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Topographically Specific Effects of ELF-1 on Retinal Axon Guidance In Vitro and Retinal Axon Mapping In Vivo

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Summary

Topographic maps, which maintain the spatial order of neurons in the order of their axonal connections, are found throughout the nervous system. In the visual retinotectal projection, ELF-1, a ligand in the tectum, and its receptors in the retina show complementary gradients in expression and binding, indicating they may be positional labels for map development. Here we show that ELF-1 acts as a repellent axon guidance factor in vitro. In vivo, when the tectal ELF-1 pattern is modified by retroviral overexpression, retinal axons avoid ectopic ELF-1 patches and map to abnormally anterior positions. All these effects were seen on axons from temporal but not nasal retina, indicating that ELF-1 could determine nasal versus temporal retinotectal specificity, and providing a direct demonstration of a cell recognition molecule with topographically specific effects on neural map development.

Introduction

The correct functioning of the nervous system depends on the establishment of a precise and complex spatial order in its neuronal connections. The initial development of these connections is believed to be guided by molecular cues (Goodman and Shatz, 1993; Garrity and Zipursky, 1995). First, axons find their target regions, guided by pathway- and target-derived cues. Then, within the target, axons have to recognize the correct area to form their specific connections. This can involve cell–cell specificity, in which recognition depends on distinguishing discrete cell types. Alternatively, it can involve topographic mapping, where an array of projecting neurons maps onto a target field so that the spatial arrangement of the neurons is maintained in the spatial order of the connections. Projecting axons in the vertebrate nervous system are typically arranged in this topographic manner, providing a way for sensory input and other information to be transferred from one area to another while preserving its original spatial order.

The visual projection from the retina to the tectum has for decades been a leading model for study of the development of specificity in neuronal connections. On the basis mainly of studies of this system, Sperry first proposed in the chemoaffinity theory that the specificity of neuronal connections could be determined by stable complementary tags on projecting axons and within target areas. In the topographic retinotectal projection, it was envisaged that the labels would most likely be in smooth gradients that could mark each point on the retina and tectum with a unique positional value, without needing a large number of cell-specific labels (Sperry, 1963).

Evidence to support the idea of complementary retinotectal mapping labels has come from numerous studies, including tissue ablation and grafting experiments, axon tracing studies, and in vitro models of mapping (reviewed by Hunt and Cowan, 1990; Goodman and Shatz, 1993; Garrity and Zipursky, 1995; Harris and Holt, 1995; Roskies et al., 1995; Tessier-Lavigne, 1995). Among the in vitro models, particularly noteworthy are those that have shown topographically specific effects on axon behavior (Walter et al., 1987; Cox et al., 1990; Roskies and O'Leary, 1994). These studies have convincingly demonstrated an activity that is linked to posterior tectal cell membranes by a glycosylphosphatidylinositol (GPI) anchor and that shows topographically specific inhibitory effects on axons from the temporal but not the nasal side of the retina.

The molecular basis of topographic specificity is not well understood. Upstream of the recognition process, homeobox genes such as LIM genes in motor neurons and *engrailed* in the tectum could act as nuclear factors that initiate topographically specific properties (Tsuchida et al., 1994; Itasaki and Nakamura, 1996; Friedman and O'Leary, 1996b). Topographic recognition itself is considered likely to depend on complementary cell–cell interaction molecules, but the identification of these molecules has long been an elusive goal. Recent evidence now suggests that members of the Eph receptor and ligand families might fulfil this function.

ELF-1 was identified as a GPI-anchored ligand that binds to the Mek4 and Sek receptors (Cheng and Flanagan, 1994). These receptors are members of the Eph family, the largest known family of receptor tyrosine kinases (reviewed by Brambilla and Klein, 1995; Friedman and O'Leary, 1996a). In the chick retinotectal system at the time of mapping, the *ELF-1* gene is expressed in the tectum and the *Mek4* receptor gene in the projecting retinal ganglion cells. Both are in gradients along matching axes that map to one another (Cheng et al., 1995). In addition, a functional test of binding activities using alkaline phosphatase (AP) fusions of ELF-1 and Mek4 showed that each can detect a matching gradient of binding sites in the reciprocal field, providing direct evidence for the gradient complementarity that would be predicted from the chemoaffinity theory. ELF-1 and Mek4 therefore have properties consistent with complementary positional labels for retinotectal mapping (Cheng et al., 1995).

Several other Eph family ligands have been identified (Brambilla and Klein, 1995; Tessier-Lavigne, 1995; Friedman and O'Leary, 1996a). One of them, RAGS/AL-1 (Winslow et al., 1995; Drescher et al., 1995), shows RNA expression in a gradient in the chick tectum and was

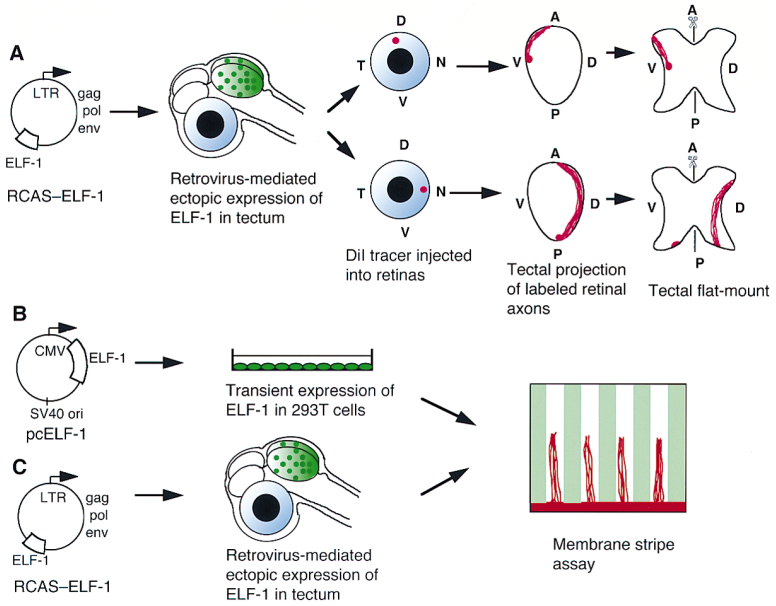

Figure 1. Procedures to Test Effects of ELF-1 on Retinal Axon Mapping In Vivo and Retinal Axon Guidance In Vitro

ELF-1 expression is illustrated in green, the retina in blue, and dye-labeled axons in red. (A) For the in vivo studies, RCAS–ELF-1 retrovirus was injected into the midbrain region of embryos before retinal axons reached the tectum. Subsequently, at a stage when the retinotectal topographic map is nearing completion, axons were labeled with DiI at one of two sites in the retina: on the temporal side of the midline or near the nasal pole. The projection pattern of the axons was later analyzed in the tectum. Each diagram illustrates a single tectal lobe. All axons enter at the anterior end of the tectum. In a normal embryo, axons labeled at the temporal retinal site map to a position just anterior to the tectal midline, whereas axons labeled at the nasal site map to the posterior end.

(B and C) For in vitro studies, the stripe assay for axon guidance was used. Retinal axons from the temporal or nasal side of the retina were given a choice of alternating stripes of membrane carpet. To test the effects of ELF-1 expressed in a cell line with little or no endogenous cross-reacting ligand or receptor, we prepared the membrane carpets from 293T cells with or without transient transfection by pcELF-1. To test effects of ELF-1 expressed in the natural context of the tectum, membranes were prepared from tecta with or without infection by retrovirus RCAS–ELF-1.

demonstrated to be a repellent for retinal axons in vitro (Drescher et al., 1995). However, the gradient appeared limited to the posterior part of the tectum, and the repellent effect was seen equally on temporal and nasal retinal axons, with no topographic specificity detectable at a range of concentrations (Drescher et al., 1995).

