THE EPHRINS AND EPH RECEPTORS IN NEURAL DEVELOPMENT

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ABSTRACT
The Eph receptors are the largest known family of receptor tyrosine kinases. Initially all of them were identified as orphan receptors without known ligands, and their specific functions were not well understood. During the past few years, a corresponding family of ligands has been identified, called the ephrins, and specific functions have now been identified in neural development. The ephrins and Eph receptors are implicated as positional labels that may guide the development of neural topographic maps. They have also been implicated in pathway selection by axons, the guidance of cell migration, and the establishment of regional pattern in the nervous system. The ligands are anchored to cell surfaces, and most of the functions so far identified can be interpreted as precise guidance of cell or axon movement. This large family of ligands and receptors may make a major contribution to the accurate spatial patterning of connections and cell position in the nervous system.

INTRODUCTION
The functioning of the nervous system depends on its precise and complex spatial organization. Creating this organization during development involves solving two problems: first, arranging the cells in the correct locations, and second, establishing the correct pattern of neuronal connections. In both of these processes, it seems clear that the correct spatial organization could not be created without some form of cell-cell communication and, moreover, that this signaling itself must be spatially precise.
Ligands that bind to receptor tyrosine kinases are known to have important roles in development. These ligands and receptors can be divided into subfamilies based on structural homologies and, in at least some cases, obvious similarities in function (van der Geer et al 1994). Examples in neural development are the neurotrophins and their trk family receptors, the platelet-derived growth factors and their receptors, the *Drosophila* sevenless receptor and its ligand boss, and the fibroblast growth factors and their receptors (Barres & Raff 1994, Zipursky & Rubin 1994, Lewin & Barde 1996, Lumsden & Krumlauf 1996).

The Eph receptors are by far the largest known subfamily of receptor tyrosine kinases, with at least 14 members identified thus far in vertebrates (Table 1, Figure 1). Remarkably, however, all the Eph receptors were initially identified as orphan receptors without known ligands, and until recently, their functions were poorly understood. The first clue for roles in neural development or physiology came from studies showing that almost all the receptors are expressed in the developing or adult nervous system (Tuzi & Gullick 1994). More recently, a family of ligands for the Eph receptors has been cloned, called the ephrins, with eight members found thus far in vertebrates (Table 1, Figure 1). Specific

![Figure 1](image_url)  
*Figure 1* Structure and sequence homology of the Eph receptor and ephrin families. Cys-rich, a unique cysteine rich domain found in the Eph receptors; FNIII, fibronectin type III motif; TM, transmembrane domain; GPI, glycosyl phosphatidylinositol membrane anchor; Core, a conserved core sequence in the ligands containing four invariant cysteines. Ephrin-A ligands have a GPI anchor, while ephrin-B ligands have a transmembrane domain. The sequence homology trees were derived by alignment of the extracellular domains of the receptors, or the conserved core sequences of the ligands, using the Clustal program. Ligand and receptor structures are drawn to the same linear scale.
Table 1 The Eph receptor and ephrin families

<table>
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<tr>
<th>Receptors</th>
<th>Ligands</th>
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<tr>
<td>New name</td>
<td>Previous names</td>
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<tr>
<td>EphA1</td>
<td>Eph, Esk</td>
</tr>
<tr>
<td>EphA2</td>
<td>Eck, Myk2, Sek2</td>
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<tr>
<td>EphA3</td>
<td>Cek4, Mek4, Hek, Tyro4; Hek4</td>
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<td>EphA4</td>
<td>Sek, Sek1, Cek8, Hek8, Tyro1</td>
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<tr>
<td>EphA5</td>
<td>Etk1, Bsk, Cek7, Hek7, Rek7</td>
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<tr>
<td>EphA6</td>
<td>Etk2, Hek12</td>
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<td>EphA7</td>
<td>Mdk1, Hek11, Ehk3, Ebk, Cek11</td>
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<tr>
<td>EphA8</td>
<td>Eck; Hek3</td>
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<tr>
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<td>Elk, Cek6, Net; Hek6</td>
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<tr>
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<td>Cek5, Nuk, Erk, Qek5, Tyro5, Sek3; Hek5, Drt</td>
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<td>Cek10, Hek2, Mdk5, Tyro6, Sek4</td>
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<tr>
<td>EphB4</td>
<td>Htk, Myk1, Tyro11; Mdk2</td>
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<tr>
<td>EphB5</td>
<td>Cek9; Hek9</td>
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<td>EphB6</td>
<td>Mep</td>
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</table>

