Many zebrafish mutants have specific defects in axon guidance or synaptogenesis, particularly in the retinotectal and motor systems. Several mutants have now been characterized in detail and/or cloned. A combination of genetic studies, in vivo imaging and new techniques for misexpressing genes or blocking their function promises to reveal the molecules and principles that govern wiring of the vertebrate nervous system.

**Abbreviations**

ace acerebellar
ast astray
CaP caudal primary motor neuron
diw diwanka
GFP green fluorescent protein
HMS horizontal myoseptum
mao macho
MIP middle primary motor neuron
noi no isthmus
RGC retinal ganglion cell
Robo Roundabout
RoP rostral primary motor neuron
Sema semaphorin
smu smooth-muscle-omitted
sop sofa potato
spc space cadet
sty stumpy
syu sonic-you/sonic hedgehog
unp unplugged
yet you-too/gli2

**Introduction**

The past decade has brought great strides in our molecular understanding of axon guidance and synaptogenesis, particularly through genetic analysis of invertebrate models and in vitro analysis of vertebrates. Such analyses have revealed an apparently canonical set of gene families that are conserved across the animal kingdom [1]. Given this knowledge, three questions stand out. First, how do the known genes act in the animal to guide particular axons? Second, how many axon guidance genes remain to be found? Third, does the relative complexity of the vertebrate nervous system arise from using old gene families in new ways, or by virtue of vertebrate-specific gene families? Studying the zebrafish promises to yield answers to these questions.

The zebrafish embryo has several well-known experimental advantages. For example, external fertilization provides early embryonic access. Its nervous system is simple and well-characterized (reviewed in [2,3*]). Its optical transparency allows in vivo imaging of cell movements (e.g. see [4]) or ablation of identified cells to perturb development or behavior [5,6]. Finally, cells or tissues can be transplanted to test the autonomy of gene action (e.g. see [7,8*]).

Recent genetic screens have identified many genes necessary for axon pathfinding and synaptogenesis [9–12]. It has now become relatively routine to clone mutant genes, by using candidate gene, positional cloning or insertional mutagenesis approaches [13]. Three new technical advances show particular promise for analyzing gene function: the development of transgenic lines that express green fluorescent protein (GFP) in specific sets of neurons [14*]; the use of a heat-shock promoter to misexpress genes in specific cells [15*]; and the use of antisense morpholino oligonucleotides — whose modified backbone provides increased stability and reduced toxicity — to knock out gene function. [16*].

In this review, we discuss recent studies that use this sizeable bag of tricks to analyze how axons find and form synapses with their targets, particularly in the retinotectal and motor systems.

**Retinotectal pathfinding**

Most retinal ganglion cell (RGC) axons project to the primary visual center in fish — the contralateral optic tectum [17,18] (Figure 1a). Retinal axons project to the optic nerve head in central retina and exit the eye. They then grow along the ventral surface of the optic stalk (which later becomes the optic nerve), cross the ventral midline of the forebrain, turn dorsocaudally, and project to the optic tectum in the dorsal midbrain. There, they branch extensively, terminating topographically according to their point of origin in the retina.

In several species, specific guidance molecules are known to function in the eye (e.g. netrin/DCC [deleted in colorectal cancer]), at the midline (GAP-43 [growth-associated protein-43], ephrinB/EphB5), and along the anteroposterior axis of the tectum (ephrinA/EphAs) (reviewed in [19–21]), but many segments of the pathway remain unexplained molecularly. A direct screen for retinotectal projection defects has yielded a large set of mutants, each with specific defects at one or two points [10,22,23] (Figure 1b–h). Many of these perturb brain or eye patterning, whereas others may affect axon guidance more directly.