While all the known properties of ELF-1 are consistent with it being a positional label for mapping, direct studies of its effects on retinal axons could help to test this idea and clarify its mechanism of action. Here we asked whether ELF-1 can influence axon behavior in two different assay systems. Using the stripe assay as an in vitro model of mapping, we tested ELF-1, expressed either in cultured cells or in tectal membranes, for effects on axon guidance. To study topographic mapping in vivo, we modified the tectal pattern of ELF-1 by retrovirus-mediated gene expression. In both in vitro and in vivo assays, ELF-1 exerted a strong, topographically appropriate avoidance effect on temporal retinal axons. These results provide a direct demonstration of a cell–cell signaling factor with topographically specific effects either on an in vitro model or on neural map development in vivo. They also support the idea that ELF-1 could be a positional mapping label of the type predicted by the chemoaffinity theory and could determine nasal versus temporal specificity in the retinotectal system.

Results

Effects of ELF-1 on Topographic Mapping In Vivo

If ELF-1 is a retinotectal mapping label, it would be expected that experimental changes in the tectal ELF-1 expression pattern should produce corresponding changes in retinal axon projection pattern. Here, we used a retroviral overexpression approach to change the ELF-1 pattern in chick tectum (Figure 1A). This approach has the advantage that in the chick the retinotectal system is the primary visual projection and has been characterized extensively. Moreover in birds, in contrast to mammals, mapping labels act during the embryonic period to create a well-ordered topographic map even before the phase of activity-dependent refinement (Roskies et al., 1995). This should help to test for changes in the map induced by changes in putative labels.

The RCAS–ELF-1 retrovirus was injected into the midbrain/hindbrain region of the neural tube of chick embryos at embryonic day 2 (E2) in ovo. Expression of ectopic ELF-1 was later detected by affinity probe in situ (Cheng et al., 1995) using probes with the Mek4 receptor extracellular domain fused to either an AP tag (Mek4–AP probe) or an immunoglobulin Fc tag (Mek4–Ig probe) (Cheng and Flanagan, 1994; Cheng et al., 1995). Tecta infected with RCAS–ELF-1 showed a patchy distribution of exogenous tectal ELF-1 expression, superimposed on the normal anteroposterior gradient of endogenous ligand (Figures 2A–2D). These patches may represent infection events followed by local spreading of the replication-competent virus. The ELF-1 patches could be detected using either the Mek4–AP probe with chromogenic staining (Figures 2A–2D) or the Mek4–Ig probe followed by fluorescein-coupled secondary reagents. The fluorescence detection was less sensitive and suffered from fluorochrome bleaching, but allowed double-label fluorescence experiments to detect ELF-1 and dye-labeled axons simultaneously, as described below. The ectopic ELF-1 patches were at different densities in different embryos, ranging from approximately half the tectal surface (Figure 2A), through more typical patterns with a lower density of patches (Figures 2B and 2D), to some embryos that showed no difference from controls (data not shown). The intensity of staining within

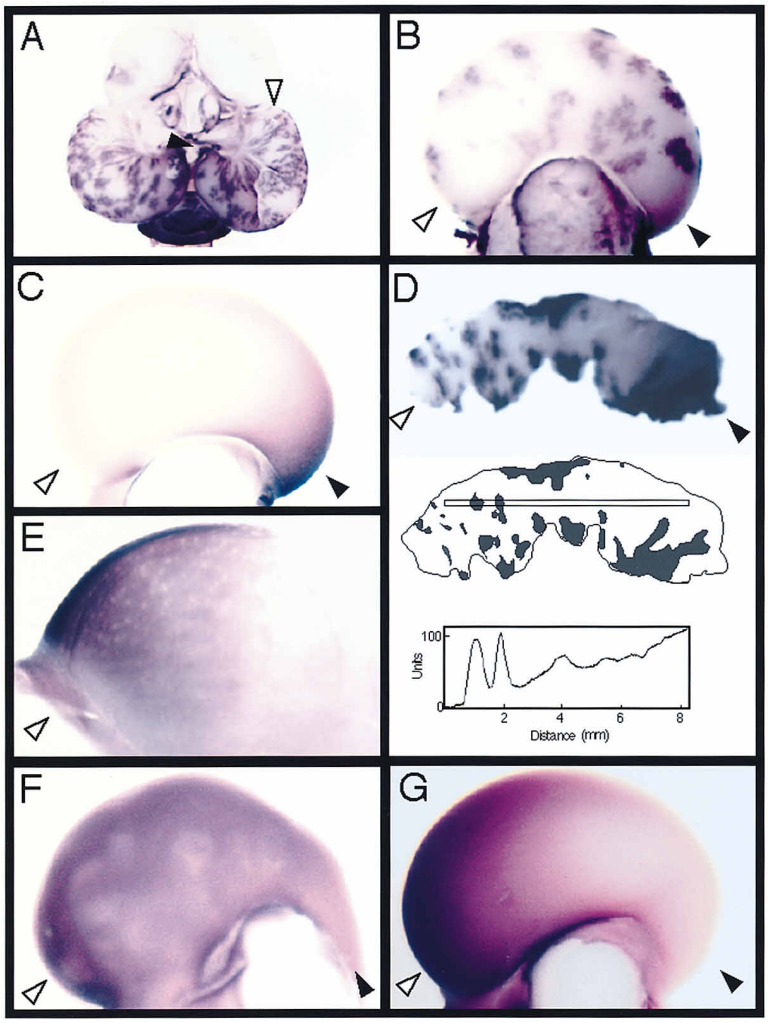

Figure 2. Ligand and Receptor Distribution in the Tectum Following Infection with the RCAS–ELF-1 Virus

The posterior end of the tectum is indicated by a closed triangle and the anterior end by an open triangle.

(A)–(D) show ligand distribution detected with a Mek4–AP soluble receptor fusion probe.

(A) Brain of an RCAS–ELF-1-infected embryo at E11. The two tectal lobes are splayed apart, so that each is viewed dorsomedially. Patches of ectopically expressed ELF-1 in the tectum are superimposed over the endogenous ligand gradient.

(B) Tectum of a typical RCAS–ELF-1-infected embryo at E16, viewed laterally.

(C) A matched uninfected control tectum. By this late stage of mapping, endogenous ligand levels have declined.

(D) An RCAS–ELF-1-infected tectum from an E9 embryo, flat mounted by making incisions along the ventral margin. A densitometric scan was taken along the box shown on the diagram.

(E)–(G) show receptor distribution detected with an ELF1–AP soluble ligand fusion probe.

(E and F) Anterior part of tecta from RCAS–ELF-1-treated embryos at E12 and E14, respectively, viewed anterolaterally. Patches of low binding activity for ELF1–AP are superimposed on the normal receptor gradient.

(G) Uninfected control tectum, showing the normal smooth receptor gradient.

ectopic patches was higher in posterior than anterior tectum, which may be a simple additive effect of endogenous and exogenous ligand. Densitometric estimates indicate that the staining intensity in ectopic patches in the anterior half of the tectum was approximately comparable with the endogenous staining level in posterior tectum (Figure 2D).