*The column on the left shows the new nomenclature for mammals and birds (Eph Nomenclature Committee 1997). Previous names are listed by publication date with full-length sequences shown first. Names after a semicolon indicate hypothetical orthologs, or proposals to rename a sequence that had previously been published. The name Eph is derived from an erythropoietin producing human hepatocellular carcinoma cell line, which expresses the first of these receptors to be identified, while the name ephrin is derived from Eph receptors interacting proteins, or from the ancient Greek epheiros, an overseer or controller. There are two subfamilies, A and B, defined by sequence homologies (Figure 1), by membrane anchorage of the ligands (Figure 1), and by the preferential binding of A ligands to A receptors and B ligands to B receptors (Table 2). Ligands and receptors are numbered sequentially according to the first report of an essentially full-length sequence. References for receptor or ligand identification are as follows: Eph, Hirai et al 1987; Esk, Lickliter et al 1996; Eck, Lindberg & Hunter 1990, Ruiz & Robertson 1994; Ganju et al 1994; Myk1,2, Andres et al 1994; Sek2,3,4, Becker et al 1994; Cek4, Mek4, Sajjadi et al 1991; Hek, Boyd et al 1992; Tyro1,4,5,6,11, Lai & Lemke 1991; Sek, Gilardi-Hebenstreit et al 1992; Cek5,7,8,9,10, Sajjadi & Pasquale 1993; Cek7, Sierer & Verderame 1994; Hek7,8,11, Fox et al 1995; Etk1-L, Miospiere et al 1993; Bsk, Zhou et al 1994; Mdk1,5, Consek et al 1995a,b; Ehk3, Valenzuela et al 1995; Elk, Ellis et al 1995; Cek11, Aragoni & Nieto 1996; Elk, Chan & Watt 1991; Park & Sanchez 1997; Elk, Leewin et al 1988; Net, Tang et al 1995; Cek5, Pasquale 1991; Nuk, Henkemeyer et al 1994; Erk, Chan & Watt 1991, Kiyokawa et al 1994; Qek5, Kenny et al 1995; Htk2, Bohme et al 1993; Htk, Bennett et al 1994; Mep, Gurniak & Berg 1996; B61, Holzman et al 1990, Bartley et al 1994; ELF-1, Cheng & Flanagan 1994a; Etk-L, Elk-L, EFL-2,3, Davis et al 1994; LERK-3,4, Koziolosky et al 1995; AL-1, Winslow et al 1995; RAGS, Drevscher et al 1995; LERK-2, Beckmann et al 1994; Cek5-L, Shao et al 1994; Htk-L, Bennett et al 1995; ELF-2, Bergemann et al 1995; NLERK-2, Nicola et al 1996; Elk-L3, EFL-6, Gale et al 1996a; ELF-3, Brambilla et al 1996, Bergemann et al 1997.

functions in neural development have also been identified. The ephrins can act as axon guidance molecules and are implicated in the mapping of axonal connections within target regions, as well as in pathway selection on the way to targets. The ephrins have also been shown to influence regional neural patterning. In this review, we discuss the identification and molecular properties of the ephrins and their Eph receptors, followed by recent evidence for their functions in neural development.
IDENTIFICATION OF RECEPTORS AND LIGANDS

The classical approach to identifying new cell-cell signaling factors, pioneered with nerve growth factor (Levi-Montalcini 1987), is to identify an effect on cell behavior and then to use this as a bioassay to trace the molecule during purification or cloning steps. This bioassay approach has been highly successful, but it also has limitations. Most obviously, it is necessary for a convenient assay to be available. At least as important, for strategies involving purification, it is generally necessary for the factor to be active in a soluble form.

To date, none of the ephrins or their Eph receptors have been identified by isolating an activity through the bioassay approach. Moreover, in striking contrast to other families of tyrosine kinases and their ligands, none of them has yet been identified through activity as an oncogene or transforming gene. Instead, all the receptors and ligands have been identified through their molecular properties.

The receptors have so far been identified by approaches based on the conservation of sequence or catalytic activity in the kinase domain. The first to be described, EphA1, was identified by screening a cDNA library with a kinase domain probe by low-stringency cross-hybridization (Hirai et al 1987). Cross-hybridization or polymerase chain reaction of kinase domain sequences was subsequently used to isolate most of the other members of the Eph receptor family, in several cases through searches that were specifically targeted to neural tissues (Lindberg & Hunter 1990, Lai & Lemke 1991, Chan & Watt 1991, Gilardi-Hebenstreit et al 1992; see also references in Table 1). Several additional Eph receptors have been identified through the catalytic activity of the kinase by screening bacterial cDNA expression libraries with antiphosphotyrosine antibodies (Letwin et al 1988, Pasquale & Singer 1989, Pasquale 1991, Sajjadi et al 1991, Henkemeyer et al 1994, Zhou et al 1994). EphA3 was also identified using a monoclonal antibody to a lymphoid cell surface antigen (Boyd et al 1992).

Identification of the ligands lagged several years behind that of the receptors. Ligands for receptor tyrosine kinases generally have weak sequence conservation, making it impractical to use sequence homology to identify new families. The Eph ligands were instead first identified by soluble receptor affinity methods. In this approach, first applied to receptors in other classes (Aruffo et al 1990, Flanagan & Leder 1990), the receptor extracellular domain is fused to a tag, such as alkaline phosphatase or the immunoglobulin Fc region, to produce a soluble affinity probe that can be used to detect the ligand. In 1994, soluble receptor approaches were used to identify four ligands for Eph receptors: ephrin-A1 (Bartley et al 1994) and soon afterwards ephrin-A2, -A3, and -B1 (Beckmann et al 1994, Cheng & Flanagan 1994a, Davis et al 1994, Shao et al 1994). It turned out that ephrin-A1 had also been identified previously
as a cytokine-inducible cDNA of unknown function (Holzman et al 1990). Since then, four more ligands have been identified by soluble receptor methods (Bennett et al 1995, Kozlosky et al 1995, Winslow et al 1995) or through the appearance of homologs in sequence tag databases (Bergemann et al 1995, Cerretti et al 1995, Gale et al 1996a, Nicola et al 1996). One of these ligands, ephrin-A5, was also identified independently in a screen for molecules potentially involved in retinotectal development, through its properties as a glycosyl phosphatidylinositol (GPI)–anchored protein expressed in posterior chick tectum (Drescher et al 1995).

