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Zebrafish mutations affecting retinotectal axon pathfinding

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SUMMARY

We have isolated mutants in the zebrafish *Danio rerio* that have defects in axonal connectivity between the retina and tectum. 5-day-old fish larvae were screened by labeling retinal ganglion cells with DiI and DiO and observing their axonal projections to and on the tectum.

82 mutations, representing 13 complementation groups and 6 single allele loci, were found that have defects in retinal ganglion cell axon pathfinding to the tectum. These pathfinding genes fall into five classes, based on the location of pathfinding errors between eye and tectum. In Class I mutant larvae (*belladonna, detour, you-too, iguana, umleitung, blowout*) axons grow directly to the ipsilateral tectal lobe after leaving the eye. Class II mutant larvae (*chameleon, bashful*) have ipsilaterally projecting axons and, in addition, pathfinding mistakes are seen within the eye. In Class III mutant larvae (*esrom, tilsit, tofu*) fewer axons than normal cross the midline, but some axons do reach the contralateral tectal lobe. Class IV mutant larvae (*boxer, dackel, pinscher*) have defects in axon sorting after the midline and retinal axons occasionally make further pathfinding errors upon reaching the contralateral tectal lobe. Finally, Class V mutant larvae (*bashful, grumpy, sleepy, cyclops, astray*) have anterior-posterior axon trajectory defects at or after the midline.

The analysis of these mutants supports several conclusions about the mechanisms of retinal axon pathfinding from eye to tectum. A series of sequential cues seems to guide retinal axons to the contralateral tectal lobe. Pre-existing axon tracts seem not to be necessary to guide axons across the midline. The midline itself seems to play a central role in guiding retinal axons. Axons in nearby regions of the brain seem to use different cues to cross the ventral midline. Mutant effects are not all-or-none, as misrouted axons may reach their target, and if they do, they project normally on the tectum. The retinotectal pathfinding mutants reveal important choice points encountered by neuronal growth cones as they navigate between eye and tectum.

Key words: retinal ganglion cell, axon guidance, tectum, *Danio rerio*

INTRODUCTION

How proper neuronal connectivity is established during development is a fundamental issue in neurobiology. During embryogenesis, neuronal growth cones extend along distinct and stereotypical pathways to reach their targets. Guidance molecules along the pathway are thought to provide the information necessary for a growth cone to navigate to its target (reviewed in Goodman and Shatz, 1993; Holt and Harris, 1993). While genetics has been successfully employed to study invertebrate axon guidance (reviewed in Goodman and Shatz, 1993; Holt and Harris, 1993), including in the visual system (Martin et al., 1995), the identification of molecules that function in vertebrate axon guidance has been achieved mostly using in vitro assays. While several candidate molecules have been identified (for example, see Reichardt and Tomaselli, 1991; Kennedy et al., 1994), the in vivo function of these molecules has been difficult to demonstrate.

We have used the zebrafish, *Danio rerio*, to dissect axonal pathfinding genetically in a vertebrate. We have focused on the formation of one axonal pathway, the axon projections between retinal ganglion cells (RGCs) in the eye and their target in the brain, the optic tectum. The retinotectal system has been well characterized in lower vertebrates, and provides a good system for studying both the process of axon pathfinding to a target and the process of topographic connectivity within the target (reviewed in Holt and Harris, 1993; Chien and Harris, 1994).

In forming the optic nerve and tract, RGC axons follow a distinct pathway along the ventral and lateral surface of the diencephalon (Stuermer, 1988; Burrill and Easter, 1995). Axons from the two eyes cross each other at the ventral midline

of the diencephalon to form the chiasm. Retinal axons grow near, but not on, pre-existing axons of the tract of the post-optic commissure and the postoptic commissure as they grow toward and cross the ventral midline (Burrill and Easter, 1995; Fig. 9A). Upon reaching the contralateral tectal lobe, retinal axons project topographically to form an extremely accurate map of the visual world within the brain (Kaethner and Stuermer, 1992).

In order to discover genes that affect retinotectal axon connectivity, mutant zebrafish families were screened by labeling retinal ganglion cells of fixed 5-day-old larvae with DiI and DiO and observing their axonal projections to and on the tectum (Baier et al., 1996). 114 mutants were identified with defects in either the axon trajectory between eye and tectum (pathfinding mutants), or with altered projection patterns on the tectum (topographic mutants). This paper describes the pathfinding mutants, while the topographic mutants are described in an accompanying paper (Trowe et al., 1996).

We have divided the pathfinding mutants into five classes, based on the position of RGC axon pathfinding errors. The characterization of the pathfinding errors made in these mutants indicates important choice points encountered by a retinal ganglion cell growth cone as it navigates from eye to tectum. Further analysis of the mutants and the eventual cloning of the mutant genes promises to uncover molecular mechanisms responsible for high fidelity axonal pathfinding in the vertebrate brain.

MATERIALS AND METHODS

Zebrafish were reared and crossed as described elsewhere (Haffter et al., 1996a). Embryos were kept at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). Those embryos that were to be used for antibody labeling were grown in the presence of 0.2 mM PTU (1-phenyl-2-thiourea) to prevent pigment biosynthesis (Westerfield, 1989).

For dye injections, 5-day-old larvae were fixed overnight in 4% paraformaldehyde at room temperature, then mounted in 1.2% low-melting-temperature agarose made in phosphate-buffered saline (PBS) diluted 1:3 in water (1/3×PBS). Retinal ganglion cells were labeled with DiI and DiO, as described in an accompanying paper (Baier et al., 1996). DAPI (5 μg/ml in 1/3×PBS) was poured on the plates after dye injections to allow the visualization of the tectal neuropil. DAPI labels the DNA of the cell-body-rich region surrounding the tectal neuropil, thus outlining the neuropil with blue fluorescence. Photoconversion of the DiI label was done by incubating the labeled fish in 0.5 mg/ml DAB in ⅓× PBS for 15 minutes, mounting under a coverslip, and exposing to fluorescent light on a compound microscope using a 20× objective. The embryos were dehydrated through an ethanol series, rinsed in acetone and put into a 50:50 mixture of acetone/araldite to evaporate overnight. The fish were then mounted in 100% araldite and sectioned.

Two antibodies were used to characterize embryonic axon projections. Antibodies generated against acetylated tubulin (anti-AT) label most axons in the developing embryo (Chitnis and Kuwada, 1990; Wilson et al., 1990), while the ZN-5 monoclonal antibody (Trevarrow et al., 1990) labels a subset of neurons and axons that includes the retinal ganglion cells and their axons (Westerfield, 1989). ZN-5 appears to recognize an immunoglobulin superfamily surface molecule called neurolin, the fish homolog of DM-GRASP/BEN/SC-1 (Kanki et al., 1994; Laessing et al., 1994).

For immunohistochemistry, embryos were fixed for 1 hour in 4% paraformaldehyde, rinsed and incubated in blocking solution for 1 hour (PBS + 0.5% Triton X-100 + 0.2% BSA + 5% normal goat serum). The primary antibody [mouse anti-acetylated tubulin (Sigma), 1:1000, or mouse ZN-5, 1:1000] was added and embryos were incubated overnight at 4°C. After washing in PBS + 0.5% Triton X-100, embryos were again incubated in blocking solution, then a goat anti-mouse peroxidase-conjugated secondary antibody was added, followed by incubation overnight at 4°C. After extensive washing, embryos were incubated in 0.5 mg/ml DAB and then reacted in 0.003% H_2O_2. Embryos were cleared in 70% glycerol overnight, mounted under coverslips and viewed using differential interference contrast optics.

RESULTS

In wild-type 5-day-old fish larvae, retinal ganglion cell (RGC) axons have grown to the contralateral tectal lobe (Fig. 1A-C). Within the eye, axons originating from RGC somata grow toward the center of the eye and exit at the ventral fissure. After exiting the eye, RGC axons run along the base of the diencephalon and cross the midline, then turn dorsally and slightly posteriorly to the contralateral tectal lobe (Fig. 1A-C). No axons project to the ipsilateral tectal lobe at this age (Stuermer, 1988; Burrill and Easter, 1994). Axons are ordered within the optic nerve and tract according to the position of their cell bodies in the retina (Fig. 1C; Stuermer, 1988). After passing the midline, axon bundles from dorsal and ventral areas of the retina separate into dorsal and ventral brachia and take these different branches of the optic tract to the contralateral tectal lobe. Ventral RGC axons form the dorsal brachium (Fig. 1C, arrowhead) and grow to the dorsal region of the contralateral tectal lobe (medial in these dorsal views), while dorsal RGC axons form the ventral brachium (Fig. 1C, arrow) and grow to the ventral region of the contralateral tectal lobe (lateral). On the tectum, axons project topographically to form a reversed map of the retinal image of the visual world. Nasal/dorsal axons project to the posterior/ventral tectum (Fig. 1A-C, green axons), while temporal/ventral axons project to the anterior/dorsal tectum (Fig. 1A-C, red axons).

