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The Pax protein Noi is required for commissural axon pathway formation in the rostral forebrain

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SUMMARY

No-isthmus (Noi) is a member of the zebrafish Pax family of transcriptional regulators that is expressed in restricted domains of the developing CNS. In the developing eye and optic nerve, the Noi$^+$ cells are primitive glial cells that line the choroid fissure and optic stalk/nerve to its junction with the optic tract. This pattern of Noi expression is retained in the adult, defining the optic nerve astroglia, which wrap the left and right nerves separately at the midline, thus forming the bodily crossed optic chiasm found in fish. In embryos carrying mutations in the *noi* gene, the choroid fissure fails to close, glial cells of the optic nerve fail to differentiate and optic axons exhibit abnormal trajectories exiting the eye and at the midline of the diencephalon. Optic axons select inappropriate pathways into the contralateral optic nerve, rostrally towards the anterior commissure and along the ipsilateral optic tract. Noi$^+$ cells also border the pathway of axons in the postoptic commissure, which is located adjacent to the optic chiasm. These postoptic commissural axons are defasciculated and also exhibit pathfinding defects in *noi*$^-$ embryos. These results indicate that Noi is required in cells that line the pathways taken by optic and non-optic commissural axons for guidance across the midline of the diencephalon. We find that expression of two members of the Netrin family of axon guidance molecules and the signalling protein Sonic hedgehog is disturbed in *noi*$^-$ embryos, whereas several members of the Eph family of receptors and ligands show no obvious alterations in expression at the diencephalic midline.

Key words: Pax, axon, Noi, zebrafish, CNS, glial cell, forebrain, commissural axon

INTRODUCTION

During vertebrate embryonic development, retinal ganglion cell axons navigate over considerable distances from their site of origin in the eye to their targets in the diencephalon and midbrain. Within the retina, axons extend in radial fascicles towards the optic nerve head, the location at which all of the axons coalesce before leaving the eye. At the stage when the first axons exit the eye, the retina is still connected to the forebrain by the optic stalk, a transitory structure that provides the substratum for axons to extend from the eye to the diencephalon. The route out of the retina is provided by the choroid fissure, an opening within the optic cup that allows growth cones access from the optic nerve head onto the optic stalk. Later in development, the retina on either side of the choroid fissure fuses around the exiting axons. During the period of retinal axon outgrowth, the optic stalk is gradually replaced by the optic nerve and it is presumed that the epithelial cells of the stalk either die or give rise to glial cells within the nerve.

Once at the midline of the diencephalon, the optic axons establish the optic chiasm and the precise behaviour of retinal growth cones at this location varies between species. In vertebrates with binocular vision, retinal axons diverge with temporal axons remaining ipsilateral and nasal axons extending across the midline towards contralateral targets (Guillery et al., 1995). In species without binocular vision, the majority of axons decussate and extend towards contralateral targets. Several cellular components of the midline tissue may be involved in the regulation of retinal growth cone behaviour. First, the position at which optic axons exit from the optic stalk and enter the optic tract is adjacent to a pre-existing tract termed the postoptic (or supraoptic) commissure (Guillery et al., 1995). Ablation of neurons in the vicinity of this tract prevents chiasm formation in mice (Stretevan et al., 1995) but appears to have little effect upon optic axons in *Xenopus* (Cornell and Holt, 1992). In addition to neurons and axons, retinal growth cones in mammals also encounter specialised glia at the midline (Marcus et al., 1995). It is upon contact with these glial cells that ipsilateral and contralateral axons diverge from each other (Marcus et al., 1995; Marcus and Mason, 1995; Wang et al., 1995).

Considerable effort has been directed at elucidating the molecular nature of the guidance mechanisms that underlie the establishment of patterned connections between the eye and

brain (Tessier-Lavigne and Goodman, 1996). One promising line of research has been to generate lines of zebrafish that carry mutations affecting the establishment of the retinotectal projection (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). In this study, we examine the function of a paired box (Pax) containing transcription factor called No-isthmus (Noi) during eye development in zebrafish, focussing upon the role of this protein in regulating the formation of the postoptic commissure (POC), optic nerve and chiasm.

Pax proteins constitute a family of transcription factors characterised by a DNA-binding element termed the paired box. Mutations in several Pax genes lead to human syndromes. For instance, individuals with mutations in *PAX-6* exhibit a variety of eye defects associated with Aniridia and Peter's anomaly (Chalepakis et al., 1993) and individuals heterozygous for a mutation in the *PAX-2* gene exhibit severe visual impairment and optic nerve coloboma, a condition in which the choroid fissure fails to close (Sanyanusin et al., 1995). Comparable visual system defects are also observed in mice lacking the *Pax-2* gene (Keller et al., 1994; Torres et al., 1996; Favor et al., 1996). *noi* is a zebrafish gene that has considerable sequence homology and similar expression domains to the mouse *Pax-2* gene (Nornes et al., 1990; Krauss et al, 1991; Püschel et al., 1992). However, it remains uncertain whether *noi* is the zebrafish orthologue of *Pax-2* as *Pax-2* belongs to a group of three closely related genes that includes *Pax-5* and *Pax-8*, and to date *noi* is the only member of this subfamily to have been identified in zebrafish (see Discussion in Brand et al., 1996).

Several lines of fish carrying mutations in the *noi* gene have been isolated in a large-scale screen for genes affecting early development in zebrafish (Brand et al., 1996). Homozygous *noi*$^{-}$ embryos were originally identified on the basis of severe midbrain defects consistent with *noi* expression in the caudal midbrain. In this study, we show that Noi activity is required within the developing visual system to establish the normal pattern of axon outgrowth from the retina to the midline. We find that the Noi^{+} cells within the optic stalks give rise to a population of reticular astrocytes that are present throughout the life of the fish. In the absence of functional Noi protein, the choroid fissure fails to fuse and axons show abnormal trajectories out of the retina. Glial cell differentiation in the optic nerve is also compromised and axons show severe pathfinding deficits approaching, and at, the optic chiasm. Pathfinding defects are also made by earlier decussating axons that establish the POC adjacent to the prospective chiasm. We conclude that Noi is a key regulator of commissural axon pathway formation at the midline of the diencephalon. We investigate several candidate genes that may act downstream of Noi and find that both *netrin* and *Sonic hedgehog* (*shh*) expression is altered in *noi*$^{-}$ embryos whereas several members of the Eph family of receptors and ligands appear to be unaffected.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28.5°C on a 14 hours light/10 hours dark cycle. Embryos were staged according to Kimmel et al. (1995) or in days postfertilisation at 28.5°C for older embryos. To prevent pigment formation, embryos were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma).

Two *noi* alleles *noi*^{tu29a} and *noi*^{ty31a} were analysed (Brand et al., 1996). Based upon analysis of midbrain defects, *noi*^{tu29a} is the stonger of the two alleles and no protein is detected by anti-Noi antibody staining in homozygous mutant embryos (Brand et al., 1996). Homozygous *noi*^{ty31a} embryos exhibit a weaker midbrain phenotype and some Noi protein is detected. In agreement with the analysis of midbrain defects, homozygous *noi*^{tu29a} embryos exhibited more consistent and severe axonal pathfinding defects in the visual system than homozygous *noi*^{ty31a} embryos. Unless otherwise stated, the data presented in this study is from analysis of homozygous *noi*^{tu29a} embryos and when we use the term '*noi*$^{-}$ embryos' we are referring to homozygous *noi*^{tu29a} embryos.

Immunohistochemistry

Embryos and larvae were anaesthetised in 0.03% MS222 (Sigma), fixed (4% formalin in Pipes buffer – Wilson et al., 1990) for 3 hours at room temperature, washed in 0.1 M phosphate buffer and either embedded for cryosectioning (Macdonald et al., 1994), or further processed as outlined below. Adult zebrafish were killed by over-anaesthetisation and CNS tissue fixed overnight at 4°C, washed in 0.1 M phosphate buffer and embedded for cryosectioning.

Standard antibody protocols were used (Wilson et al., 1990). For primary incubations, anti-acetylated alpha tubulin (Sigma) was diluted 1:1000, anti-Noi (Mikkola et al., 1992) 1:3000, anti-band 7 cytokeratin was used at 1:100 on tissue treated with 70% ethanol, 20% glacial acetic acid for 3-5 minutes. Embryos beyond prim-20 stage were usually fixed in 2% trichloroacetic acid and treated for 5 minutes in 0.25% trypsin on ice. For plastic sectioning, antibody-labelled embryos were dehydrated to 100% methanol, embedded in JB4 resin (Agar Scientific) and sectioned at 5-10 μm using a tungsten knife on a Jung 2055 Autocut.

dil labelling

Embryos were fixed and immobilised in 3% methyl cellulose. diI dissolved in dimethylformamide was pressure injected into the eyes and photoconversion was performed as in Wilson and Easter (1991).

Analysis of gene expression

Standard methods for in situ hybridisation were used (Macdonald et al., 1995). *net1* and *net2* were PCR amplified using primers to regions conserved between *C. elegans* UNC-6 and chicken Netrins (Strähle et al., 1997). *net1* sequence is published in Strähle et al. (1997) and *net2* sequence is accessible in GenBank (accession number AF002018). *L2* was PCR amplified as described in Brennan et al. (1997) and sequence is accessible in GenBank (accession number Y12928). Other probes were made as described in Macdonald et al. (1994), Barth and Wilson (1995), Brennan et al. (1997), Cooke et al. (1997) and Toyama et al. (1995).