To test for effects of ectopic ELF-1 on topographic mapping, we marked small groups of axons in the retina by anterograde labeling with the fluorescent lipophilic dye DiI so that their projection patterns could subsequently be traced in the tectum. The protocol is illustrated in Figure 1A. Two sites were labeled in different embryos, one on the temporal side of the retina and the other on the nasal side. The temporal site was not at the extreme temporal edge, because those axons normally enter the tectum at its anterior end and terminate there, making it difficult or impossible to detect further repulsion. Temporal axons closer to the retinal midline map near the middle of the tectum, so repellent effects of ELF-1 should be detectable.

In normal embryos without viral infection, or after infection with the negative control virus RCASBP/AP, axons labeled at the temporal site showed a reproducible projection pattern, following paths across the tectum that were essentially parallel and usually clustered and all terminating at the topographically appropriate site on the anterior side of the tectal midline (Figures 3A and 3E; Table 1). In contrast, in most embryos infected with the RCAS–ELF-1 virus, axons labeled at the temporal site showed highly aberrant projection patterns (Table 1; typical examples shown in Figures 3B, 3C, 3F, and 3G). In these aberrant cases, axons projected to topographically abnormal sites, which were always more anterior than the normal termination region. They also showed obvious abnormalities in their pathways across the tectum, appearing generally less well organized, showing irregular turns, and frequently diverging rather than following parallel paths. When axons were labeled at the nasal retinal site, in uninfected embryos the projections followed parallel paths across the tectum and terminated near the posterior tectal pole, as expected. Infection with RCAS–ELF-1 made no detectable difference (Figures 3D and 3H; Table 1).

To relate the axon projection patterns more directly to the distribution of ELF-1, we performed double labeling of axons and ELF-1. For the highest magnification views, labeling is shown using the Mek4–AP probe with

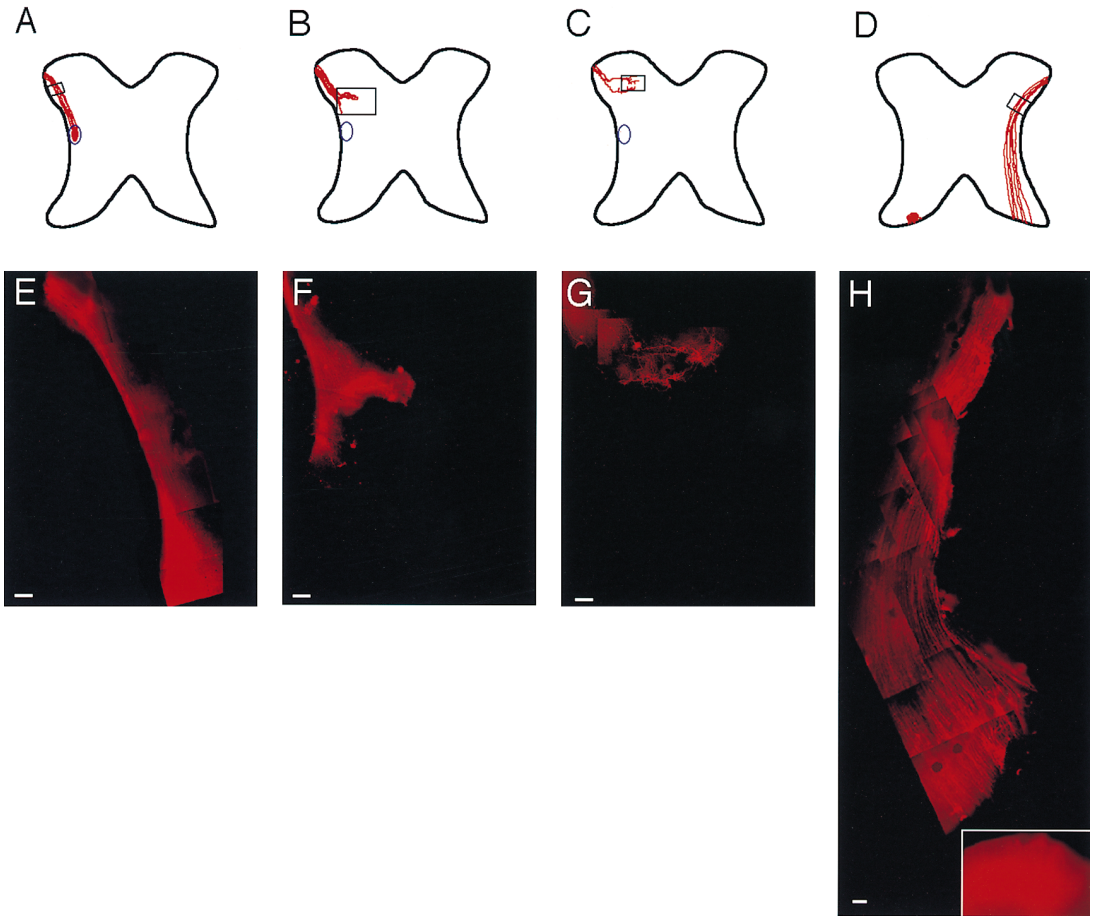

Figure 3. Effects of Tectal ELF-1 Overexpression on Retinal Axon Projections

Photographs and corresponding diagrams show projection patterns of axons labeled with DiI as described in Figure 1A. Rectangles on the diagrams indicate areas shown in Figure 4.

(A and E) Temporal axons in an embryo infected with RCASBP/AP control virus. The projection pattern is indistinguishable from that in a normal uninfected embryo. Axons follow parallel pathways across the tectum and terminate at the topographically appropriate site.

(B, C, F, and G) Temporal axons in two representative embryos infected with RCAS–ELF-1. Axons enter as usual at the anterior end of the tectum, but do not reach the topographically appropriate termination region, indicated by an oval outlined in blue. Pathways across the tectum are also highly abnormal, showing irregular turns and sometimes diverging into two or more groups that extend in different directions. In the embryo shown in (B), a large number of axons were labeled, while (C) is an example in which only a few labeled axons are visible.

(D and H) Nasal axons in an embryo infected with RCAS–ELF-1. The termination zone was separated by the incision made to flatten the tectum and is shown in a separate box; circular dark areas are caused by small bubbles in the mounting medium. The projection pattern is indistinguishable from uninfected control embryos. Scale bars represent 250 μm.

chromogenic staining, which gave more reliable detection of the boundaries of ELF-1 patches. It was apparent that temporal axons avoided the patches of ELF-1, with individual axons sometimes giving the appearance of turning to do so (Figures 4M and 4N). The sites of the turns are not always in direct contact with the edges of the ELF-1 patches, but this is not unexpected since both in vitro and in the normal tectum axons tend to retract following contact with topographically inappropriate areas (Nakamura and O'Leary, 1989; Cox et al., 1990).

For most of the double-labeling experiments, we used double fluorescence to avoid the risk of opaque chromogenic stains obscuring axon fluorescence. The results showed that temporal retinal axons consistently followed paths that avoided patches of ELF-1 (Figures 4B, 4C, 4F, 4G, 4J, and 4K) but were unaffected by control virus patches (Figures 4A, 4E, and 4I). It is unlikely that the effects seen here can be explained by ELF-1 expression in retinal ganglion cells. When injecting the retrovirus, we took care to minimize spread anterior to the tectum. It is still possible for viral particles to enter the developing optic cup, and ectopic patches of Mek4–AP staining were seen covering <10% of the retinal surface in some embryos. However, the observed retinal expression could not account for the high percentage of temporal projections affected (Table 1, see first footnote) or the avoidance of tectal ELF-1 patches seen by double labeling (Figure 4).