82 mutations were found that affect pathfinding of RGCs to the tectum. Complementation testing revealed that these mutations define 19 genes with 1 to 15 alleles per gene (Table 1). The phenotypes of the mutants with defects in pathfinding can be divided into classes based on the position at which RGC axons first deviate from their normal pathway. In Class I mutants, RGC axons grow to the ipsilateral tectal lobe after leaving the eye. This class contains mutations in several genes and includes *belladonna, blowout, you-too, detour, iguana* and *umleitung* (representative examples shown in Fig. 1). Mutants of Class II (*chameleon* and *bashful*) also have ipsilateral projections, but in addition some RGC axons are misrouted within the eye (Fig. 5). In Class III mutants (*esrom, tilsit* and *tofu*), axons have varying defects in their ability to grow to the midline, but some axons do cross the midline and eventually connect to the contralateral tectal lobe (Fig. 6B). Class IV mutants (*boxer, dackel* and *pinscher*) have defects in the ordering of dorsal and ventral RGC axons in the optic tract (Fig. 6C) and some axons fail to enter the contralateral tectal lobe (Fig. 6D). Finally, Class V mutants (*bashful, grumpy, sleepy, cyclops* and *astray*) have anterior-posterior trajectory defects at or after the midline (Fig. 7).

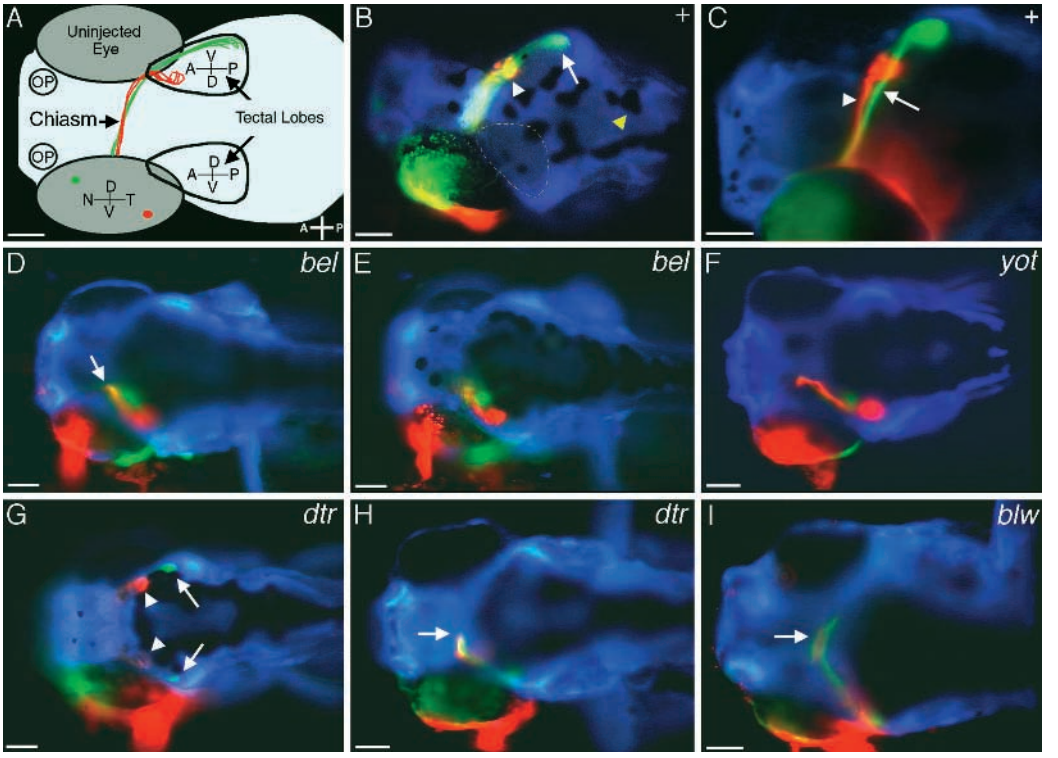

Fig. 1. Retinotectal projections in the wild type and in mutants with ipsilateral retinotectal projections. Dorsal view, anterior to the left. Fish were stained with DAPI (blue fluorescence) to outline the tectal neuropil, which is surrounded by DNA-containing cell bodies (see methods). Neuropilar regions are often difficult to see in these photographs and one neuropil is outlined in B. Black spots are melanophores in the skin (yellow arrowhead in B) (A) Diagram of an injected 5-day-old zebrafish. The left eye of each fish was injected with DiI (red) in the temporal/ventral quadrant and with DiO (green) in the nasal/dorsal quadrant except in D, E and F, where the dyes were reversed. Labeled retinal ganglion cell axons cross the brain and grow to the contralateral tectal lobe where they project topographically. (B) Wild-type projections, dorsal focal plane. On the contralateral tectal lobe, retinal ganglion cell axons project topographically with temporal/ventral RGC neurons sending axons to the anterior/dorsal tectum (arrowhead) and nasal/dorsal RGC neurons sending axons to the posterior/ventral tectum (arrow). (C) Wild-type projections, ventral focal plane; termination zones are now out of focus. Retinal ganglion cell axons leave the eye at the papilla and grow along the base of the diencephalon to the midline, where they cross completely at the optic chiasm. After the chiasm, axons sort into a dorsal (arrowhead) and a ventral (arrow) brachium and grow dorsoposteriorly to the contralateral tectal lobe. (D,E) Retinal ganglion cells project to the ipsilateral tectal lobe in *belladonna* mutants. Reverse dye injections. (D)Ventral focal plane, showing that the axons turn dorsal immediately after leaving the eye (arrow). (E) Dorsal focal plane showing normal topographic projections on the ipsilateral tectal lobe. (F) RGC axons also project to the ipsilateral tectal lobe in *you-too* mutants. (G,H) In *detour* mutants, RGC axons either project to the ipsilateral and contralateral tectal lobes in the same fish (G), or to the ipsilateral tectal lobe only (H). (G) Dorsal focal plane showing normal mapping of nasodorsal RGC axons (arrows) and ventral/temporal axons (arrowheads) from one eye on both tectal lobes. (H) In some *dtr* mutant fish, RGC axons grow to the midline (arrow) before turning back to the ipsilateral tectal lobe. (I) In *blw* mutants, RGC axons grow across the midline (arrow), then return to the ipsilateral tectal lobe. OP, olfactory placode; N, nasal; T, temporal. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 100 μm.

Class I

Ipsilateral retinotectal projections: *belladonna (bel), blowout (blw), detour (dtr), you-too (yot), iguana (igu), umleitung (uml)*

In the mutants that have ipsilateral projections, the phenotypes are variable. RGC axons from one eye either project to the ipsilateral tectal lobe only (Fig. 1D-F), or they project bilaterally (Fig. 1G). Within one fish, one tectal lobe may be innervated by both eyes while the other tectal lobe receives no retinal input (Fig. 4F). In all cases, axons that reach the ipsilateral tectal lobe are able to find their correct retinotopic position (Fig. 1D-G), indicating that mapping on the tectum is independent of pathfinding to the tectum. The ipsilateral mutants are of different types, with one group of mutants (*dtr*, *yot*, *igu* and *uml*) having general midline defects, one mutant being more specific for RGC axonal defects (*bel*), and one mutant having major defects in eye morphology (*blw*). Two additional mutants (*con* and *bal*) have axon pathfinding errors within the eye as well as ipsilateral projections and are discussed below as Class II. Mutations in four genes (*bal*, *sly*, *gup* and *cyc*) result in ipsilateral projections as well as anterior projections and these are described in Class IV.

Ipsilateral projections accompanied by midline defects: *detour (dtr), you-too (yot), iguana (igu), umleitung (uml)*

The first group of mutants with ipsilateral RGC projections also have general midline defects that are described elsewhere in this issue (Brand et al., 1996; van Eeden et al., 1996a). Briefly, midline structures such as the floorplate of the spinal cord and the notochord show different degrees of malformation. The eyes in these mutants are turned in ventrally to various degrees, consistent with a loss of midline structures in the brain.