Nomenclature

The gene termed *noi* in this study has previously been published using the nomenclature *pax2*, *paxb* and *zfpax[b]*. Although previously called *pax2*, it is not certain that *noi* is the orthologue of mammalian *Pax-2* (see Introduction and discussion in Brand et al., 1996).

RESULTS

Noi is present in the developing optic stalk, nerve and choroid fissure

Noi protein is first detected in medial cells of the prospective optic vesicles at 6-7 somites (Fig. 1A) and, by 9-10 somites, Noi^{+} cells begin to form the optic stalks (Fig. 1B). By 18 somites, the optic stalks are distinct structures (Fig. 1C) and by prim-15 stage (30 h), higher levels of protein are present in rostral and ventral regions of the stalks (Fig. 1G). In continu-

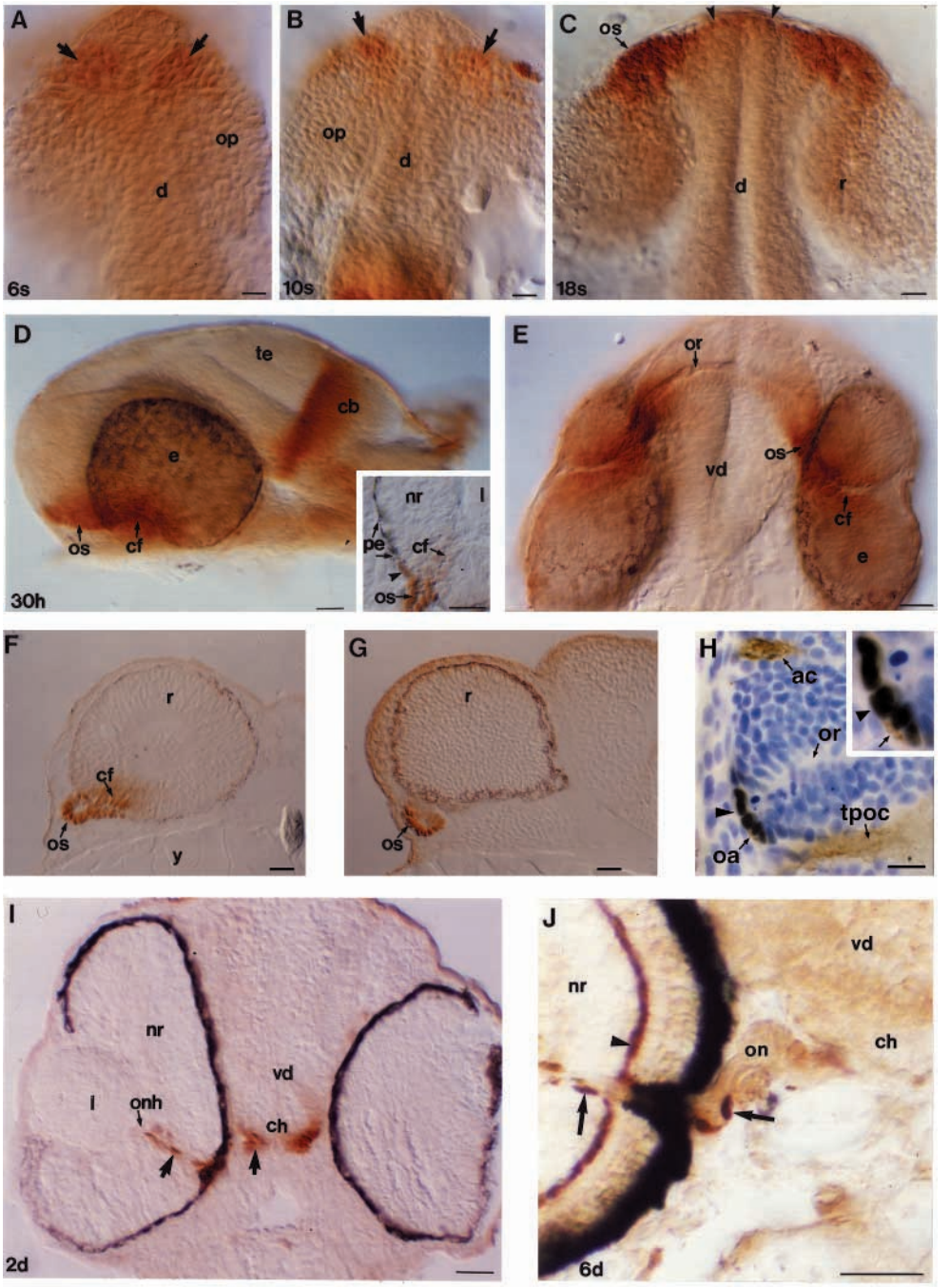


Fig. 1. Noi protein is present in the developing optic stalks and choroid fissure. Dorsal (A-C,E) and lateral (D) views of embryos with rostral to the top or left (D) and 10-14 μm sections (F-J) labelled with anti-Noi antibody (brown). (A) 6-7 somites. (B) 10 somites. Arrows (A,B) indicate Noi expression in the presumptive optic stalk region. (C) 18 somites. Weak labelling is present across the midline (arrowheads). (D,E) Prim-15. The inset panel shows Noi protein is present in cells directly adjacent to the pigment epithelium. (F,G) Lateral (F) and more medial (G) parasagittal sections through a prim-15 embryo. (H) Prim-15. Parasagittal section approaching the midline labelled with anti-Noi and anti-tubulin antibodies. The optic axons approach the midline on the superficial surface of the Noi$^+$ cells (arrowhead). The inset panel shows a high magnification view of the Noi$^+$ cells (arrowhead) and the optic axons (arrow). (I) 2 day. Noi$^+$ cells (arrows) line the optic nerve both within the retina and between the retina and the diencephalon. (J) 6 day. Arrows indicate the Noi$^+$ nuclei within the neural retina at the optic nerve head and on the surface of the optic nerve. The arrowhead indicates non-specific labelling within the retina. Abbreviations: ac, anterior commissure; cb, cerebellum; cf, choroid fissure; ch, chiasm; d, diencephalon; e, eye; l, lens; nr, neural retina; oa optic axons; onh, optic nerve head; on, optic nerve; op, optic primordia; or, optic recess; os, optic stalks; pe, pigmented epithelium; te, tectum; vd, ventral diencephalon; tpoc, tract of the postoptic commissure; y, yolk. Scale bars: 25 μm except H, 12.5 μm.

ity with the stalks, Noi is detected across the midline in a small number of cells superficial to the optic recess (Fig. 1C), upon which the first optic axons extend as they approach the chiasm (Fig. 1H). Noi expression continues in these regions throughout the stages at which the optic stalk is replaced by the optic nerve (Fig. 1I) and, by 6 days, Noi is present in cells with large nuclei associated with the surface of the nerve (Fig. 1J). Abruptly at the transition between optic chiasm and optic tract, Noi expression ceases and no other cells in the diencephalon contain Noi (Fig. 1I,J).

In addition to expression in the optic stalk/nerve, Noi is present in cells of the ventral retina that line the choroid fissure from the presumptive optic nerve head through the retina and in continuity with the optic stalk/nerve (Fig. 1D-F). The Noi$^+$ cells within the retina abut the pigment epithelial cell layer, but contain no pigment themselves (Fig. 1D inset). By 2 days, the choroid fissure has closed around the retinal axons that have exited onto the optic stalk. At this and later stages, Noi$^+$ cells continue to line the pathway taken by the optic axons through the retina (Fig. 1I,J). Given the phenotype of *noi*$^-$ embryos described below, it is important to note that retinal ganglion cells do not express Noi (Fig. 1I).

Noi is detected in astrocytes within the optic nerve but not the optic tract

In order to follow the fate of the Noi$^+$ cells, mature optic nerves

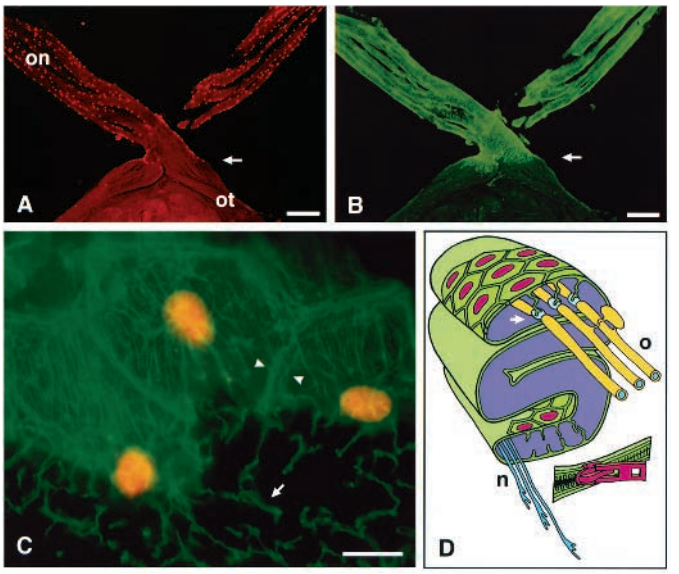


Fig. 2. Noi$^+$ cells differentiate as reticular astrocytes unique to the optic axon pathway. 15 μm sections of optic nerves labelled with anti-Noi (red) and anti-cytokeratin (green) antibodies. (A,B) Horizontal section showing the optic nerves crossing at the chiasm and joining the optic tracts. (A) Noi distribution in nuclei of the optic nerve. (B) cytokeratin distribution. Arrows indicate the beginning of the optic tracts and the limits of Noi and cytokeratin staining. (C) High magnification view of an oblique double-labelled section of the optic nerve showing reticular astrocytes forming the glia limitans. The Noi$^+$ nuclei (yellow) are surrounded by a lattice of cytokeratin filaments (green) joined to the hexagonally shaped cell outlines (arrowheads). Processes from the cells (arrow) drop vertically into the fibre layer below. (D) Schematic drawing of a mature optic nerve showing its ribbon structure. Reticular astrocytes (green) with Noi$^+$ nuclei (red) have processes contacting the axons at the nodes of Ranvier (arrow). The processes of the astrocytes may help 'zipper' up each fold of the ribbon nerve. Abbreviations: n, nerve axons; o, oligodendrocytes; on, optic nerve; ot, optic tract. Scale bars: (A,B) 200 μm; (C) 10 μm.