Unlike temporal axons, nasal axons showed no evidence of ELF-1 avoidance and passed through patches of high ELF-1 expression without any obvious effects (Figures 4D, 4H, and 4L). The results therefore show

Table 1. Effect of ELF-1 Overexpression in the Tectum on Retinal Axon Mapping

Labeling Site in Retina	Expression Vector in Tectum	Aberrant Projections	Normal Projections
Temporal	RCAS–ELF-1	16	6[a]
Nasal	RCAS–ELF-1	0	21[b]
Temporal	RCASBP/AP	0	11[b]
Nasal	RCASBP/AP	0	12[b]
Temporal	None	0	8[b]
Nasal	None	0	4[c]

The experimental protocol is summarized in Figure 1A, and representative results are shown in Figures 3 and 4. In projections that were scored as aberrant, axons followed clearly abnormal pathways across the tectum, appearing disorganized and showing irregular turns, and some or all of the axons projected to topographically inappropriate sites. RCASBP/AP (Fekete and Cepko, 1993) is a control virus containing AP rather than *ELF-1* cDNA, but otherwise the same. Staining for the AP marker showed that this virus gave a similar pattern of expression in the tectum.

[a] When the tecta with normal projection patterns were tested for ELF-1 expression, 5 of them showed no detectable ectopic ELF-1. In the one remaining case, patches of ectopic ELF-1 were seen, but none of the patches was located along the projection path of the axons.

[b] $p < 0.0005$, by Fisher's exact test, for the group with temporal axons and RCAS–ELF-1 infection versus each of the other groups.

[c] $p < 0.015$.

that ELF-1 ectopically expressed in the tectum during mapping is sufficient to cause a topographically specific avoidance by temporal retinal axons.

Gradient Complementarity: Effect of ELF-1 Overexpression on Tectal Receptor Distribution

Our previous studies showed complementary binding gradients, with a Mek4–AP probe detecting a gradient of ligand in the tectum, and an ELF1–AP probe detecting receptor on cultured retinal axons and also in the tectum at times after retinal axons have grown in. The receptor and ligand gradients are in opposite orientations, leading us to suggest ELF-1 might have avoidance activity for axons with high receptor density (Cheng et al., 1995). If so, it would be expected that patches of retrovirally overexpressed ELF-1 would be avoided by axons with high receptor levels, leading to holes in the normally smooth tectal receptor gradient.

Consistent with this prediction, in embryos infected with the RCAS–ELF-1 virus, the tecta showed patches of low binding activity for ELF1–AP superimposed on the normal tectal receptor gradient (see Figures 2E–2G). In these experiments we cannot rule out the possibility that the patches of low binding activity for ELF1–AP could simply be due to blocking or down-regulation of receptors caused by local high ELF-1 levels. Nevertheless, the results are consistent with our predictions of gradient complementarity and with the observed effects

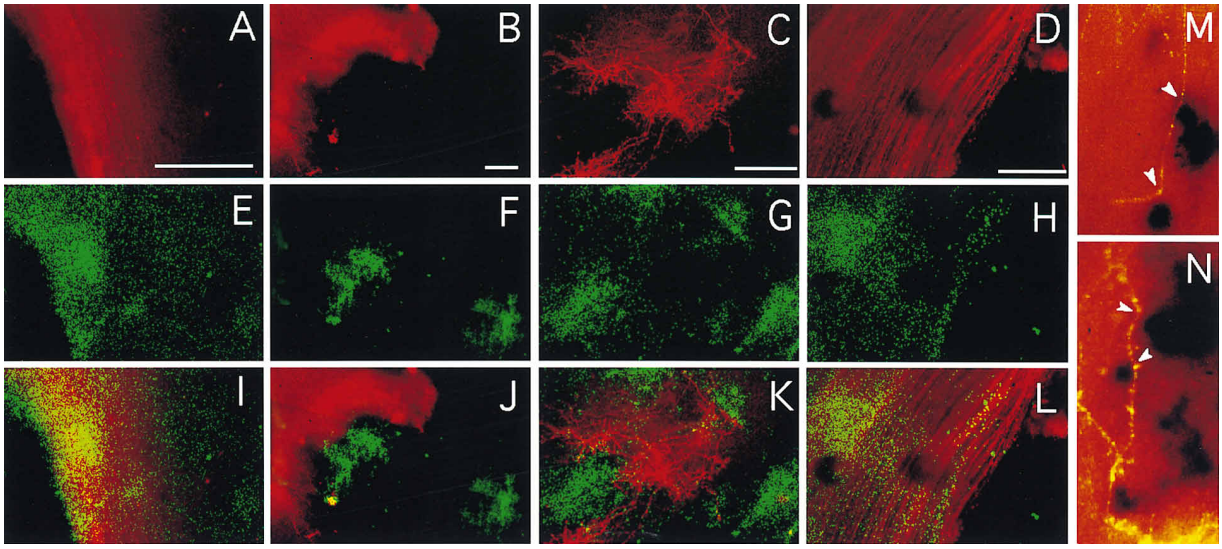

Figure 4. Double Labeling of Axons and Patches of Ectopic Expression

(A)–(D) show DiI-labeled axons from the rectangular boxes indicated in Figures 3A–3D, respectively. Scale bars represent 250 μm. (E)–(H) are the same fields, showing staining for retrovirally expressed proteins. (E) shows antibody staining for the AP marker of the RCASBP/AP control virus, while (F)–(H) show staining for ELF-1 expressed from RCAS–ELF-1. (I)–(L) show superimposition of the two images above. Yellow areas result from overlapping red and green fluorescence.

(A, E, and I) Temporal axons pass through patches of control virus with no apparent effect.

(B, F, J and C, G, K) Temporal axons avoid ELF-1 patches. In (B), (F), and (J), a larger number of axons are labeled, and a termination zone, visible here as a brighter fuzzy area, has formed just anterior to the ELF-1 patch.

(D, H, and L) Nasal axons pass through ectopic ELF-1 patches with no apparent effect; dark circles are caused by small bubbles in the mounting medium.

(M and N) Higher magnification views of individual DiI-labeled temporal axons avoiding ectopic patches of ELF-1, showing turns (arrows). Axons are visible in orange-red. ELF-1 patches here are stained using Mek4–AP with opaque chromogenic staining products and are visible as dark areas against a background of red tectal autofluorescence.

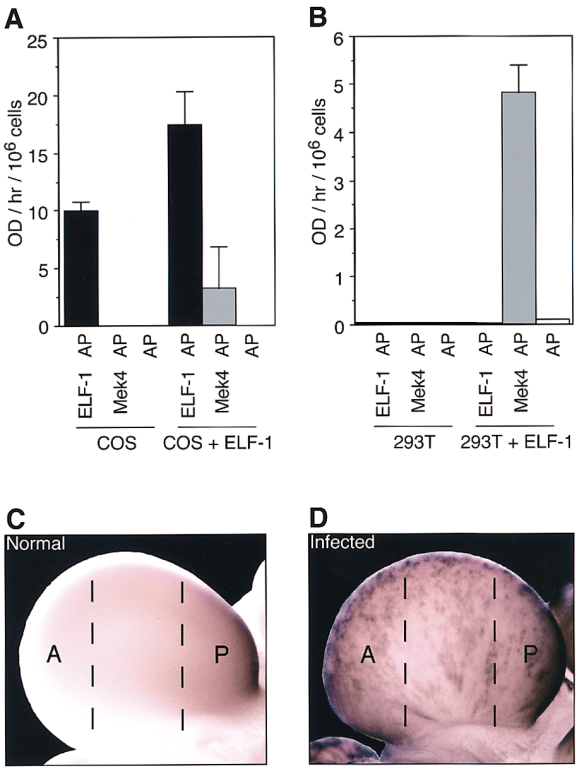

Figure 5. Receptor and Ligand Expression on Cell Lines and Tecta Used to Prepare Membranes for the Stripe Assay

(A and B) Quantitation of binding to the cell surface of COS or 293T cell lines, respectively, with or without transient transfection by plasmid pcELF-1. Cells were treated with ELF1–AP or Mek4–AP fusion proteins or with unfused AP as a control. Error bars show standard deviations. Unlike COS cells, 293T cells show low levels of endogenous ligand and receptor sites (<100 per cell).