Why have none of the ephrins or Eph receptors been identified by isolating an activity with a bioassay? As we see further below, there may be at least two reasons. First, the guidance functions associated thus far with the family do not necessarily lend themselves to bioassays that are convenient compared with, for example, a cell proliferation assay. Second, the ligands are all membrane associated and appear to lose their activity when cleaved from the membrane (Davis et al 1994). The Eph family thus provides a striking example of where modern molecularly based techniques have played a key role in identifying an entire new set of cell-cell signaling factors, thereby allowing new approaches to study biological questions.

MOLECULAR PROPERTIES

Structural Features

RECEPTOR STRUCTURE The Eph receptors are characterized by an extracellular region with a unique cysteine-rich motif extending over its amino-terminal half, followed by two fibronectin type III motifs (Figure 1). Three exons matching these three motifs are found in the genomic structure (Connor & Pasquale 1995). As in other receptor tyrosine kinases, there is an intracellular domain presumed to be involved in signal transduction, and a single transmembrane domain. The Eph receptors form a particularly closely related subfamily of receptor tyrosine kinases, with sequence identities of approximately 65–90% in the kinase domain, and approximately 30–70% in the extracellular domain.

LIGAND STRUCTURE All the ligands share a conserved core sequence of approximately 125 amino acids, including 4 invariant cysteine residues, probably corresponding to a receptor binding domain. This is followed by a membrane anchorage domain, taking the form of a GPI anchor for five of the ligands (ephrin-A1 to -A5) or a transmembrane domain for three of them (ephrin-B1 to -B3) (Figure 1). The ligands have close homology relationships, with approximately 30–70% identity in the core sequence. This close conservation is perhaps even more striking than the homology among the receptors, since
other classes of ligands that bind to receptor tyrosine kinases typically have low levels of sequence identity apart from a few key residues.

**PHYLOGENETIC COMPARISONS** Ephrins and Eph receptors have been cloned from several vertebrate species, with the most extensive coverage in human, mouse, and chicken. In the amniotes that have been studied—mammals and birds—it is possible to unambiguously assign orthologs (homologs diverging by speciation rather than by gene duplication), and the overall number of ligands and receptors seems to be conserved (Figure 1). Several receptors and ligands have also been cloned from frog (Xenopus laevis) and zebrafish (Brachydanio rerio). At least some of these seem to be orthologs of particular mammalian molecules, and there is probably a similar overall number of ligands and receptors (Macdonald et al 1994; Winning & Sargent 1994; Xu et al 1994, 1995, 1996; Brandli & Kirschner 1995; Jones et al 1995; Scales et al 1995; Taneja et al 1995; Weinstein et al 1996; Winning et al 1996; Brennan et al 1997; Cooke et al 1997; Smith et al 1997).

Ephrins and Eph receptors have been identified both in flies and in nematodes (A Chisholm, J Culotti, J Thomas & SL Zipursky, personal communications). This indicates that the family is ancient and may date to the origin of metazoans. On the other hand, considering the large numbers and especially close sequence relationships among the vertebrate ligands and receptors, a major expansion may have accompanied the evolution of vertebrates and may be related to the acquisition of higher neural function.

**Membrane Anchorage of the Ligands May Produce a Tightly Localized Signal, Allowing Precise Functions in Spatial Patterning**

The need to generate precise three-dimensional pattern in development implies a requirement for cell-cell signaling that is equally precise. This may explain why, although the first growth factors were identified as soluble molecules, it now appears that many, if not most, ligands for receptor tyrosine kinases can be found in forms that are not freely diffusible, being anchored either to cell surfaces or to extracellular matrix (Massague & Pandiella 1993, Taipale & Keski-Oja 1997).

All the known members of the ephrin family are membrane associated. The GPI linkage found in the A ephrins is a feature not yet seen in any other families of ligands that bind to receptor tyrosine kinases, though the functional significance of this particular type of linkage in the ephrins remains to be determined. The transmembrane linkage in the B ephrins, on the other hand, is a feature found in many other ligands for receptor tyrosine kinases (Massague & Pandiella 1993). Examples include the kit ligand, colony stimulating factor-1,
and numerous members of the epidermal growth factor family, all of which are produced as transmembrane molecules and can also be cleaved to produce active soluble molecules. The functional significance of the transmembrane versus soluble forms is not certain, but in the case of the kit ligand, genetic evidence indicates that the membrane anchorage is essential for normal biological activity in vivo, and it has been proposed that ligand presentation at the cell surface may serve to spatially localize the signaling activity (Brannan et al 1991, Flanagan et al 1991).

In the case of the ephrins, this surface localization may be especially tight. Artificial soluble forms produced by truncation outside the membrane are incapable of receptor activation, whereas the same ligands will activate their receptors when presented on a cell surface, or when the soluble forms are artificially clustered with dimeric tags or antibodies (Davis et al 1994). This presumably reflects a requirement for these ligands to be in a dimeric or oligomeric form to activate the receptor, which in turn may reflect a requirement for the Eph receptors to be dimerized or clustered to transduce a signal, as demonstrated for other classes of receptor (Lemmon & Schlessinger 1994). The apparently stringent requirement for ephrins to be expressed at the cell surface for receptor activation is unique among known ligands for receptor tyrosine kinases and may be a critical feature allowing especially precise functions of this family in spatial patterning.