We examined some of these mutants in more detail by photoconverting the DiI label into a visible reaction product and making transverse sections at the level of the optic nerve and chiasm (Fig. 2). *dtr* and *yot* mutants have defects in the cartilage cells at the base of the brain. In both *dtr* and *yot* these cells, which are part of the trabeculae, have not fused to form the ethnoid plate, as they have in the wild type and in *bel* mutants (Brand et al., 1996). The sections show that the RGC

Table 1. Genes involved in retinotectal pathfinding

Gene	Number of alleles	Alleles	Other phenotypes	Other references
Class I: Ipsilateral retinotectal projections				
belladonna (bel)	1	*tv42z*	Eye	–
blowout (blw)	1	*tc294z*	Eye	–
chameleon (con)	See below			
detour (dtr)	3	*te370, tm276, ts269*	Spinal cord, curly tail	a
iguana (igu)	2	*tm79, ts294e*	Spinal cord, somites	a
umleitung (uml)	1	*ty54z*	Curly tail	–
you-too (yot)	2	*ty17, ty199*	Spinal cord, somites	a, b
Class II: Pathfinding errors within the eye				
bashful (bal)	See below			
chameleon (con)	5	*tf18b, th6d, tm15, tu214, ty60*	Ipsilateral, curly tail, neural tube	a
Class III: Reduced midline crossing				
esrom (esr)	14	*tb241, te250e, te275z, te279b, te376b, tf4z, tg5f, tg265, th36b, th222, tj236b, tn207b, tp203, ts208*	Xanthophores	c, d
tilsit (til)	1	*ty130b*	Xanthophores	d
tofu (tof)	1	*tq213c*	Xanthophores	d
Class IV: Missorting of axons in the optic tract				
boxer (box)	8	*tm4, tm70g, tg308c, te242d, tm317c, tw24z, tp67z, to232z*	Jaw, fins	c, e, f
dackel (dak)	3	*tf205z, to273b, tw25e*	Jaw, fins	c, e, f
pinscher (pic)	1	*to216*	Jaw	c
Class V: Anterior-posterior pathfinding errors				
bashful (bal)	15	*tp82, tp86, tm220, tr259b, tf235b, tv36, tt206, tm267, tf209b, to265, tc245, tq210c, tb244f, tc248f, tr203b*	Ipsilateral errors in eye, notochord, hindbrain	g
grumpy (gup)	7	*tg210, ti228b, tj229, tl17b, tm61, tp42, tx221*	Ipsilateral, notochord, hindbrain	g
sleepy (sly)	9	*te223, te333b, tf215b, ti263, ti272, to216, tm89, tp16c, ts33*	Ipsilateral, notochord, hindbrain	g
cyclops (cyc)	2	*te262c, tf219*	Ipsilateral, ventral midline	h
astray (ast)	4	*te284z, te378, ti272z, tl231*	None	–

References: a, Brand et al. (1996); b, van Eeden et al. (1996); c, Trowe et al. (1996); d, Odenthal et al. (1996b); e, Schilling et al. (1996); f, Granato et al. (1996); g, Odenthal et al. (1996a); h, Heisenberg et al. (1996).

axons turn dorsally on the ipsilateral wall of the diencephalon after leaving the eye (arrows). This is a substrate for RGC axons, normally taken after axons cross the midline. Axon growth up the ipsilateral diencephalon indicates that dorsal and posterior cues can function independently of cues guiding axons to and across the midline. Brain defects are most extreme in *yot*, which has an enlarged third ventricle and reduced neuropil in the diencephalon (Fig. 2D). *igu* and *uml* mutants have similar retinotectal and midline phenotypes. All of these fish are easily identified because they remain tightly curled after hatching. They also fail to develop swim bladders and die within the first two weeks.

Ipsilateral projections without major midline defects: *belladonna (bel)*

In *bel* mutants, RGC axons grow dorsally on the ipsilateral diencephalon immediately after leaving the eye (Figs 1D,E, 2B, 4D), as in midline-defective mutants. Midline structures in the trunk such as floorplate and notochord appear normal (not shown). The *bel* mutation has fewer developmental defects than the other ipsilateral mutants, and some *bel* homozygous fish (10-40%) are viable. *bel* homozygous adults have defects in establishing buoyant equilibrium and are often darkly pigmented; some appear blind. A few individuals showed erratic swimming behavior.

The *bel* mutant eye has a visible phenotype starting at day 3, for which the mutant was named. An abnormal gap is seen between the lens and the pigmented epithelium and iridophores, causing the eye to appear as if it had a dilated pupil (Fig. 3D). Adult fish have similar defects in the arrangement of pigmented epithelium around the lens and, in some cases, tumor-like growths protrude out of the eye (not shown). The lens appears normal, however.

Because of this eye defect, we examined the differentiation of the retinal ganglion cells in *bel* using the ZN-5 antibody (Trevarrow et al., 1990), which labels RGCs and their axons. RGCs begin to differentiate at approximately 30 hours of development, and they quickly form a ring around the lens in the wild type (Burrill and Easter, 1995; Laessing and Stuermer, 1995). ZN-5 labeling of the RGCs in *bel* homozygotes at 48 hours of development shows a normal pattern of cells around the lens (Fig. 3). Thus the 'large pupil' defect of *bel* appears

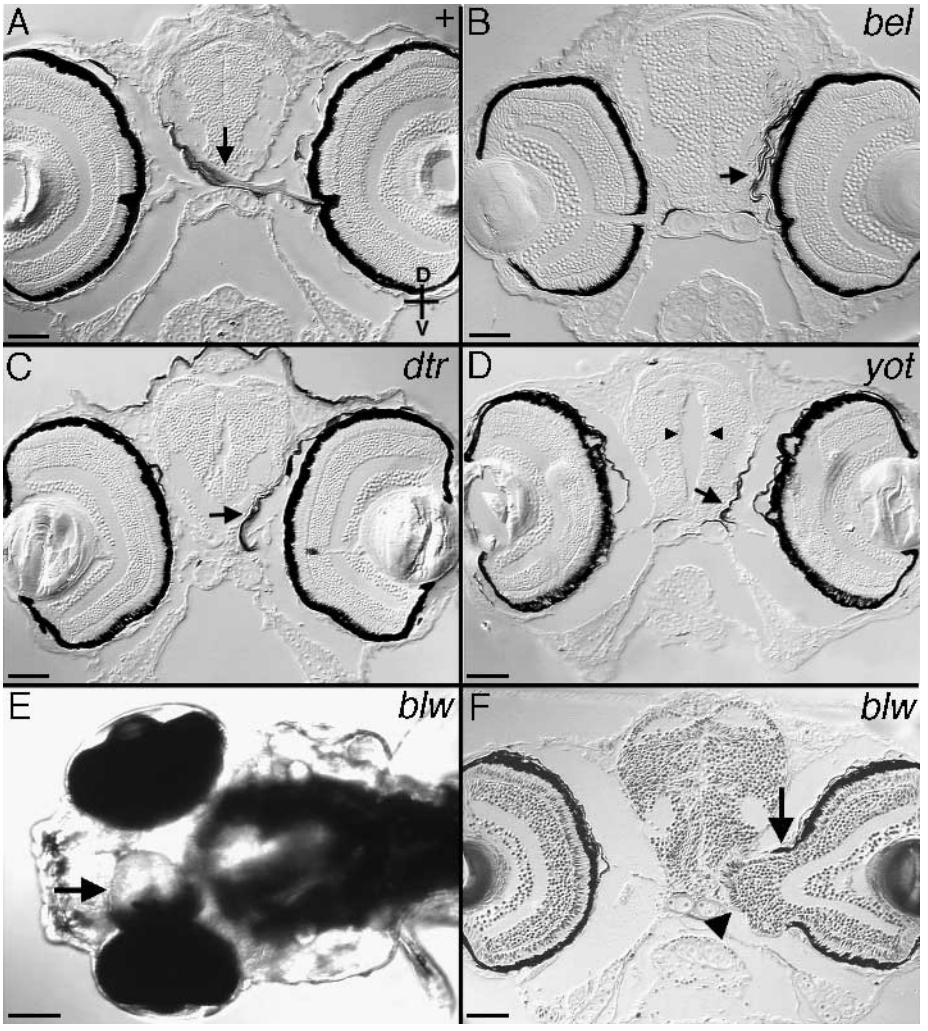

Fig. 2. Sections showing the optic nerve and chiasm of wild type and mutants with ipsilateral retinotectal projections. All panels show cross sections through the eyes, optic nerve and optic chiasm of 5-day-old fish; dorsal is up. DiI-labeled temporal/ventral retinal ganglion cells and their axons are visualized after photoconversion of the fluorescence into a brown reaction product. (A) RGC axons in the wild type grow across the midline (arrow) then turn dorsally on the diencephalon toward the contralateral tectal lobe. (B) In *belladonna* mutants, axons project dorsally along the ipsilateral diencephalon wall (arrow). (C) In *detour* mutants, axons also grow along the ipsilateral diencephalon (arrow) to reach the ipsilateral tectal lobe. (D) In *you-too* mutants, the diencephalic ventricle is enlarged (arrowheads). RGC axons again reach the ipsilateral tectal lobe via the ipsilateral diencephalon wall (arrow). (E) One (or both, not shown) eye in *blowout* mutants extends into the diencephalon (arrow). (F) A section through a *blw* mutant embryo showing that the abnormal eye structure contains the normal eye layers, including the pigment epithelium (arrow) and the photoreceptor layer (arrowhead). D, dorsal; V, ventral. Scale bars, 50 μm (A-D, F), 100 μm (E).