were examined. We found that Noi is present in a population of astroglia that constitute about 25% of cells in the optic nerve. The Noi$^+$ cells extend from the optic nerve head to the junction of the optic nerve with the optic tract (Fig. 2A). This distribution coincides with that of a population of teleost glial cells termed reticular astrocytes (Maggs and Scholes, 1990) that are characterised by intermediate filaments composed of cytokeratins, not GFAP (Markl and Franke, 1988; Giordano et al., 1989). Labelling with antibodies against cytokeratins (Maggs and Scholes, 1986, 1990) and against Noi confirmed that the zebrafish optic nerve contains a network of reticular astrocytes distributed in the same pattern as the Noi$^+$ nuclei (compare Fig. 2A and B). Analysis of sections confirmed that the large disc-shaped Noi$^+$ nuclei are located within cytokeratin$^+$ astrocyte somas that line the surfaces of the ribbon-shaped optic nerve and form a thin epithelial monolayer of hexagonal cells just beneath the limiting basal lamina (Fig. 2C,D).

The astrocytes form a network of cytokeratin-containing processes running predominantly vertically between the optic axons (Fig. 2C,D). These processes need not be extensive to establish contact with all of the optic axons as the thickness of each fold of the ribbon optic nerve is little greater than the diameter of the astrocyte cell somas (approx. 35 μm). The remaining 75% of optic nerve cells were cytokeratin$^-$ and Noi$^-$, had smaller more spherical nuclei, and consisted mainly of oligodendrocytes and microglia, which were identified by electron microscopy (EM, data not shown).

Thus, Noi is expressed by cytokeratin-containing reticular astrocytes that form the *glia limitans* and internal glial network of the zebrafish optic nerve. The persistence of a population of Noi$^+$ cells throughout development and into adulthood indicates that these glia are likely to be the descendants of the early optic stalk cells.

noi$^-$ embryos exhibit coloboma

To elucidate the function of Noi in cells lining the optic axon pathway, we examined embryos homozygous for mutations in the *noi* gene (Brand et al., 1996; and see Materials and Methods).

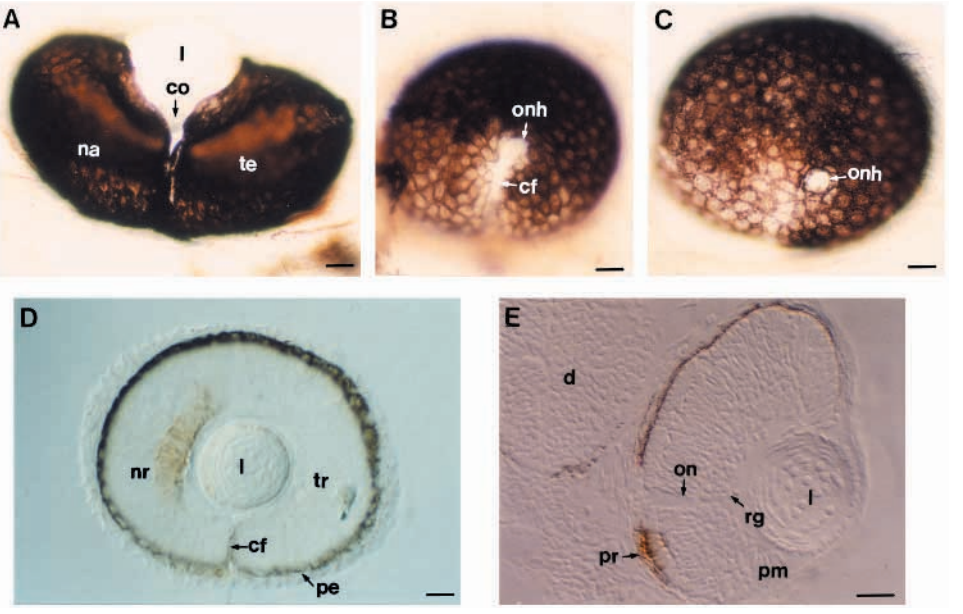


Fig. 3. The choroid fissure fails to close in *noi*$^-$ embryos. (A-C) are whole eyes and (D,E) are 10 μm sections. (A) Ventral view of the eye cup in a 6 day *noi*^{ty31a} embryo illustrating the coloboma. (B,C) Views of the back of a eye cup of *noi*$^-$ (B) and wild-type (C) 2 day embryos. (D) Parasagittal section of the eye of a 2 day *noi*$^-$ embryo showing ingression of the pigment epithelium into the retina at the site of the unfused choroid fissue. (E) Transverse section of a 44 h *noi*$^-$ embryo labelled with the photoreceptor-specific antibody, FRet43 (brown, arrow). The earliest photoreceptors differentiate adjacent to the optic nerve head as in wild-type embryos. Abbreviations: cf, choroid fissure; co, coloboma; d, diencephalon; l, lens; na, nasal retina; onh, optic nerve head; on, optic nerve; pe, pigment epithelium; pm, proliferating margin; pr, photoreceptors; rg, retinal ganglion cell layer; te, temporal retina. Scale bars; A-D, 50 μm; E, 25 μm.

The most obvious eye phenotype of *noi*⁻ embryos was that the choroid fissure failed to close, resulting in a coloboma (Fig. 3). In wild-type embryos, the pigmented and neural retina fuse around the exiting optic axons at the junction between ventral nasal and ventral temporal retina (Fig. 3C). In *noi*⁻ embryos, the fissure remains open (Fig. 3A,B) and pigment epithelial cells ingress into the retina along the open fissure (Fig. 3D).

The *noi* mutation had no obvious cell non-autonomous effects upon retinal neuron differentiation adjacent to the domain of Noi expression. For instance, the first photoreceptors to differentiate do so close to the choroid fissure (Larison and Bremiller, 1990) and these cells appeared normal in *noi*⁻ embryos (Fig. 3E) as did other aspects of neuronal differentiation and retinal lamination (Fig. 3E and data not shown).

Axons have abnormal trajectories leaving the eye in *noi*⁻ embryos

Noi⁺ cells line the pathway of optic axons from the optic nerve head to the optic tract. To determine if Noi is involved in guiding axons in these regions, axons were labelled with antibodies and diI in wild-type and *noi*⁻ embryos. Within the retina of wild-type embryos, radial fascicles of retinal ganglion cell axons converge at the optic nerve head (which is located at the dorsalmost limit of the choroid fissure) to form a single bundle of axons as they pass out of the retina (Fig. 4A,C). In *noi*⁻ embryos, the axons still converge towards the choroid fissure, but fail to coalesce at the optic nerve nerve head and frequently exit the eye as two or more discrete bundles on one or both surfaces of the fissure (Fig. 4B,D).

In wild-type embryos, the optic stalk decreases in size over time as it is replaced by the optic nerve. By 3 days, the stalk is no longer apparent and the optic nerve consists primarily of tightly packed unmyelinated optic axons surrounded by a thin membrane of flattened Noi⁺ glial cells (Fig. 4E). This transformation fails in *noi*⁻ embryos, so that a stalk-like structure is retained, with large numbers of nuclei surrounding the fibres in disorganised clumps (Fig. 4F). Furthermore, the axons are less tightly bundled and the superficial basal lamina is poorly formed (EM data not shown).

Retinal ganglion cell axons exhibit pathfinding defects at the midline of *noi*⁻ embryos

Labelling of *noi*⁻ embryos with anti-tubulin antibodies showed that many axons are misrouted between the optic chiasm and the anterior commissure (compare Figs 4A to 4B and 5A to 5B). To determine if optic axons were amongst those misrouted at the midline, they were labelled with diI (n=47 eyes) or antibodies specific to retinal ganglion cell axons (n=10 embryos). Optic axon defects were detected in all *noi*⁻ embryos, but the severity and precise nature of the defects varied from embryo to embryo. Not all defects were observed in all embryos and the proportion of axons selecting the various inappropriate pathways also varied between embryos.