**Receptor-Ligand Binding Interactions and Affinities**

An important way to understand the potential biological functions of the ephrins and their receptors is to identify which ligands bind which receptors. For the ephrin family, this is an unusually complex issue. There are many receptors and ligands, and there is a high degree of binding promiscuity, with individual ligands binding multiple receptors and vice versa. Some simplification comes from the division into A and B subgroups (Table 1, Figure 1), since the EphA receptors show a strong preference for binding the ephrin-A ligands, and the B receptors for B ligands (Table 2). However, even this simplification must be treated with caution, since there are wide variations in affinity within each subgroup. Moreover, there may be some moderately high affinity binding between subgroups, for example in the case of EphA4 and ephrin-B2 (Table 2).

It is also important to remember that all the binding affinities have been measured with one partner in an artificial soluble form (almost always linked to a dimeric tag of immunoglobulin or alkaline phosphatase). In the native state, when the ligands and receptors are both confined to interacting cell surfaces, the binding is likely to be highly multivalent and cooperative. In other words, the unfavorable entropy change of binding should be less than in free solution, so the interaction is expected to have a higher avidity. Therefore
## Table 2  Binding properties of Eph receptors and ephrins

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<td>18d</td>
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<td>0.2–0.5f</td>
<td>1.19m</td>
<td>2.41m</td>
<td>—</td>
<td>4.3/1.4d</td>
<td>NBDm</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>20.1m</td>
<td>1e</td>
<td>0.86p</td>
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<td>3.96m</td>
<td>8.6p</td>
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<td>1.47m</td>
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<tr>
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<td>—</td>
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<td>0.42n</td>
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<td>NBD3.2</td>
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even receptor-ligand pairs with weak affinities in solution might be biologically significant. Provisionally, it seems reasonable to assume that at least the higher-affinity receptor-ligand pairs, with dissociation constants around 1 nM, may be biologically relevant.

In principle, ligand binding can be activating, inactivating, or antagonistic for receptor signaling. An example is provided by the *Drosophila* receptor tyrosine kinase DER, which has both activating and antagonistic ligands (Schweitzer et al 1995). Such possibilities have not yet been thoroughly tested for the Eph family. In most cases, high-affinity binding appears to correlate with receptor autophosphorylation (Davis et al 1994, Brambilla et al 1995, Shao et al 1995, Winslow et al 1995, Gale et al 1996b). However, in at least one case, ephrin-B2 with EphB2, high-affinity binding may not result in receptor activation, suggesting the intriguing possibility of inhibitory or antagonistic effects (Brambilla et al 1996). Also, in view of the close homologies within both ephrin and Eph receptor families, there might be receptor-receptor heterodimerization, as found for other receptor tyrosine kinases (Carraway & Cantley 1994), or even ligand-ligand heterodimerization. The potential effect of heterodimers on function has not yet been addressed.

**Signaling Through the Receptors**

The downstream signaling pathways for the Eph receptors have not yet been well characterized. By analogy with other receptor tyrosine kinases, signaling is presumed to involve modulation of the kinase activity, as well as binding of intracellular targets through phosphotyrosine-containing motifs on the receptor (van der Geer et al 1994). The Eph receptors have several conserved cytoplasmic tyrosine residues, and several proteins have been identified that can interact with the cytoplasmic domains in vitro, or in yeast two-hybrid systems, including the lipid kinase PI3-K (Pandey et al 1994), the adaptor proteins Grb2, Grb10, and SLAP (Pandey et al 1995a, Stein et al 1996), and the cytoplasmic tyrosine kinase p59fyn (Ellis et al 1996). It remains to be seen whether these interactions have functional significance in vivo. The kinase domain sequence of the Eph receptors is unusual in having some sequence features typical of receptor tyrosine kinases, and others typical of cytoplasmic tyrosine kinases (Hanks & Quinn 1991). This suggests the possibility of novel downstream signaling mechanisms, a possibility that could also be consistent with the unique biological functions of these receptors.

**Intracellular Interactions and Signal Transduction by the Cytoplasmic Tails of the Ligands**

Since their identification more than 10 years ago, the existence of transmembrane ligands for receptor tyrosine kinases has raised the possibility that the
ligand intracellular domain might be capable of cytoplasmic interactions, and might even transduce a signal into the ligand-bearing cell (Pfeffer & Ullrich 1985, Massague & Pandiella 1993). However, there is very little conservation of sequence in comparisons between different ligands, and although there has been some indication of potential roles (Bosenberg et al 1992, Brannan et al 1992, Cheng & Flanagan 1994b, Shum et al 1994), the biological functions of ligand cytoplasmic tails are not yet well understood.

In striking contrast to other transmembrane ligands of receptor tyrosine kinases, the transmembrane ephrins show a close sequence conservation of their cytoplasmic tails. The tails are well conserved throughout their length of approximately 85 amino acids, and the last 33 amino acids are especially well conserved, showing a remarkable 90–100% identity (Bennett et al 1995, Bergemann et al 1995, Gale et al 1996a, Nicola et al 1996). Indeed, the cytoplasmic tail is the best conserved region of the entire molecule, strongly implying functionally significant interactions.

Do the sequences give any specific clue to potential interactions? The cytoplasmic tails have several candidate phosphorylation sites, including tyrosine residues in sequence contexts that could bind to SH2 domains. Also, like other transmembrane ligands, the transmembrane ephrins have a valine residue at the carboxy terminus. This valine residue is required for extracellular proteolysis of transforming growth factor-α (Bosenberg et al 1992) but not required for the equivalent cleavage of kit ligand (Cheng & Flanagan 1994b). Recent studies have now shown that a carboxy-terminal valine residue can be part of a four–amino acid motif that binds PDZ domains (Sheng 1996, Songyang et al 1997). It will be interesting to see whether the transmembrane ephrins actually do bind PDZ-containing proteins and what the functional significance of such interactions may be.

