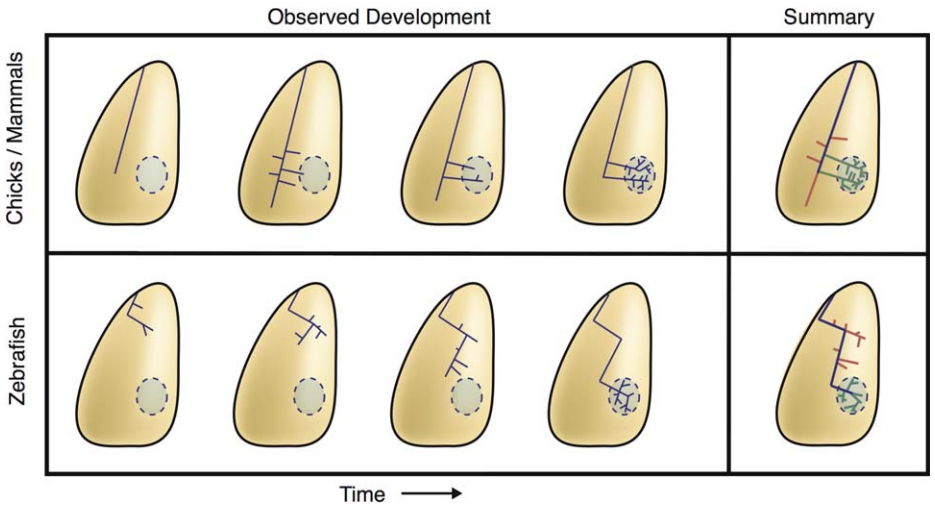

Figure 2 Pathfinding branch patterns differ between species. In zebrafish, successive rounds of branching lead growth cones closer to their targets. In chicks and mammals, axons grow straight to the caudal edge of the tectum and extend side branches. Branches that encounter the target (dashed blue oval) are maintained and elaborated (green, final frame) while others are pruned (red, final frame). Although there are differences between the methods of pathfinding, the molecules and mechanisms used are conserved between species.

The correct position is identified along the axon's length through collateral branching. Excess branches at incorrect locations are pruned back while the branches in the correct area elaborate arbors (Roskies et al., 1995; Nakamura and Sugiyama, 2004; Triplett, 2014). In chicks and mammals, the retina and the optic tectum do not undergo such dramatic growth after embryonic development as compared to zebrafish, and rearrangements are not thought to play a significant role after a critical period of developmental plasticity. These differences notwithstanding, fundamental aspects of retinotectal development are conserved across vertebrates, including the initial molecular guidance of axons through gradients of ephrins and Eph receptors, and arbor refinement through activity. As a result, manipulations—whether chemical, genetic, or environmental—that can be more easily delivered in the zebrafish model system, remain useful for identifying mechanisms in mammalian retinotopic map formation.

MANIPULATIONS AFFECTING EARLY MAP ESTABLISHMENT

Map Formation in the Absence or Reduction of Activity

Initial studies to block neural activity during development used tetrodotoxin (TTX) to remove activity from the entire CNS, including the retina and tectum. Due to the entirely external development of zebrafish (in contrast to mammals) knocking out neural activity with TTX injections is a simple process and the results can be easily monitored. When observing the gross structure of the map, the loss of activity does not prevent the axons from arborizing topographically in the tectal neuropil (Stuermer et al., 1990). The arbor areas are similar between controls and TTX treated zebrafish at 70–100 hpf (Kaethner and Stuermer, 1992), showing no sign of arbor enlargement, unlike in other species treated with TTX such as frogs (Reh and Constantine-Paton, 1985). However, the lack of effect might be a function of the age the arbors were measured at, as older larvae treated with TTX (from 4 dpf onward) have larger, more diffuse arbors (Gnuegge et al., 2001), as we will discuss in a later section.

The structure and dynamic movements of individual axons in a TTX treated environment have been examined through time-lapse imaging *in vivo*. The way growth cones typically move, by pausing, exploring, then launching forward, was unchanged with TTX (Kaethner and Stuermer, 1994). However, small changes in the pathfinding behavior of axon branches, including the branches covering smaller areas during pathfinding, can be seen upon close inspection (Kita et al., submitted). No major changes to guidance were observed in either study. A two-step model is thus still suitable for the map development in zebrafish. The initial guidance of axons to their rough target is mostly

activity independent and based heavily on molecular cues. After that, control of branching dynamics and activity-dependent processes worked together to refine arbors into more precise connections.