(C and D) Tecta of E10 embryos without infection or infected with RCAS–ELF-1, stained with Mek4–AP to show ligand distribution. Two serial viral injections ensured ectopic expression covering most of the tectal surface. Broken lines show approximate tectal regions used to prepare membranes for the stripe assay. A, anterior; P, posterior.

of ectopic ELF-1 on the projection patterns of DiI-labeled axons.

Stripe Assay for Axon Guidance with ELF-1 Expressed in Cultured 293T Cells

The membrane stripe assay provides an in vitro model of retinotectal mapping and allows one to test for effects of specific activities on axon guidance (Walter et al., 1987). Here, we used the stripe assay to test for effects of ELF-1 on axon guidance, using either transiently transfected cell lines to isolate ELF-1 from other tectal components (see Figure 1B) or retrovirally infected tecta to express ELF-1 in its more complex, biologically relevant context (see Figure 1C).

In preliminary experiments, ELF-1 was expressed in COS cells, a cell line commonly used for transient transfection. Effects of ELF-1 on retinal axons in the collapse assay were detected, but were weak and variable (data not shown). When COS cells were tested for binding of ELF1–AP fusion protein, they were found to display very high levels of endogenous receptors for ELF-1 (Figure 5A). It therefore seems that experiments using COS cells to test the effects of ELF-1 may be difficult to interpret. For the experiments shown here, we used 293T, a human embryonic kidney cell line expressing simian virus 40 (SV40) T antigen, which provides an alternate system for transient expression from SV40-based vectors. 293T cells display little or no binding activity for either Mek4–AP or ELF1–AP (Figure 5B).

When given a choice of membranes from ELF-1-transfected or mock-transfected 293T cells, temporal axons showed a preference for membranes without ELF-1 transfection (Figure 6A). This preference was abolished by treatment of the membranes with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI anchors, consistent with the GPI linkage of ELF-1 (Figure 6H). Unlike temporal axons, nasal axons did not show a lane preference (Figure 6B). These experiments therefore showed a position-dependent response to ELF-1, indicating that it acts as a specific temporal axon repellent.

Stripe Assay for Axon Guidance with ELF-1 Expressed in Tectal Membranes

If ELF-1 is responsible at least in part for the topographically specific effects of normal tectal membranes in the stripe assay, it would be expected that alterations in the distribution of tectal ELF-1 would cause corresponding changes in the stripe preferences of retinal axons. Therefore, we assessed the influence of ELF-1 on axon behavior using membranes prepared from tecta infected with the RCAS–ELF-1 virus (see Figure 1C). For these experiments, each embryo was injected on E1.5 and E2.5 to enhance the level of infection, in contrast with the in vivo mapping experiments, in which care was taken to limit the spread of the virus (compare Figures 5C and 5D with Figures 2B and 2C). Membranes were prepared from the anterior and posterior thirds of normal or infected tecta, while the middle third was stained with Mek4–AP to confirm overexpression of ELF-1.

When retinal axons were given a choice of membranes from the anterior third of RCAS–ELF-1-infected tecta or uninfected tecta, temporal axons showed a preference for the uninfected membranes (Figures 6C and 6E). Nasal axons showed no preference (Figures 6D and 6F). These in vitro results with exogenous ELF-1 expressed in the natural context of the tectum are similar to those obtained with ELF-1 expressed in 293T cells and reinforce the idea that ELF-1 selectively affects the growth preferences of axons in a topographically specific manner.

If ELF-1 is responsible for the difference between anterior and posterior thirds of the normal tectum detected in the stripe assay, it would be expected that raising the level of ELF-1 in anterior tectum relative to that in posterior tectum should abolish this difference. To test this, temporal axons were given a choice in the stripe assay between membranes from the anterior third of RCAS–ELF-1-infected tectum versus the posterior third of infected or uninfected tectum. As predicted, the axons showed no preference for either membrane type (Figure 6G). Good axon outgrowth was nevertheless

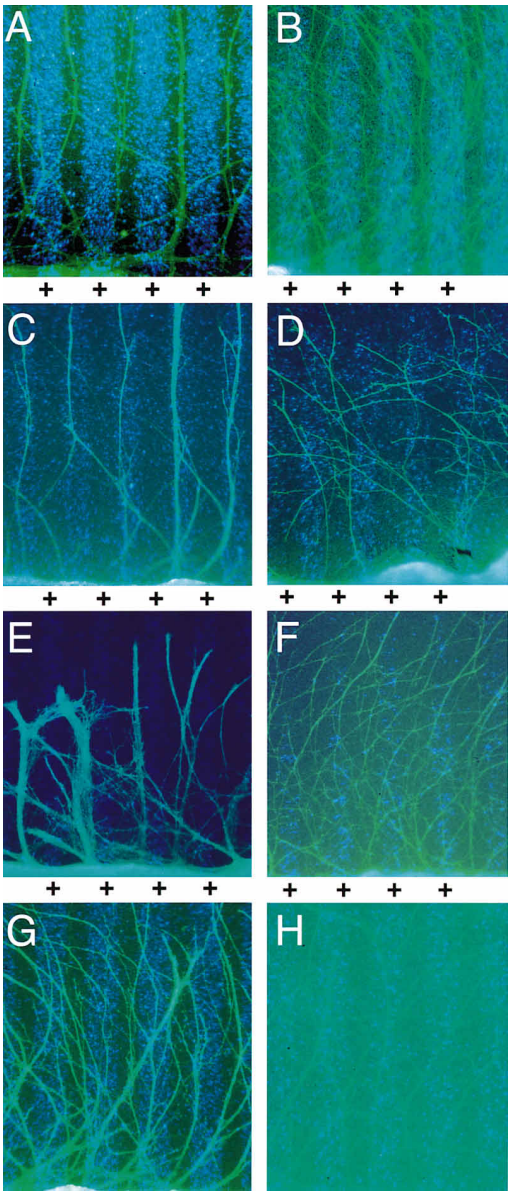

Figure 6. Effects of ELF-1 on Retinal Axon Outgrowth in the Stripe Assay

Retinal axons are visible in green, and one set of lanes is marked by blue fluorescent microspheres.

(A and B) Temporal and nasal axons, respectively, grown on the same carpet of alternating lanes of 293T cell membranes, either mock transfected or transfected with pcELF-1 (marked by a plus and microspheres). Temporal axons show a strong preference for lanes without ELF-1; nasal axons show no preference.

(C and D) Temporal and nasal axons, respectively, grown on the same carpet of lanes from the anterior third of tecta, either RCAS–ELF-1 infected (marked by a plus) or uninfected (with microspheres).