not to result from the failure of RGCs to differentiate around the lens.

Ipsilateral projections accompanied by eye defects: *blowout (blw)*

blw mutant embryos also have ipsilaterally projecting RGC axons. Unlike the mutants described above, axons in *blw* embryos were seen to project well beyond the midline before turning back to the ipsilateral side (Fig. 1I). 5-day-old *blw* mutant larvae have gross defects in eye morphology, with extra eye tissue extending from the center of the eye medially into the brain (Fig. 2E,F). This extra tissue looks like an evagination or 'blowout' of the eye and contains pigment epithelium and photoreceptor layers (Fig. 2F). The eye phenotype can be in one or both eyes. RGC axons seem to grow along the abnormal eye structure for a short distance before they turn back to the ipsilateral tectal lobe. In individuals with only one abnormal eye, RGC axons from the abnormal eye project to the contralateral tectal lobe, as in the wild type.

Earlier phenotypes

To better understand the defects associated with ipsilateral projections, the axon scaffold was examined at earlier stages of development (Fig. 4). Anti-acetylated tubulin (anti-AT) labeling of *bel, uml* (Fig. 4) and *dtr* (not shown) mutant embryos at 36 hours after fertilization shows that commissural axons of the tract of the post-optic commissure fail to cross the midline, while other commissural axons in the forebrain are less disturbed. The anterior commissure appears normal or is somewhat reduced, and the dorsally located posterior commissure appears normal (not shown). Other axon pathways that have been examined in *bel, uml* and *dtr* appear normal (not shown). The effects of these mutations therefore seem to be restricted to the region in which retinal axons and post-optic commissure axons cross the ventral diencephalon.

There is variation in the axonal phenotype in *bel* and *uml* mutants. In some *bel* fish, RGC axons approach the midline but do not cross it, while axons in the tract of the post-optic commissure do not appear to grow toward the midline (not shown). In other fish, RGC axons stop soon after leaving the eye, while tracts of the post-optic commissure axons spread toward the midline (Fig. 4C). In *uml* the post-optic commissure fails to form, while RGC axons show various degrees of growth toward the midline (Fig. 4E), and even cross the midline occasionally, as would be expected from the 5-day-old phenotypes.

Labeling of 48-hour *bel* embryos with ZN-5 shows the RGC axons projecting dorsally immediately after leaving the eye (Fig. 4D). This is the most frequent phenotype for all ipsi-

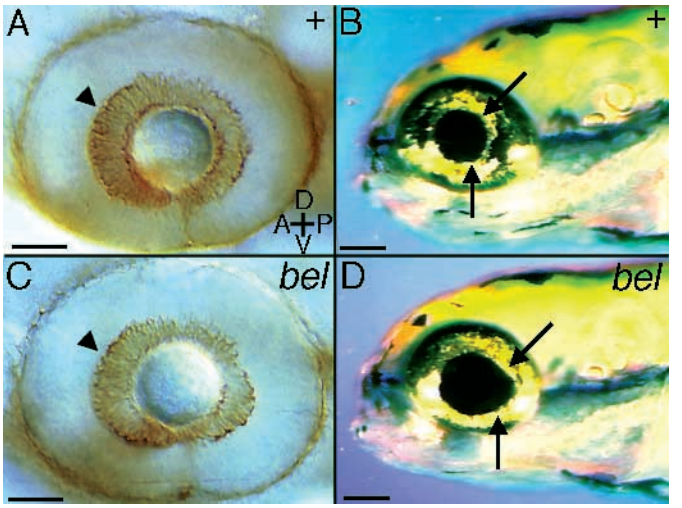

Fig. 3. *belladonna* mutant eye phenotype. Wild-type (A) and *belladonna* (C) eyes at 48 hours labeled with the ZN-5 antibody to visualize retinal ganglion cell bodies. The retinal ganglion cells (arrowheads) and the entire eye appear normal at this age, thus the mutation does not appear to affect RGC differentiation. At 5 days iridophores and the pigmented epithelium are adjacent to the lens in the wild type (B). In *belladonna* mutants (D), gaps appear between the lens and some parts of the pigmented epithelium (arrow). The lens appears normal however. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 50 μm (A,C), 100 μm (B,D).

lateral mutants. In a few individuals homozygous for *dtr*, *uml* and *yot*, axons from both eyes project to one tectal lobe (Fig. 4F). The retinal ganglion cells in all of these mutants appear normal and are positioned normally within the eye (Figs 3C, 4D,F).

Class II

Pathfinding errors within the eye: *chameleon (con), bashful (bal)*

Two of the mutants with ipsilateral projections and midline defects (*con*, *bal*) also have RGC pathfinding errors within the eye (Fig. 5). No such errors were seen in the other mutations examined to date (*dtr*, *yot*, *uml*, *bel*, *esr* and *ast*). In both *con* and *bal* mutants, RGC axons sometimes fail to converge in the center of the eye to form the 'spoked' pattern (Easter et al., 1984) seen in the wild type (Fig. 5A). In *con* mutants, axons often fail to leave the eye, and instead tend to grow along the equator of the eye in either the anterior or posterior direction (Fig. 5B). When axons do leave the eye in *con* homozygotes, they project either to the ipsilateral or the contralateral tectal lobe. *con* has extreme midline defects that are described elsewhere in this issue (Brand et al., 1996).

Homozygous *bal* larvae also occasionally have defects in axonal pathfinding within the eye. This eye phenotype is variable, and is more common in the strongest alleles. Instead of growing radially inward, RGC axons grow in a disorganized way within the eye (Fig. 5D). Misrouted RGC axons eventually leave the eye in these *bal* fish and project to either the ipsilateral or contralateral tectal lobe, or they grow anteriorly into the telencephalon (see Class V). RGC differentiation seems to be disrupted in *bal*, as RGC somata do not form normally in some quadrants of the eye (Fig. 5C).