Early Development of the Map does not Require Spontaneous Activity

A difference between zebrafish and some other model systems is that there is no evidence for early spontaneous neural activity in zebrafish. As zebrafish develop so rapidly, they can use visual stimuli around them to refine retinotectal connections from the time that they are first made. The initial connections at 72 hpf can generate tectal activity in response to visual stimuli (Niell and Smith, 2005). In contrast, other species, including rats, mice, rabbits, turtles, and chicks, set up the retinotectal/retinocollicular connections before eye opening and thus before patterned visual input is possible (Wong, 1999). These systems compensate for a lack of early visual experience by generating spontaneously occurring retinal activity waves, where activity moves through neighboring cells and assists in setting up circuits throughout the visual system (Wong, 1999; Firth et al., 2005; Ackman et al., 2012; Kirkby et al., 2013; Ackman and Crair, 2014; Burbridge et al., 2014). When these waves are disrupted, the arbors are larger than usual and cannot be remodeled even by later visual experience (McLaughlin et al., 2003). However, despite these early differences between the model organisms, activity-based mechanisms may be used in similar ways to refine the topographic map based on visual experience (Fraser, 1992; Ruthazer and Cline, 2004; O'Leary and McLaughlin, 2005; Benjumeda et al., 2013).

Competition is not Required for Early Map Arrangement

Competition between axons does not contribute to an axonal arbor's initial position in the retinotectal map in zebrafish (Gosse et al., 2008). The *lakritz* mutants, lacking RGCs, generate a tectal neuropil devoid of any visual connections. To create chimeric embryos, small numbers of wildtype cells carrying a fluorescent mGFP gene under the control of a retinal promoter (Brn3c, expressed by 50% of RGCs) can be transplanted into *lakritz* host blastulae. Some of the resulting chimeric larvae display solitary fluorescent RGC arbors. Instead of arborizing anywhere—or everywhere, as the entire tectal space is open—arbors grow to a retinotopically appropriate position in the tectum. There they elaborate arbors that are larger than usual and more branched, but competition with other ingrowing axons is not necessary prior to that refining step. Thus in zebrafish, it appears that only axon-target interactions, rather than axon–axon interactions (Lemke and Reber, 2005), are necessary for the initial topographic arrangement. The density of axons is important in altering the final arbor size and shape, but not for pathfinding.

Surgical Manipulations

Some of the most interesting questions about the formation of the retinotectal map arose from the results of surgical manipulations (Udin and Fawcett, 1988; Goodhill and Richards, 1999; Goodhill and Xu, 2005). The connections form a precise point-to-point map of visual space but remain adaptable to manipulations of both the retina and tectal surfaces. In other species, including frogs and goldfish, it was found that maps could expand or compress to cover the available space. Removing half the retina saw the remaining RGC axons connect to their correct half of the tectum initially, but later expand to fill the whole area (Attardi and Sperry, 1963; Schmidt et al., 1978). If mismatched halves of the retina and tectum are ablated, a topographic map can still form (Horder, 1971). If half the tectum is removed, the RGCs form a compressed map on the remaining half (Yoon, 1971; Sharma, 1972). If the adult retina is rotated, then the visual map is rotated as well (Sperry, 1943).

Similarly, in zebrafish, removing half the retina before axons reach the tectum shows that axons continue to their defined target, rather than elaborating arbors in the first available space they encounter (Stuermer, 1988). When the nasal portion of the retina is removed, temporal axons terminate topographically in the rostral portion of the tectum and do not initially extend further into the vacant caudal half. Likewise, when the temporal retina is removed, the axons bypass the empty rostral tectum to arborize in the caudal half. Similar specification occurs along the mediolateral axis of the tectum; when the ventral retina is removed, all dorsal RGC axons arborize on the lateral tectal surface, and similarly if the dorsal retina is removed, all of the ventral axons connect to the medial surface of the tectum (Stuermer, 1988). These experiments confirmed that the map in zebrafish, despite being small and fast to form, follows similar organizing principles to the models studied earlier. Interesting advances in this area are still possible using imaging and being able to watch the individual axons react to the altered environment.

Although surgeries on the tectum showed that goldfish form a compressed map if half the target area was removed (Schmidt, 1983), dissections are not necessary to show the same principle in the much smaller zebrafish. Zebrafish with mutated Radar (a TGFβ-

related factor) genes have small ventralized eyes (Gosse and Baier, 2009). Radar (Gdf6a) is part of the signaling cascade that gives dorsal regions of the retina the correct identity. Because the ventral RGCs project to the medial tectum, a compressed map is formed in the medial half, while the lateral half of the tectum remains empty (Gosse and Baier, 2009). Zebrafish mapping therefore shows the same ability to compress when necessary. This study also illustrates the usefulness of various mutant strains of zebrafish.

