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Two Eph receptor tyrosine kinase ligands control axon growth and may be involved in the creation of the retinotectal map in the zebrafish

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SUMMARY

The isolation and characterisation of two zebrafish Eph receptor ligand cDNAs which we have called zfEphL3 and zfEphL4 is described. These genes are expressed in the presumptive midbrain of developing embryos from 6 somites. By 24 hours L3 is expressed throughout the midbrain including the region of the presumptive tectum whereas L4 is strongly expressed in the midbrain caudal to the presumptive tectum. At later stages of development L3 is expressed in a graded fashion throughout the tectum and L4 is maintained at its posterior margin. Growth cone collapse and pathway selection assays demonstrate that both these proteins have a collapse activity for retinal ganglion cells. When faced with a choice of substrate on which to grow, temporal axons from chick retinal ganglion cells selectively avoided membranes from Cos cells transfected with L3, whereas nasal axons did not. Both temporal and nasal axons avoided membranes from Cos cells transfected with L4. The expression patterns together with the functional data suggest that although both ligands may be able to guide retinal ganglion cells axons in vitro, they have different roles in the guidance of retinotectal projections in vivo. The expression of L3 is consistent with a role in the guidance of retinal ganglion cells to their targets on the tectum whereas that of L4 suggests a role in delineating the posterior boundary of the optic tectum.

Key words: Eph ligands, retinotectal map, zebrafish, axon guidence

INTRODUCTION

During neural development axons make precise connections with target cells. In the vertebrate brain the most favoured system for studying how such connections are made has been that of the retinotectal projection in which axons from the nasal retina project to the posterior tectum, axons from temporal retina project to the anterior tectum, ventral axons project to dorsal tectum and dorsal axons to ventral tectum, thus making a precise topographic map. In the search for key proteins which could regulate the formation of such a map, a number of families of proteins, including the netrins (Kennedy and Tessier-Lavigne, 1995) and semaphorins (Kolodkin, 1996) have been shown to play a role in controlling axon guidance. Most recently, the Eph receptor tyrosine kinases (Van der Geer et al., 1994) and their ligands (Pandy et al., 1995) have also been implicated in the control of axon outgrowth and targeting within the retinotectal system (Drescher et al., 1995; Cheng et al., 1995). Drescher et al. (1995) have isolated a 25 kD protein from chick posterior tectal membranes, which shows significant homology to the B61-like ligands for the Eph family and which in in vitro experiments has the properties of an inhibitory guidance protein for retinal ganglion cell growth cones. They have called this protein RAGS for repulsive axon guidance signal. In situ hybridisations with RAGS show it to be expressed in a graded fashion across the tectum being more strongly expressed towards the posterior pole. Similarly, in mouse and chick, Cheng and Flanagan (1994) and Cheng et al. (1995) have shown that the Eph ligand elf-1 is expressed in a gradient across the tectum again being more strongly expressed at the posterior pole and that misexpression of this protein in the tectum leads to errors in retinal ganglion cell axon targeting (Nakamoto et al., 1996). A potential elf-1 receptor, MEK-4, is itself expressed in a graded manner across the chick retina with the highest expression at its temporal pole. These findings suggest that these ligands and their receptors may act in concert to guide retinal ganglion cells to their targets on the tectum. As suggested by Cheng et al. (1995), if elf-1 is capable of guiding retinal ganglion cells the existence of complementary gradients of the receptor within the projection neurons of the retina and the ligand within the target region fulfills Sperry's criteria for the mechanism of axon guidance and the formation of topographic maps (Sperry, 1963).

We have been studying the role of Eph receptor signalling in different aspects of zebrafish development (Xu et al., 1994; Macdonald et al., 1994, 1995; Xu et al., 1995, 1996). The

zebrafish shows various advantages for the study of developmental neurobiological problems because of its rapid development, its relatively simple nervous system – including the presence of identified neurons, and its transparency (Eisen, 1991). The retinotectal connection has been well characterised and there is evidence that the growing retinal ganglion cell axons show growth characteristics upon the tectum consistent with the presence of a specific axonal growth control mechanism (Stuermer, 1988; Kaethner and Stuermer, 1992; Burrill and Easter, 1994). In this study we report the isolation and characterisation of zebrafish homologues of Eph receptor ligands which we designate ZfEphLX. The two ligands reported are possibly the homologues of Elf-1 (ZfEphL3) and RAGS (ZfEphL4) and their expression is described over the first 3 days of development. Their expression in the midbrain, together with the results of in vitro functional assays, is consistent with a role in guiding retinal ganglion cells to their targets on the tectum and in preventing axons entering the caudal midbrain. ZfEphL3 shows a graded pattern of expression across the midbrain, being more strongly expressed at the posterior pole. ZfEphL4 is expressed at the caudal margin of the tectum and the posterior midbrain. Both ligands show growth cone collapse activity when presented to growing chick retinal ganglion cell axons. Furthermore, when faced with a choice of substrate on which to grow, temporal axons from chick retinal ganglion cells selectively avoided L3 transfected membranes whereas nasal axons did not. Axons from both nasal and temporal retina avoided membranes from Cos cells transfected with L4. The widespread expression of these ligands and their receptors elsewhere in the embryo suggest that they may play a role in axon guidance in other regions of the developing nervous system although other roles in cell specification are also possible (Xu et al., 1995).

MATERIALS AND METHODS

Isolation of zebrafish B61 like ligands by degenerate PCR

Degenerate primers designed to conserved regions (sense sequence VFWSSN and antisense sequence EKFQLFT) within the sequences of B61 and AL-1 (Bartley et al., 1994) were used in polymerase chain reactions to amplify fragments from a neurula stage zebrafish library. 1 μl of a 1/5 dilution of the library was used in a 50 μl reaction together with 150 pmole of each of the sense and antisense primer. The annealing temperature used was 48°C. The PCR fragments were cloned into pKS bluescript, sequenced and those fragments showing homology to Eph family ligands, of which there were four, used to screen the neurula stage library. 0.5×10^6 plaques were screened at moderate stringency (65°C, 0.5× SSC) with each fragment in turn. Likely full length clones were sequenced as described previously (Fox et al., 1995). The sequences have been submitted to GenBank and have the following accession numbers: ZfEphL3, Y09668; ZfEphL4, Y09669.

In situ hybridisation on whole mounts

Whole-mount in situ hybridisation was performed according to the method of Xu et al. (1994).

Preparation of Cos cell membranes for collapse and stripe assays

Expression constructs were prepared for each of the ligands L1, L3 and L4 in the CS2+ vector. Kozak sequences (GGAGAGATGC, which was the L4 Kozak sequence) for these constructs were included by PCR in an attempt to ensure uniformity of expression within the cells. Thus all ligands had the same promoter, Kozak sequence and poly A tail. Cos 7 cells were transiently transfected with each of these ligands or with a CS2+β-gal construct using the calcium phosphate method. Transfection efficiencies were checked by β-gal reaction or by a chimeric receptor alkaline phosphatase assay (RAP assay; Cheng and Flanagan, 1994).