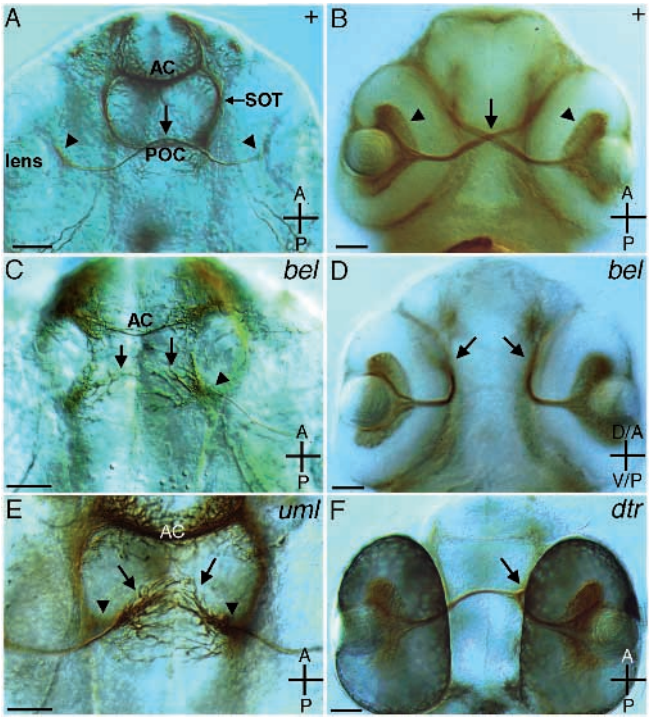

Fig. 4. ZN-5 and anti-AT antibody labeling of wild type and mutants with ipsilateral retinotectal projections. (A, C and E) 36-hour embryos labeled with the anti-acetylated tubulin antibody that recognizes most axons. (B, D and F) 48-hour embryos labeled with the ZN-5 antibody that recognizes RGCs and their axons. (A) At 36 hours in the wild type, RGC axons have crossed the midline and grow along their contralateral counterparts (arrow). Arrowheads show retinal ganglion cell bodies. (B) At 48 hours in the wild type, the optic chiasm is well formed (arrow) and RGC axons extend to the contralateral tectal lobes. RGCs are positioned near the lens (arrowheads). (C) In *belladonna* mutants at 36 hours, the POC does not form (arrows), while the AC forms relatively normally. RGC axons remain near the ipsilateral eye (arrowhead). (D) In *belladonna* mutants at 48 hours, RGC axons project dorsally immediately after leaving the eye (arrows). (E) In some 36-hour *umleitung* mutant embryos, RGC axons grow toward the midline (arrows). The axons of the tract of the post-optic commissure remain lateral (arrowheads) and no POC forms. In other fish, RGC axons seem to turn dorsally immediately after leaving the eye (not shown). (F) In some *detour* mutant embryos, RGC axons from one eye project ipsilaterally (arrow), while those from the other eye project contralaterally. Axons can be followed to the tectal lobe on the right in this figure, while no axons project to the other tectal lobe. The result is that both eyes project to the same tectal lobe. AC, anterior commissure; POC, post-optic commissure; SOT, supraoptic tract. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 50 μm (A,B,D,F), 25 μm (C,E).

Class III

Reduced midline crossing: *esrom (esr), tilsit (til), tofu (tof)*

Three genes, *esrom*, *tilsit* and *tofu*, were found that affect the ability of RGC axons to grow to and beyond the midline. In these mutants, many RGC axons fail to cross the midline and end up in an aggregate of axons near their point of exit from the ipsilateral eye (Fig. 6B). Those axons that do cross the midline and reach the contralateral tectal lobe frequently stop prematurely on

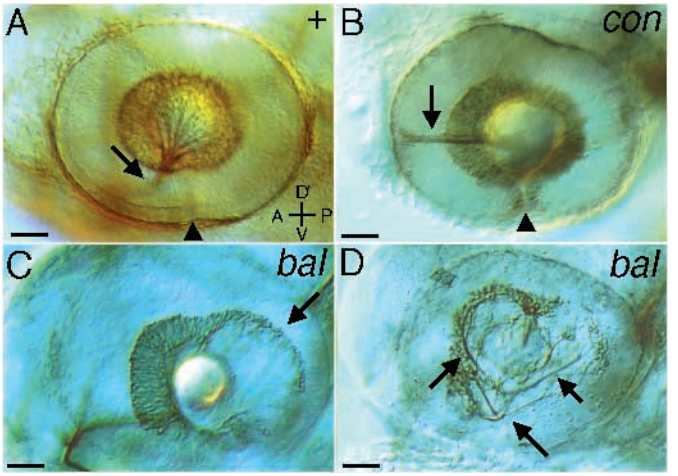

Fig. 5. Pathfinding errors within the eye. Lateral views of eyes labeled with ZN-5 at 48 hours of development. Arrowheads mark the ventral fissure. (A) In the wild type, RGC axons aggregate in the ventral medial portion of the eye and exit at the optic nerve head (arrow). (B) In *con* mutants, RGC axons often do not exit the eye and instead grow along the equator of the eye in both anterior (arrow) and posterior (out of focus) directions. (C) RGC cell bodies do not surround the lens normally in *bal* mutants. In these fish temporal RGCs are not normally formed (arrow). Lateral focal plane. (D) Some *bal* mutants also have misrouted axons within the eye (arrows). These axons are disorganized within the eye, but do exit the eye and eventually project into the brain. Medial focal plane. A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars, 50 μm.

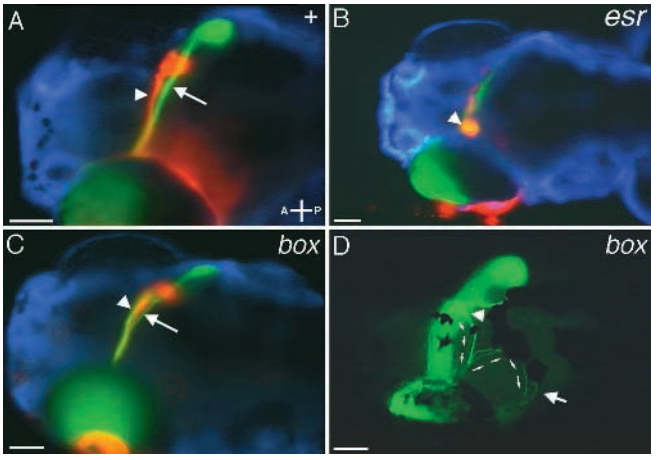

Fig. 6. Retinotectal projections in mutants with reduced midline crossing and midline sorting errors. Ventral focal planes. (A) Wild-type projections showing ventral (arrow) and dorsal (arrowhead) brachia of the optic tract. (B) In *esrom* (and *tilsit* and *tofu*, not shown) mutants a large percentage of retinal ganglion cell axons fail to cross the midline. Instead they form an aggregate of fibers (arrowhead) just lateral to the papilla. (C) In *boxer* mutants, axons from dorsally located retinal ganglion cells do not sort correctly at the optic chiasm. Normally, all axons from dorsal RGCs follow the ventral brachium to the ventral side of the tectum. In *boxer* (and *dackel*, not shown), these axons split and follow both the ventral (arrow) brachium and the dorsal (arrowhead) brachium. Axons arriving at the wrong (dorsal) side of the tectum nonetheless find their appropriate ventral target region (see Trowe et al., 1996). (D) In some cases nasal/dorsal RGC axons that grow in the inappropriate (dorsal) brachium fail to enter the contralateral tectal lobe in *boxer* mutants (arrowhead). These axons (small arrows) project to the ipsilateral tectal lobe by crossing the dorsal midline and terminate in their appropriate retinotopic position (arrow). A, anterior; P, posterior. Scale bars, 100 μm.

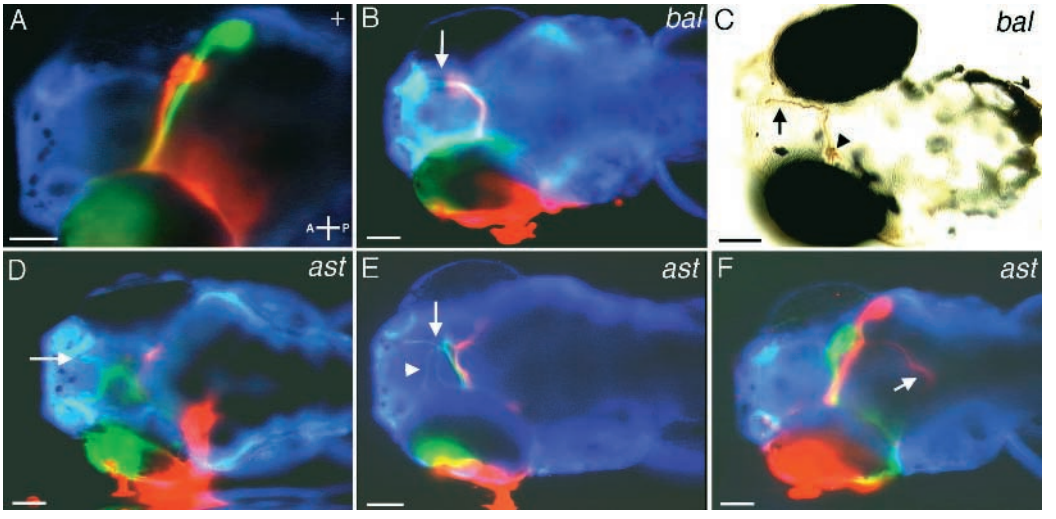

Fig. 7. Retinotectal projections in mutants with anterior/posterior pathfinding errors. Ventral focal plane. (A) Wild-type projections. (B) In *bashful* mutants, axons project anteriorly into the forebrain, where they grow along the edge of the telencephalon (arrow) and occasionally make a complete circuit of the forebrain. (C) In some cases axons project to the ipsilateral tectal lobe in *bashful*. In these fish, RGC axons project both anteriorly (arrow) and turn (arrowhead) toward the ipsilateral tectal lobe (not seen in this focal plane). The DiI label was photoconverted to make the labeled RGC axons visible using DIC optics. (D-F) In *astray* mutants, axons grow aberrantly to one of three general destinations. After crossing the midline, axons either grow anteriorly into the anterior telencephalon (D, arrow), fan out toward the contralateral eye (E, arrow) and/or return to the ipsilateral side of the brain (E, arrowhead), or grow ventrally under the tectum to the hindbrain (F, arrow) (reverse dye fill). A majority of axons find their correct target region in the tectum in F. In these cases, axons fail to turn dorsally after crossing the midline and remain at the same dorsal/ventral level as the optic nerve. A, anterior; P, posterior. Scale bars, 100 μm.

the tectum. Mutations in *esr*, *til* and *tof* do not appear to affect the formation of the early axon scaffold (not shown).