MOLECULAR CUES DRIVE INITIAL RETINOTECTAL MAP FORMATION

The factors that have the most influence on the developing topographic map in zebrafish are the molecular cues expressed by the RGC axons themselves and by the tectal cells. As the axons grow into the tectum, they are guided toward a particular location on the rostrocaudal and mediolateral axes by many different guidance cues.

Zebrafish have contributed to the discovery of new molecules involved in creating the retinotectal map through the first large-scale forward genetic screen in a vertebrate system (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). In the screen, hundreds of new mutant lines were created through *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis. The mutants displayed interesting phenotypes resulting from individual genetic mutations (reviewed in Hutson et al., 2004; Nüsslein-Volhard, 2012). In the screen, 114 mutants were found in the retinotectal pathway (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). There has since been a gradual process of identifying the mutated genes and mechanisms causing the observed phenotypes (Culverwell and Karlstrom, 2002; Hutson and Chien, 2002a). Among the identified retinotectal mutations, four uniquely affected the arrangement of the topographic map on the tectum called *macho*, *gnarled*, *who cares*, and *nevermind* (Trowe et al., 1996). Following the success of this first screen, a second was performed using genetically encoded Brn3c::mGFP to drive fluorescence in a subset of RGCs rather than injecting dye into fixed larvae. Through the visualization of *in vivo* development, more subtle mutants and disruptions in timing of retinotectal map formation were discovered (Xiao et al., 2005). The mutants *fuzzy wuzzy*, *beyond borders*, and *breaking up* identified RGC axons that leave the tectal neuropil while axons in *vertigo*, *tarde demais*, and *late bloomer* delay their entry into the tectum. Mutations in *dragnet*, which disrupted the vertical layers the RGC axons entered the tectum in were later traced to collagen IV (Xiao and Baier, 2007; Xiao et al., 2011). However, the other mutations from this screen remain unidentified and a potential source of new molecules of interest.

Zebrafish have also been used to examine the expression and effects of well-known guidance molecules. Ephrin ligands and Eph receptors are the most widely known recognition molecules for the retinotectal mapping process, and they are conserved among vertebrates. When taken in combination, patterns of Ephs and ephrins provide molecular markers for tectal locations and retinal origins, and contribute physical labels to topographic map organization. Detailed discussions of the Eph/ephrin proteins can be found in recent reviews (e.g., Lisabeth et al., 2013; Nikolov et al., 2013; Klein and Kania, 2014). Briefly, these partners are important in both rostral-caudal and medial-lateral mapping.

Several Eph receptors and ephrin ligands are expressed in the retina and the tectum of zebrafish (Fig. 3). On the tectum, ephrin-A2, ephrin-A3b, ephrin-A5a, and EphB3a are expressed in low-rostral to high-caudal gradients (Brennan et al., 1997; Picker et al., 1999; Thisse and Thisse, 2004, 2005; French et al., 2007; Erickson et al., 2010). EphB4a and ephrin-B1 are also expressed in the tectum (Thisse et al., 2001; Liu et al., 2004; Thisse and Thisse, 2005). Ephrin-A5b and ephrin-B2a are expressed only at the caudal edge of the tectum (Brennan et al., 1997; Picker et al., 1999; Wagle et al., 2004) while ephrin-B2 is expressed along the dorsal midline (Liu et al., 2004). An area of ephrin-B3b is expressed in the mediocaudal tectum (Erickson et al., 2010).

The zebrafish retina also contains Ephs and ephrins. The ventral retina expresses EphB2 and EphA4b (Veien et al., 2008; Gosse and Baier, 2009; Erickson et al., 2010). The dorsotemporal retina is marked by Ephrin-B2a (Veien et al., 2008), Ephrin-A5a is nasal (Thisse et al., 2001; Liu et al., 2004) while EphA7 is expressed temporally (Taneja et al., 1996). EphB3a is also expressed by RGCs during development but restrictions in area have not so far been described (Thisse and Thisse, 2004). We now detail how these and other molecules contribute to the formation of the retinotectal map.