(E and F) As in (C) and (D), except that the infected membranes (marked with a plus and microspheres) were laid down second instead of first. Regardless of the order of lane deposition, temporal axons showed a strong preference for lanes without ELF-1, while nasal axons showed no preference.

(G) Temporal axons show no preference when grown on a carpet of alternating lanes from the posterior third of normal tecta versus the anterior third of tecta infected with RCAS–ELF-1 (marked by a plus and microspheres).

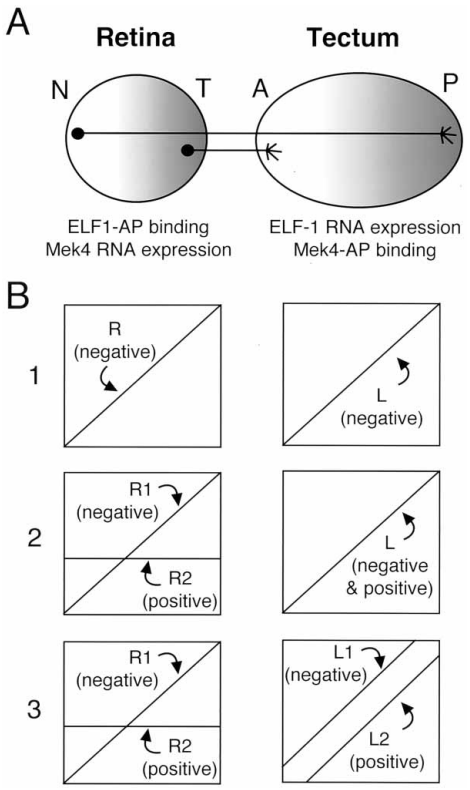

Figure 7. ELF-1 and the Mechanism of Retinotectal Mapping

(A) Complementary gradients of ELF-1 and its receptors in relation to retinotectal mapping. All retinal axons enter the tectum at its anterior end and grow toward the posterior end. Temporal axons project to anterior tectum and nasal axons to posterior tectum. Topographic order is also established on the dorsoventral axis (not shown). Shading illustrates expression and binding gradients for Mek4, a receptor in the projecting retinal ganglion cells, and ELF-1, a GPI-linked ligand in tectum.

(B) Models for the mechanism of topographic specification are discussed in the text. Gradients in vivo are not necessarily linear. R, receptor; L, ligand.

seen, consistent with the lack of inhibition of temporal axon growth by a membrane carpet containing only normal posterior tectal membranes (Walter et al., 1987), reinforcing the idea that ELF-1 acts by repelling, rather than generally inhibiting, the growth of temporal axons.

Discussion

Topographically Specific Properties of ELF-1

The retinotectal projection has long been a favorite model for study of the development of specific neural connections within the target. We recently reported that ELF-1 has suitable molecular properties, and expression and binding patterns, to make it a candidate label for topographic specificity in the retinotectal map (Figure 7A; Cheng and Flanagan, 1994; Cheng et al., 1995). The

(H) Treatment of both the pcELF-1-transfected (marked by a plus and microspheres) and mock-transfected 293T cell membranes with PI-PLC abolished the growth preference of temporal axons.

purpose of the present study was to test this idea further, as well as to study the mechanism of ELF-1 action, using assays of axon behavior.

The repellent activity of ELF-1 shown here in the stripe assay places it in a category of other molecules shown to act as repellent axon guidance factors: SemaIII/collapsin, netrin-1, and RAGS (Kolodkin et al., 1993; Luo et al., 1993; Colamarino and Tessier-Lavigne, 1995; Drescher et al., 1995; reviewed by Keynes and Cook, 1995). The further ability of ELF-1 to discriminate between axons from different positions in the projecting neuronal field is a unique feature for a guidance factor and an essential requirement for a topographic mapping factor.

The effects of ELF-1 in the stripe assay appear similar to those previously reported for normal posterior tectal membranes, which repel temporal but not nasal retinal axons (Walter et al., 1987). Moreover, raising the level of ELF-1 in anterior tectal membranes by retroviral overexpression abolished the usual preference of temporal retinal axons in the stripe assay for anterior over posterior membranes. These results do not rule out the possibility that other cross-reacting Eph ligands could have some role in mapping, but they do support the idea that ELF-1 could be partly or entirely responsible for the nasal versus temporal specificity of the repellent effect shown by normal tectal membranes in the stripe assay.

The stripe assay provides an elegant way to test for effects on axon guidance and a useful model for analysis of topographic mapping in vitro (Walter et al., 1987). However, it is clear that in the transition to in vitro models, structural organization, molecular components, and cellular responses can all be modified or lost. For example, in vitro assays have failed to detect evidence of mapping labels for the retinotectal dorsoventral axis, although in vivo studies indicate they exist. Even on the anteroposterior axis, very different activities can be detected depending on the methods used to prepare the tectal membranes (Boxberg et al., 1993). Also, when in vitro assays are used to assess molecules expressed in cell lines, the cellular expression context is not necessarily inert. For example, the 293T cells used here display little or no receptor for ELF-1, whereas COS cells show high receptor levels, which are likely to affect the results.

To investigate the effects of ELF-1 on topographic mapping, it is therefore important to characterize its effects during map development in the embryo. To do this, we took a novel in vivo approach to the study of complementary labels, using a chick retroviral expression system to alter the tectal pattern of ELF-1. The results show topographically specific effects of ELF-1 during retinotectal mapping in vivo, with no effect seen on nasal axons, whereas temporal axons avoided ectopic ELF-1 patches and projected to abnormally anterior positions.

The effects detected for ELF-1 in the in vitro and in vivo assays are clearly very compatible. It is unlikely that these effects are caused by ELF-1 inducing an intermediate molecule rather than acting on axons directly. First, retinal axons are known to have receptors that bind ELF-1 with a topographically appropriate distribution (Cheng et al., 1995; Figure 7A). Second, the activity detected here in the stripe assay is removed by PI-PLC treatment, consistent with the GPI linkage of ELF-1. Third, ELF-1 is active following expression either in tecta or by transient transfection in 293T cells, a human kidney cell line that showed no detectable expression of ELF-1-binding receptors that might mediate an induction. It is also noteworthy that 293T cells displayed no detectable ligand sites for Mek4, making it unlikely that the actions of ELF-1 detected here require cooperation with RAGS or other cross-reacting Eph ligands.

The results described here show ELF-1 is sufficient to cause topographically specific effects on retinal axons. It has not yet been shown whether ELF-1 is necessary for mapping in the normal tectum or whether other factors might have redundant functions, conclusions that would presumably require ELF-1 to be removed from the organism. Nevertheless, since ELF-1 shows expression at appropriate times and places in the embryo, and since it is sufficient to cause topographically specific effects in vitro and in vivo, it now seems highly probable that ELF-1 makes at least some contribution to topographic specificity, and indeed it could be the major determinant of nasal versus temporal specificity in retinotectal mapping.

Other Eph Receptors and Ligands in the Retinotectal System

Several other Eph family ligands and receptors are expressed in the retinotectal system. Mek4, a receptor that binds ELF-1 (Cheng and Flanagan, 1994), shows matching expression and binding patterns, consistent with the idea that ELF-1 and Mek4 could be complementary recognition labels of the type predicted by Sperry (Cheng et al., 1995; Figure 7A). Other Eph family receptors such as Sek (Cheng et al., 1995) are also expressed in the projecting retinal ganglion cells. Although Sek is not in an obvious gradient, it too might have some role in mapping.