5-day-old *esr*, *tof* and *til* mutant larvae have reduced xanthophore pigmentation and appear white rather than yellow under a dissecting microscope (Odenthal et al., 1996b). *esr* and *til* homozygotes do not form swim bladders and the mutations are lethal. Homozygous *tof* fish are viable.

Class IV

Missorting of axons in the optic tract: *boxer (box), dackel (dak), pinscher (pic)*

Mutations in three genes affect the sorting of axons in the optic tract without affecting mapping on the tectum. In *box*, *dak* and *pic* mutants, axons from dorsally located RGCs, which normally all grow in the ventral brachium, diverge after the midline. Some of the axons grow appropriately in the ventral brachium, but some dorsal axons grow inappropriately in the dorsal brachium (Fig. 6C; Trowe et al., 1996). As a result these dorsal axons arrive at an inappropriate dorsal position on the tectum.

Occasionally, retinal axons make further pathfinding errors upon reaching the contralateral tectal lobe in *box*, *dak* and *pic* mutants. In these cases, after reaching the inappropriate (dorsal) region of the contralateral tectal lobe, a few axons from dorsal RGCs grow across the dorsal midline to reach the ipsilateral tectal lobe (Fig. 6D, arrowhead). When these axons enter the ipsilateral tectal lobe they are able to find their appropriate retinotopic target site. These genes also affect jaw and fin development (Schilling et al., 1996; van Eeden et al., 1996b).

Class V

Anterior-posterior pathfinding errors: *bashful (bal), grumpy (gup), sleepy (sly), cyclops (cyc), astray (ast)*

After crossing the midline, wild-type axons turn dorsally and slightly posteriorly to grow along the wall of the diencephalon and eventually reach the contralateral tectal lobe (Fig. 2A, 7A). Five genes, *bashful*, *grumpy*, *sleepy*, *cyclops* and *astray*, affect this dorsal/posterior turn.

bashful (bal), grumpy (gup), sleepy (sly), cyclops (cyc)

In most *bal* mutant embryos, axons grow anteriorly rather than dorsally and posteriorly after they cross to the contralateral side of the diencephalon (Fig. 7B). The anterior path taken by the aberrant axons is just inside the pia of the diencephalon and telencephalon (not shown). *bal* axons fail to turn dorsally and often remain in the same dorsoventral plane as the optic nerve and chiasm.

Some *bal* mutant embryos have ipsilateral retinotectal projections. A sub-population of axons turns at or before the midline and grows dorsally and posteriorly to the ipsilateral tectal lobe where they map correctly. An example of a fish in which axons grew both anteriorly and to the ipsilateral tectal lobe is shown in Fig. 7C. In this fish, some axons turned before the midline and projected ipsilaterally, while another population of axons grew anteriorly to the front of the telencephalon.

The *bal* mutation also affects earlier development. At 24 hours the anterior notochord has not differentiated normally and the hindbrain has a bumpy appearance. Hindbrain axon projections are disorganized (Haffter et al., 1996b). *gup* and *sly* mutants have early midline defects similar to, but more severe than, *bal*. *gup* and *sly* mutants were not found in the original retinotectal screen, probably because most fish die before day 5. Re-examination of weak alleles of *sly* and *gup* mutant embryos that survive to day 5 revealed anterior and ipsilateral RGC projections in a small percentage of injected fish. A mild *cyclops* allele found in the Tübingen screen also has ipsilateral and anterior projections of RGC axons.

astray (ast)

Fish larvae homozygous for the *astray* mutation have anterior-posterior defects in retinotectal pathfinding. RGC axons in *ast* homozygotes grow to a number of different locations in 5-day-old larvae. Three general patterns of axon projections are seen. Axons either project anteriorly into the telencephalon (Fig. 7D), fan out after the midline toward the contralateral eye (Fig. 7E), or follow a ventral/posterior path to the hindbrain (Fig. 7F). In most cases, some RGC axons do project to their normal location on the contralateral tectal lobe. We have seen normal axon projections on all regions of the tectum, indicating that it is not one subset of RGC axons that is consistently lost in *ast*.

While several different axon pathfinding errors are seen in the *ast* mutants, there is nonetheless a limited number of pathways taken by aberrantly projecting RGC axons. These secondary pathways only partially follow preexisting axonal tracts. The anterior projecting axons in Fig. 7D follow the midline for a short distance where no axon pathway exists. They split at the level of the anterior commissure, then grow anteriorly, probably along the tract of the anterior commissure. These axons terminate in the anterior telencephalon in the region of the developing olfactory bulb. Axons in the 'fanning' pattern (Fig. 7E) also wander anteriorly after the midline. They grow in proximity to the tract of the post-optic commissure, but many axons leave the tract and return to the ipsilateral side of the brain.

In *ast* mutants, the first errors made by the RGC axons occur at the midline, as shown by anti-AT labeling of 36-hour embryos (Fig. 8). In wild-type embryos, RGC axons meet at the midline and seem to grow for a short distance along their contralateral counterparts before turning dorsally toward the tectum (Fig. 8A). In *ast* mutants, RGC growth cones grow to the midline apparently normally, but do not fasciculate with or grow along their contralateral counterparts. These growth cones remain on the ventral surface of the brain and do not turn dorsally toward the contralateral tectal lobe. Three examples are shown in Fig. 8. In these examples, growth cones either stop near the midline and branch extensively (Fig. 8B,D) or they project abnormally on the ventral diencephalon (Fig. 8C). Other axon tracts in *ast* embryos, particularly the post-optic commissure and the anterior commissure, form normally. The *ast* defect thus seems to be specific for RGC axons.

No gross morphological defect has yet been found in homozygous *ast* embryos. Homozygous mutants appear to be viable on the basis of two criteria. Firstly, all 5-day-old embryos from heterozygous parents develop swim bladders and begin to feed. Secondly, simple counting of offspring surviving from the mating of two heterozygous parents indicates that more than three quarters of the progeny survive to adulthood. We are presently determining whether homozygous *ast* mutant adults can be identified and whether they are fertile.

DISCUSSION

General findings

The retinotectal pathfinding mutants are shown schematically in Fig. 9 and listed in Table 1. In the mutants of all the genes described, a subset of RGC axons does successfully navigate to one of the tectal lobes. This indicates that some guidance mechanisms still function in the mutants, allowing axons to approach their appropriate target organ. This partial effect on axon pathfinding can be explained in three ways. Firstly, guidance cues seem to be sequentially arranged between eye and tectum. Axons that somehow (perhaps by chance) bypass an early guidance block in a mutant are still able to read subsequent cues and reach the contralateral tectal lobe. One example of this is *con*. In *con* mutant embryos axons often fail to leave the eye. When they do leave the eye, however, these axons are able to recognize guidance cues on the diencephalon and project to either the ipsilateral or contralateral tectal lobe.

A second explanation for the partial effects of the mutations is that combinations of multiple guidance cues may function at any one given pathway choice point. The elimination of one of a combination of cues would result in some, but not all, axons making errors. This may be the case for *box*, *dak* and *pic*. In embryos mutated at these loci some, but not all, dorsal RGC axons grow into the inappropriate dorsal branch of the optic tract. Combinatorial effects have also been shown in vitro. Antibody blocking experiments show that adhesion molecules can work in combination to promote axon outgrowth (Neugebauer et al., 1988).

Finally, these mutations may not be null mutations, so partial effects might be due to the presence of partially functional protein products that facilitate pathfinding for some of the axons.