Cos cell membranes were prepared as previously described (Walter et al., 1987; Drescher et al., 1995). 48 hours after transfection Cos cells were washed two times in PBS and manually harvested with a rubber policeman, into 0.5 ml per plate of complete PBS (PBS plus 1.3 mM $CaCl_2$, 0.98 mM $MgCl_2$) plus protease inhibitors. Membranes were spun down and resuspended in 0.5 ml homogenisation buffer. All membranes from one treatment were pooled at this point. The membranes were manually homogenised by titurating through a 0.2 mm guage needle and then applied to a sucrose gradient consisting of 350 μl 50% sucrose (w/w) and 150 μl 5% sucrose (w/w). Gradients were spun for 10 minutes at 28,000 rpm, 4°C in a Beckmann ultracentrifuge. After centrifugation the membrane layer was removed, washed with PBS and resuspended in 1 ml of fresh complete PBS. Protein concentration was determined according to the method of Walter et al. (1987). The membranes were then aliquoted and either used fresh or flash frozen before storing at –70°C.

Collapse assay and stripe assay

The procedures used for the collapse and stripe assays were essentially the same as previously described (Cox et al., 1990; Drescher et al., 1995). For the collapse assay retinal explants were allowed to grow overnight on a poly-D-lysine and laminin-treated surface in F12 medium. Aliquots of sucrose membrane preparations of COS cells were pelleted (23100 ***g***, 8 minutes, 4°C) and resuspended in F12 cultue medium. After sonication on ice (twice for 15 seconds at 30 W; Branson sonicator) the working concentration was adjusted and a 200 μl membrane suspension was carefully applied to the retinal explants. Axonal growth cones were assessed using a charge coupled device (CCD) camera. By using a computer controlled scanning stage, 15 growth cones (8 of the temporal and 7 of the nasal explants) could be simultaneously observed in a single experiment by time-lapse photography. Pictures were taken under manual control every 2-5 minutes, starting about 15 minutes before and ending 30 minutes after application of the mebrane vesicles. Pictures were digitized and stored on disc. For analysis, the complete sequence was reloaded using the NIH Image 1.55 program.

The stripe assay experiments also followed previously published protocols (Walter et al., 1987) with the following modification. Before preparation of the membrane stripes, nucleopore filters were incubated in 20 g/ml laminin in Hanks medium for 2-3 hours at 37°C. Afterwards, filters were washed in Hanks medium and stored in the same medium until use. In the stripe assay experiments, in which mock transfected COS cell membranes were tested against various dilutions of RAGS containing membranes, both membrane types were diluted using untransfected Cos cells.

Whole-mount RAP in situs

Receptor alkaline phosphatase in situ reactions were performed on whole-mount embryos according to the method of Cheng and Flanagan (1994). A chick embryo kinase 4 receptor alkaline phosphatase (Cek4/AP) fusion protein was used to probe 24 hour zebrafish embryos. Embryos were incubated in CEK4/AP medium for 90 minutes then washed 6 times in HBHA (Hank's buffered saline plus 0.5 mg/ml BSA, 0.1% NaN_3, 20 mM Hepes, pH 7.0), treated for 2.5 minutes with acetone-formaldehyde fixative (60% acetone, 3% formaldehyde, 20 mM Hepes, pH 7.0), washed three times with HBS (150 mM NaCl, 20 mM Hepes, pH 7.0) and then incubated at 65°C for 15 minutes to inactivate endogenous cellular phosphatases. After rinsing in alkaline phosphatase reaction buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) the embryos were stained in the same buffer containing 0.17 mg/ml BCIP and 0.33 mg/ml NBT.

RESULTS

Isolation of L3 and L4

ZfEphL3 and ZfEphL4 were isolated from zebrafish embryonic cDNA by PCR using primers designed from conserved sequences of human Eph ligands B61 and AL-1 (Bartley et al., 1994). Full length cDNAs were subsequently isolated by screening a neurula stage library. Sequence analysis (Fig. 1) showed these proteins to be homologous to other members of the Eph ligand family (Pandey et al., 1995), each having conserved cysteines in the putative receptor binding region and a GPI linkage signal. L3 has highest homology (58% identical at the amino acid level) to Elf-1/Cek7 ligand (Cheng and Flanagan, 1994; Shao et al., 1995) and L4 highest homology (76% identical at the amino acid level) to RAGS/AL1 (Drescher et al., 1995; Winslow et al., 1995). These homologies increase to 69% and 86% respectively if one excludes the 5′ signal sequence and the signal for GPI linkage. A further ligand which is the likely zebrafish B61 homologue, which was termed L1, was also isolated (manuscript in preparation). This ligand was found not to be expressed within the CNS and was therefore used as a control for the purposes of the collapse and stripe assay experiments.

Expression of the ligands

The expression of both genes was assessed by in situ hybridisation in whole embryos.

(i) Expression of L3 and L4 up to the prim 5 stage (24 hours; Kimmel et al., 1995) in the CNS

L3. Transcripts for L3 first appear at the six-somite stage in a broad domain with borders in the forebrain and at the presumptive hindbrain/midbrain boundary. During development this initial L3 domain resolves into three stripes (compare Fig. 2A and 2B), by 20 hours the most anterior stripe is present in the caudal diencephalon, the middle stripe has expanded and is expressed throughout the midbrain (Fig. 2B) and the third stripe is in the anterior hindbrain. The positioning of these stripes was assessed by anatomical landmarks and by performing double in situs with *pax2* which is expressed in a stripe at the caudal midbrain and in the eyestalks (Krauss et al., 1991). In the double labelled specimens, at 15-24 hours the *pax2* stripe sits between the two caudal L3 stripes (Fig. 2B,C). At 24 hours L3 is also expressed in the hindbrain as narrow stripes at the centre of each rhombomere and in the otic vesicle (Fig. 2E). Expression is also evident at prim 5 (24 hours) in cells around the region of the otic vesicle (Fig. 4A). In the forebrain the initial stripe becomes more restricted to a region immediately beneath the forming epiphysis in the caudal/dorsal diencephalon (Fig. 2D).

L4. As for L3, L4 transcripts are first evident at the six-somite stage in a localised stripe of expression at the midbrain/hindbrain boundary region (Fig. 2F). In the early neural keel expression is also seen as a thin stripe in the caudal diencephalon and in the eye primordia (Fig. 2F) but not the eye stalks. Expression at the caudal midbrain is maintained and overlaps with the *pax2* domain (Fig. 2G), but expression in the eye is down regulated by 24 hours but is maintained in cells that will be nasal retinal ganglion cells (Fig. 2H). L4 can also be detected in the anterior hypothalamus (Fig. 2I) and within the otic vesicle at 24 hours (Fig. 2J).