Organizing the Gradients: The Rostrocaudal Axis

During development, guidance cues are generated by the tectum before the retinal axons arrive (Picker et al., 1999). The midbrain–hindbrain boundary

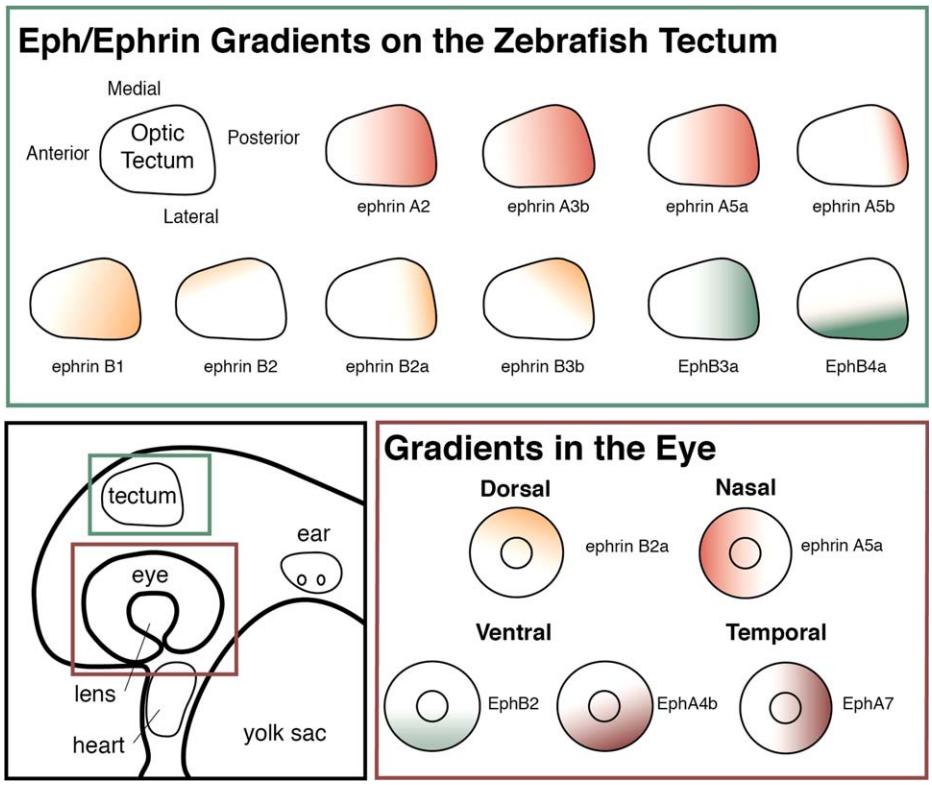

Figure 3 Known distributions of Eph receptors and ephrin ligands in the zebrafish retina (lower right) and tectum (top). Most expression patterns are based on *in situ* hybridization studies. In the future, protein localization may show further subcellular restrictions for some of these expression patterns.

(MHB), just caudal to the tectum, has polarizing effects on tectal development (Picker et al., 1999). In a strain of *acerebellar* mutant zebrafish that lack the MHB, ephrin expression on the tectum becomes altered (Brand et al., 1996). Ephrin-A5b disappears entirely, while ephrin-A5a and ephrin-A2 are expressed at low levels in uniform distributions instead of gradients (Picker et al., 1999). The RGCs that usually respond to these gradients often end up overshooting the tectum, suggesting that the ephrins act as a stop signal. When the MHB defect was shown to be due to a loss of Fgf8, it was determined that Fgf8 coordinated the patterning of the tectum (Nakamura and Sugiyama, 2004).

Despite their importance, only a few molecular gradients have had their function convincingly demonstrated. Ephrin-B2a, expressed by a subset of cells in the caudal tectum, is one example (Wagle et al., 2004). When ephrin-B2a was ectopically expressed more rostrally in the tectum, axons did not enter the neuropil (Wagle et al., 2004). Ephrin-B2a is hypothesized to be part of a signal that stops axons.

Another indication of ephrin-B2a's role comes from *gnarled* mutants, which as their name suggests, have convoluted axon paths across their tecta. Some nasodorsal axons terminate rostrally in *gnarled* mutants, and their axon arbors are so expanded in some cases that they can cover the entire tectum (Trowe et al., 1996). Ventral RGC axons project correctly and no other defects are obvious in *gnarled* mutant fish. The domain of ephrin-B2a expression is expanded in these mutants, stretching from the caudal border to the lateral edge of the tectum, which could stop a subset of RGCs from entering the tectum properly. Additionally, an ectopic population of cells is generated in the rostral tectal neuropil of *gnarled* larvae, which may cause a physical barrier for axons (Wagle et al., 2004) The mutation responsible remains a mystery, but the mutated gene likely lies upstream of ephrin-B2a expression and possibly expression of other mapping molecules as well.