**Mutants with mispatterned brains**

In midline-crossing mutants (Figure 1d), retinal axons project to both the ipsilateral and contralateral optic tecta, forebrain commissures are often defective, and the forebrain is often narrowed [10,24–27]. The three cloned mutants in this class, sonic-you/sonic hedgehog (syr) [28], you-too/gli2 (yot) [25] and smooth-muscle-omitted (smu)/smoothened (smo) [26,27],
Mutations disrupting different steps in formation of the retinotectal projection. (a) Dorsal view (right) of wild-type projection at 5 days postfertilization, after labeling groups of RGCs in the doroanterior (green) and ventroposterior (red) quadrants of the eye. Labeling is shown in the corresponding lateral view (left). Retinal axons project out of the eye, across the midline and to the contralateral optic tectum. They are topographically ordered as they exit the eye, rearrange this order shortly after the midline and terminate topographically on the tectum. (b–h) Dorsal views of mutant phenotypes. (b) In four mutants, bashful (bal), chameleon (con), spc and syu, axons sometimes fail to exit the eye. (c) Two mutants, esrom (esr) and spc have defects in elongation, with few axons reaching the tectum. (d) Ten mutants, belladonna (bel), blowout (blw), con, debur (dtr), iguana (igu), smu, spc, syu, umleitung (uml) and yot have defects in midline crossing, with projections to both ipsilateral and contralateral tecta. (e) Six mutants, ace, bal, grumpy (gup) noi, sleepy (sly) and ast, have defects in pathfinding to the tectum, with ipsilateral and anterior projections. In noi, retinal axons also project into the opposite eye and to ventral hindbrain, and show extra midline crossing. (f) Three mutants, boxer (box), dackel (dak) and pincher (pic), have defects in optic tract sorting, but normal tectal topography; box and pic also have projections to ipsilateral tectum. (g) Three mutants, ace, nevermind (nev) and who-cares (woe), have defects in optic tract sorting and defective tectal topography: along the dorsoventral axis for nev and woe, and along both axes for ace. (h) Three mutants, mao, blumenkohl (blu) and gnarled (gna), show expanded or defective tectal terminations. Orange font indicates that the mutant gene has been cloned; underlined names indicate a known defect in brain patterning. A: anterior; D: dorsal; P: posterior; V: ventral.

Retinal axons gone astray

The astray/robo2 (ast) mutant has the most dramatic known retinotectal phenotype. In addition to mistakes similar to ace and noi, ast retinal axons project into ventral hindbrain and recross both the ventral and dorsal midlines [8**,10]. Brain patterning and the forebrain commissures appear normal, however, and eye transplants show that ast acts eye-autonomously. Cloning of ast shows that it is a defect in Robo2—a homolog of the Drosophila axon guidance receptor Roundabout (Robo) [8**,31] — which is expressed in RGCs during axon outgrowth. As robo2 is also expressed in mouse RGCs [32–34], it is likely to be essential for retinal axon guidance in all vertebrates.

We have used high-resolution in vivo imaging of growth cone behavior to try and elucidate the function of ast. We found that ast growth cones make frequent pathfinding errors and fail to correct them, whereas wild-type growth cones make occasional pathfinding errors (to our surprise), but always...
correct them. Importantly, ast affects retinal growth cone morphology, behavior and pathfinding before, at and after the midline (LD Hutson, C-B Chien, unpublished data).

This contrasts with Drosophila commissural axons. In these axons, Robo is downregulated (and therefore nonfunctional) at the midline, and pathfinding defects in robo mutants are only seen after midline crossing [31]. What accounts for this difference? Perhaps it is because Robo ligands are expressed in different patterns. In Drosophila, commissural axons must cross a midline band of Slit, a repulsive Robo ligand [35]; in contrast, vertebrate slits are expressed in domains adjacent to, but not crossing, the retinal axon pathway [32–34] (Hutson LD, C-B Chien, unpublished data).