In sequential and combinatorial guidance systems, the fidelity of pathfinding is reduced by the elimination of one guidance cue. This has also been observed in the zebrafish hindbrain and spinal cord, where ablation of the floorplate or other axon tracts, either genetically or surgically, results in pathfinding errors in only a fraction of commissural axons (Chitnis and Kuwada, 1991; Bernhardt et al., 1992; Hatta, 1992; Patel et al., 1994). Creating double mutants in the zebrafish should help in the elucidation of the interactions of different axon guidance systems.

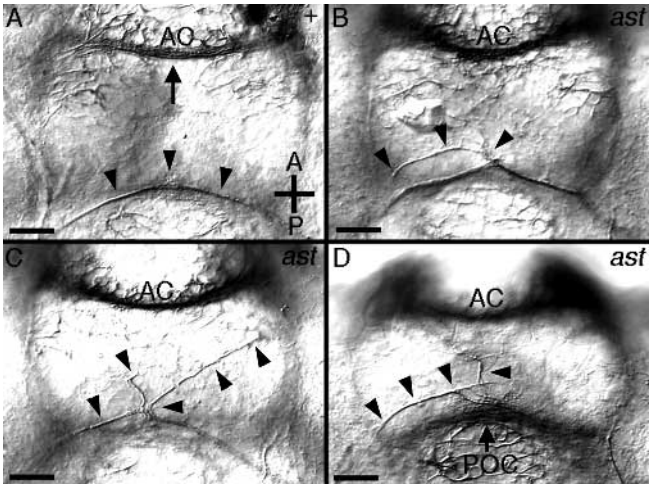

Fig. 8. Anti-acetylated tubulin labeling of *astray* mutants**.** All panels show a ventral view of the ventral brain commissures at 36 hours of development. (A) In the wild type, the retinal ganglion cell axons grow near the POC (just dorsal to this focal plane). At 36 hours the first RGC axons (arrowheads) have reached the midline and fasciculated with their contralateral homologues. The anterior commissure is at the top of the photo (arrow) and the TPOC axons are lateral in a more dorsal focal plane. (D-D) Three examples of errors made at the midline by RGC growth cones in *astray* mutants. In all embryos, the POC has formed normally (most easily seen in D, arrow). RGC axons (arrowheads) grow abnormally on the ventral diencephalon. Instead of growing along their contralateral counterparts, RGC axons spread on the ventral diencephalon (B,D). In some cases axons from the two eyes clearly remain separate and appear to avoid each other (C). AC, anterior commissure; POC, post-optic commissure. Scale bars, 25 μm.

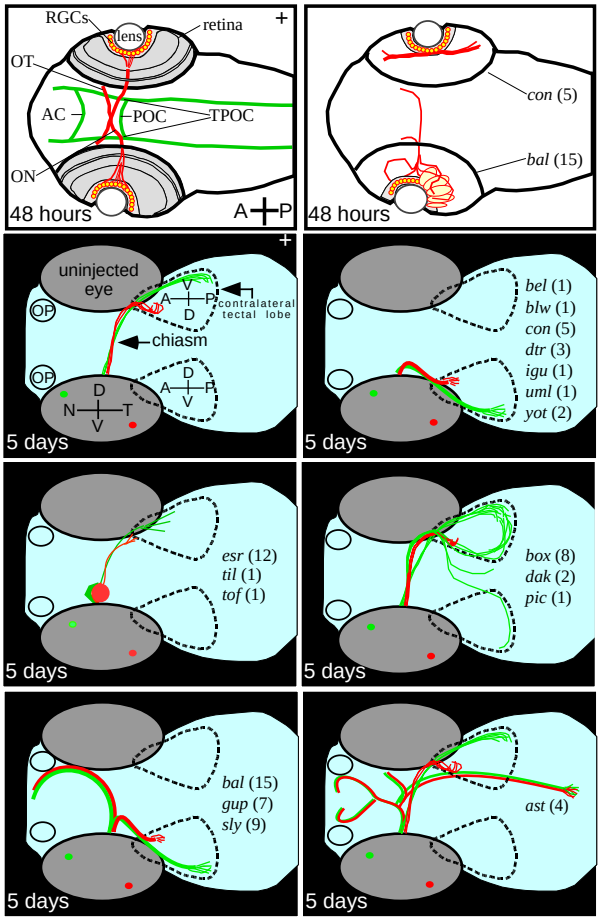

Fig. 9. Schematic overview of the retinotectal pathfinding mutants. Diagrams of each of the pathfinding phenotypes are shown, with the number of alleles of each gene found in the screen in parentheses after the mutant name. 48-hour diagrams represent a ventral view while 5-day diagrams represent a dorsal view. The embryonic scaffold is included in the 2-day wild-type diagram. For *ast*, three axon projection patterns seen in different fish have been combined into one diagram. ON, optic nerve; OT, optic tract; AC, anterior commissure; TPOC, tract of the post-optic commissure; POC, post-optic commissure; OP, olfactory placode; RGCs, retinal ganglion cells; N, nasal; T, temporal; A, anterior; P, posterior; D, dorsal; V, ventral.

Pathfinding in the eye

In the wild-type eye, RGC axons grow radially inward toward the optic nerve head to form a spoked pattern of axon bundles (Easter et al., 1984). It has been shown that this ordered pathfinding in the chick eye requires cues present on retinal basal lamina and endfeet of neuroepithelial cells (Halfter, 1989). In our screen, two genes (*con*, *bal*) were found that affect pathfinding in the eye, each in a different way.

The *con* locus seems to affect the ability of RGC axons to leave the eye. *con* mutant embryos have severe midline defects, and the eyes are turned inward ventrally as if midline structures fail to form (Brand et al., 1996). It seems likely that early defects include malformation of the optic stalk region, with the final result that the exit point for RGC axons fails to form normally in the eye.

Axons in *con* embryos that fail to exit the eye tend to form large fascicles that extend to the periphery of the eye along the equatorial region. It has been suggested that axons in the CNS grow along borders of gene expression domains to form the embryonic axon scaffold (Wilson et al., 1993). A similar phenomenon might be occurring for axons that are unable to exit the eye in *con*, revealing a functional border within the retina. In fact, some genes are expressed in restricted regions of the eye (reviewed in Holt and Harris, 1993), including radar, a TGF-β family member expressed in the dorsal region of the retina (Rissi et al., 1995).

bal seems to affect RGC differentiation, as the pattern of RGCs around the lens is often disrupted in strong *bal* alleles. The ordered growth of axons toward the optic nerve head is also disrupted in these individuals. Axons in *bal* mutants wander as they grow inward toward the optic nerve head, as if radial guidance cues were affected. Axons eventually do leave the eye, at which point they either make further pathfinding errors (see below), or in some cases project normally to the contralateral tectal lobe.

Pathfinding to the midline

At least 14 genes affect growth of axons toward and across the midline. Mutations in these genes cause RGC axons to grow to the ipsilateral instead of the contralateral tectal lobe, or to stop before reaching the midline. Among the mutants with ipsilateral projections, different genes seem to affect midline growth in different ways, suggesting that we have isolated mutations in several different guidance mechanisms.

Normally, RGC axons grow toward the midline alongside, but not on, the preexisting axon bundles of the tract of the post-optic commissure or the post-optic commissure itself (Burrill and Easter, 1995). In *dtr* and *uml* mutants, RGC growth cones were observed to grow across the midline in the absence of the post-optic commissure. RGC axons thus do not seem to need the post-optic commissure axons to cross the midline. This is in agreement with the results of heterochronic transplantation experiments in *Xenopus*, which show that RGC axons are able to grow to the tectum even when the tract of the post-optic commissure has not yet formed (Cornel and Holt, 1992).

What guides these axons toward the midline? One possibility is that growth toward the midline is influenced by chemoattractive molecules. Signaling by ventral midline cells, specifically cells of the floorplate, is known to be important in spinal cord commissural axon outgrowth (reviewed in Colamarino and Tessier-Lavigne, 1995). Secreted molecules that attract spinal cord commissural axons toward the midline, the netrins, have recently been identified in vertebrates (Serafini et al., 1994). The elimination of a general chemoattractant could not account for the specificity of the pathfinding defects, however, and it is likely that the ipsilateral mutant phenotype is due to the disruption of specific signaling that normally attracts retinal ganglion cell axons to the midline. In the absence of such a midline attraction system, RGC growth cones would find a secondary pathway and turn dorsally on the diencephalon.