It has now been shown directly that the transmembrane ephrins apparently can transduce a signal across the membrane (Holland et al 1996, Bruckner et al 1997). Both ephrin-B1 and ephrin-B2 become phosphorylated on tyrosine residues after ligand-bearing cells are either cocultured with cells carrying the EphB2 receptor or treated with a soluble fusion protein consisting of the EphB2 extracellular domain fused to an immunoglobulin tag. These ligands also contain phosphotyrosine in embryos. In solution, the ligands can be phosphorylated by v-src, suggesting a possible model in which the extracellular interaction of B ephrins with their receptors might result in intracellular phosphorylation of the ligands by src-like cytoplasmic kinases (Holland et al 1996, Bruckner et al 1997).

It is not yet known what the signal transduced through the transmembrane ligands might do biologically. However, the results of targeted disruption of
the EphB2 receptor gene, discussed further below, may be relevant. EphB2 disruption results in defects in axonal connections, but surprisingly, deletion of only the kinase domain of EphB2 does not cause the same abnormalities. Moreover, the affected neurons do not express the EphB2 receptor but do express transmembrane ephrins. These findings suggest the intriguing possibility that the transmembrane ephrins might transduce signals for axon guidance into the ligand-bearing cell (Henkemeyer et al 1996).

Although by convention the Eph kinases are referred to as receptors and the ephrins as ligands, the idea of bidirectional signaling raises the question of which is the ligand and which is the receptor.

ROLES IN THE DEVELOPMENT OF NEURONAL CONNECTIONS

During development, the initial pattern of connections between neurons is believed to be established by molecular guidance cues (Tessier-Lavigne & Goodman 1996). First, axons must find their correct 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 recognition within the target can involve cell-cell specificity, involving recognition of 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 their connections.

The first clue that the Eph family might have some function in axons came from immunolocalization studies showing that Eph receptors are present on developing axon tracts (Pasquale et al 1992, Henkemeyer et al 1994, Soans et al 1994). More specific evidence for functions in axon guidance followed identification of the ligands, with the demonstration of effects on cortical axon fasciculation (Winslow et al 1995) and with evidence for functions in retinotectal topographic mapping (Cheng et al 1995, Drescher et al 1995). Several other potential roles in axon guidance have also now been identified. The retinotectal projection is discussed first below. It provides a model particularly well-suited to understand the functions of the ephrins, not only because of recent work at the molecular level, but also because of the extensive background of outstanding work on the retinotectal system, which for more than 50 years has been a favorite model to understand the development of neural specificity (Figure 2).

Retinotectal Topographic Mapping

INITIAL EVIDENCE FOR THE EXISTENCE OF TOPOGRAPHIC LABELS There is a vast literature on retinotectal mapping that cannot be fully cited here. For
Figure 2  Expression of ephrins and Eph receptors in relation to retinotectal topographic mapping.  
(a) Objects in the outside world form an inverted image on the retina. The image is then transferred to the optic tectum.  
(b) Mapping of the retinal image onto the tectum relies on the topographic arrangement of the projecting axons. Retinal axons pass through the optic nerve and optic tract and all enter the tectum at its anterior end. Nasal axons project to posterior tectum, and temporal axons to anterior tectum. Similarly, dorsal axons project to ventral tectum, and ventral axons to dorsal tectum (not shown).  
(c) Eph ligands and receptors may serve as complementary positional labels that help guide the axons to their correct termination point, based on their point of origin. The ligands and receptors illustrated may be involved in mapping the anteroposterior axis. Other receptor and ligand patterns are also present in the developing retinotectal system, including EphB receptors and ephrin-B ligands in dorsoventral gradients. See the text for references and further details.


In 1943, Roger Sperry published his classic experiments on the axonal projection from the retina to the tectum of the midbrain, the primary visual projection in nonmammalian vertebrates. These experiments involved cutting the optic nerve of the newt and rotating the eye 180°. The result, after axon regeneration, was that the animals behaved as if they saw the world upside down and back to front. For example, if they were presented with a piece of meat in front, they would whirl and strike backwards. Since their response was maladaptive, it seemed inconsistent with the prevailing models of specificity being determined purely by neural activity, and from this simple experiment Sperry first proposed the ideas that became known as the chemoaffinity theory: that neuronal specificity could be determined by stable complementary tags on projecting neurons.
and their target regions. He further proposed that these positional labels were likely to take the form of gradients that could mark each position with its appropriate latitude and longitude (Sperry 1943, 1963). This use of gradients could have two major advantages over individual cell-specific labels. First, it could be far more economical, requiring only a few labeling molecules. Second, because a gradient could be sensed throughout the target, it could tell the axons not only where to terminate, but which direction to go if they were in the wrong place, without needing to search the target randomly.

The chemoaffinity theory was subsequently tested in many experiments involving ablation or grafting of parts of the retinotectal system. Some results were consistent with the theory, though others indicated an unexpected plasticity. For example, after ablation of a half retina, some experiments showed innervation of half the tectum as predicted, whereas other experiments, in different species or after longer times, showed innervation of the whole tectum. These differences resulted in a good deal of controversy. In retrospect, however, the key conclusion seems to be that, since regeneration can be guided in a position-specific manner, the chemoaffinity labels must exist, even if under other conditions, the labels or the responses to them can be modulated (Fraser & Hunt 1980, Jacobson 1991). Further support for chemoaffinity labels came from labeling experiments using modern dye tracers, showing that developing axons can find their correct tectal termination point, even if they are deflected along the way, and even in the presence of inhibitors of neural activity—although neural activity subsequently refines the map (Jacobson 1991, Holt & Harris 1993).