Components of signaling cascades from receptors are necessary for interpreting guidance cues. Downstream of the Eph/ephrin-A interactions, focal adhesion kinase (FAK) is a molecule necessary for

proper arbor positioning. When FAK is blocked, axons travel further caudally than they would otherwise. FAK likely acts to stabilize existing adhesions between cells and prevents new ones from forming at the leading edge of the growth cone, resulting in the inhibition of forward movement of axons (Woo et al., 2009).

The Mediolateral Axis

Gradients besides those of ephrins and Eph receptors also contribute to mapping. Semaphorin 3D (Sema3D), a secreted guidance molecule, provides positional information along the mediolateral axis of the tectum (Liu et al., 2004) in addition to an earlier role regulating RGC midline crossing (Sakai and Halloran, 2006). Sema3D is expressed in the lateral tectum, where dorsal RGCs arborize. Ventral axons are repelled by the expression of Sema3D *in vivo*, potentially through the receptors neuropilin-1a and −1b (Liu et al., 2004; Wolman et al., 2004). Knock-down of the Sema3D protein allows ventral RGC axons to arborize in the lateral tectum, disrupting the map (Liu et al., 2004). Therefore, Sema3D acts along the mediolateral axis in a manner similar to the ephrins' role in rostrocaudal guidance.

As with other facets of retinotectal mapping, mutants from the original forward genetic screen have shed light on possible mechanisms of mediolateral guidance. In *nevermind* mutants, the termination zone of nasodorsal axons expands along the mediolateral axis of the tectum, and more axons terminate medially than laterally. Ventral RGC axons target their medial termination zone correctly, but take abnormal, erratic paths to get there (Trowe et al., 1996). The *nevermind* mutation maps to Cyfip2 (Cytoplasmic FMRP (Fragile X mental retardation protein) interacting protein 2) which acts on the retinotopic map through an unknown mechanism (Pittman et al., 2010).

Who cares mutants also have mediolateral mapping disruptions. The termination zones for nasodorsal axons are abnormal, with two separate target zones appearing in the caudolateral (posteroventral) and caudomedial (posterodorsal) tectum. Axons distribute equally between the two areas. Ventral RGC axons still terminate retinotopically in the medial tectum, as in wild type fish (Trowe et al., 1996). However, in this case, the genetic cause has not yet been discovered.

The zebrafish model has thus contributed an extensive array of expression patterns and yet-to-be-identified genetic mutants to the existing guidance cue field. Mutants gave novel insight into the tectum, such as *acerebellar* fish providing the first evidence for the MHB as an organizing center for tectal gradients. Zebrafish also provide an easily manipulated system where genetic knockdowns and subsequent imaging can determine how these many small effects can add together and integrate. The relatively recent teleost genome duplication in zebrafish provides an example of how proteins become functionally diverse and expressed in different areas after the constraints are lifted by duplication (e.g., ephrin-A5 in mammals compared to ephrin-A5a and ephrin-A5b in zebrafish). Additionally, using zebrafish as a vertebrate model in forward screens has provided unbiased ways to look for new genes involved in map formation. Many of these genes still await identification and full descriptions.

ELABORATING THE TERMINAL ARBORS: AN INTERPLAY OF ACTIVITY AND MOLECULAR CONTROL

Synapses Contribute to Branch Length and Stability in the Arbor

When the retinotectal connection is first forming at 3–4 dpf there is rapid branch turnover, however, branches become more stable by 9–10 dpf (Meyer and Smith, 2006). The branches stabilize when synapses are present and often terminate at a synaptic site. Many synapses form in a "trial and error" process before the correct connections are found and maintained, with incorrect connections initiating synapse disassembly before the branch is retracted (Meyer and Smith, 2006). Axons also preferentially extend branches from young synapses close to the branch tips rather than older synapses. The projection of new branches from newly formed correct synapses creates dense arbors in the correct topographic area. Between the selective addition locations and selective maintenance of branches, synapses contribute to local guidance of arbor growth.

An intriguing relationship between arbor growth and number of synapses was identified in zebrafish (Hörnberg et al., 2013). An RNA-binding protein known as Hermes is expressed exclusively in RGCs. When Hermes expression is lost, the number of branches per arbor is reduced, while the stability, lifetime, and retraction rates of branches are unaffected. Under a putative homeostatic control mechanism, the remaining branches produce more densely packed synapses. The increased number of synaptic puncta on the remaining branches enhances early visual behaviors. Hundreds of mRNA molecules are translated in growing axons and the precise mRNA regulated by Hermes is not yet known.