The fact that only the post-optic commissure and the optic nerve are affected by *bel, dtr* and *uml* suggests that these genes affect local guidance cues necessary only in the region where these two axon tracts form. What kind of cues might be responsible for determining the location of the optic nerve and post-optic commissure? As mentioned above, gene expression borders often correlate well with the positions of axon tracts in the developing CNS (Wilson et al., 1993). Several gene expression boundaries correspond with the position of the post-optic commissure and optic nerve in the developing zebrafish brain. In fact, the post-optic commissure forms at the interface between *pax2* expression and *Shh* expression, a zone in which *nk2.2* is also expressed (Barth and Wilson, 1995). These expression borders may somehow be responsible for establishing specific cues that guide post-optic commissure and RGC axons in the diencephalon. The pathfinding genes may affect the establishment of these borders, the translation of border information into guidance cues, or the ability of axons to read these guidance cues.

An alternative explanation for the specificity of these mutations is that specific guidepost cells are necessary for guiding RGC and post-optic commissure axons across the midline. Recent experimental work in mouse points to the existence of a set of neurons (Sretavan et al., 1995) and glia (Marcus et al., 1995) at the position of the mammalian chiasm that may act as guideposts for growing RGC axons. When specific neurons are ablated, RGC axons often project ipsilaterally (Sretavan et al., 1995). This suggests that the RGC axons may encounter guidance cues, in the form of specific guidepost cells, that other commissural pathways do not encounter.

The secondary pathway chosen by the ipsilaterally projecting RGC axons, the ipsilateral diencephalon, is the pathway normally followed by axons from the other eye in forming the optic tract. In amphibians it has been shown that guidance cues are present on the wall of the diencephalon (reviewed by Chien and Harris, 1994). These cues appear to be associated with neuroepithelial cells or the extracellular matrix of the diencephalic wall, as rotations of this substrate cause RGC growth cones to be deflected in the direction of rotation (Harris, 1989). The zebrafish mutants show that these dorsal cues can be interpreted by growth cones even in the absence of normal midline crossing. In the wild type, midline signals must overpower these dorsal signals until a growth cone crosses the midline. If the midline signaling is disturbed, the dorsal cues are still available and are readable by the RGC growth cones.

In *esr, tof* and *til* mutants many RGC axons do not cross the midline but, instead, form ball-shaped aggregates of axons between the eye and the midline. This is the only example in which RGC axons seem to stop growing rather than take a secondary pathway. It seems likely that axon outgrowth is generally reduced in these mutants. *esr*, *tof*, and *til* may affect cytoskeletal elements that are also necessary for pigment granule distribution within xanthophores (Odenthal et al., 1996b).

Crossing the midline and turning dorsally

Growth cones of all axons that cross the midline must change their growth behavior in two ways once they reach the midline. Firstly, they must ignore the cues that guided them toward the midline in order to continue growth away from the midline. Secondly, they must recognize and be guided by cues that were ignored while growing toward the midline. This change in behavior might be achieved by specific interactions at the midline that serve to respecify the growth cone by, for instance, altering the expression of adhesion molecules on growth cone membranes. Such respecification has been shown for commissural axons in the spinal cord that express different surface molecules on each side of the midline (Dodd et al., 1988). Midline interactions between contralateral homologs have been shown to be important for midline crossing of invertebrate commissural axons (Myers and Bastiani, 1993), and might be responsible for growth cone respecification.

In the wild-type zebrafish embryo, the two converging bundles of RGC axons seem to recognize each other at the midline and fasciculate (Fig. 8A). After a brief period of growth along the axons of their contralateral counterparts, RGC growth cones then separate from the contralateral optic tract to grow dorsally along the contralateral diencephalon wall. Mutations in *ast* appear to disrupt this midline interaction between contralateral homologs, as the growing optic nerves fail to fasciculate and do not grow normally across the midline.

Growth cones in *ast* mutant embryos fail to turn dorsally after the midline and instead often grow along the midline for some distance (Fig. 7D). If RGC axons do cross the midline in *ast* mutant embryos, they often turn back toward it (Fig. 7E,F) Both of these behaviors would be expected if RGC growth cones were not respecified at the midline. Since axons in *ast* embryos fail to interact normally with their contralateral counterparts, perhaps these growth cones are not respecified at the midline.

Mutations at the *bal* locus result in both ipsilateral and anterior RGC axon projections. Midline signaling is disrupted in this mutant as well, as homozygous *bal* embryos have defects in the formation of midline structures earlier in development (Haffter et al., 1996b). A weak *cyclops* allele isolated in the Tübingen screen shows RGC axon defects similar to those seen in *bal*, with both anterior and ipsilateral retinotectal projections. The weakest alleles of two other genes, *sly* and *gup*, occasionally also show this same retinotectal phenotype. *sly* and *gup* have early midline defects similar to, but more extreme than, *bal*. The similarity in both midline and eye phenotypes between *bal*, *sly* and *gup* mutants suggests that these genes may all affect a single molecular pathway.

The anterior pathway taken by axons in *bal* is distinct from the pathways taken in *ast*. Anteriorly projecting axons seem to grow near the outer edge of the diencephalon and telencephalon and may be simply following the boundary of the brain itself.

Forming dorsal and ventral branches of the optic tract

After turning dorsally on the contralateral diencephalon, RGC axons sort into a dorsal and a ventral branch of the optic tract (Stuermer, 1988). Ventral RGC axons are primarily found in the dorsal branch and dorsal RGC axons make up the ventral branch. In *box*, *dak* and *pic* mutant embryos, this sorting of axons is disrupted. Dorsal RGC axons inappropriately enter both branches, while ventral RGC axons seem unaffected and grow properly in the dorsal branch. Upon reaching the contralateral tectal lobe the missorted dorsal RGC axons are able to find their correct target region within the tectum, despite the fact that they arrive at the wrong position on the tectum (Trowe et al., 1996). This shows that some guidance cues on the tectum are independent of guidance cues to the tectum. Both guidance systems, however, may be important for high-fidelity pathfinding, as in some cases axons that arrive at an inappropriate position on the contralateral tectal lobe make further pathfinding errors and cross to the ipsilateral tectal lobe.

Conclusion

The retinotectal pathfinding mutants found in this large-scale zebrafish screen have provided insights into the nature of RGC pathfinding from eye to tectum. We can make several general conclusions based on mutant phenotypes. (1) A sequence of guidance cues directs axons toward their target. Assuming that at least some of the alleles are null mutations, not all of these cues are absolutely necessary for RGC axon guidance, as a subset of axons almost always finds its way to the correct target despite the loss of any one guidance cue. (2) Pre-existing axons of the tract of the post-optic commissure appear not to be necessary for RGC pathfinding to the tectum as some RGC axons can reach their target when this scaffold is disrupted. This confirms earlier experimental results. (3) The ventral midline appears be an important and complex choice point for axons growing to the contralateral tectal lobe, as a large number of mutations show pathfinding errors associated with midline defects. (4) Axons in nearby regions of the brain seem to use different cues to cross the ventral midline (anterior commissure versus post-optic commissure).

The molecular nature of the guidance cues responsible for accurate axon pathfinding between eye and tectum is still unresolved. The strength of the genetic approach is that we may now be in a position to identify genes that are responsible for specific pathfinding phenomena. The molecular characterization of the mutants should allow us to couple molecules with functions in vivo to achieve a clearer picture of how axons are guided to their correct targets in the vertebrate brain.

The success of this project was the result of the efforts of many people, all of whom deserve our thanks. We thank the following people for mounting and/or injecting a staggering number of zebrafish larvae during the screen: Christian Bayertz, Eckard Dominik, Dr Uwe Drescher, Carmen Gitter, Claudia Handwerker, Ingrid Horschke, Julita Huf, Dr Hiroyuki Ichijo, Kurt Knauf, Dr Sigrun Korsching, Ulrike Nell, Elke Ober, Thomas Stroh, Dr Thomas Voigt and Franco Weth. Thanks to the MPI workshop, under the leadership of Franz Endress, for the exceptional injection machinery, and to Jürgen Jung and Dr Jürgen Löschinger for help with computers and optics. We are indebted to the members of the Department of Developmental Genetics for the fish work that made the identification of the retinotectal mutants possible (see Contents, this issue). Finally, thanks to Drs Chi-Bin Chien, Steve Easter, Alfred Gierer, Suresh Jesuthasan, Claudia Stürmer and Steve Wilson for critical reading of the manuscript.

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