(ii) Expression of L3 and L4 within the visual system

In order to determine if the ligands are expressed in cells of the visual system during the establishment of neuronal connectivity, we examined L3 and L4 expression both in the eye and the midbrain tectum. In the zebrafish the retinal ganglion cell axons first leave the retina at 32 hours and arrive at the tectum about 10 hours later (Stuermer, 1988). It is clear that both ligands are expressed in different regions of the visual system at stages earlier than the retinotectal projection is established. L3 is first expressed in the eye in the retinal ganglion cells at 30 hours. This expression is maintained in cells throughout the period of retinal ganglion cell growth (Fig. 3A). In contrast, L4 is initially broadly expressed in the eye primordium from 6 somite stage (Fig. 2F). By 24 hours this expression has become restricted to neuroepithelial cells in the nasal retina (Fig. 2H), a pattern that is maintained throughout the period of retinal ganglion cell axonal outgrowth (Fig. 3E).

Both L3 and L4 are expressed in the presumptive tectum prior to and during the period of innervation by retinal ganglion cells (Figs 2 and 3). At all time points examined the expression

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zf L3  .......MEL SLVVFTVVCW VSVWSDD..D RIISDRHAVY WNSSNSRFW.
 Elf1  ..MAPAQRPL LPLLLLLLPL RARNEDP..A RANADRYAVY WNRSNPRFQV
zf L4  .....MLQAE MIVFVGVILW MCVFSQEPSS KVMADRYAVF WNRTNPRFQ.
  Al1  .....MLHVE MLTLVFLVLW MCVFSQDPGS KAVADRYAVY WNSSNPRFQ.
  B61  .......... ....MEFLW. .APLLGLCCS LAAADRHTVF WNSSNPKFR.

zf L3  .....QGEYT VAVSINDYLD VYCPYYESPQ PHS.RMERYI LFMVNHDGYL
 Elf1  SAVGDGGGYT VEVSINDYLD IYCPHYGAPL PPAERMERYI LYMVNGEGHA
zf L4  .....RGDYH IDVCINDYLD VYCPHYEDSV P.EERTERYV LYMVNYDGYS
  Al1  .....RGDYH IDVCINDYLD VFCPHYEDSV P.EDKTERYV LYMVNFDGYS
  B61  .....NEDYT IHVQLNDYVD IICPHYEDHS VADAAMEQYI LYLVEHEEYQ

zf L3  TCEHRMRGFK RWECNRPQSP DGPLRFSEKF QLFTPFSLGF EFRPGHEYYY
 Elf1  SCDHRQRGFK RWECNRPAAP GGPLKFSEKF QLFTPFSLGF EFRPGHEYYY
zf L4  TCDHTAKGFK RWECNRPHSP NGPLKFSEKF QLFTPFSLGF EFRPGREYYY
  Al1  ACDHTSKGFK RWECNRPHSP NGPLKFSEKF QLFTPFSLGF EFRPGREYFY
  B61  LCQPQSKDQV RWQCNRPSAK HGPEKLSEKF QRFTPFTLGK EFKEGHSYYY

zf L3  ISSPHPNHAG KPCLKLKVYV KPTSS..... .......... ..........
 Elf1  ISATPPNLVD RPCLRLKVYV RPTNE..... .......... ..........
zf L4  ISSMITETGR RSCLKLKVFV RPPNGCEKTI GVHDRVF.VD DKVDNALEPR
  Al1  ISSAIPDNGR RSCLKLKVFV RPTNSCMKTI GVHDRVFDVN DKVENSLEPA
  B61  ISKPIHQHED R.CLRLKVTV SGKITHS... .......... ...PQAHVNP

zf L3  ..G.YESPEP F....LTDQS QRC..GADGP CLAVLMLLLV FLLAGV*
 Elf1  ..TLYEAPEP I....FTSNS SCS..GLGGC HLFLTTVPVL WSLLGS*
zf L4  DDTSHE.AEP S....RSDVS TSGLRHQTSR PLLALLLLCI SLYLLL*
  Al1  DDTVHESAEP S....RGENA AQTPR..IPS RLLAILLFLL AMLLTL*
  B61  QEKRLAADDP EV......RV LHSIGHSAAP RLFPLAWTVL LLPLLLLQTP*
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Fig. 1. Alignment of the full length protein sequences of zebrafish ligands L3 and L4 with that of B61, Elf-1 and Al 1. The signal sequence is overlined and the putative GPI linkage signal and attachment is underlined. The conserved residues are marked with a ° and the conserved cysteines are boxed.

of L3 extends further anteriorly than does that of L4. Thus at 24 hours L3 is expressed throughout much of the midbrain whereas L4 is restricted to its caudal margin. By 36 hours the expression of L3 is markedly graded across the region of the presumptive tectum (Fig. 3B). By 3 days expression of L3 can be detected throughout the dorsal midbrain except in the region of the most anterior optic tectum (Fig. 3C). This expression domain covers much of the forming tectal neuropil (Fig. 3C). L4 is still expressed at the caudal extent of the midbrain but by 3 days can be detected as far anteriorly as the caudal margins of the optic tectum (Fig. 3F).

Expression of L3 and L4 elsewhere in the CNS

In order to assess whether L3 and L4 maybe involved in axon guidance in regions of the CNS other than the visual system we examined expression throughout the CNS. At the prim 5 stage (24 hours) L3 is expressed in the rhombomeres of the hindbrain. These bands of expression are in the middle of each segment (Figs 2E, 7C). Both ligands are also expressed by 24 hours in cells in the otic vesicle (Fig. 2E,J). L3 transcripts are also in mesenchymal cells around the otic vesicle at 24 hours (Fig. 4A) and subsequently, by 48 hours, in the branchial arches (Fig. 4B,C), making it very likely that these cells seen at early stages in the lateral hindbrain region are migrating neural crest.

Identification of sites of ligand protein using Cek4/AP binding

The in situ labelling of ligand following binding to a Cek 4 receptor-alkaline phosphatase fusion protein (RAP) is a