In zebrafish, retinal axons form an almost entirely crossed projection (Burrill and Easter, 1994), with axons from one eye passing adjacent to axons from the other eye within the optic chiasm (Fig. 5C). In contrast, in *noi*⁻ embryos, the axons from the two eyes coursed directly into each other (Fig. 5D). At the midline of wild-type embryos, optic axons from the two eyes form discrete fascicles adjacent and rostral to the axons in the POC (Fig. 5E). In *noi*⁻ embryos, the axons from the two eyes

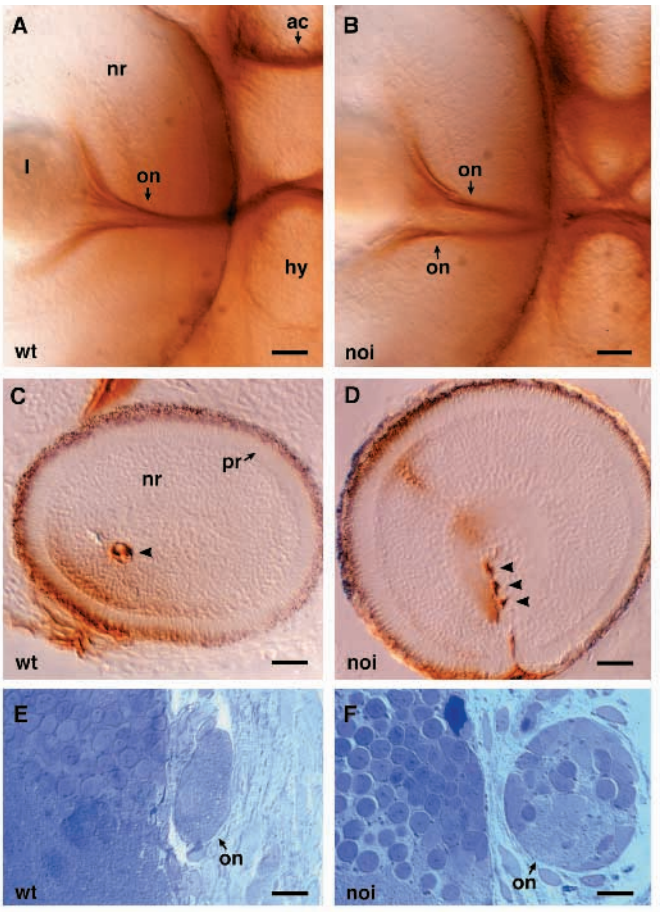


Fig. 4. Optic axons have abnormal trajectories out of the eye in *noi*⁻ embryos. Ventral views (A,B) or parasagittal sections (C-F) of 2 day embryos. (A,B) Anti-tubulin labelling (brown) of retinal and other axons. In the wild-type embryo, the retinal axons coalesce to form a single fascicle (arrow) as they exit the eye, whereas in the *noi*⁻ embryo, several retinal axon fascicles are present. (C,D) Parasagittal sections through the retinae of embryos in which retinal ganglion cell axons have been labelled with DiI. Arrowheads indicate axons exiting the eye at the optic nerve head in the wild-type embryo and as several discrete fascicles along the open choroid fissure in the *noi*⁻ embryo. (E,F) Parasagittal 1 μm sections through the optic nerve. In the wild-type embryo the nerve mainly consists of axons with large sub-pial nuclei. In the mutant, the optic nerve is an enlarged and disorganised structure with many nuclei located within the body of the nerve. Abbreviations: ac, anterior commissure; hy, hypothalamus; l, lens; nr, neural retina, on, optic nerve axons; pr, photoreceptors. Scale bars: (A-D) 30 μm; (E,F) 10 μm.

do not form discrete fascicles and are not consistently positioned with respect to the axons of the POC (Fig. 5F). Unilateral diI labelling confirmed that at least some optic axons had access to the pathway offered by the opposite optic nerve, in that axons from one eye were observed to extend along the contralateral optic nerve and into the opposite eye (Fig. 5K). Several other aberrant pathways were selected by optic axons. For instance, optic axons were frequently observed to join the ipsilateral optic tract (compare Fig. 5G to 5H and see Trowe et al., 1996) and, in some cases, the majority of the retinal axons remained ipsilateral (Fig. 5I,J). Once within the optic tracts, retinal axon extension appeared normal in that axons

were appropriately directed towards the midbrain (Fig. 5H and data not shown). Finally, in confirmation of the anti-tubulin labelling, some optic axons extended rostrally from the chiasm towards the anterior commissure (Fig. 5L).

The optic axon defects in *noi*⁻ embryos are preceded by pathfinding defects within the POC

About 12-14 hours before the first optic axons leave the eye, a small number of axons pass contralaterally in the POC (Wilson et al., 1990; Chitnis and Kuwada, 1990). When the optic axons later enter the diencephalon, they do so adjacent to the POC but separated from it by fine neuroepithelial/glial cell processes (Burrill and Easter, 1995; Marcus and Easter, 1995). The trajectory of the first axons to pass contralaterally within the POC is adjacent to the ventral boundary of expression of Noi within midline cells superficial to the optic recess (Fig. 6A,B and

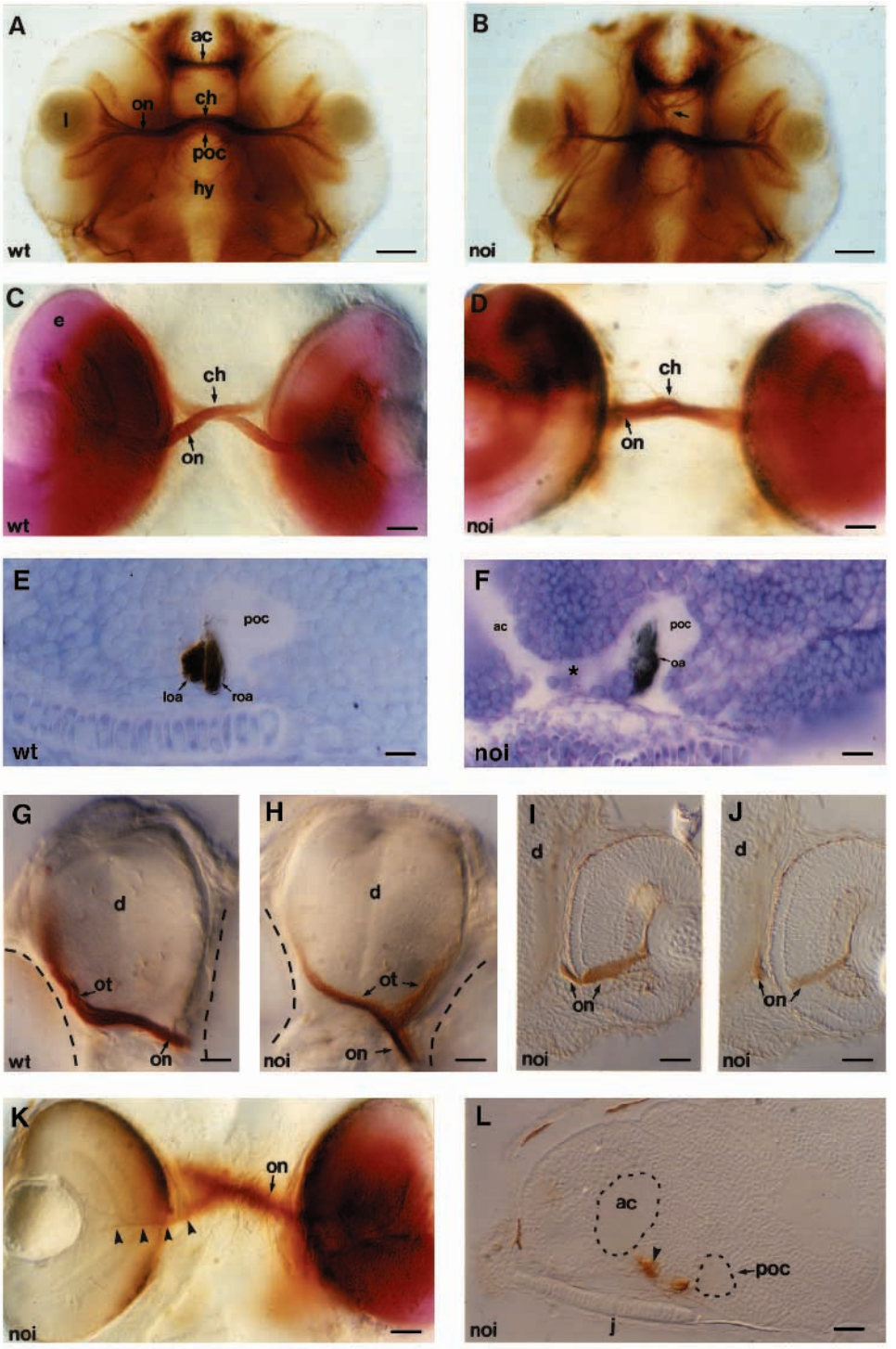


Fig. 5. Optic axons show severe pathfinding defects at the midline in *noi*⁻ embryos. Ventral views of whole embryos (A-D,K) and sections of embryos at 2 days of development (except L). (A,B) Wild-type and *noi*⁻ embryos labelled with anti-tubulin antibody (brown). Many abnormally positioned axons (arrow) are present between the POC and AC of the *noi*⁻ embryo. (C-L) Wild-type and *noi*⁻ embryos in which ganglion cell axons (brown) were labelled with diI or with Zn5 antibody. (C,D) In the wild-type embryo, the optic axons from the two eyes cross completely in the optic chiasm whereas in the mutant, the optic axons from one eye run into the axons from the other eye. (E,F) 10 μm sagittal sections at the chiasm. In the wild-type embryo, axons from the two eyes are discrete fascicles adjacent to the POC. In the *noi*⁻ embryo, optic axons from the two eyes are not discrete and are located among ectopically positioned axons between the POC and anterior commissure (asterisk). (G,H) Thick transverse sections through the diencephalon. In the wild-type embryo all of the axons from one eye pass contralaterally whereas in the *noi*⁻ embryo, optic axons extend in both the ipsilateral and contralateral optic tracts. The original positions of the eyes, which have been removed, are indicated by dashed lines. (I,J) 10 μm successive transverse sections through the retina of a *noi*⁻ embryo labelled with the antibody Zn5 which recognises retinal ganglion cell axons (brown). In this embryo, the retinal axons turn dorsally upon exiting the eye and join the ipsilateral optic tract. (K) Ventral view of a *noi*⁻ embryo in which retinal axons from one eye have been diI labelled. At the midline, some of the axons extend along the contralateral optic nerve and enter the contralateral eye (arrowheads). (L) Parasagittal 10 μm plastic section through a 3 day *noi*⁻ embryo in which diI was injected into both eyes. Some retinal axons (brown, arrowhead) are about to enter the anterior commissure. The limits of the anterior and postoptic commissures are indicated by dots. Abbreviations: ac, anterior commissure; ch, chiasm; d, diencephalon; e, eye; hy, hypothalamus; j, jaw; l, lens; oa, optic axons (loa and roa, from left and right eyes); on, optic nerve; ot, optic tract; poc, postoptic commissure. Scale bars, (A,B) 50 μm; (C,D,G-L) 25 μm; (E) 10 μm; (F) 12.5 μm.