Retinotectal plus: space cadet and macho

Originally, space cadet (spc) mutants were described as having two disparate phenotypes: retinotectal pathfinding defects and faulty escape behavior [9,36••] (Figures 1b–d,2). Recent work has shown that these defects may share a common basis. Normally, spiral fiber neurons form synapses on Mauthner neurons (large hindbrain neurons that control the escape response), presumably modulating their activity (Figure 2a). In spc mutants, the spiral fibers do not innervate the Mauthner neurons (Figure 2b), and the spc behavioral defect can be phenocopied by microsurgical transection of axon tracts that include the spiral fibers (Figure 2a). Therefore, spc may be required for axon pathfinding of spiral fibers as well as retinal axons.

The macho (mao) mutant also has apparently disparate phenotypes: enlarged retinotectal arbors and touch insensitivity [9,23]; (Figure 1b). These have been shown to have a common biophysical underpinning. The touch-response defect is caused by lack of sodium current in the sensory Rohon–Beard cells [37]. mao RGCs also lack sodium current, and injecting the sodium channel blocker tetrodotoxin into the eye or the optic tectum can phenocopy the enlargement of arbors seen in mao mutants [38••]. Thus, mao provides a genetic recapitulation of classic tetrodotoxin-blocking experiments [39], and may prove useful for studying other activity-dependent processes.

Motor axon pathfinding

Zebrafish trunk motor neurons develop in two waves. In each hemisegment, three identified primary motor neurons, named CaP, MiP and RoP (for caudal, middle and rostral primary), are formed 9–10 hours after fertilization, and about 30 secondary motor neurons are formed a few hours later [3•]. All three primary motor neurons extend their axons within the spinal cord to a shared exit point, and then follow a shared ‘common pathway’ to reach the horizontal myoseptum (HMS), which is marked by a set of specialized muscle pioneer cells. Here they pause before diverging to distinct targets (Figure 3a,b).

The cellular interactions used for CaP/MiP/RoP axon guidance have been studied extensively [3•]. Each primary motor neuron can project correctly even with the other two ablated, but their axons do require myotome-derived signals. Although the secondary motor axons can navigate correctly when the primary motor neurons are ablated [5], they do follow the paths of the primary axons [4], and perturbations that affect the primary axons can also affect the secondary axons [40•]. Therefore, primary and secondary motor axons may share some pathfinding mechanisms.

Several axon guidance molecules are known to be expressed by the motor neurons and myotome [3•]. Among these, chondroitin sulfate proteoglycans [41], GDNF (glial cell line derived neurotrophic factor) [42], semaphorin (Sema) 3A1 (formerly SemaZ1a) [15•] and Sema3A2 (formerly SemaZ1b) [43] can impede ventral motor axon outgrowth when injected or overexpressed. In addition, enzymatic removal of chondroitin sulfate proteoglycans [41] and function-blocking antibodies to Neurulin [44] cause aberrant branching and outgrowth of primary or secondary motor axons, respectively. More definitive experiments are required before the roles of these molecules in motor axon guidance are understood.

Motor axon mutants

Three known mutants specifically disrupt primary motor axon pathfinding without affecting muscle, notochord or floor plate development [9,12]. diwanka (die) and unplugged (unp) were found using antibody staining to re-screen mutants found in a large-scale embryonic motility screen, whereas stumpy (sty) was found in a smaller screen that used antibody staining as the primary assay. Each mutant has a distinct defect in motor axon navigation (Figure 3c–e), suggesting that they disrupt different signaling pathways.
Mutations that specifically disrupt pathfinding by primary motor axons. (a,b) (a) Transverse and (b) lateral views showing wild-type projections of the three primary motor neurons, RoP (R; green), MiP (M; red) and CaP (C; blue), in each hemisegment. Axons from RoP, MiP and CaP project ventrally from the spinal cord (sc) along the common pathway to the HMS, and then diverge. RoP invades the myotome at the level of the HMS, whereas MiP retracts its common pathway projection and instead projects to dorsal myotome. CaP continues on and projects to ventral myotome. Not shown is VaP, the variable primary motor neuron, which is present in about 50% of segments and usually dies. Also shown in (a) are the adaxial cells (orange), which have an essential role in guiding motor axons. These cells originate adjacent to the notochord (not), migrate laterally and eventually differentiate to form slow muscle fibers. (c–e) Lateral views of mutant phenotypes. (c) In diw, all three primary motor axons either fail to project into, or stall within, the common pathway. RoP occasionally bypasses the spinal cord–exit point altogether, projecting caudally within the spinal cord. (d) In unp, CaP often stalls and extends aberrant branches at the HMS. Occasionally, RoP also branches aberrantly at the HMS, sometimes sending one projection into ventral myotome. MiP projects normally. (e) In sty, CaP initially stalls at the HMS, but then sometimes extends further into the ventral myotome. MiP and RoP initially project normally, but later show subtle defects such as reduced branching.