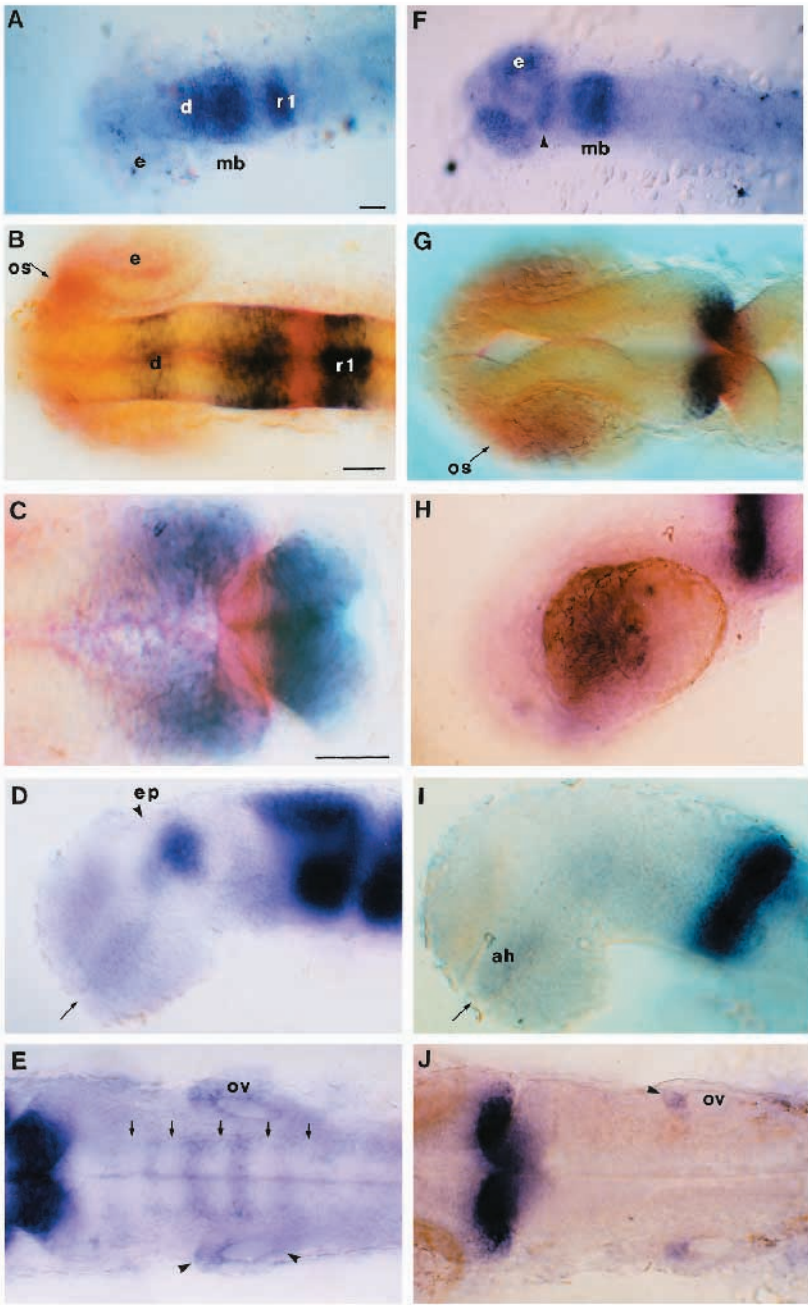

Fig. 2. Expression patterns of L3 (A-E) and L4 (F-J) in the developing brain assessed by in situ hybridisation. In all pictures anterior is to the left. A and B are dorsal views of 8- and 20-somite stage embryos showing the resolution of the initial expression pattern into three distinct stripes. The red colour in B is expression of *pax2* which marks the mid-hindbrain boundary and is also expressed in the eye stalks. (C) The boundary region at 24 hours at higher magnification illustrating expression of L3 in the anterior hindbrain and graded expression in the midbrain. (D) A lateral view of a 24 hour embryo showing the expression of L3 in the diencephalon ventral to the epiphysis (arrowhead) and in the telencephalon. The arrow marks the position of the postoptic commissure. (E) Expression of L3 in the hindbrain also occurs in the rhombomeres and the otic vesicles (arrowheads). The small arrows mark the positions of the rhombomere boundaries. (F,G) Dorsal views of 8- and 24-somite embryos showing the expression of L4 in the brain. In F transcripts are evident in the eyes and in the diencephalon and midbrain. However, by the 24-somite stage expression is reduced to the region immediately anterior to and possibly overlapping the *pax2* domain (G), which appears in red. (H) A lateral view of an embryo at 24 hours showing a limited amount of expression in the nasal half of the eye. (I) A lateral view of a 24-hour embryo with the eye removed. Expression is clear in the caudal midbrain but limited expression is also evident in the forebrain. The arrow marks the position of the postoptic commissure. (J) A dorsal view of the hindbrain region at 24 hours showing limited expression of L4 in the otic vesicle (arrowhead). e, eye; d, diencephalon; mb, midbrain rudiment; r1, rhombomere 1; os, optic stalk; ep, epiphysis; ov, otic vesicle; ah, anterior hypothalamus. Scale bars, 50 μm (A and F) (C) (B,G,H,D,I,E,J).

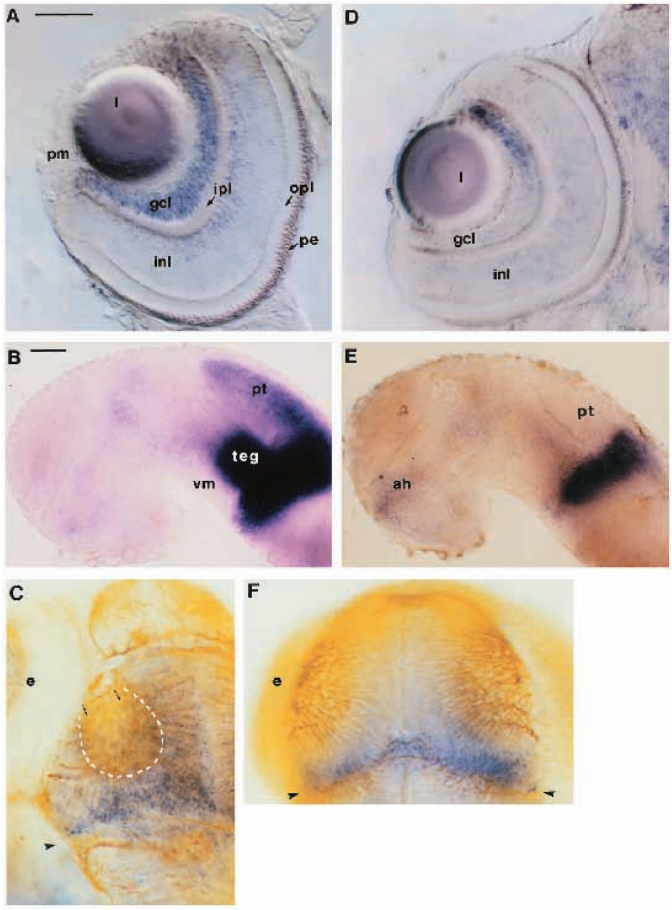

Fig. 3. Expression of L3 (A-C) and L4 (D-F) in the visual system. Anterior is up in all pictures except B and E in which it is to the left. (A,D) Horizontal sections revealing the expression of L3 and L4 respectively in the retinal ganglion cells of the eye at 72 hours. Note that L4 expression is restricted to ganglion cells in the nasal half of the retina and appears to be graded from a high point in the most nasal retinal ganglion cells in contrast to L3 which is expressed in all retinal ganglion cells. Weak expression of L3 is detected in the innermost cells of the inner nuclear cell layer which is the site of the amacrine cells. (B) Lateral view of a 42-hour old embryo showing graded expression of L3 in the tectum and strong expression in tegmental regions of the midbrain. Expression of L4 at the same stage (E) shows that it is not obviously graded in the tectum but is expressed in the ventral-posterior midbrain region and in the anterior hypothalamus in the forebrain. (C,F) Dorsal views of the midbrain at 3 days showing the expression of L3 and L4 respectively by in situ hybridisation in blue, and the axons of the tectum stained by anti-tubulin antibodies in orange. L3 (C) extends into the optic tectum (outlined) and extends caudally to the posterior margin of the midbrain (arrowhead). L4 expression (F) is considerably less extensive, extending only as far as the posterior margin of the optic tectum. Arrowheads show the posterior extent of the midbrain. Labels as for Fig. 2 and; gcl, ganglion cell layer; inl, inner nuclear cell layer; onl, outer nuclear cell layer; pm, proliferating margin; pe, pigmented epithelium; l, lens ; ipl, inner plexiform layer; opl, outer plexiform layer; pt, presumtive tectum; teg, tegmentum; vm, ventral midbrain. Scale bar, 50 μm.