Suppression of Activity Alters Arbor Size and Function

Several studies have gathered different reports of the effects of activity loss on the refinement of the RGC arbors, whether single axons are silenced or all of them. When single RGC axons are silenced with tetanus toxin light chain, the silent synapses still allow the stabilization of branches and the generation of an axon arbor (Ben Fredj et al., 2010). However, arbors with silent synapses extend longer branches and become larger overall at 5–7 dpf, though they contain similar numbers of branches to controls. Silencing the neighboring arbors rescues the phenotype. Arbor growth arrest and refinement are therefore considered to be both activity driven and competitive (Ben Fredj et al., 2010).

Hua et al. (2005) also showed that individual axons' activity levels are important, however, when silenced the RGCs displayed a contrasting phenotype. Neurons were hyperpolarized and made less likely to fire through overexpression of Kir2.1, an inward rectifying potassium channel, or silenced through a dominant-negative VAMPm SNARE protein, which prevents neurosecretory vesicle release at synapses. When a single neuron is silenced among active neighbors, the total length of branches in its arbor falls to about two-thirds the length of controls at 5 dpf (Hua et al., 2005). When TTX is used to globally suppress activity, the smaller phenotype of a single silenced arbor is rescued. Arbor length and complexity return to control levels, implicating competition, rather than activity in isolation, as the key factor. However, the results contrast with the larger arbors typically seen in other work.

Mutant zebrafish have also been identified with activity defects that affect the size of the RGC arbors. During normal development, zebrafish alter their expression of sodium channels to allow classes of neurons to begin firing. At 27 hpf, the sodium channel $Na_V1.6$ is upregulated to supplement the larval expression of $Na_V1.1$, allowing the embryos to become touch sensitive (Ribera and Nüsslein-Volhard, 1998; Pineda et al., 2005). The first ENU screen identified an activity-dependent mapping phenotype with its basis in this mechanism (Granato et al., 1996; Trowe et al., 1996). In *macho* mutants, the $Na_V1.6$ channels are not upregulated at the appropriate time, leading to unrefined retinotectal mapping in addition to the touch insensitivity. The RGCs end up effectively silenced without the $Na_V1.6$ channels, and the silencing alters the later stages of map refinement (Gnuegge et al., 2001). In *macho* mutant larvae, the nasodorsal axons do not travel as far, and thus terminate more rostrally than normal (Trowe et al., 1996). Axons also have expanded terminal arbors (Gnuegge et al., 2001). Blocking all activity with TTX at 4–6 dpf phenocopies this trait rather than returning all arbors to the size of controls.

blumenkohl mutants also present with visual impairments and diffuse topographic maps (Trowe et al., 1996; Neuhauss et al., 1999). RGCs are the only cells in the visual circuit that express Vglut2a, a vesicular glutamate transporter important for release of the neurotransmitter glutamate at the synapse (Smear et al., 2007). The *blumenkohl* mutation causes a deletion in Vglut2a that abolishes functional expression. Without the Vglut2a transporter to mediate glutamate packing into synaptic vesicles, the RGCs release less glutamate per action potential and fatigue quickly, such that they cannot maintain high frequency firing patterns evoked by quickly moving objects (Smear et al., 2007). These synaptic effects lead to a behavioral deficit where the larvae cannot hunt small prey or orient to quickly moving gratings, although they respond normally to larger prey and slower gratings (Smear et al., 2007). There are also corresponding structural changes in individual RGC arbors. The arbors expand in branch length, number and area, with electrophysiological data suggesting that they increase the number of synaptic terminals. Although overall topography is maintained, the arbor expansion degrades the map by increasing the receptive field of the tectal cells, making them less responsive to smaller stimuli. The two mutants described above, *macho* and *blumenkohl*, are excellent examples of how phenotypes discovered in forward genetic screens can be described at the structural, molecular, electrophysiological, and behavioral levels creating comprehensive characterizations of novel phenotypes.

Previous reviews have commented on the opposing effects, especially between the two papers that silence single axons (Hua et al., 2005; Ben Fredj et al., 2010), suggesting that the different methods used for silencing may cause the different arbor sizes (Gibson and Ma, 2011). However, both tetanus toxin light chain, which cleaves VAMP2 (Ben Fredj et al., 2010), and the dominant negative VAMPm SNARE protein (Hua et al., 2005) converge at a synaptic vesicle release mechanism, though there could be differing levels of remaining activity (Ben Fredj et al., 2010). Both papers also show phenotypic rescue when the entire system is silent; a result not seen in the activity mutants. One variable could be in the timing. Much like how the response to TTX changes from being indiscernible from 2 to 4 dpf to creating enlarged projections from 4 to 6 dpf, the relative size