Figure 3

The diw mutants show accordion-like contraction in response to a touch stimulus, rather than the side-to-side tail motion seen in wild-type [9], and all three primary motor axons show defects (Figure 3c). Most RoPs arrest at the spinal cord–exit point and fail to enter the common pathway, whereas most MiPs and CaPs stall along the common pathway before reaching the HMS [7]. Analyzing genetic mosaics created by cell transplantation shows that diw function is required in the adaxial cells—a set of slow-muscle precursors whose path of migration seems to prefigure the common pathway [7]. This suggests that adaxial cells either secrete, or instruct the somite to produce, a signal that attracts growth cones along the common path.

The unp mutants are initially immotile but can later swim [9]. All three primary motor axons exit the spinal cord and pathfind correctly to the HMS, but thereafter CaP and RoP fail to follow their normal pathways, whereas MiP projects normally. Most CaP axons, which normally project to ventral myotome, stall at the HMS, occasionally forming inappropriate branches. Most RoP axons, which normally pause at the HMS, either branch abnormally or project into the ventral myotome. Secondary motor axons show defects very similar to those of CaP and RoP [40*] (Figure 3d). Transplant experiments show that like diw, unp is required in adaxial cells [45**], suggesting that adaxial cells help to specify not only the common but also the cell type-specific pathways.

In sty mutants, the common pathway appears normal, but extension of the primary motor neurons along their individual pathways, together with secondary motor axon extension, is affected [12]. In spite of these defects, sty has no obvious motility defect. In a strong sty allele, most CaP axons stall at the HMS, whereas the rest stall before reaching their targets in ventral myotome. MiP and RoP axons in sty pathfind correctly to the HMS but subsequently exhibit various branching and pathfinding defects [12]; (Figure 3e). Transplant experiments show that sty is required both in the motor neurons and in the surrounding cells, suggesting that Stumpy might be a homophilic cell-surface protein [46**].

Visualizing synaptogenesis

Two recent studies have analyzed synaptogenesis in vivo. Jontes et al. [47] used confocal time-lapse microscopy to visualize encounters between Mauthner axons and their motor neuron targets. They showed that the presynaptic axon does not always play an active role; instead, synapses sometimes form after dendritic filopodia from the postsynaptic motor neuron reach out to contact a passing Mauthner axon.

Ono et al. [48*] have looked at motility mutants from a class that completely lacks muscular contraction [9]; their results suggest that these mutants have defects in muscle function or neuromuscular transmission. Muscles in sofa potato (sop) lack functional nicotinic acetylcholine receptors [48*]. The in vivo function of rapsyn, which clusters acetylcholine receptors, has been tested using a transgenic line that expresses a rapsyn–GFP fusion in muscles. In sop mutants, rapsyn–GFP forms clusters but does not localize to synapses, suggesting that acetylcholine receptors themselves are required to localize rapsyn to the synapse [48*]. Such in vivo imaging will be useful indeed for further studies of synaptogenesis.

Conclusions

The zebrafish retinotectal and primary motor axon projections have revealed much about the tissue interactions and cellular behavior that underlie their axon pathfinding. Studies of retinotectal and motor synaptogenesis have just
The molecular mechanisms underlying zebrafish pathfinding and synaptogenesis are starting to be understood, especially as the mutants are cloned. Vigorous efforts to clone the remaining pathfinding mutants are underway in several laboratories, and will be helped greatly by the sequence of the zebrafish genome, which is scheduled to be fully completed by late 2003.