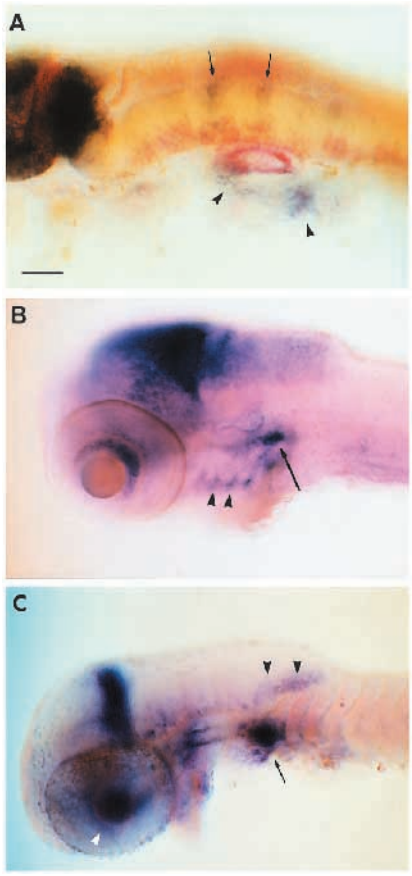

Fig. 4. Wider expression of L3 and L4. (A) Hindbrain expression of L3 includes some mesenchymal cells. These are shown (arrowheads) at 24 hours migrating with respect to the otic vesicle. This expression is separate from the rhombomeric patterns (arrows). (B) At 48 hours L3 is expressed in groups of cells in the branchial arches (arrowheads) and in a structure associated with fin attachment (arrow). (C) At 48 hours L4 is expressed in cells in the caudal hindbrain (black arrowheads), in cells of the nasal retina (white arrowhead) and in a structure associated with fin attachment (arrow). Scale bar, 50 μm.

method for the spatial localisation of bound ligand protein (Cheng and Flanagan, 1994). This does not discriminate the different ligands but will identify any ligand able to bind to Cek 4. This receptor was selected because it is expressed in the eye and is the homologue of Mek 4 (Sajjadi et al., 1991), the mouse receptor which is a likely endogenous partner for elf-1 (Cheng et al., 1995). By partial sequence and expression pattern criteria the most likely zebrafish homologue of Cek4/Mek4 identified to date is rtk2 (Xu et al., 1994; Macdonald et al., 1995). Rtk2 was not used for these experiments because the complete cDNA is not yet available.

Nearly all sites which show expression of L3 or L4 by RNA in situ hybridisation also show active protein as indicated by RAP in situ (Fig. 5). The combined expresssion of L3 and L4 can account for all regions of Cek4/AP binding in the CNS at 24 and 30 hours. The pattern of binding changes during this period with a signal appearing in the temporal region of the eye (Fig. 5A,B). This binding in the complete eye reflects the expression of L3 in the retinal ganglion cells of the retina as the retinal ganglion cells differentiate. The only region where binding was expected but not seen was at the caudal domain of L3 and L4 expression in the midbrain-hindbrain boundary region. Binding occurs in this location at 24 hours but fades

Table 1. The percentage identity at the amino acid level between zebrafish ligands L3 and L4 and published B61 like ligands for Eph receptors

	B61	Elf1	Al1	Lerk3	Lerk4
zf L3	45.0	58.4	54.0	44.9	44.1
zf L4	42.0	52.0	76.0	37.6	41.8

away by 30 hours suggesting that the ligand may be compromised in some way.

A strong region of RAP signal also occurs in the region of the diencephalon immediately ventral to the postoptic commissure. This is evident at 24 hours and significantly stronger at 34 hours (Fig. 5C). By the time retinal ganglion cell axons cross the midline, ligand is also localised at the site of the optic chiasm (Fig. 5D).

Assessment of function of L3 and L4 in growth cone collapse and stripe assays

To assess the probability that L3 and L4 are involved in axon guidance in the visual system we used the in vitro growth cone collapse assay and stripe assay as previously described for the chicken visual system (Bonhoefer and Huf, 1982; Walter et al., 1987; Cox et al., 1990). Initial experiments using cell cultures demonstrated that the zebrafish ligands do recognise chicken Eph receptors. Using the alkaline phosphatase tag system we showed that L1, a ligand not expressed in the CNS, L3 and L4 all bind to Cek 4 (Fig. 6), 7 and 8 (data not shown), all of which are expessed in the chick visual system (Sajjadi and Pasquale, 1993). The colour reaction was slower to develop with Cos cells transfected with L1 compared to L3 or L4 (overnight as opposed to 2 hours). This suggests that either the protein level of L1 was lower or the L1 protein had a decreased affinity for the CEK4 receptor compared to L3 and L4. Either way, a difference is supported by the observation that membranes from Cos cells transfected with L1 had no effect on the behaviour of retinal ganglion cells in either the collapse or stripe assay.

The function of L3 and L4 was assessed using the growth cone collapse and stripe assays. In the growth cone collapse assay membranes from Cos cells transfected with either L3 or L4 caused collapse of axons from retinal ganglion cells (Table 2). In the stripe assay in which retinal axons are faced with a choice of growing over membranes derived from Cos cells transfected with either β-galactosidase as a control or ligands L1, L3 or L4, specific patterns of axon growth were seen with L3 and L4 (Fig. 7). In both cases temporal axons preferred to grow over control membranes than ligand transfected membranes. In most cases nasal axons were also inhibited from growing over membranes from Cos cells transfected with L4 but nasal axons were unaffected by membranes from L3 transfected Cos cells. The relative lack of effect of membranes from Cos cells transfected with L3 on growth of nasal axons in these experiments may result from a lower protein level of L3 relative to L4 or the L3 protein may have a decreased affinity for the Eph receptor/s present on the retinal ganglion cells. Alternatively nasal axons may be sensitive to some other protein carried by the Cos cells which prevented them from responding to the L3 protein. Nonetheless there was a clear difference in the behaviour of axons from nasal and temporal retina when faced with a choice of growth on membranes from control Cos cells or those from Cos cells transfected with L3 (Fig. 7). The photomicrograph of axons growing on L3 transfected Cos cell membranes presented in Fig. 7 shows this clearly and this pattern was reliably reproduced 3 times and on each occasion three to six cultures were assessed.