Macdonald et al., 1994). Invariably, there are 4-5 cell diameters between the optic recess and the POC (Fig. 6A-C; Wilson and Easter, 1991). Noi expression is robust in 3-4 of these cells (Fig. 1H, 6A-C) but the row of cells directly in contact with the first axons to cross the POC has little or no Noi expression (Fig. 6B,C).

In wild-type embryos, the POC is a tight fascicle of 5-10 axons by the prim5 stage (Fig. 6D). In *noi*$^{-}$ embryos, the territory between the POC and the anterior commissure is still present and axons still navigate towards the midline. However, they are less tightly fasciculated and some axons invariably extend rostrally instead of passing contralaterally (Fig. 6E) – navigational errors of this sort were never observed in wild-type embryos. None of the neurons that extend axons through the POC at these stages express Noi (Mikkola et al., 1992) and so the pathfinding errors can be attributed to defects in Noi^{+} cells along the pathway. Thus by the stage that the first optic axons join the optic tract, there is already axonal disorganisation at the midline of the diencephalon (compare Fig. 6F to G).

In summary, all of the phenotypic defects in *noi*$^{-}$ embryos occur within or adjacent to the domains of cells that normally express Noi, consistent with this protein being required in pathway cells for the regulation of commissural axon guidance across the midline.

shh and *netrin* expression are altered in *noi*$^{-}$ embryos

Netrins are a family of secreted, laminin-related proteins that have growth-promoting, chemotropic and chemorepulsive activities upon a variety of axons (Tessier-Lavigne and Goodman, 1996). *netrin-1* appears to be expressed in a domain of the chick eye comparable to that of Noi in the fish eye (Kennedy et al., 1994) and, in mice, Netrin-1 has growth-promoting effects upon retinal ganglion cell axons (Wang et al., 1996). Furthermore, neurons that have axons in the POC probably express Netrin receptors (Keino-Masu et al., 1996) and thus it is likely that many or all of the axons affected by mutations in *noi* can respond to Netrin guidance cues. Given these observations, we examined whether *netrin* expression is altered in *noi*$^{-}$ embryos.

Two members of the zebrafish Netrin family, *net1* and *net2* were isolated (Strähle et al., 1997 and see Materials and Methods) and their expression analysed in wild type and *noi*$^{-}$ embryos between 28-somite and prim25 stages. In the trunk, *net1* is expressed most predominantly in the floorplate whereas *net2* has a broader expression in the ventral spinal cord and adjacent mesoderm (Strähle et al., 1997 and unpublished observations). Within rostral regions, *net1* and *net2* have similar expression domains although *net1* expression is more diffuse than that of *net2*. *net2* is expressed strongly and *net1* very weakly in cells lining the choroid fissure, in a domain similar to that of Noi (Fig. 7A and data not shown). Within the forebrain neuroepithelium, intense expression of both *netrin* genes is present in cells positioned between the optic recess and the anterior commissure directly adjacent and dorsal to Noi^{+} cells in the same region (compare Fig. 7D and F to C).

In *noi*$^{-}$ embryos, *net2* expression was considerably reduced in the choroid fissure (Fig. 7B) and *net1* expression was barely detectable (data not shown). The reduction in expression reflected a decreased number of cells expressing *net2* and probably also a reduction in the intensity of labelling of expressing cells. At the midline of the forebrain of *noi*$^{-}$ embryos, *netrin* expression remained high in cells between the optic recess and the anterior commissure (compare Fig. 7D to E and F to G).

A second family of proteins that have been shown to mediate guidance of retinal and other commissural axons are the Eph family of receptor tyrosine kinases and ligands (Tessier-Lavigne and Goodman, 1996). *L4* is a ligand of this family that

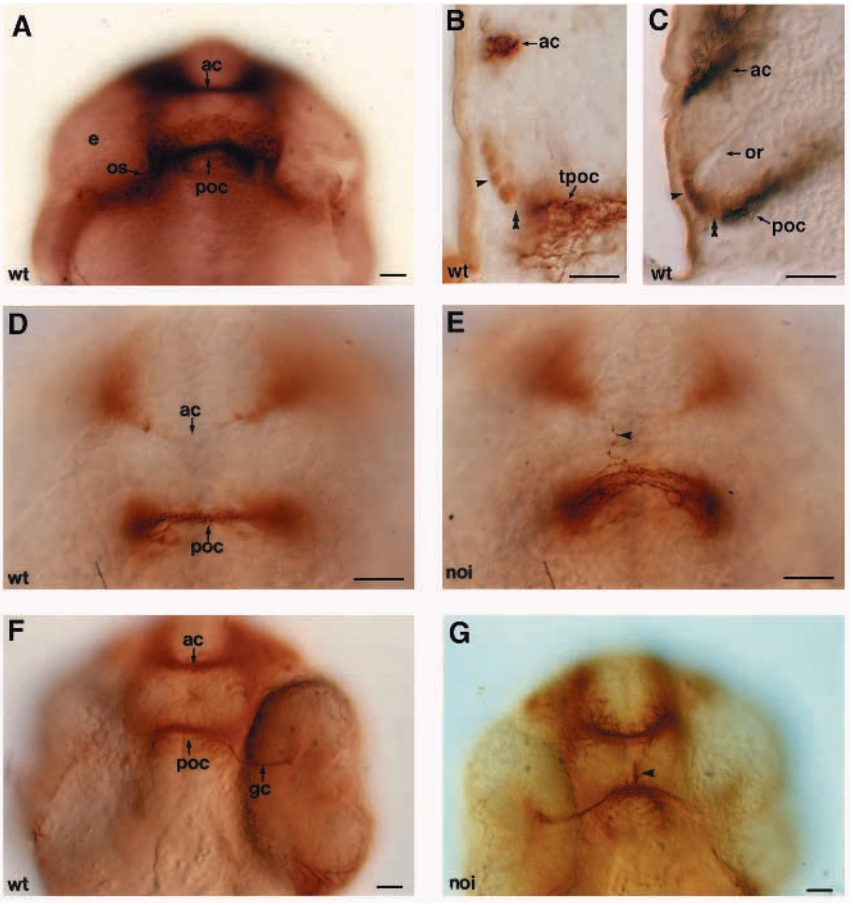
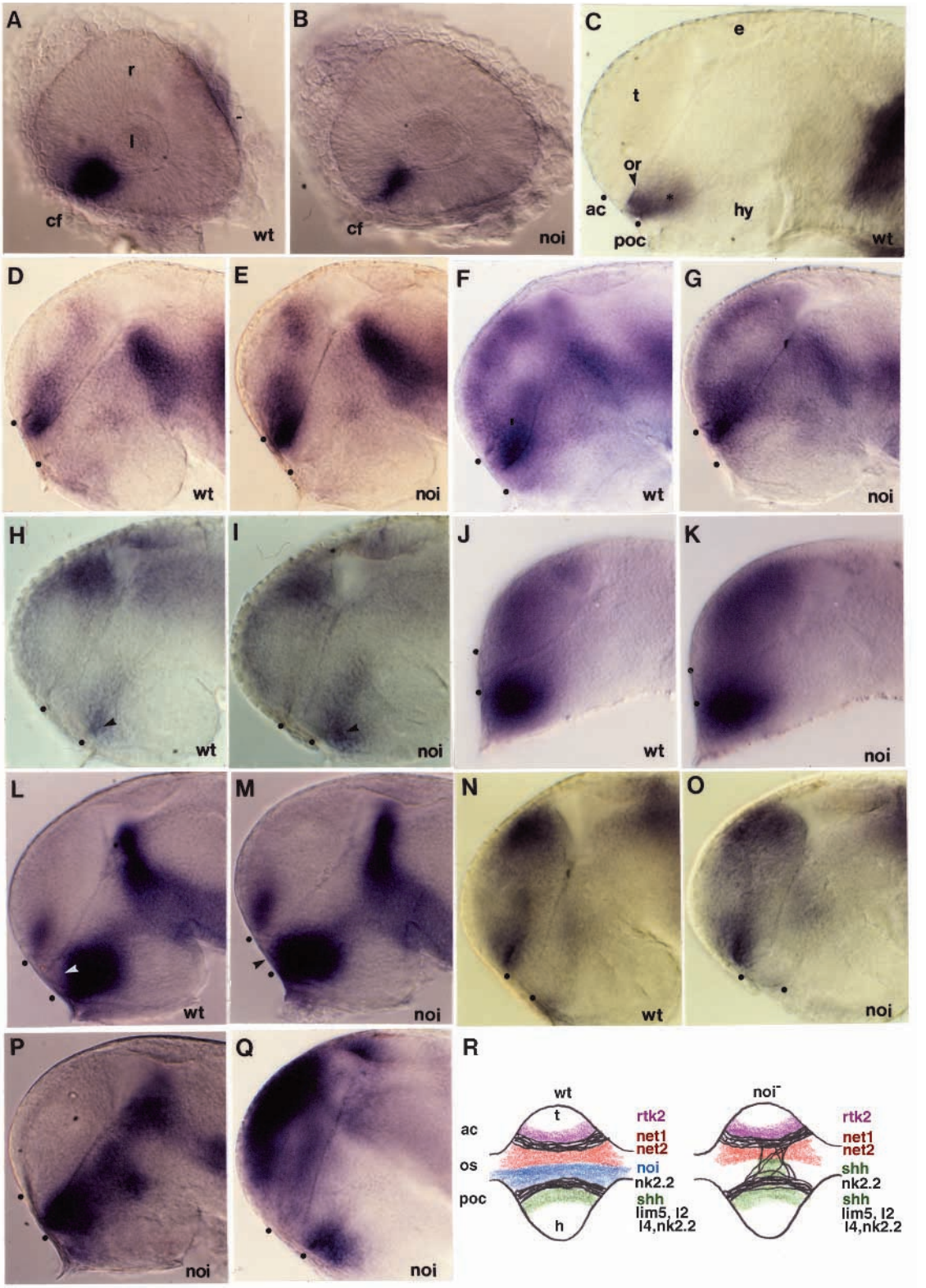