We predict that nearly all the remaining pathfinding mutants will be cloned within the next two years. As mutant cloning becomes less of a hurdle, zebrafish researchers can concentrate on analyzing existing mutants and screening for new ones. The genetic, molecular and imaging tools available in zebrafish will allow us to discover how our favorite genes really work in vivo, and how they contribute to the wiring of the vertebral nervous system.

Update

The data demonstrating that ast is required for both preventing and correcting small-scale pathfinding errors by retinal axons throughout the ventral forebrain are now in press [51**]. Also described in this paper are the forebrain expression patterns of the putative Robo2 ligands, slit2 and slit3. Both slits are expressed on one or both sides of the retinotectal pathway, consistent with a role in shaping the pathway through the forebrain, presumably by signaling through ast.

Acknowledgements

Thanks to our zebrafish colleagues for illuminating discussions and for sharing unpublished data; we apologize to those whose work has been omitted owing to space limitations. The authors are supported by the National Institutes of Health (grant F32-EY07017 to LD Hutson, and R01-EY12873 to C-B Chien).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


38. Gruenge L, Schmid S, Neuhaus SC: Analysis of the activity-deprived zebrafish mutant macho reveals an essential requirement of neuronal activity for the development of a fine-grained visuotopic map. J Neurosci 2001, 21:3542-3548. The authors show that enlarged retinotectal arbor in ma mutants are due to an activity-dependent defect. Perforated-patch recording shows that RGCs lack almost all sodium current, and tetrodotoxin injections can pharmacop the enlarged arbor phenotype.


40. Zhang J, Malayaman S, Davis C, Granato M: A dual role for the zebrafish unplugged gene in motor axon pathfinding and pharyngeal development. Dev Biol 2001, 240:560-573. A further analysis of unp (see [42]) shows that there is also a secondary motor phenotype that is quite similar to the CaP/Rob phenotype. Intriguingly, there is also a subtle jaw phenotype, suggesting that unp may act in cell migration as well as axon guidance.


45. Zhang J, Granato M: The zebrafish unplugged gene controls motor axon pathway selection. Development 2000, 127:2099-2111. A detailed analysis of unplugged shows that it is specifically required for CaP and Rob axon pathfinding along their cell-type-specific pathways. As for diw (see [7]), transplant experiments show that unp function is required in the adaxial cells, emphasizing their importance for motor axon guidance.

46. Beattie CE, Melanson E, Eisen JS: Mutations in the stumpen gene reveal intermediate targets for zebrafish motor axons. Development 2000, 127:2653-2662. Here, the sty mutant is analyzed in detail. It shows specific defects in primary and secondary motor axons, especially CaP axons. These axons stall at two specific points, which seem to be intermediate targets where the axons need sty function to grow further. Transplants show that sty is required both in axons and in neighboring cells.


48. Ono F, Higashijima S, Shcherbatko A, Fetcho JR, Brehm P: Paralytic zebrafish lacking acetylcholine receptors fail to localize rapsyn clusters to the synapse. J Neurosci 2001, 21:5439-5448. The authors start to analyze the paralyzed mutants, a class that has previously been ignored. They find that relaxed fiber is defective in excitation-contraction coupling, whereas som lacks nicotinic acetylcholine receptors on muscles.


Note added in proof

The work referred to in the text as (LD Hutson, C-B Chien, unpublished data) is now in press:

51. Hutson LD, Chien C-B: Pathfinding and error correction by retinal axons: the role of astray/robo2. Neuron 2002, in press. Timelapse imaging and fixed tissue analysis of retinal growth cones in both wild-type and astray mutants reveals that Astray/Robo2, rather than regulating midline crossing as in Drosophila, shapes the retinotectal pathway through the ventral diencephalon. It does so by both preventing and correcting errors that arise at a low rate during normal embryonic development.