The association of ligand expression and axon outgrowth in other regions of the embryo

To gain more information about the association of ligand expression patterns and the sites of axon outgrowth, normal embryos were labelled to reveal ligand by in situ hybridisation, and to reveal axons by staining with either anti-acetylated tubulin or HNK-1 antibodies. As L3 is more extensively expressed, we have gained more information from examination of this ligand. Having established that the wider expression does correspond to active protein using RAP we assessed the expression of the ligand at 24 hours in the hindbrain, midbrain

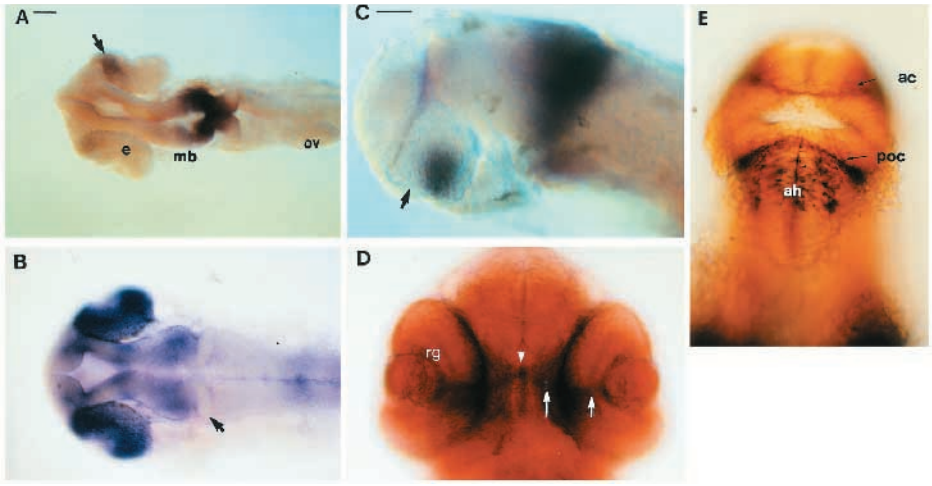

Fig. 5. In situ binding of Cek4/RAP fusion protein to the zebrafish embryo to identify active ligand. Anterior is to the left in all panels except D in which anterior is up and E which is an anterior view of the embryo. (A) Dorsal view of a 20-somite embryo showing binding in the nasal region of the eye which corresponds to L4 (arrow), in the midbrain and the anterior hindbrain (L3 and L4). (B) By 30 hours binding is present throughout the eye reflecting the expression of L3 and L4 in the forming retinal ganglion cells, but is distinctly absent in the anterior hindbrain (arrow). (C). Lateral view of an embryo at 30 hours with the eye removed. The ligand domain is present in the anterior hypothalamus immediately ventral to the postoptic commissure (arrow). (D) A ventral view of Cek4/AP binding in a 48-hour embryo showing binding to the optic nerve (arrows). The optic chiasm can be seen(arrowhead). (E). A view of a 30-hour embryo from the anterior pole showing RAP binding of Cek4/AP to cells of the anterior hypothalamus (in blue) and the axons of the postoptic commissure stained with anti-tubulin antibodies (in brown). Labels as for Fig. 2 and os, optic stalk; poc, post optic commissure; ac, anterior commissure, rg, retinal ganglion. Scale bar equals 50 μm (A and B) (C-E).

and forebrain with respect to the formation of the early axon scaffold (Fig. 8 and see Wilson et al., 1990).

From a survey of the growth of axons relative to the L3 expression domains it is not evident that the ligand always acts to deter axon growth. There are two regions where expression is associated with sites of axon growth. In the first, in the forebrain, expression is in the diencephalon, immediately beneath the epiphysis, which is the site of origin of the epiphyseal projection (dorsoventral diencephalic tract). On examination, this early axon pathway is established through the middle of this L3 expression domain (Fig. 8D). In the second example, in the hindbrain, the rhombomeric bands of L3 lie in the middle of each rhombomere as shown by their location adjacent to the HNK-1 positive neurons of the reticulospinal complex (Fig. 8C; Hatta, 1992). These are the regions through which the axons of these neurons will grow, suggesting that this ligand is not involved in negative control of reticulospinal axon growth.

In contrast, in addition to the retinotectal projection there are a number of regions where axons grow around the borders of expression of L3. Thus, the posterior commissure lies between the caudal diencephalic expression domain and the rostral tectal and ventral midbrain expression domains. More strikingly, at the caudal end of the midbrain the projection of the trochlear nerve runs directly through the space between the two L3 bands of expression. Finally, the ventral nucleus of the longitudinal fasciculus and its tract are located ventral to the tegmental expression domain in the midbrain. Antibody staining also shows that axons are excluded from the L4 zone at the caudal midbrain (Fig. 3D).

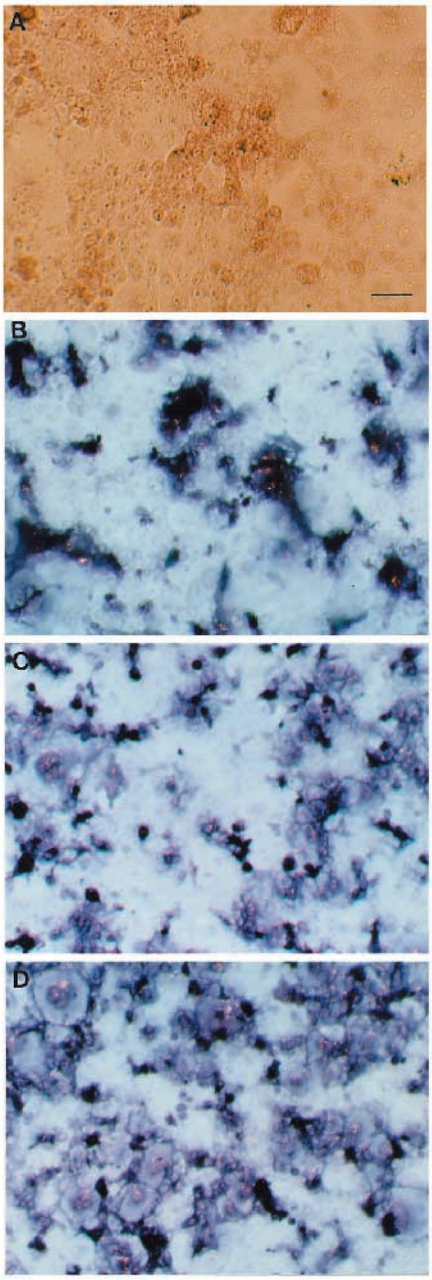

Fig. 6. Transfection of ligands into Cos cells showing comparable transfection rates. These are CEK4/RAP in situs to Cos cells tranfected with β-gal (A), L1 (B), L3 (C) and L4 (D). Scale bar, 50 μm.

