Making the connection: retinal axon guidance in the zebrafish

James Culverwell and Rolf O. Karlstrom

Genetic screens in zebrafish have identified a large number of mutations that affect neural connectivity in the developing visual system. These mutants define genes essential for accurate retinal axon guidance in the eye and brain and the characterization of these mutants is helping to define the cellular and molecular mechanisms that guide axons in the vertebrate embryo. The combination of zebrafish genetic and embryological approaches promises to greatly increase our understanding of how multiple guidance mechanisms establish the complex neural interconnectivity of the vertebrate brain.

Keywords: mutants/pathfinding/growth cone/tectum/forebrain/genetic screens

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The retinotectal system in developmental biology

The visual system in lower vertebrates has long been a favorite model system for developmental biologists trying to understand how the brain gets wired. During the past half century, classical embryological experiments in frogs have helped to define various aspects of the assembly of the connections between retinal ganglion cells (RGCs) in the eye and their primary target in the brain, the optic tectum. By analyzing behaviors that resulted from surgically induced defects, Roger Sperry and others were able to define the functionality and formation of the retinotectal projection. This work resulted in a pivotal paper by Sperry where he summarized the work and clarified the chemoaffinity model for the formation of ‘neurospecificity’ that we still use today.1 In more modern terms, this model states that axons are guided to their appropriate targets by guidance cues present in the growth environment. We now know that these environmental cues take the form of molecules that help guide the motile tip of an axon, the growth cone, as it grows in the embryo. Guidance molecules may attract or repel growth cones and may be either diffusible or bound to the growth substrate.2 Most importantly, multiple guidance cues act on an individual growth cone to direct it to the correct target. The modern challenge is to identify the full suite of guidance cues acting in the embryo, and to elucidate how growth cones integrate these cues to achieve the precise and reproducible neuronal connections that make a functioning brain.

The zebrafish retinotectal projection, like that of the frog, forms as RGCs in the eye extend axons that connect to the optic tectum [Figure 1(A)]. In the eye, RGC axons grow in an organized way to the optic disc, where they exit the eye and grow along the optic stalk/optic nerve to the optic chiasm. In lower vertebrates such as zebrafish and frogs, all retinal axons cross the midline where they enter the optic tract, resort, and grow dorsally to the contralateral tectal lobe.3,4 Upon reaching the tectum, each retinal axon terminates at a position that reflects the position of its cell body in the eye, forming a topographic projection or sensory ‘map’ on the tectum. Neurons from the dorsal eye send axons to the ventral part of the tectum while ventral retinal neurons project dorsally. Nasal (anterior) retinal neurons project to the posterior tectum and temporal (posterior) neurons project anteriorly [Figure 1(A)].