Fig. 6. Axons in the postoptic commissure show pathfinding defects in *noi*$^{-}$ embryos. Frontal/ventral views of whole embryos (except B,C) labelled with anti-tubulin antibody (all panels) and anti-Noi antibody (A-C). (A-C) Frontal view (A) and parasagittal 10 μm sections (B,C) of prim10 embryos. The panel shown in C is closer to the midline than B. Noi^{+} cells (arrowheads) are adjacent to axons in the TPOC and POC. There may be a row of Noi^{-} cells between the domain of Noi expression and the axons (double arrowheads). (D-G) Ventral views of wild-type and *noi*$^{-}$ embryos at prim5 (D,E) and prim20 (F,G) stages. The POC is a tight fascicle in wild-type embryos (D,F) whereas it is less fasciculated and axons aberrantly extends towards the anterior commissure in *noi*$^{-}$ mutants (arrowheads in E,G). Abbreviations: ac, anterior commissure; e, eye; gc, ganglion cell axons; or, optic recess; os, optic stalk; poc, tpoc, postoptic commissure and its tract. Scale bars, 25 μm; except B, 20 μm.



is expressed in a gradient in the tectum and a domain that borders the POC/optic chiasm on the opposite (ventral) side to Noi (Fig. 7H and Brennan et al., 1997). Examination of *L4* expression in wild-type and *noi*$^{-}$ embryos showed that its hypothalamic expression domain was retained in mutant embryos (Fig. 7I). *L2* is a related ligand (see Experimental Procedures) expressed in the eye and midbrain, and in a broader, more diffuse domain ventral to the POC (Fig. 7J). The domain of expression of *L2* ventral to the POC appeared to be unaffected in *noi*$^{-}$ embryos (Fig. 7K). We also failed to detect any ectopic midline expression of Eph receptors *rtk1*, *rtk2*, *rtk6* and *rtk7* (Fig. 7N,O and data not shown; Cooke et al., 1997). For

Fig. 7. *netrin* and *shh* expression is altered in *noi*⁻ embryos. Lateral views of eyes (A,B) and whole brains of wild-type and *noi*⁻ embryos hybridised with various digoxigenin-labelled probes to genes expressed at the midline. The positions of the commissures indicated in this figure are invariant between embryos and by focussing up and down through the brain of whole embryos, it is possible to unambiguously determine the position of the expression domains with respect to the commissural axons (which are visible using DIC optics) although this relationship is less obvious on photographs taken at a single focal plane. (A,B) *net2* expression in the choroid fissure. Expression is greatly reduced in the *noi*⁻ embryo. (C) *noi* expression in midline cells between the optic recess and the POC. The out of focus staining (asterisk) is in *noi*+ cells lateral to the midline. In all subsequent panels, the positions of the anterior commissure and POC are indicated by dots. (D,E) *net2* expression in cells between the optic recess and anterior commissure. (F,G) *net1* expression in cells between the optic recess and anterior commissure. (H,I) *L4* expression (arrowheads) in cells ventral to the POC. (J,K) *L2* expression in cells ventral to the POC. The embryos shown in this panel are around prim-5 stage. *L2* expression is still present at prim15-25 stages but is weaker and slightly more diffuse. (L,M) *shh* expression. In the wild-type embryo, *shh* expression is excluded from the midline cells between the optic recess and the POC (arrowhead) that express *noi* (compare to C). In the *noi*⁻ embryo, these cells ectopically express *shh* (arrowhead). (N,O) *rtk2* expression dorsal to the anterior commissure. (P) *nk2.2* expression in cells between the optic recess and the POC and ventral to the POC in a *noi*⁻ embryo. This pattern of expression is similar to wild type (see Barth and Wilson, 1995). (Q) *lim5* expression in cells ventral to the POC in a *noi*⁻ embryo. The pattern of expression is similar to wild type in cells ventral to the the POC. (R) Schematic simplified representations of frontal views (as in Fig. 6A) of gene expression domains and commissural pathway formation in the rostral forebrain of prim10-20 stage wild type and *noi*⁻ embryos. The domain of Noi expression dorsal to the POC is shown in blue. The domain of *netrin* gene expression is shown in red, the domain of *rtk2* expression in purple and the domain of *shh* expression in green. Other genes expressed in these same territories are indicated to the right of the drawings. In the *noi*⁻ embryo, Noi expression is absent, *shh* expression extends dorsally and midline expression of *netrin* is unaffected. Abbreviations: ac, anterior commissure; cf, choroid fissure; e, epiphysis; hy, hypothalamus; l, lens; or, optic recess; os, optic stalk; poc, postoptic commissure; r, retina; t, telencephalon.

several genes including *L2* (at stages later than shown in Fig. 7), *L4* and the *netrins*, labelling intensity of expressing cells adjacent to the POC was higher in *noi*⁻ embryos than in wild-type embryos (for instance compare Fig. 7D to E and H to I) although we do not know if this reflects true differences in levels of expression.

The secreted protein Shh is expressed in cells directly ventral to the POC such that the dorsal boundary of *shh* delineates the pathway of axons across the midline (Barth and Wilson, 1995 and Fig. 7L). In *noi*⁻ embryos, *shh* is abnormally expressed in the cells between the optic recess and the POC that would normally contain Noi (compare Fig. 7M to L and C). The ectopic expression is restricted to midline cells and no *shh* expression is detected further laterally in the optic stalks. Thus the POC axons in *noi*⁻ embryos are misrouted at the position at which they encounter ectopic *shh* expression. The abnormal domain of *shh* expression could either arise through ectopic expression of the gene in midline cells or through abnormal morphogenetic movement of *shh*-expressing cells at the midline. We favour the first possibility for two reasons. First, in *noi*⁻ embryos in which *noi* RNA was detectable by in situ hybridisation, we observed that *noi*⁻-expressing cells are still present across the midline (data not shown). Second, if midline morphogenesis was disrupted, we would have expected to observe comparable alterations in the domains of *L4*, *L2* and *lim5* (see below) expression.

Finally, we examined the expression of two transcription factors that have been suggested to be involved in neuronal/axonal patterning in the forebrain. *nk2.2* is expressed at the midline and in the proximal optic stalks, and overlaps with *shh* in the rostral hypothalamus (Barth and Wilson, 1995). Although Shh has been shown to spatially regulate *nk2.2* expression and *shh* expression is altered in *noi*⁻ embryos, no obvious alterations in the midline domain of *nk2.2* expression were observed (Fig. 7P). *lim5* is expressed in cells just ventral to the POC (Toyama et al., 1995) and expression of this gene is disrupted in several other zebrafish lines that exhibit retinotectal pathfinding defects (Rolf Karlstrom, personal communication). As with *nk2.2*, we did not detect any obvious ectopic expression of *lim5* in *noi*⁻ embryos (Fig. 7Q).

DISCUSSION

Many studies have sought to characterise the cellular environment that influences retinal axon guidance at the optic chiasm, and neurons, other axons and primitive glial cells have all been implicated in this process (Guillery et al., 1995). We show here that the Pax protein Noi is required in primitive glial cells for guidance of both optic and non-optic axons across the midline in the postoptic commissure and chiasm.

Noi function in the developing retina

The cells that express Noi in the developing choroid fissure almost certainly correspond to a previously described population of glial cells upon which retinal axons converge into a single fascicle as they leave the eye (Bodick and Levinthal, 1980). This process is disturbed in *noi*⁻ embryos in that axons frequently fail to coalesce at the optic nerve head and consequently leave the eye as several discrete fascicles along the open choroid fissure. We have shown that *netrin* expression around the choroid fissure is severely reduced in *noi*⁻ embryos, raising the possibility that proteins of this family may be involved in guiding axons out of the eye and onto the optic stalk. Consistent with a role for Netrins in regulating retinal axon extension, it has recently been shown that Netrin-1 promotes the outgrowth of mouse retinal ganglion cell axons (Wang et al., 1996). Although optic axons often take inappropriate trajectories out of the eye in *noi*⁻ embryos, they nevertheless still navigate towards the choroid fissure. Recent organ culture studies suggest that retinal axons may be guided within the retina independent of long-range chemotropic activity from the optic nerve head (Halfter, 1996). Furthermore, Wang et al. (1996) failed to find any long range chemotropic activity of Netrin-1 for mouse retinal axons.