Since the 1970s, several in vitro assay systems have been developed to model retinotectal mapping. The initial assays were based on the idea that the chemoaffinity labels could be adhesion molecules. For example, tectal membranes preferentially adhere to nasal retinal neurites (Halfter et al 1981), and dorsal retinal cells preferentially adhere to ventral retina (Gottlieb et al 1976). More recently, elegant studies pioneered by Bonhoeffer’s group have used assays that directly test the growth responses of retinal axons. These assays have included the stripe assay for axon guidance, where retinal axons are allowed to choose between alternating stripes of membrane vesicles derived from anterior or posterior tectum (Walter et al 1987), the growth cone collapse assay, where axons are treated with suspended vesicles of tectal membranes (Cox et al 1990), and the stripe assay for axon branching, where the retinal axons are allowed to grow at right angles to the alternating membrane stripes (Roskies & O’Leary 1994). These studies have convincingly demonstrated an activity that is linked to posterior tectal cell membranes by a GPI anchor and that shows topographically specific inhibitory effects on axons from the temporal but not the nasal side of the retina. The results have thus provided compelling evidence for the

Taken together, the in vivo and in vitro studies have provided strong evidence for topographic labels. The molecular identification of these recognition labels has, however, proven elusive. Numerous antigens or molecules have been proposed, and some remain candidates for a role in mapping, including TOPAP, RGM, and TRAP (Trisler et al 1981, Stahl et al 1990, McLoon 1991, Savitt et al 1995, Muller et al 1996). The first evidence for a potential involvement of the ephrins came from two reports: one showing graded expression and axon repellent effects of ephrin-A5 (Drescher et al 1995), and the other showing complementary gradients in expression and binding for ephrin-A2 and its receptor EphA3 (Cheng et al 1995).

Ephrins andTheir Receptors as Topographically Specific Labels

Currently there are three types of evidence that ephrins and Eph receptors may act as topographically specific labels: (a) expression gradients across the retina or tectum, (b) binding gradients detected by soluble receptor or ligand fusion proteins, and (c) experiments showing topographically specific effects of ephrins on retinal axon growth in vitro and in vivo. These three areas are discussed further below.

Complementary gradients in expression and binding Sperry’s chemoaffinity theory postulates positional labels in gradients. These gradients are formally analogous to those in other developmental fields such as the Drosophila embryo (St Johnston & Nusslein-Volhard 1992), although with the additional requirement that the topographic labels must be complementary in two separate fields. Although the topographic labels need not be simple gradients, some form of complementarity is essential, because the projecting field is being mapped onto the target: There must be not only a differential distribution of labels across the tectum, but also a differential responsiveness across the retina—otherwise all the retinal axons would map to the same place.

Ephrin-A2 was initially identified as a ligand for the EphA3 and EphA4 receptors expressed in the embryonic mouse midbrain (Cheng & Flanagan 1994a). It was subsequently shown in the chick, a species where retinotectal mapping has been studied extensively, that the ephrin-A2 gene is expressed in the tectum and the EphA3 receptor gene is expressed in the retina, where it localizes to the projecting retinal ganglion cells. Both genes are expressed at the time of mapping, both are in gradients, and the gradients are along matching axes that map to one another (Figure 2) (Cheng et al 1995).

Ephrin-A2 and EphA3 also show complementarity in a functional analysis of binding activity. This was assessed by the affinity probe in situ technique
EPHRINS IN NEURL DEVELOPMENT

(Cheng & Flanagan 1994a), where soluble receptor or ligand fusion protein probes are used to determine the in situ distribution of cognate binding sites in the embryo. This technique showed that ephrin-A2 and EphA3 probes can each detect a matching gradient of binding sites in the reciprocal field. This result cannot simply be predicted from the expression patterns of individual ligands and receptors, because of the high binding promiscuity in the ephrin family. These binding experiments thus provided direct evidence for the gradient complementarity that would be predicted from the chemoaffinity theory (Cheng et al 1995).

A separate tectal ligand, ephrin-A5, was cloned as a ligand for the receptor EphA5 (Winslow et al 1995), and independently as a GPI-linked protein in posterior tectum, in a screen for molecules potentially involved in retinotectal mapping (Drescher et al 1995). RNA in situ hybridization showed that the ephrin-A5 gene is expressed in a gradient within the posterior part of the tectum (Figure 2) (Drescher et al 1995). Unlike ephrin-A2 RNA, which is expressed broadly through the layers of the tectum, ephrin-A5 RNA is expressed mainly in the ventricular zone, on the opposite side from the stratum opticum where the retinal axons enter. However, consistent with the idea that it may be expressed on radial glial fibers, immunolocalization studies show ephrin-A5 protein distribution through all tectal layers, including the superficial layers where the axons enter (Monschau et al 1997).