RAGS, a ligand closely related to ELF-1, was reported to be in a gradient limited to the posterior tectum and to have in vitro repellent activity with indistinguishable effects on temporal and nasal axons (Drescher et al., 1995). While it remains possible that RAGS could show a topographic discrimination not detected in those studies, its activity seems clearly different from that detected in posterior tectal membranes, which had no repellent effect on nasal axons (Walter et al., 1987). The different activities described for ELF-1 and RAGS seem very consistent with their localizations. *RAGS* RNA is in the ventricular layers of the tectum, so it was proposed that it could be involved in retinotectal development if it is in glial cells, which send processes to the superficial layers where mapping occurs (Drescher et al., 1995). *ELF-1* RNA is distributed fairly uniformly among different cellular layers of the tectum, consistent with expression in neurons and possibly also glia (J.-H. Zhang, D. Cerretti, J. G. F., and R. Zhou, submitted; H.-J. C. and J. G. F., unpublished data). These patterns seem to correlate well with a recent in vitro study showing that tectal neurons, like ELF-1, have topographically specific repellent effects, whereas tectal glial/neuroepithelial cells, like RAGS, have an inhibitory effect with no topographic specificity (Davenport et al., 1996). Interestingly, comparison of *ELF-1* and *AL-1/RAGS* RNA expression in

the mouse shows that at the time of mapping *ELF-1* is expressed prominently within the superior colliculus (optic tectum), whereas *AL-1/RAGS* is expressed most prominently beyond its posterior end (J.-H. Zhang, D. Cerretti, J. G. F., and R. Zhou, submitted). Taking activities and expression patterns together, all the data for ELF-1 seem consistent with a role in determining nasal versus temporal specificity, long considered the most obvious hallmark of retinotectal topography. For RAGS/AL-1, it remains conceivable that there could be a role in refining topographic specificity near the posterior end of the tectum. However, its major role might be to prevent axons from projecting too far by acting as a repellent for both nasal and temporal axons as they approach and then reach the posterior limit of the tectum.

In addition to the anteroposterior axis, Eph receptors and ligands might be involved in establishment of order along the retinal dorsoventral axis. The Cek5/Qek5/Nuk receptor is expressed in ventral retina (Henkemeyer et al., 1994; Holash and Pasquale, 1995; Kenny et al., 1995), while ELF-2, a ligand that binds this receptor, is expressed in dorsal retina (Bergemann et al., 1995; M. J. H. and J. G. F., unpublished data). However, strongest protein expression seems to be in the retinal inner plexiform layer, and no matching ligand has yet been reported in the tectum. While it is not yet clear whether these molecules could be involved in retinotectal mapping, their asymmetric expression does suggest possible functions in patterning visual connections.

ELF-1 and the Mechanism of Retinotectal Mapping

Our previous studies left open several possibilities of how ELF-1 might act on axons. Because ELF-1 and Mek4 are in countergradients with opposite orientations (Figure 7A), we suggested that ELF-1 acting through Mek4 might have a negative or repellent effect on retinal axons (Cheng et al., 1995). However, other models could be constructed, including an attractant role for ELF-1 (Cheng et al., 1995; Tessier-Lavigne, 1995). We can now refine the possible models on the basis of our direct observations of ELF-1 action on retinal axon behavior. Because ELF-1 can act as a repellent, models in which it acts only as an attractant can presumably be excluded. Figure 7B illustrates some of the models that remain. Because an ELF1–AP probe detects higher receptor levels on temporal than nasal axons (Cheng et al., 1995), the examples include at least one receptor with a temporal (high) to nasal (low) distribution.

In the first model (Figure 7B), ELF-1 acts as a negative signal only. Temporal axons with high concentrations of a topographic receptor would be most sensitive to the repellent signal, whereas nasal axons would show little or no sensitivity. Some force would have to drive axons across the tectum, or they would all be repelled to the anterior pole. This force could be supplied by a positive signal in the tectum (models 2 and 3; Gierer, 1987) or could be an intrinsic tendency of the axons to fill the available space due to axon–axon repulsion or directional axon growth.

The second and third models in Figure 7B are extensions of the first, with additional signaling molecules added, as in the theoretical models of Gierer (1987). In model 2, ELF-1 acts as both a negative and a positive signal. Two (or more) corresponding negative and positive receptors would have different signaling characteristics and would be in different retinal distributions. Axons would then come to rest at the point in the tectum where positive and negative signals cancel out. This model seems intriguingly consistent with the different retinal distributions of known receptors that bind ELF-1, including Mek4 and Sek (Cheng et al., 1995). Our observations here remain consistent with this model, but provide no clear support for it, since ELF-1 showed no obvious attractant effect on nasal axons.

The third model in Figure 7B is another extension of model 1 in which a second, positive ligand would encourage growth of axons into the tectum. This ligand and its receptor could both be in gradients, or alternatively one or both could be uniformly distributed (as illustrated), with topographic specificity supplied only by the negative labels. There is some evidence to support this third model. Following a modification of the preparation procedure for tectal membranes, nasal axons preferred posterior membranes in the stripe assay and their survival was enhanced by these membranes, suggesting a possible positive factor (Boxberg et al., 1993). Also, when *engrailed* homeobox genes were retrovirally overexpressed in the tectum, temporal axons terminated in aberrantly anterior locations, while nasal axons branched and formed termination zones in ectopic locations (Itasaki and Nakamura, 1996; Friedman and O'Leary, 1996b). The effect on temporal axons could be consistent with an induction of ELF-1, while that on nasal axons may suggest induction of another signaling molecule with very different actions.

Each of these models includes a negative signal transmitted from ELF-1 through a topographic receptor. Here we make a further proposal: that topographic order could result when all axons receive an equivalent amount of negative signal from the receptor, and that this could be achieved simply by the law of mass action. For a simple interaction of receptor (R) and ligand (L), the concentration of signaling complex (RL) would be given by the standard binding equation $[RL] = K_A[R][L]$. Thus, a constant amount of signal could result either from high receptor and low ligand (anterior tectum) or from low receptor and high ligand (posterior tectum). The equation could be adapted to fit different combinations of receptors and ligands. A particularly interesting aspect of this mass action model is that it could only work with countergradients. Then, if countergradients are needed, it could only work for a repellent rather than an attractant signal. Finally, if the signal is repellent, the system would be expected to work best if axons enter the tectum at the low end of the gradient, where none of them would be strongly repelled, and grow toward the high end. Thus, starting from molecular principles, the mass action model could explain countergradients, repulsion, orientation of gradients, and direction of growth, features that previous models have considered arbitrary.

A model different from those in Figure 7B would be that ELF-1 could define only the correct termination site, without repellent or attractant effects. Such models seem less likely in view of the repellent effects of ELF-1

observed here and because we saw no evidence of nasal axons preferring to terminate at high levels of ELF-1. In addition, such models do not take advantage of a major theoretical advantage of repellent or attractant gradients, which can tell axons not only where to terminate, but which direction to go in if they are in the wrong place.

In all of these models, ELF-1 could most easily contribute to a smooth and continuous map if, in normal mapping, it causes a smoothly graded response by axons from different retinal positions. Our experiments here do not test the effects of ELF-1 gradients. They also do not directly address the issue of graded responsiveness in vivo, and the assays may not be ideal for this purpose. In the stripe assay, axons throughout the nasal third of the retina appeared unresponsive to ELF-1. However, even normal posterior tectal membranes produce an abrupt rather than graded transition in the stripe assay, with temporal axons responsive and nasal axons unresponsive, and since this differs from in vivo mapping it is presumably one of the artifacts of the in vitro system (Walter et al., 1987). In the in vivo assay used here, axons from temporal retina were responsive to ELF-1, while axons labeled near the far nasal edge were unresponsive, consistent with either graded or discontinuous responsiveness. During normal mapping in vivo, a graded response to ELF-1 seems the simplest model in view of the graded expression and binding patterns shown by ELF-1 and Mek4 (Cheng et al., 1995; Figure 7A), but a discontinuous response is also possible. In either case, ELF-1 would have suitable properties to contribute to topographic specificity, and the basic principles discussed in the models above would apply.