It has now been shown that the choroid fissure fails to close in *noi*⁻ fish as well as humans and mice carrying mutations or targeted deletion of *Pax-2* (Sanyanusin et al., 1995; Torres et al., 1996; Favor et al., 1996) indicating that Pax gene function in this process is highly conserved. The closure of the choroid fissure presumably involves formation of cell-cell junctions

between Noi$^+$ cells lining the nasal and temporal sides of the fissure. Such affinity between Noi$^+$ cells is consistent with our observations that these cells form a confluent layer of tightly apposed glial cells on the surface of the nerve. To our knowledge, nothing is yet known about any other molecules that mediate fusion of the choroid fissure and might therefore be targets for regulation by Noi/Pax-2.

Noi is required for glial cell differentiation in the optic nerve

Noi$^+$ cells in the mature zebrafish optic nerve are reticular astrocytes, a form of glial cell with epithelial characteristics that are restricted to the optic nerves of teleost fish (Maggs and Scholes, 1986; 1990; Markl and Franke, 1988; Giordano et al., 1989). As similar cytokeratin$^+$ cells are present throughout the CNS in cyclostomes (Merrick et al., 1995), they may be a primitive form of glial cell. The continuous presence of Noi$^+$ cells during development suggests that the reticular astrocytes originate from the neuroepithelial cells of the optic stalk. The optic stalk cells first transform into a thin glia limitans surrounding the optic nerve, before developing strong cytokeratin antigenicity during later maturation when the optic fibres become myelinated. The mature reticular astrocytes appear to act morphogenetically to form the folded ribbon structure of the optic nerve in fish by tightly enclosing successive cohorts of new retinal axons during growth (see also Maggs and Scholes, 1990). The initial steps of astrocyte differentiation failed in *noi*$^-$ embryos, leaving disorganised clumps of neuroepithelial-like cells surrounding an otherwise well-formed unmyelinated optic fibre array.

The presence of Pax-2$^+$ cells in mammals (Nornes et al., 1990; Püschel et al., 1992) indicates that the immature optic stalk glia of mammals are likely to initially develop similarly to those in fish. However, preliminary data showing that Pax-2 immunoreactivity is lost postnatally in the mouse optic nerve (J. S., unpublished observations) suggests that fish and mammalian glial cells either express different proteins as they differentiate or that the Pax-2$^+$ primitive glia are replaced by other GFAP$^+$ astrocytes during mammalian optic nerve maturation.

Noi and midline axon guidance through the POC

One of the most striking aspects of the *noi*$^-$ phenotype is the severe disruption of axons at the midline in the POC and optic chiasm. In the zebrafish forebrain and midbrain, there are initially only four locations at which axons cross the midline: the POC and the anterior, posterior and ventral tegmental commissures (Chitnis and Kuwada, 1990; Wilson et al., 1990). Each of these commissures is established by a small number of axons that traverse the neuroepithelial midline at invariant locations and for three of the four commissures, their location coincides with, or is adjacent to, boundaries of regulatory gene expression domains within the neuroepithelium (Macdonald et al., 1994; Barth et al., 1995; Fig. 7R). How then, might these boundaries affect commissure formation? We suggest that transcription factors expressed within each of the domains adjacent to the commissure may regulate the expression of different combinations of cell surface and/or secreted proteins that discourage growth cone exploratory behaviour. In this way, decussating axons would be funnelled across the midline as a tight fascicle at the interface between the two domains (Fig. 7R). Indeed, analysis of some of the earliest axons to pass through the POC has shown that the commissural axons become more tightly fasciculated and their growth cones much less exploratory as they approach the midline (Wilson and Easter, 1991).

Noi is expressed in cells dorsal to the POC and we suggest that one of its functions may be to regulate proteins that make the domain of Noi expression unfavourable to growth cone exploration. In support of this possibility, early non-optic axon pathways are established rostral, dorsal and ventrocaudal to the optic stalks, but growth cones do not extend onto the stalks or the midline tissue between them (Chitnis and Kuwada, 1990; Wilson et al., 1990). On the opposite, ventral, side of the POC, cells express L2 and L4, members of a family of Eph ligands that have been shown to repel advancing growth cones (Tessier-Lavigne and Goodman, 1996; Brennan et al., 1997). Thus, L2 and L4 may prevent commissural axons from wandering ventrally into the hypothalamus, in the same way that we propose Noi$^+$ cells may normally discourage migration dorsally towards the anterior commissure.

In *noi*$^-$ embryos, POC axons wander dorsally towards the anterior commissure but were never observed to project ventrally into the hypothalamus, consistent with *L2* and *L4* expression being maintained in cells ventral to the commissure. In contrast to *L2* and *L4*, *shh* expression no longer retains its expression boundary ventral to the POC and spreads into midline cells between the optic stalks in *noi*$^-$ embryos. Thus the majority of axon pathfinding errors occur where growth cones encounter cells ectopically expressing *shh*. Given that there are no reports of Shh directly influencing growth cone behaviour, it is perhaps unlikely that Shh itself contributes to the pathfinding errors. However, Shh is a potent signalling protein and so it is possible that there may be abnormal expression of other, as yet unidentified, guidance molecules in response to ectopic Shh at the midline. The observation that *shh* expression is expanded in the absence of Noi suggests that, while Shh may promote *noi* expression (Ekker et al., 1995; Macdonald et al., 1995), Noi activity may, conversely, limit *shh* expression. If so, this may have the effect of sharpening the expression boundaries between *shh* and *noi* either side of the POC.

Finally, as Netrins have chemorepulsive activities for certain classes of axons (Tessier-Lavigne and Goodman, 1996) and both *net1* and *net2* are expressed in axon-free territory between the anterior commissure and the POC, it is possible that Netrins may discourage axons from entering this territory. However, we favour the alternative possibility that Netrins are involved in promoting growth towards the midline while other proteins direct POC and retinal axons contralaterally. Such a scenario is more consistent with analysis of *noi*$^-$ embryos in which *netrin* expression is maintained at the midline and axons still approach the commissure. Furthermore, those axons that do have abnormal trajectories in *noi*$^-$ embryos frequently navigate into the domain of *netrin* expression between the optic recess and the anterior commissure.

Noi and retinal axon guidance

Retinal axons arrive at the chiasm about 12 hours after the first POC axons have traversed the midline. The most parsimonious explanation for the retinal axon pathfinding defects in *noi*$^-$ embryos is that optic axons navigate along the misdirected

POC axons. However, we do not favour this interpretation as only a minority of misdirected retinal axons select a rostral trajectory towards the anterior commissure thereby 'following' the POC axons. More frequently aberrant axons were detected in the ipsilateral optic tract or contralateral optic nerve. The reason that optic axons do not select these pathways in wild-type embryos may be because they do not usually have access to them. DiI labelling and electron microscopic analysis of axons at the midline have shown that axons from the two eyes and axons of the POC are segregated in wild-type embryos (this study; Burrill and Easter, 1995; Marcus and Easter, 1995). This segregation appears to be lost in *noi*$^{-}$ embryos in that the optic axons from the two eyes are not separated at the midline. Therefore, the Noi^{+} cells may normally funnel retinal axons straight across the midline, preventing them from having access to the substrata offered by axons from the opposite eye or the ipsilateral optic tract.

Analysis of mice carrying a targeted deletion of the *Pax-2* gene (Torres et al., 1996) has revealed potential differences between the functions of Pax-2^{+} cells in the mouse visual system and Noi^{+} cells in the fish. In the mouse, it is suggested that just prior to outgrowth of retinal axons, Pax-2^{+} cells in the optic stalks intercalate across the midline, splitting the domain of *shh* expression into two territories and creating a favourable substratum at the midline for retinal axons to form the optic chiasm. In the absence of *Pax-2*, it has been proposed that the optic stalk cells do not intercalate across the midline leading to a deletion of the optic recess area and consequent inability of optic axons to form an optic chiasm. In fish, the domain of *shh* does not become subdivided into two territories by Noi^{+} cells (which are present across the midline long before optic axons decussate) and gene expression analysis in *noi*$^{-}$ embryos suggests that the optic recess area is still present.

In mice it is proposed that Pax-2^{+} cells offer a favourable substratum for retinal axons (Torres et al., 1996) and in fish, in contrast to the POC axons, retinal axons do extend upon the Noi^{+} cells. It is possible that Noi^{+} cells could discourage invasion by POC axons yet later provide a favourable substratum for the retinal axons. However, although the optic axons extend along the Noi^{+} cells, this does not necessarily imply that the surface of these cells offers a preferred substratum for axonal extension. The optic axons form a tight fascicle along the stalk and at the midline suggesting that axons prefer to extend among their neighbours than upon the Noi^{+} primitive glial cells of the optic stalk. Furthermore, once at the midline, optic axons invariably select pathways within the diencephalon rather than remaining on the Noi^{+} cells.