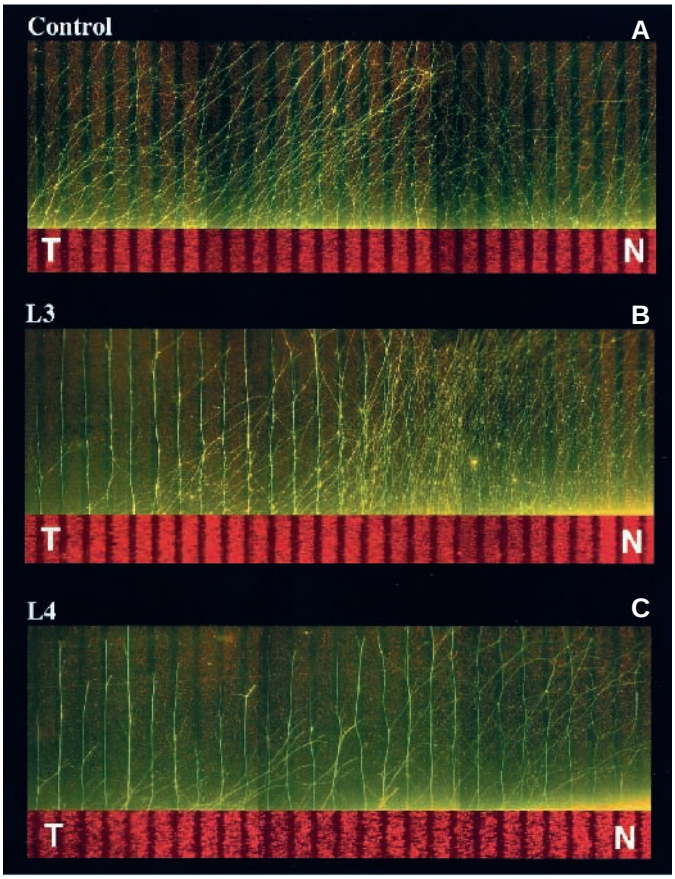

Fig. 7. Results of the stripe assay in which chick retinal ganglion cell axons are allowed to grow across a carpet of membranes from Cos cells which have been transfected either with a control (β-galactosidase; A), L3 (B), L4 (C). The red and black stripes beneath each panel represent the stripes with the control membranes in the black lanes. The retinal explants are placed over the striped substrates with the nasal pole (N) on one side and the temporal pole (T) on the other. Selectivity occurs with membranes from Cos cells transfected with L4 or L3 such that temporal axons fail to grow over membranes carrying these proteins. Nasal axons are apparently less sensitive to L3 and will grow over membranes from cells expressing this protein (C). No distinction is evident with the β-galactosidase (A) or L1 (not shown) transfected membranes.

Table 2. Primary data from the growth cone collapse assay

	Amount of membrane per dish				
	10 μg	20 μg	30 μg	40 μg	50 μg
β-gal					
temporal	1/7 (26)	0/10	3/9 (3)		0/9
nasal	1/6	2/8 (20)	1/7 (7)		0/5
L1					
temporal		1/7 (18)			0/6
nasal		0/8			0/5
L3					
temporal	1/9(24)	4/6 (20)	5/9 (25)	2/7 (16)	
nasal	0/7	1/4 (28)	0/6	0/7	
L4					
temporal	11/15 (14)	16/16 (5)	10/10 (5.5)		
nasal	4/16 (17)	5/10 (12)	5/7 (21)		

Different amounts of membranes from Cos cells transfected with CS2+β-gal (control) or one of three ligands, were used to assess growth cone collapse. Each experiment involved either temporal- or nasal-derived retinal ganglion cells. The number of growth cones collapsing is given compared to the number assessed and the mean time taken for this group to collapse in minutes is shown in brackets.

DISCUSSION

Since Sperry's formulation of the theory of neuronal specificity, largely based on his work on the amphibian and fish retinotectal projection (Sperry, 1963), there has been a search for the molecular basis of such a mechanism. Recent work suggests that Eph receptor signalling is involved in this process (Drescher et al., 1995, Cheng et al., 1995). We show in this study that not only are two of the zebrafish Eph ligands expressed in the tectum in a manner that is consistent with this proposal but that both have a function in the modulation of axon growth that is based on a repulsive activity. This feature is common to several other families of proteins involved in axon guidance including the semaphorins (Kolodkin, 1996) and the netrins (Kennedy and Tessier-Lavigne, 1995).

There are a number of features of the expression of L3 and L4 that suggest a role in creation of the retinotectal connection. Both are expressed prior to and during the period of innervation by retinal ganglion cell axons. Their expression in the midbrain is nested suggesting that they could act coordinately in the control of axon growth on the tectum. L3 is graded with a caudal high point and the transcripts are detected to the anterior extent of the tectum. In contrast, the L4 expression domain is discrete, being restricted to the posterior margin of the tectum. We suggest that retinal ganglion cells from the temporal half of the retina express high concentrations of the appropriate L3 receptor and that these axons are progressively inhibited as they extend into the territory of increasing L3 protein in the tectum. The majority of temporal axons then cease growing and make connections in the anterior tectum. By contrast, the nasal axons, which may express the receptor in lower amounts, or not at all, are able to reach the caudal tectum. This model is consistent with what is known about the development of the zebrafish retinotectal map. During its establishment retinal ganglion cell axons make very few mistakes as they grow across the tectum. It appears from time-lapse studies of axons growing in the tectum that growth cones grow directly towards a small region of the field prior to making local exploratory movements (Kaethner and Stuermer, 1992), a behaviour that is consistent with the presence on the tectum of spatially orchestrated guidance cues. The model is also consistent with the fact that both L3 and L4 are expressed much