Another question is how the axons could detect a gradient. Models have been proposed in which the axonal growth cone might detect a tiny concentration difference across its ends (Gierer, 1987; Walter et al., 1990). However, mapping is not necessarily determined only by guidance at the axon tip. In birds and mammals, axons typically overshoot, with recognition at the correct anteroposterior position involving collateral branching and retraction of overshooting segments (Nakamura and O'Leary, 1989; Roskies and O'Leary, 1994; Roskies et al., 1995). It is also notable that receptors that bind ELF1–AP are located on cultured retinal axons along their entire length (Cheng et al., 1995). We therefore now propose that the positional information in the tectum could be read not only by the growth cone, but by integrating or distinguishing signals along the entire length of each axon. This model of detection by a long segment of the axon could provide a much easier way to decode positional information in a gradient.

Eph Ligands and Receptors and Spatial Patterning

The Eph family includes at least 7 ligands and 12 receptors, and almost all the receptors are known to be expressed in the nervous system (Brambilla and Klein, 1995; Friedman and O'Leary, 1996a). In view of the properties of ELF-1 in the retinotectal system, it seems plausible that other Eph ligands could also have roles in neural map specification. The Eph family may also have other analogous functions in spatial patterning during nervous system development, for example sharpening the boundaries of embryological domains (Xu et al., 1995) or controlling axon pathfinding or cell migration. Eph family ligands and receptors are also expressed in nonneuronal tissues and could have the potential in many systems, during development and later, to provide spatially precise positional information.

Experimental Procedures

Plasmid and Virus Constructs

pcELF-1 contains chicken *ELF-1* cDNA sequences from nucleotide 34 to 642 (Cheng et al., 1995; GenBank accession L40932) between the BamHI and EcoRI sites of pcDNAI (Invitrogen). RCAS–ELF-1 contains chicken *ELF-1* cDNA sequences from nucleotide 37 to 642, with an NcoI site created by changing the first two codons to atgggg, inserted between the NcoI and EcoRI sites of the shuttle vector pSlax-13 (Riddle et al., 1993) and then transferred into the ClaI site of retroviral vector RCASBP (Hughes et al., 1987; Fekete and Cepko, 1993). Retroviral titers were 3×10^8 to 5×10^8 ml^{-1}. Viral strains A and B were both used and gave similar expression patterns.

Assay of ELF-1 during In Vivo Mapping

Retroviral stock with dye tracer was injected into the neural tube of E2 (stage 9–12) chick embryos in windowed eggs (Fekete and Cepko, 1993). Affinity probe in situ with chromogenic substrates was performed as described on tissue whole mounts, using Mek4–AP or ELF1–AP probes (Cheng and Flanagan, 1994; Cheng et al., 1995). For fluorescent staining, tissues were treated with a Mek4–Ig probe (Cheng et al., 1995) and then with biotin-conjugated anti–human IgG (Pierce), followed by fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and finally were fixed in 4% paraformaldehyde. Tecta were spread for fluorescence microscopy in glycerol with 240 mM n-propyl gallate, 22 mM diazabicyclo-octane, and 230 mM p-phenylenediamine (Sigma) as bleaching inhibitors. Retinal axons were anterogradely labeled with DiI (Molecular Probes) on E14–E16 either by localized injection of a 10% solution in dimethylformamide or by placing crystals into an incision in the retina. Labeled axons were analyzed 36–48 hr later, at stage 40–42 (windowing and other manipulations cause some developmental delay), a stage when the basic topographic order of the map is reaching completion (Nakamura and O'Leary, 1989). Examination of the eye, oriented by the optic fissure, confirmed that the DiI applications were at the expected site within an angle of approximately $\pm 5°$. Tecta were spread by making an incision at the two ends and photographed under a Zeiss Axiophot microscope, and Adobe Photoshop was used to assemble overlapping photographs. Not all embryos survived the procedure: approximately one third survived windowing and retroviral injection long enough be injected with DiI, and of these approximately one half survived to analysis.

In Vitro Stripe Assay

The membrane stripe assay (Walter et al., 1987) was used as modified by Roskies and O'Leary (1994). Tectal membranes were prepared from E9–E10 embryos infected with the RCAS–ELF-1 retrovirus at E1.5 and E2.5 (stages 10–11 and 14–15, respectively) or from uninfected embryos. 293T cell (DuBridge et al., 1987) membranes were prepared 30–40 hr after transfection with pcELF-1 or control plasmid using the calcium phosphate method. Transfection efficiencies were 50%–80% as determined by Mek4–AP binding. Quantitation of ELF1–AP, Mek4–AP, and AP binding to cell lines was as described previously (Cheng and Flanagan, 1994), with proteins at 5 nM. Since in preliminary experiments retinal axons grew poorly on 293T membranes, 293T membranes were mixed with membranes from the anterior third of E9–E10 tecta to create a more permissive growth substrate. Anterior tectal membranes were selected because they do not inhibit or repel either temporal or nasal retinal axons (Walter et al., 1990; A. Roskies, G. C. F., T. M., and D. D. M. O'L., unpublished data). The proportion of 293T cell membranes used varied from 10% to 75%, and retinal axons showed the same growth specificity across this dilution range. Pretreatment of the anterior tectal membranes with PI-PLC to remove endogenous GPI-

anchored proteins did not affect the growth preference of temporal axons. Membrane carpets consisted of alternating 90 μm wide lanes of the different membrane types on a Nucleopore filter. Latex microspheres that fluoresce blue when exposed to UV illumination were added to one of the membrane preparations. Retinal strips (300 μm wide) from the temporal or nasal third of E6 retinas, cut parallel to the optic fissure, were placed ganglion cell side down onto the membrane carpets. After 36 hr, axons were labeled by incubating the cultures in 33 μM carboxyfluorescein diacetate, succinimidyl ester (a fluorescent vital dye; Molecular Probes) for 5 min. All outgrowth in each case was photographed under FITC illumination. Growth preference for each explant was scored independently by three investigators on a graded 0 to 4 scale, in which 0 was no bias for either set of lanes and 4 was a very strong bias for one set, and the mean score was calculated. Only cases that met the following criteria were scored: lanes were distinct as indicated by the blue microsphere marking; axons were labeled adequately; and outgrowth was adequate to judge growth preferences. With 293T membranes, preference was seen with 18 of 20 temporal (mean score 2.25 ± 0.27 SEM) and 1 of 13 nasal explants (mean score 0.23 ± 0.23 SEM); with tectal membranes, 7 of 8 temporal (mean score 2.19 ± 0.41 SEM) and 0 of 5 nasal (mean score 0.00 ± 0.00 SEM). In each case in which a preference was observed, it was for the lanes that did not contain ELF-1-transfected 293T or infected tectal membranes. Preference was not affected by whether the ELF-1-containing membranes were laid down in the first or second set of lanes. Temporal and nasal explants showed a different response to ELF-1, with $p < 0.0001$ for 293T and $p < 0.002$ for tectal membranes.

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