The zebrafish model system, due to its experimental, optical, and genetic accessibility, is helping us understand the mechanisms underlying the ‘neurospecificity’ that helps establish this neural connection. We are now able to use the power of zebrafish genetics to revisit many of the questions posed by Sperry in the very same system. Genetic screens are revealing many of the defects seen by Sperry, but these defects
Figure 1. Mutations affecting the zebrafish retinotectal projection. (A) Schematic dorsal view of the 5-day wildtype retinotectal projection. RGC axons project out of the eye into the optic nerve, cross the midline of the diencephalon at the optic chiasm (arrow), enter the optic tract, and grow dorsally to the contralateral tectal lobes. Labeling of dorsal/nasal axons (green) and ventral/temporal (red) axons shows that RGC axons are topographically ordered in the optic nerve and tract and project topographically on the tectum. Fluorescent pictures show examples of mutant phenotypes found in the retinotectal screen. Arrows point to aberrant retinal axon projections. (B) In six mutants, axons occasionally make errors within the eye. (C) Ten mutants disrupt midline crossing, with axons projecting to the ipsilateral (incorrect) tectal lobe where they find their correct topographic position. Axons occasionally also cross the midline in these mutants (dotted lines). (D) Mutations in five genes lead to dramatic pathfinding errors in the forebrain. In different mutants, axons grow rostrally, ipsilaterally, or into the contralateral eye. (E) In any individual ax/robo-2 mutant, axons can make multiple pathfinding errors, including rostral and ipsilateral growth and recrossing of the midline. (F) In two mutants, few axons cross the midline. Those axons that reach the contralateral tectum often do not find their correct topographic position. (G) In three mutants, dorsal retinal axons fail to sort completely into the ventral optic tract. Despite this missorting, axons map correctly in the tectum. Occasional ipsilateral projections are also seen in these mutants. (H) Three mutations lead to defects in dorsal/ventral and/or anterior/posterior mapping. (I) Three mutations cause retinal axons to have expanded termination fields with corresponding mapping errors. Underlining indicates the gene affected in the mutant has been identified (see Table 1 for mutant name and gene name).
Table 1. Retinotectal axon guidance mutants at a glance (listed alphabetically)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene</th>
<th>Axon defects</th>
<th>Other defects</th>
<th>References</th>
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<td>Gire, ear, FB comm., heart, MHB</td>
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<td>Path. FB</td>
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<td>Path. FB + eye</td>
<td>HB, NC, som.</td>
<td>5, 11, 12</td>
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<td>bellalineosal</td>
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<td>hyperaesthetic</td>
<td>Unknown</td>
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<td>6, 52</td>
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<td>house</td>
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<td>container</td>
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<td>5, 16, 29, 57-59</td>
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<td>cyclops</td>
<td>Nodal-related-2</td>
<td>Curb, cyclopia, FP</td>
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<td>gnuppo</td>
<td>jf-1-lasen</td>
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<tr>
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<td>Unknown</td>
<td>Topography</td>
<td>Na+ currents, pig, touch resp.</td>
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<td>Unknown</td>
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<td>Mot.</td>
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<td>nso oзнамен</td>
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<td>Smoothen</td>
<td>Path. FB</td>
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<td>space cadet</td>
<td>Unknown</td>
<td>Path. eye + HB</td>
<td>Mot., spiral fibers in HB</td>
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<tr>
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<td>sonic you</td>
<td>sonic hodag</td>
<td>Path. FB + eye</td>
<td>Gire, curb, fin, mot., som., Vib</td>
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<td>gh2</td>
<td>Curb, circ., jaw, mid lens, mot., som., vFB</td>
<td>5, 16, 17, 28, 53, 67-69, 75, 82</td>
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Notes: Abbreviations: circ., circulation; comm., commissure; ect., ectopic; FB, forebrain; HB, hindbrain; LFP, lateral floorplate; MHB, midbrain–hindbrain boundary; mot., motility; NC, notochord; OKR, optokinetic response; path., pathwayfinding; pig., pigment; resp., response; som., somite, and vFB, ventral forebrain.
are the result of loss of gene function rather than surgical manipulation. These mutants will thus provide a molecular explanation for well-described axon guidance and mapping phenomena.

In a zebrafish retinotectal screen done in the 1990s, Dr Friedrich Bonhoeffer’s lab identified over 30 retinal projection mutants by labeling RGC axons with the lipophilic dyes DiI and DiO\(^{5–7}\) (Table 1). Errors were seen at a variety of positions in the pathway between the eye and tectum (Figure 1). These mutants can be grouped into those affecting pathfinding in the eye (‘eye exit’ mutants), pathfinding across the forebrain to the tectum (‘pathfinding mutants’), and the formation of topographic connections on the tectum (‘topography mutants’) (reviewed in References 7, 8). The retinotectal mutants help to define choice points encountered by retinal axons as they grow to their final target in the tectum. Many of these mutants, along with some mutants found in related screens, have now been extensively characterized, and many of the affected genes have been identified. In this review we examine what has been learned about retinal axon guidance from this ‘first generation’ of genetic screens.