While further analysis will be necessary to determine if significant differences between the midline phenotypes of *Pax-2*$^{-}$ mice and *noi*$^{-}$ fish do exist, there are indeed major differences in the optic chiasmata between these species. In fish, the optic fibres cross the midline of the diencephalon in separate channels lined by immature glial cells (Marcus and Easter, 1995). During optic nerve maturation, the primitive astrocytes in each optic nerve form their own glia limitans, thereby freeing each optic nerve from the diencephalic midline so that each nerve joins the brain contralaterally. In mammals, by contrast, the chiasm remains embedded in the ventral surface of the brain, enabling optic fibres from the two eyes to mingle and form the binocular decussation. In the future, it will be important to determine the identity of Pax2^{+}/Noi^{+} cells in species other than fish to extend comparative analytical studies of midline axon guidance in fish, frogs, chicks and mice in order to fully understand the role of primitive glial cells in axonal migration across the midline.

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REFERENCES

Baier, H., Klostermann, S., Trowe, T., Karlstrom, R. O., Nusslein-Volhard, C. and Bonhoeffer, F. (1996). Genetic dissection of the retinotectal projection. *Development* **123**, 415-425.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish *nk2.2* is influenced by *sonic hedgehog/vertebrate hedgehog-1* and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Bodick, N. and Levinthal, C. (1980). Growing optic nerve fibers follow neighbors during embryogenesis. *Proc. Natl. Acad. Sci.* **77**, 4374-4378.

Brand, M., Heisenberg, C.-P., Jiang, Y.-J., Beuchle, D., Lun, K., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D., Kelsh, R., Mullins, M., Odenthal, J., van Eeden, F. J. M. and Nusslein-Volhard, C. (1996). Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain. *Development* **123**, 179-190.

Brennan, C., Monschau, B., Lindberg, R., Guthrie, B., Drescher, U., Bonhoeffer, F. and Holder, N. (1997). Two Eph receptor tyrosine kinase ligands control axon growth and may be involved in the creation of the retinotectal map in the zebrafish. *Development* **124**, 655-664.

Burrill, J. D. and Easter, S. S., Jr. (1994). Development of the retinofugal projections in the embryonic and larval zebrafish (Brachydanio rerio). *J. Comp. Neurol.* **346**, 583-600.

Burrill, J. D. and Easter, S. S., Jr. (1995). The first retinal axons and their microenvironment in Zebrafish: cryptic pioneers and the pretract. *J. Neurosci.* **15**, 2935-2947.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P. and Gruss, P. (1993). Pax: gene regulators in the developing nervous system. *J. Neurobiol.* **24**, 1367-1384.

Chitnis, A. B. and Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10**, 1892-1905.

Cooke, J. E., Xu, Q., Wilson, S. W. and Holder, N. (1997). Characterisation of five novel zebrafish Eph-related receptor tyrosine kinases suggests roles in patterning the neural plate. *Dev, Genes & Evol.* in press,

Cornell, E. and Holt, C. (1992). Precocious pathfinding: retinal axons can navigate in an axonless brain. *Neuron* **9**, 1001-1011.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate *hedgehog* proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Favor, J., Sandulache, R., Neuhäuser-Klaus, A., Pretsch, W., Chatterjee, B., Senft, E., Wurstr, W., Blanquet, V., Grimes, P., Spörle, R. and Schughart, K. (1996). The mouse *Pax2*^{1new} mutation is identical to a human *PAX2* mutation in a family with renal-coloboma syndrome and results in developmental detects of the brain, ear, eye and kidney. *Proc. Natl. Acad. Sci. USA* **93**, 13870-13875.

Giordano, S., Glasgow, E., Tesser, P. and Schecter, N. (1989). A type II keratin is expressed in glial cells of the goldfish visual pathway. *Neuron* **2**, 1507-1516.

Guillery, R. W., Mason, C. A. and Taylor, J. S. H. (1995). Developmental determinants at the mammalian optic chiasm. *J. Neurosci.* **15**, 4727-4737.

Halfter, W. (1996). Intraretinal grafting reveals growth requirements and guidance cues for optic axons in the developing avian retina. *Dev. Biol.* **177**, 160-177.

Karlstrom, R. O., Trowe, T., Klostermann, S., Baier, H., Brand, M.,

Crawford, A. D., Grunewald, B., Haffter, P., Hoffmann, H., Meyer, S. U., Muller, B. K., Richter, S., van Eeden, F. J. M., Nusslein-Volhard, C. and Bonhoeffer, F. (1996). Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123**, 427-438.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S.-Y., Culotti, J. G. and Tessier-Lavigne, M. (1996). *Deleted in colorectal cancer* (*DCC*) encodes a netrin receptor. *Cell* **87**, 175-185.

Keller, S. A., Jones, J. M., Boyle, A., Barrow, L. L., Killen, P. D., Green, D. G., Kapousta, N. V., Hitchcock, P. F., Swank, R. T. and Meisler, M. H. (1994). Kidney and retinal defects (*Krd*), a transgene-induced mutation with a deletion of mouse chromosome 19 that includes the *Pax2* locus. *Genomics* **23**, 309-320.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425-435.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991). Expression of the zebrafish paired box gene *pax[zf-b]* during early neurogenesis. *Development* **113**, 1193-1206.

Larison, K. D. and Bremiller, R. (1990). Early onset of phenotype and cell patterning in the embryonic zebrafish retina. *Development* **109**, 567-576.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121**, 3267-3278.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. W. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13**, 1039-1053.

Maggs, A. and Scholes, J. (1986). Glial domains and nerve fiber patterns in the fish retinotectal pathway. *J. Neurosci.* **6**, 424-438.

Maggs, A. and Scholes, J. (1990). Reticular astrocytes in the fish optic nerve: macroglia with epithelial characteristics form an axially repeated lacework pattern, to which nodes of Ranvier are apposed. *J. Neurosci.* **10**, 1600-1614.

Marcus, R. C., Blazeski, R., Godement, P. and Mason, C. A. (1995). Retinal axon divergence in the optic chiasm: uncrossed axons diverge from crossed axons within a midline glial specialization. *J. Neurosci.* **15**, 3716-3729.

Marcus, R. C. and Easter, S. S., Jr. (1995). Expression of glial fibrillary acidic protein and its relation to tract formation in embryonic zebrafish (*Danio rerio*). *J. Comp. Neurol.* **359**, 365-381.

Marcus, R. C. and Mason, C. A. (1995). The first retinal axon growth in the mouse optic chiasm: axon patterning and the cellular environment. *J. Neurosci.* **15**, 6389-6402.

Markl, J. and Franke, W. W. (1988). Localization of cytokeratins in tissues of the rainbow trout: fundamental differences in expression pattern between fish and higher vertebrates. *Differentiation* **39**, 97-122.

Merrick, S. E., Pleasure, S. J., Lurie, D. I., Pijak, D. S., Selzer, M. E. and Lee, V. M. (1995). Glial cells of the lamprey nervous system contain keratin-like proteins. *J. Comp. Neurol.* **355**, 199-210.

Mikkola, I., Fjose, A., Kuwada, J. Y., Wilson, S., Guddal, P. H. and Krauss, S. (1992). The paired domain-containing nuclear factor *pax*[b] is expressed in specific commissural interneurons in zebrafish embryos. *J. Neurobiol.* **23**, 933-946.

Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U. and Gruss, P. (1990). Spatially and temporally restricted expression of *Pax2* during murine neurogenesis. *Development* **109**, 797-809.

Püschel, A. W., Westerfield, M. and Dressler, G. R. (1992). Comparative analysis of Pax-2 protein distributions during neurulation in mice and zebrafish. *Mech. Dev.* **38**, 197-208.

Sanyanusin, P., Schimmenti, C. A., McNoe, L. A., Ward, T. A., Pierpont, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the *PAX2* gene in a family with optic nerve colobomas, renal anomalies and vesicoureteral reflux. *Nat. Genet.* **9**, 358-364.

Strähle, U., Fischer, N. and Blader, P. (1997). Expression and regulation of a *netrin* homologue in the zebrafish embryo. *Mech. Dev.* **62** 147-160.

Stretavan, D. W., Pure, E., Siegel, M. W. and Reichardt, L. F. (1995). Disruption of retinal axon ingrowth by ablation of embryonic mouse optic chiasm neurons. *Science* **269**, 98-101.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

Torres, M., Gomez-Pardo, E. and Gruss, P. (1996). *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-3391.

Toyama, R., Curtiss, P. E., Otani, H., Kimura, M., Dawid, I. B. and Taira, M. (1995). The LIM class homeobox gene *lim5*: inplied role in CNS patterning in *Xenopus* and zebrafish. *Dev. Biol.* **170**, 583-593.

Trowe, T., Klostermann, S., Baier, H., Granato, M., Crawford, A. D., Grunewald, B., Hoffmann, H., Karlstrom, R. O., Meyer, S. U., Muller, B., Richter, S., Nusslein-Volhard, C. and Bonhoeffer, F. 0. (1996). Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, Danio rerio. *Development* **123**, 439-45.

Wang, L.-C., Dani, J., Godement, P., Marcus, R. C. and Mason, C. A. (1995). Crossed and uncrossed retinal axons respond differently to cells of the optic chiasm midline in vitro. *Neuron* **15**, 1349-1364.

Wang, L.-C., Rachel, R. A., Marcus, R. C. and Mason, C. A. (1996). Chemosuppression of retinal axon growth by the mouse optic chiasm. *Neuron* **17**, 849-862.

Wilson, S. W. and Easter, S. S. J. (1991). Stereotyped pathfinding by growth cones of early epiphysial neurons in the embryonic zebrafish epiphysis. *Development* **112**, 723-746.

Wilson, S. W., Ross, L. S., Parrett, T. and Easter, S. S., Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. *Development* **108**, 121-14.

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