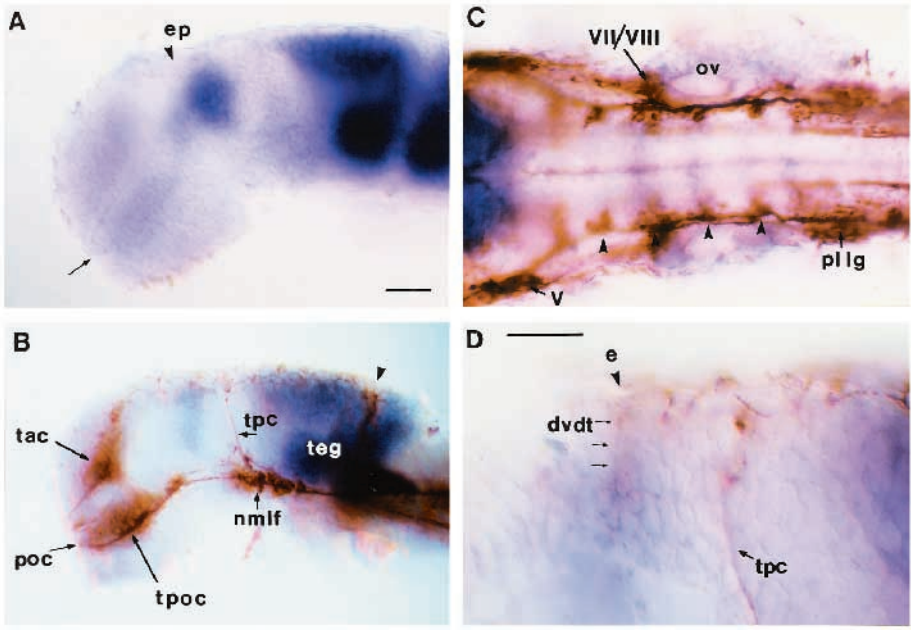

Fig. 8. The relationship of L3 expression to the formation of axon tracts in the brain. (A) At 24 hours L3 is expressed in the caudal diencephalon beneath the epiphysis and more weakly in the anterior hypothalamus. The arrow indicates the preoptic commissure. (B) In a comparable staged embryo double labelled with HNK-1 antibody the major tracts can be seen. The nucleus of the medial longitudinal fasciculus and its tract run ventral to the tegmental expression in the midbrain, the trochlear nerve (arrowhead) runs between the expression domains at the mid-hindbrain border and the tpoc runs in at the fore-midbrain border in a region where the gene is not expressed. (C) Dorsal view of a 24-hour embryo double labelled with HNK-1. The sites of formation of the reticulospinal neurons in the middle of each rhombomere lie adjacent to the regions of L3 expression. (D) High power view of the region of L3 expression ventral to the epiphysis at 28 hours. The projection axons from the epiphysis, the dvdt (arrows), extend into the expression domain. Labels as for Fig. 2 and; tac, tract of the anterior commissure; post optic commissure; tpoc, tract of the post optic commissure; tpc tract of the posterior commissure; nmlf; nucleus of the medial longitudinal fasciculus; V, trigemminal ganglion; pllg, posterior lateral line ganglion; dvdt, dorsoventral diencephalic tract. Scale bars, 50 μm (A,B) (C,D).

earlier in the tectum than the time at which the retinal ganglion cell axons arrive. Indeed, the midbrain expression for both is evident from the six-somite stage when the neural keel is still undergoing morphogenesis in the zebrafish (Papan and Campos-Ortega, 1994).

A considerable amount is known about the control of midbrain polarity and it is likely that the nested expression domans of L3 and L4 are under the control of a signalling system involving the secreted proteins FGF8 (Crossley et al., 1996) and Wnt1 and transcription factors of the Pax and Engrailed families (Itasaki and Nakamura, 1996; reviewed by Joyner, 1996). In the zebrafish, three different *engrailed* genes are expressed in a coordinate fashion over the mid-hindbrain boundary region (Ekker et al., 1992) and in the chick, Logan et al. (1996) have shown that Eph ligands in the midbrain are regulated by Engrailed. In *Xenopus,* Retaux et al. (1996) have reduced the levels of *engrailed* expression in the caudal midbrain using antisense oligonucleotides. This causes abnormal growth of axons from the tectum into the more caudal hindbrain/midbrain territory. This phenotype could result from a suppression of L4 expression in this region leading to an exuberance of axons into the area which is normally devoid of axons. This would suggest that the normal function of L4 is to create an axon free zone at the caudal extent of the midbrain rather than be involved in forming the retinotopic map. It is also possible that a third ligand with the expression characteristics of RAGS in the chick tectum exists in the zebrafish.

Two observations suggest that the ligands may also play a role in the formation of the optic nerve. Firstly L3 and L4 are expressed in the retinal ganglion cell layer at the time of axon outgrowth. L4 expression is localised to the nasal retina where it is graded with a high point in the most nasal ganglion cells whilst L3 is expressed in all nasal and temporal neurons. It is possible that the ligands function during axon growth to maintain order in the optic nerve, at least to keep nasal and temporal axons apart, a process in which the differential expression of the two ligands may play a key role. Secondly, the presence of an Eph ligand at the optic chiasm is revealed by the Cek 4/RAP treated embryos (Fig. 5D). In the zebrafish, the retinal ganglion cells from one eye all decussate at the chiasm so it is an important site for the maintenance of axonal order. The site of binding coincides with the expression of L3 and L4 in the anterior hypothalamus (compare Fig. 2D and Fig. 5C,D), and lies immediately ventral to the location of the postoptic commissure and the location of the axons of the forming optic nerve which reach the chiasm at 34 hours (Burrill and Easter 1994). This domain of ligand expression, which is evident at 24 hours and strong at 34 hours (Fig. 5C,D) could be responsible for maintaning the tract and not allowing axons to spread into the anterior diencephalon.

It is important to compare the results achieved in this study with those obtained in chicken and mouse embryos. In the chick, the L4 homologue, RAGS, has an expression pattern that is less restrictive than the comparable L4 expression domain in the posterior region of the tectum. RAGS transcripts are present in tectal cells extending more anteriorly (Drescher et al., 1995). The expression patterns of L3 and chick elf-1 seem comparable (Chang and Flanagan, 1995). It is very likely that the coordinate expression of these ligands are performing similar tasks in the zebrafish and the chick. Mouse elf-1 again has a similar graded expression extending to the rostrocaudal extent of the midbrain although the mouse RAGS expression pattern has not been described. However, it remains unclear whether axons entering the zebrafish tectum will interact with the Eph ligands in the same manner as they do in chick and mouse. This is because the possible zebrafish Mek 4/Cek 4 homologue, rtk2 (Xu et al., 1994; Macdonald et al., 1995) is not expressed in a gradient in the temporal half of the eye, rather the RNA is distributed apparantly evenly over this region. Furthermore, the zebrafish receptor rtk 1 (Xu et al., 1994, 1996), homologue of the second receptor known to be present in the mouse and chick retina, Cek 8/Sek 1 (Cheng and Flanagan, 1995) is not expressed in the zebrafish eye. In addition, a second zebrafish receptor, ZDK1, the homologue of the mouse MDK1 receptor, is also expressed in the temporal region of the eye (Taneja et al., 1996). However, to date we have not found a zebrafish Eph receptor which is expressed in the nasal retina. Thus while the topographic map in the zebrafish, chick and mouse may be created using the Eph receptor system the details of this process may be different in the fish and the amniotes.

Thus far we have concentrated on the role of L3 and L4 in the visual system. However, it is evident, especially for L3, that these ligands may play a role in the navigation of axons in other regions of the CNS. Clearly, inhibition of axonal growth will only occur if axons or their targets express the appropriate receptors. It is not surprising, therefore, that an analysis of axon growth relative to the expression patterns of L3 throughout the brain shows that some axons appear to avoid such expression patterns and others do not. It will be important to match the expression of Eph ligands and receptors to understand how this signalling system is involved in the widespresd creation of topographic neuronal projections and neuronal circuits.

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