Pathfinding within the eye: eye exit mutants

Several identified mutations interfere with the ability of retinal axons to grow toward the optic disc and exit the eye\(^{3,10}\) [Figure 1(B)]. In lavishful (bal), grumpy (gup), and sleepy (shy) mutants retinal axon growth is disorganized in the eye, with axons wandering circumferentially before reaching the optic nerve head. bal, gup, and shy share an array of axon phenotypes as well as similar defects in notochord and hindbrain development\(^{11,12}\) which suggested that these genes act in the same genetic pathway. This hypothesis was recently confirmed when gup and shy were shown to encode the \(\beta 1\) and \(\gamma\) subunits, respectively, of the extracellular matrix (ECM)/axon guidance molecule Laminin\(^{13}\); bal may also encode a component of Laminin (D. Stemple, personal communication). RGCs express Integrins, receptors for Laminin, and Laminin is known to play a role in retinal axon growth in the eye (reviewed in Reference 14). The bal, gup, and shy phenotypes confirm that Laminin plays a crucial role in fasciculation and directed axon growth toward the optic disc. These laminin subchain mutant phenotypes are surprisingly specific given the widespread roles played by Laminin in development (see Reference 15), and these mutants should be extremely useful in helping to explain how retinal growth cones integrate cues from the ECM as they grow out of the eye.

con and syu also disrupt retinal axon growth in the eye and midline patterning\(^{16}\). syu encodes the midline secreted morphogen Sonic Hedgehog (Shh), and con has been shown to disrupt Hedgehog (Hh) signaling\(^{10}\). Since Hh signaling plays a major role in patterning the ventral forebrain and optic vesicles\(^{16,17}\), these eye exit axon phenotypes might be due to early eye and optic stalk patterning defects. Alternatively, since RGC differentiation depends on Hh signaling\(^{18}\), these pathfinding errors may reflect defects in the retinal cells themselves. Finally, space cadet (spc) mutations result in a third, distinct, eye exit phenotype. In spc mutants, RGC axons often fail to leave the eye, despite well-organized growth to the optic disc\(^{19}\).

Pathfinding in the forebrain

Crossing the midline

A large number of identified pathfinding mutations affect the ability of axons to grow toward and across the midline of the forebrain [Figure 1(C)]. In these mutant fish, retinal axons grow to the ipsilateral rather than the contralateral tectal lobe. Despite being on the wrong side of the brain, retinal axons find their correct topographic target, confirming classic work which showed that guidance mechanisms within the tectum can operate independently of mechanisms guiding axons across the midline\(^{20}\). Similar results have been found in fly mutants with defective midline axon crossing\(^{21}\) showing that the remarkable ability of growth cones to make ‘appropriate’ connections on the wrong side of the embryo after following alternate pathways is evolutionarily conserved. In the fly commissuranceless (comm) mutant, axons completely fail to cross the midline\(^{22}\); similar to the phenotype seen in the zebrafish midline mutants. Midline pathfinding is also defective in fly, worm, and mouse embryos mutant for the netrin guidance molecule [reviewed in Reference 25]. So far, none of the zebrafish midline crossing mutants have been shown to encode these well-characterized guidance molecules. Instead, these mutations all affect the formation of the ventral forebrain, and thus these midline pathfinding defects appear to be caused by mispatterning of the growth substrate.

All of the identified zebrafish midline mutations, with the possible exception of bal, may affect Hh
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signaling in the forebrain. The cyclops (cyc) mutation reduces ventral forebrain tissues (leading to cyclopia), and mild alleles of cyc have ipsilaterally projecting retinal projections. cyc encodes the TGF-β signaling molecule Nodal related-2 (Ndr-2),24,25 which is needed for Hh signaling in the ventral neural tube26 and for the formation of the ventral forebrain.27 As mentioned above, cyc encodes Shh itself,10 while smu encodes the Hh receptor complex protein Smoothened.9 We recently showed that dtr and yot encode Gli1 and Gli2, respectively, transcription factors that interpret Hh signals in responding cells,17 (R. Karlstrom et al., unpublished results). The proteins encoded by con, igu, and uml have not been identified, but all of these mutants have phenotypes consistent with defects in Hh signaling.26,28 (O. Tyurina and R. Karlstrom, unpublished results). Our analysis shows that these mutations disrupt specific instructive (both attractant and repellent) and permissive (cellular) guidance cues (M. Barresi and R. Karlstrom, unpublished results). In particular, it is known that the Hh signaling regulates netrin expression in the vertebrate ventral midline.29,30 Indeed, among other defects, we have seen that netrin expression is selectively reduced in the zebrafish Hh pathway mutants (M. Barresi and R. Karlstrom, unpublished results). Our analysis suggests that the midline mutants will be extremely valuable in characterizing the molecular and cellular nature of the retinal axon growth substrate in the forebrain.