of silenced arbors could change with time and development of the larvae (Debski and Cline, 2002). The arbors were previously seen increasing from 3 to 7 dpf and remaining steady between 7 and 10 dpf (Niell et al., 2004). However, the functional receptive fields are similar between 4 and 8–9 dpf but show a significant expansion at 6 dpf (Zhang et al., 2011). The difference observed in silenced arbors at 5 dpf (Hua et al., 2005) and 6–7 dpf (Ben Fredj et al., 2010) may be superimposed on various growth stages present in the zebrafish retinotectal system. Another caveat is that different types of RGCs, now being categorized based on structure, connections, and morphology (Lowe et al., 2013; Robles et al., 2013, 2014), may also have different modes of growth, intracellular components, and responses to activity loss, and stochastically, could be differentially represented in different papers or by different expression techniques. On a final note, fish lines are not as inbred as mouse lines, and may have slightly different genetic backgrounds between labs (Shinya and Sakai, 2011).

Activity Matching, Plasticity, and Retrograde Signaling

Once synapses are created and are active, matched activity becomes an important component of map refinement and maintenance. The activity of synaptic partners can be compared, and if the RGC's action potential is part of the input that causes the tectal cell to subsequently depolarize then the partners' activity is considered matched. NMDA receptors detect synchronized activity occurring presynaptically and postsynaptically, and much work has been done on this in *Xenopus* (e.g., Cline and Constantine-Paton, 1989; Cline, 1991; Debski and Cline, 2002). After treating zebrafish with the NMDA antagonist MK801 from 3 to 4 dpf, the total length and the area of axon arbors increases. However, the number of branches, the rates of branch addition and deletion, and overall branch lifetimes are unaltered (Schmidt et al., 2000). These changes cause a wider spacing between branches to cover the larger area. This suggests that activity matching between the RGC axons and the tectal cell dendrites is a crucial component of pruning. The branches furthest away from the center of the arbor are retracted—or not extended as far in the first place—to concentrate axon coverage and synapses to matched activity in the central target region (Schmidt et al., 2000).

Matched activity can be interpreted by the presynaptic axons through retrograde messengers. Both the existence and identity of retrograde factors released from the tectal cells used to be debated (reviewed in Schmidt, 2004). Zebrafish were used both to provide evidence for this process and to determine the molecular details of this mechanism. When the activity of presynaptic and postsynaptic cells match, NMDA receptors open to allow calcium to enter the cell. The calcium triggers phospholipase A2 (cPLA2) to release arachidonic acid (AA). When released at the postsynaptic tectal site AA diffuses back to the RGC axon. AA activates presynaptic proteins, including GAP43 and protein kinase C (PKC), which alter cytoskeleton dynamics to cause stabilization of branches and synapses (Leu and Schmidt, 2008). Some of the effect comes through PKC regulation of adhesion molecules, but AA can also act on actin polymerization directly to stabilize the synaptic structure (Zhang and Benson, 2001; Schmidt et al., 2004). Applying AA directly to the tectum stabilizes branches and decreases dynamic events to lower than normal levels (Schmidt et al., 2004). If the retrograde signaling process is disrupted, branch activation and elimination events double in frequency and axons do not gain a mature appearance (Schmidt et al., 2004; Leu and Schmidt, 2008). In this way, retrograde signals act as a stop signal that stabilizes the existing branch and prevents further outgrowth after proper synapse establishment with a tectal cell displaying similar firing patterns as the RGC (Schmidt, 2004).

AA might not be the only retrograde signal used during map development. BDNF can also induce axonal branching *in vivo* and is a necessary molecule for arborization in several species including *Xenopus* and chick (Herzog et al., 1994; Cohen-Cory et al., 1996; Du and Poo, 2004; Hu et al., 2005; Sanchez et al., 2006). By 72 hpf, BDNF is produced in the zebrafish tectum (De Felice et al., 2014). One of BDNF's receptors, TrkB, can also activate the same pathways that create AA (Leu and Schmidt, 2008) suggesting that despite different effects and strengths (Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999), the retrograde signals may converge on similar pathways. This work with retrograde signaling shows that zebrafish can be a useful model both for observing effects at the cellular level and for investigating molecular pathways.

Guidance Factors and Structural Proteins can Shape Arbors Independent of Activity

In forming the retinotectal map, the guidance of an axon to the correct area of the tectum is followed by the elaboration of an arbor. The shape and size of the arbors can affect the scale and function of the map, and can be easily observed by labeling RGCs *in vivo* or in fixed zebrafish tissue. New molecular elements

controlling branching patterns have been discovered in the zebrafish tectum, where guidance molecules and structural stabilization components can both contribute to eventual branch structure.