Although ephrin-A2 and ephrin-A5 are both in tectal gradients, their distributions are different (Figure 2). Ephrin-A2 in chicken and mouse, as well as zf-L3, a likely ortholog in zebrafish, are all expressed most highly at the posterior end of the tectum (or its mammalian equivalent the superior colliculus) and diminish in a smooth gradient to the anterior end (Cheng et al 1995, Zhang et al 1996, Brennan et al 1997; D Feldheim, P Vanderhaeghen & J Flanagan, unpublished data). Ephrin-A5, on the other hand, shows a more posterior expression. In chick, it is in a gradient in the posterior part of the tectum but is not readily detected in the anterior part (Drescher et al 1995, Monschau et al 1997). In mouse, the highest expression is in the inferior colliculus, posterior to the retinal target region, with lower expression extending into the superior colliculus (Donoghue et al 1996, Zhang et al 1996; D Feldheim, P Vanderhaeghen & J Flanagan, unpublished data). In zebrafish, zf-L4, an apparent ortholog of ephrin-A5, is in a band rather than a gradient and is posterior to the optic tectum (Brennan et al 1997).

Topographically specific repulsion of retinal axons

The first direct evidence that ephrins can affect axon growth came from studies of ephrin-A5. It was shown in a coculture assay that ephrin-A5 expressed on glial cells promotes
fasciculation of cortical axons (Winslow et al. 1995), an effect that may be due to axon repulsion by the glial cells (Tessier-Lavigne 1995, Meima et al. 1997). In the retinotectal system, it was shown using the in vitro stripe assay that ephrin-A5 can act as a repellent for retinal axons. Initially, identical effects were seen on nasal and temporal axons at a range of concentrations, so it was concluded that ephrin-A5 is not likely to be a topographically specific mapping label (Drescher et al. 1995). A subsequent reevaluation indicated that at some concentrations ephrin-A5 has a higher activity on temporal than nasal axons, although it always showed pronounced effects on both nasal and temporal axons (Monschau et al. 1997).

The effects of ephrin-A2 have also been tested on retinal axons. Using the stripe assay, it was shown that ephrin-A2 can act as an axon repellent guidance factor in vitro, with effects on temporal but not nasal retinal axons (Nakamoto et al. 1996), as previously reported for natural tectal membranes in the stripe assay (Walter et al. 1987). In vivo, when the tectal ephrin-A2 pattern was modified by retroviral overexpression in developing chick embryos, retinal axons avoided ephrin-A2 patches and mapped to abnormally anterior positions. These effects were seen on temporal axons but not axons from the nasal pole of the retina, providing the first demonstration of a cell recognition molecule with topographically specific effects on neural map development (Nakamoto et al. 1996).

EPHRINS AND THE MECHANISM OF MAPPING The available evidence on ephrins and their receptors, summarized above, makes it very likely that this family plays some role as positional labels in retinotectal mapping. However, much remains to be learned about the creation of the map. Below, we discuss some of the models and questions raised by the current information.

Why two tectal ligands? Ephrin-A2 and ephrin-A5 are both axon repellents and are both in anteroposterior gradients. Why have both? For ephrin-A2, the current evidence on expression pattern, binding pattern, and in vitro and in vivo activities all seem consistent with the idea that this molecule could play a role in determining nasal versus temporal retinotectal specificity. For ephrin-A5, the current data suggest two models, not mutually exclusive. First, ephrin-A2 and ephrin-A5 may somehow cooperate in generating the overall topographic map. For example, two overlapping gradients might allow a greater precision of mapping than a single one. Second, in view of the expression of ephrin-A5 at or beyond the posterior end of the tectum, and in view of its repellent effects on both temporal and nasal axons, ephrin-A5 may serve as a barrier that prevents all retinal axons from terminating beyond the limits of the tectum. Consistent with both these models, targeted disruption of the ephrin-A5 gene in mice apparently causes abnormalities in the retinocollicular map, though
overall topography remains, and also causes abnormal projection of retinal axons beyond the end of the superior colliculus (J Frisen, P Yates, D O’Leary & M Barbacid, personal communication).

**Graded responsiveness** To generate a smooth and continuous map, the simplest model would be that different retinal positions should show a graded responsiveness to the tectal ligands. This feature has not yet been quantitatively demonstrated for either ephrin-A2 or ephrin-A5. Surprisingly, the response of retinal axons to normal tectal membranes in the stripe assay appeared to be not smoothly graded but discontinuous, with all temporal axons responding and all nasal axons unresponsive (Walter et al 1987). The topographically specific response to ephrin-A2 in vitro may be similar, although there is also some indication of a gradation in the response to both ephrin-A2 and ephrin-A5 (Nakamoto et al 1996, Monschau et al 1997). One possibility is that the smooth map might be constructed by the summation of discontinuous responses to more than one topographic label. However, it should also be remembered that the in vitro models lose important spatial and temporal features and cannot reproduce all aspects of mapping in vivo. Indeed, the initial stages of in vivo mapping in the chick show a discontinuity perhaps similar to the short-term assays in vitro, with all temporal axons projecting to the anterior half of the tectum and all nasal axons to the posterior half, and smooth gradation appears only later (Nakamura & O’Leary 1989, Roskies et al 1995). Ultimately, in vivo approaches may help to address these more subtle questions on the mapping mechanism.