Making the wrong connection

Like the midline mutants described above, belladonna (bel) mutants have ipsilateral retinotectal projections that map to the correct position on the ipsilateral tectal lobe31 [Figures 1(C) and 2]. Unlike the Hh pathway mutants, however, midline structures are generally unaffected in bel. The bel larvae have a reversed response to visual stimuli,31,32 indicating that retinal axons make functional connections on the wrong side of the brain. The bel phenotype thus reproduces classic embryological experiments showing a reversed visual response in frogs with surgically induced ipsilateral projections.30 Homozygous bel animals are semiviable and exhibit abnormal swimming behaviors, further suggesting that the ipsilateral projections are functional. The presence of (reversed) visual tracking, proper retinotectal mapping, and viability of homozygous bel animals all attest to the specificity of the mutation for axon guidance across the midline of the ventral forebrain.

Figure 2. Retinal axon errors in belladonna. (A) In wildtype embryos retinal axons (arrows) cross the midline to form the optic chiasm (arrowhead) by 36h of development. (B) In bel mutants retinal axons (arrows) can grow up to, but not across, the midline (arrowhead). (C) By 38h the chiasm is well formed (arrowhead) in wildtype embryos and retinal axons have grown dorsally towards the tectum (arrows). (D) By 38h, axons in bel mutants are no longer near the midline and grow dorsally along the ipsilateral diencephalon (arrows). (E) Schematic of wildtype retinal (red) and forebrain commissure (gray) axons at 38h. (F) Schematic showing range of bel retinal axon defects and defects in forebrain commissure formation (gray lines). Circled areas show regions where retinal axons were observed in different bel mutant embryos at early ages. By 48h, all axons project ipsilaterally immediately after leaving the eye. (A–D) ZN-5 antibody labeling of retinal axons. All panels show ventral views, anterior up.

Our lab examined retinal axon growth in bel mutants more carefully and we found that retinal axons can grow quite close to the midline before they turn ipsilaterally (Figure 2(B)). This observation suggests that bel disrupts axon guidance at or near the ventral midline. A careful analysis of gene expression in bel mutants revealed subtle defects in forebrain patterning that may reflect the loss or mis specification of certain cell types at the midline where retinal axons normally cross (M. Walkowicz and R. Karlstrom, unpublished results). Subtle forebrain defects, in combination with the
observation that guidance errors are usually symmetrical across the midline. This suggests that RGC guidance defects result from defects in the growth substrate immediately at the midline. The bel phenotype is remarkably similar to achiasmatic disorders in humans and dogs which affect depth perception and other binocular functions. The study of bel thus promises to shed light on the molecular and developmental mechanisms that may lead to these congenital disorders.

Going haywire in the ventral forebrain

Several identified mutations lead to particularly dramatic axon defects, with retinal axons growing throughout the ventral forebrain (Figure 1(D)). In acerebellar (ace) mutants, retinal axons project ipsilaterally and rostrally in the telencephalon. The ace locus encodes FGFR8, a secreted signaling molecule crucial for normal forebrain patterning. Elegant eye transplantation experiments were used to create genetic mosaics and show that acefgfr8 function is required in the brain (not the eye) for normal retinal axon guidance. Ipsilateral and rostral axon growth is also seen in no isthmus (noi) mutants, with additional defects in axon fasciculation. Strikingly, retinal axons from the two eyes often intermingle in noi mutants, and they sometimes grow into the contralateral eye. noi encodes pax2.1, which is expressed in glial cells of the optic stalk, cells needed to organize axons in the optic nerve.