The Slit family of guidance cues, which bind to Robo receptors, are involved in several aspects of the retinotectal development. The *astray* mutant, with a nonfunctional Robo2 receptor, shows that Slit/Robo signaling first aides RGC axon guidance in the optic tract (Karlstrom et al., 1996; Fricke et al., 2001; Hutson and Chien, 2002b). When axons do make it to the tectum, Slit/Robo signaling also guides branch patterning. When Slit/Robo signaling is disrupted, axon arbors have more branch tips, a greater complexity, and cover a larger area than wildtype axons (Campbell et al., 2007). The extra branches in the *astray*/Robo2 mutant arbors initiate early in development and have greater numbers of presynaptic sites and fewer dynamic branch tips. Slit1a/Robo2 therefore may normally prevent premature maturation, causing axons to remain in a more immature state until other factors overcome this signaling and a balanced arbor is created in a more correct and smaller area.

Structural proteins are also an essential part of growth and branching dynamics. GAP43 (neuromodulin) is membrane-linked and when phosphorylated it binds to, and stabilizes, actin (Leu et al., 2010). GAP43 is usually targeted to the end of the axon, where it first causes growth cone collapse and then initiates branching from the growth cone remnants (Leu et al., 2010). When overexpressed, GAP43 causes larger RGC arbors through faster growth, increasing the number of branches, the area covered, and total branch length. With a mutant GAP43 that cannot be phosphorylated, branching does not increase, but the growth patterns change, causing elongated, spindly, immature arbors, possibly due to an inability to initiate branches in the specific target area to create a complex, mature, and defined arbor. Transfecting a permanently phosphorylated GAP43 into cells increases branching and outgrowth. The axons arborize with several focal areas instead of just one, disrupting the retinotectal map by stabilizing incorrect connections (Leu et al., 2010). These known factors interact with many additional molecules, activity and visual experience, and downstream factors to shape the arbors.

CONCLUSIONS

Over the last several decades, studies using zebrafish have contributed to many areas of retinotectal map development. Beyond genetic approaches, easy imaging and permeability to drugs has allowed descriptions of activity and competition-based mechanisms for axon arbor refinement.

Zebrafish are a convenient model to answer questions that remain in map formation, such as how the axons read the combined gradients, and whether gradients provide stop signals rather than guidance. Several of the ephrin gradients, vital to the idea that the map is based on a coordinate system, have only been observed through *in situ* hybridization of mRNA. Visualization and measurement of the protein gradients *in vivo*, potentially over time, is a next step. Disruptions with quantified deviations from the normal gradients could be used to pin down the exact functions of each protein based on the behavior of the affected axons. Furthermore, genetic labeling techniques, combined with the system's transparency, means that many of these questions can be addressed at the level of individual axons, including their branch dynamics, rather than at the level of the retinotectal projection as a whole. Data from zebrafish are also likely to be useful to help constrain theoretical models of retinotectal map development (Goodhill and Xu, 2005; Hjorth et al., 2014).

There are several cases where work in zebrafish has been very consistent with experiments done in other systems, such as the surgical manipulations, or studies on matching activity. Continuing to pursue topographic mapping questions in zebrafish allows the use modern tools to push the limits of imaging and bring in genetic manipulations. Advances in genetics, especially in the form of genome editing tools, move zebrafish into the forefront. Genes can be efficiently altered with clustered, regularly interspaced, short palindromic repeats (CRISPRs), and the CRISPR-associated system (Cas) CRISPR/Cas (Hruscha et al., 2013; Irion et al., 2014), knocked out or knocked in with zinc-finger nucleases or transcription activator-like effector nucleases (Bedell and Ekker, 2015) and there will likely soon be efficient methods for tagging genes of interest. Other tools are optogenetics for control of neural activity (Deisseroth, 2011; Del Bene and Wyart, 2012) and synaptic specific activity reporters like Syn-GCaMP or SyR-GECO (Walker et al., 2013).

Using some of these new tools, it will be possible to revisit the silencing experiments with, for example, a green-labeled silencing construct and SyRGECO showing synaptic activity in red. This would determine if the levels of activity are different between differing silencing techniques, or if it was simply the relative activity level at each synapse correlating to arbor shape. Human mutations that affect wiring could also be knocked into zebrafish genes to determine mechanisms and test treatments. With an ever-expanding

toolbox, the zebrafish is set to be an important model system for a diverse array of experiments.

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