Retinal axons in bal, gap/f1-laminin, and sh/y-laminin mutants make numerous errors and can project ipsilaterally or rostrally into the telencephalon. Since Laminin is expressed along the retinal pathway in other vertebrates, these errors may reflect a loss of an attractive or permissive substrate that allows growth across the midline and into the optic tract. Further analysis of growth cone behaviors and laminin function in bal, gap/f1-laminin, and sh/y-laminin needed to clarify the role of Laminin mediated cell-substrate adhesion in retinal axon growth through the brain. astrey (ast) homozygous fish show no obvious visible defects and are viable. In this mutant, retinal axons make multiple errors, including ipsilateral growth, rostral growth, and recrossing of the midline (Figure 1(E)). ast seems to affect only the retinal axons; eye transplantation experiments show ast function is needed in retinal axons rather than in the growth substrate. ast encodes Robo2, a receptor expressed on retinal growth cones that binds surface molecules of the Slit family. slit2 and slit3 are expressed in stripes across the forebrain in regions where axons do not grow. It thus appears that Slit proteins may provide repulsive cues that help confine retinal axons to nonexpressing regions of the forebrain. The analysis of ast/robo2 function in zebrafish embryos reveals that vertebrates and invertebrates use similar guidance mechanisms in different ways. In the Drosophila CNS, Slit acts as a midline barrier that is crossed selectively after down-regulation of Robo receptors in crossing axons. In the vertebrate forebrain, Slit seems to channel Robo-expressing RGCs into the appropriate region of the brain. There are multiple Robo receptors and Slit ligands, each with unique expression patterns, suggesting the Robo/Slit system helps guide many different types of axons in different ways. Analysis of Slit function in zebrafish will help elucidate how this one family of guidance molecules can play multiple roles in wiring the vertebrate brain.

Fiber sorting mutants

In boxer (box), dachsel (dak), and pinscher (pic) mutants, dorsal axons do not all sort into the ventral branch of the optic tract. Arriving at the incorrect, dorsal side of the tectum, these axons then traverse the tectum to find their correct ventral target (Figure 1(G)). This observation confirms embryological studies which demonstrate that retinal axons map correctly on the tectum independent of their point of entry. The observation that these three mutants share retinal axon, jaw, and fin defects (presumably) suggests that box, dak, and pic may act in the same genetic pathway. In work focusing on the jas phenotype it was recently reported that dak encodes a member of the exostosin (EXT) enzyme family which regulates heparan
Axonshaveexpandedterminationzones. Different HSPGs can bind a variety of axon guidance molecules (including Netrins, Slits, and Laminin), guidance receptors (including DCC and Protein Tyrosine Phosphatases), and growth factors (FGFs, TGF-β) (reviewed in Reference 48). The recent finding that heparan sulfate (HS) is essential for Slit2/Robo-1 repulsive interactions supports the model that HSPGs may act as cofactors that modulate ligand/receptor binding. HS has been directly implicated in retinal axon guidance in the frog, with specific HS sequences required for retinal axon targeting. Since EXT enzymes are needed for the synthesis of numerous HSPGs, which can then interact with a wide range of axon guidance systems, determining the precise mechanisms by which EXT proteins affect retinal axons may be a challenge. One hint may come from observations that cells at the border of the dorsal optic tract express a specific sulfation enzyme, 6-O-sulfotransferase, which shows that this region may contain specific sulfated forms of HSPGs needed for axon guidance in the tract. Thus the box, dahl, and pie mutants are providing an important entry point into the study of fiber sorting in a nerve bundle, a phenomenon that remains poorly understood.

Mapping and termination mutants

The retinotectal screen was designed to find mutations that affect sensory mapping. Mutants with both dorsal/ventral and anterior/posterior mapping defects were isolated. Nevermind (nev) and abosorns (ave) affect the mapping of retinal fibers along the dorsal/ventral axis (Figure 1(H)). In both mutants, nasodorsal axons terminate both ventrally and dorsally in the tectum instead of targeting only the ventral region. In ave, axons missort in the optic nerve and tract, while sorting appears normal in nev. These dorsal/ventral mapping mutants are of great interest, as very little is known about how the dorsal/ventral map is generated. In ave/gf5 mutants, which lack a midbrain/hindbrain boundary, mapping errors occur in both the dorsal/ventral and anterior/posterior axes of the tectum. Both dorsal/nasal and ventral/nasal axons have expanded termination zones.

Other anterior/posterior mapping mutants include guardet (gnd), macho (mao), and blumenkohl (blu). In gnd mutants, nasodorsal retinal axons begin to branch prematurely in the anterior region of the tectum, with some axons terminating in this inappropriate region. In mao and blu mutants, RGC arbors are expanded to various degrees throughout the tectal neuropil. mao has another phenotype, touch insensitivity, that sheds light on these retinal axon defects. mao RGCs and rohon-beard cells lack sodium currents, and blockage of Na+ channel activity with tetrodotoxin phenocopies the RGC projection defects seen in mao. Thus the expanded retinal projections on the tectum seen in mao seems to be due to the loss of activity dependent refinement of the retinotectal map. The further study of mao promises to help us understand how neural activity refines sensory maps in the brain.

Future connections in zebrafish

Given the long and productive history of research in other model systems, why should we study axon guidance in the zebrafish? The answer lies in the unique combination of genetic and experimental tools that can be applied in zebrafish to examine axon guidance at both the cellular and molecular levels. Work in Drosophila and Caenorhabditis elegans has shown the power of random mutagenesis screens in finding axon guidance genes (see References 55, 56), and zebrafish work brings this approach into a vertebrate species. This research bridges the gap between genetically accessible but relatively simple invertebrate brains, and more complicated, yet less accessible, mammalian brains. Almost half of the mutations identified in the ‘first generation’ zebrafish screens have now been identified, and all encode previously known proteins (see Table 1). We can only hope that as more laborious positional cloning projects are finished, some of the remaining mutants will be found to encode novel or surprising genes. Even now, the zebrafish retinotectal mutants are revealing how molecular function is conserved across the animal kingdom. More importantly, they are revealing new functions for evolutionarily conserved molecules. This work will help direct research in higher vertebrates and is providing important models for human disease.

Cell transplantation and ablation studies in zebrafish have contributed enormously to our cellular understanding of axon guidance, and it is now routine to alter the growth environment in the zebrafish embryo by removing or adding cells. The ability to do these manipulations in different mutants will be critical to help define the molecular mechanisms that mediate previously defined cellular interactions. As an example of this approach, cell transplantation experiments in the zebrafish spinal cord have shown...
that specific muscle precursor cells (adaxial cells) are needed for motor axon growth into the somites,58 while ablations have shown that another set of muscle cells (muscle pioneers) are not required for motor axon guidance59 reviewed in Reference 60. In addition, the generation of genetic mosaics has shown that ast/fgf8 function is required in the brain for normal retinal axon growth,52 while ast/robo-2 function is required in RGCs.52 These genetic/embryological experiments will continue to help define the molecular mechanisms that mediate interactions between growth cones and the environment.

Coupled with genetic and experimental accessibility is the well-known optical clarity of the zebrafish embryo. Because zebrafish embryos develop so rapidly and cells can be labeled and visualized in live embryos, it is possible to directly observe the processes of axon guidance and synaptogenesis. In vivo observations of mutant growth cone behavior can uncover extremely subtle defects in axon guidance. As an example, the analysis of growth cone behavior in the ast/robo-2 mutant suggests that mutant growth cones differ from wildtype growth cones in their ability to correct pathfinding errors.41 This high resolution analysis of growth cone behavior will be an extremely powerful way to uncover real-time aspects of axon guidance that might lead to significant defects in neural connectivity.

We are now entering the ‘post-genome’ era for zebrafish. As sequencing of the zebrafish genome is completed in the next year or two, and as genetic maps continue to improve, the identification of genes important for axon guidance will become increasingly straightforward. Second generation screens in zebrafish will employ new tricks borrowed from other model systems (e.g. GFP expressing transgenic lines, insertional mutagenesis, transposon based mutagenesis, and Gal4 systems) and are already being developed to help identify new components of the axon guidance machinery. ‘Reverse genetic’ techniques to identify gene function are also evolving quickly. New tools that benefit from a sequenced genome include morpholino antisense oligonucleotides to knock down gene function (see Reference 61), laser activation of the heat-shock promoter driven constructs, misexpression genes in targeted cells, and as well as targeting genetic lesions to known genes as is done in plants.53 The next few years hold great promise for understanding axon guidance, and the zebrafish system will continue to play a central role in helping us understand this process in the most complex of all interconnected systems, the vertebrate brain.

Acknowledgements

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References

1. Making the connection

2. For the full text, please refer to the original scientific articles.