*Are permissive or attractant, as well as repellent, signals involved?* If there were repellent signals only, it might be expected that all axons would stop at the anterior end of the tectum. There are at least two potential ways to avoid this problem. First, the axons could have an intrinsic tendency to fill the available tectal space, because of their directional growth or because of axon-axon repulsion. Second, mapping could involve both repellent and attractant signals in the tectum, as proposed in the dual gradient models of Gierer (1987). Regarding the molecular mechanisms for this second idea, one possibility is that the ephrins themselves could act as both repellents and attractants. This could be consistent with the presence of multiple Eph receptors and ligands in the retinotectal system. Alternatively, in addition to the ephrins, there could be a separate set of attractant or permissive molecules. Potential candidates are adhesion molecules or BDNF, which is present in the tectum and promotes retinal axon branching (Cohen-Cory & Fraser 1994, 1995; Herzog et al 1994). In principle, such positively acting molecules might be uniformly distributed in the tectum (permissive), graded in the tectum (potentially attractant), or distributed in complementary patterns in the retina and tectum (potentially topographic labels).
How is the tectal gradient detected? Models have been proposed that show that the axonal growth cone could 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 their final position along the tectal anteroposterior axis. Recognition at the correct position involves subsequent collateral branching and retraction of the overshooting segments (Nakamura & O’Leary 1989, Roskies et al 1995). Receptors that bind ephrin-A ligands are located on cultured retinal axons along their entire length (Cheng et al 1995). It therefore seems plausible 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 (Nakamoto et al 1996).

Why countergradients? Why repulsion? A mass action model for topographic mapping Receptors and ligands bind to one another, and are generally expected to colocalize in tissues. The findings that retinotectal ephrins and their receptors are in countergradients and that their interaction causes repulsion therefore seem initially surprising. To account for these observations, we have proposed a mass action model for topographic mapping (Nakamoto et al 1996). At the end of mapping, the model proposes that all the axons receive an equivalent amount of negative signal, which is just enough to counteract their tendency to grow toward the posterior tectum, and therefore defines their termination zone. It is then proposed that the amount of negative signal is determined by the amount of receptor-ligand complex, and that this in turn is determined by the law of mass action: The simplest equation for a receptor (R) and ligand (L) would be $[RL] = K_A[R][L]$, though more complex equations are also possible. Thus, a similar amount of signal could result either from high receptor and low ligand (anterior tectum), or low receptor and high ligand (posterior tectum). A particularly interesting aspect of this mass action model is that it could only work with countergradients. Then, if countergradients are needed, it could work only for a repellent rather than an attractant signal. Thus, starting from molecular principles, the mass action model could explain why topographic mapping would be guided by labels organized as countergradients and as repellents. While the model predicts at least one set of countergradient repellents, additional components may be permissive or attractant.

UPSTREAM INDUCERS OF THE RETINOTECTAL LABELS Sperry’s proposal of activity-independent recognition labels carries the implication that there must be a genetically determined cascade of events that sets up these gradients.
In the tectum, the engrailed nuclear factors, \textit{en}-1 and \textit{en}-2, are in an anteroposterior gradient, with a high point at the isthmus where the midbrain joins the hindbrain (reviewed by Lumsden & Krumlauf 1996, Retaux & Harris 1996). Retroviral overexpression studies in chick indicate that \textit{en}-1 or \textit{en}-2 have topographically specific effects on retinotectal mapping and can induce both ephrin-A2 and ephrin-A5 (Friedman & O'Leary 1996, Itasaki & Nakamura 1996, Logan et al 1996). There must in turn be extracellular signals that set up the engrailed gradient. The signaling factor \textit{wnt}-1 is required for maintenance, though not initiation of engrailed expression (Danielian & Mcmahon 1996).

Fibroblast growth factor 8 (FGF8), when introduced on a bead into a site anterior to the normal tectum, can induce an entire new tectum with reversed polarity. Since FGF8 is normally expressed at the isthmus, and earlier in the underlying mesoderm, in normal development it might be a signal that initiates tectal development and polarity (Crossley et al 1996).

In the retina, too, there are candidate nuclear factors that may be upstream of the topographic recognition labels—including SOHo-1, CBF-1, and CBF-2—all of which show nasotemporal asymmetries (Deitcher et al 1994, Yuasa et al 1996). Retroviral overexpression of CBF-1 and CBF-2 shows that each can cause changes in retinal axon projections consistent with the idea that they may play a role in setting up nasal versus temporal differences. However, it is not yet clear whether they are upstream of Eph receptor expression (Yuasa et al 1996).

OTHER POTENTIAL ROLES OF EPHRINS IN THE RETINOTECTAL SYSTEM

In addition to EphA3, at least two other related receptors are expressed in the retinal ganglion cells, EphA4 and EphA5 (Figure 2) (Cheng et al 1995, Monschau et al 1997). Although these receptors are not in an obvious gradient across the retina in the chick, they could have some role in mapping.

As well as expression of EphA receptors in retina and ephrin-A ligands in tectum, the reciprocal pattern is also seen: EphA receptors are expressed in the tectum (Ellis et al 1995, Gale et al 1996b, Park et al 1997), and ephrin-A ligands are expressed in the retina (Marcus et al 1996, Brennan et al 1997, Monschau et al 1997). These patterns might make a contribution to orderly development of the retinotectal map. However, most areas of the nervous system have both efferent and afferent connections and might for that reason be expected to contain both receptors and ligands. Indeed, as discussed below, targeted disruption of the EphA8 gene implicates this receptor in the patterning of efferent connections leaving the superior colliculus/tectum (Park et al 1997).

The ephrin-B ligands and EphB receptors are also expressed in the retinotectal system. The patterns suggest possible roles in dorsoventral patterning, although
it is not yet clear whether they act as retinotectal labels. EphB2 is in a ventral high to dorsal low gradient in the retina (Holash & Pasquale 1995, Kenny et al 1995). Ephrin-B1 is expressed weakly in a dorsal high to ventral low gradient in the tectum (Braisted et al 1997). Since ventral retinal axons project to dorsal tectum, a role for EphB2 and ephrin-B1 as complementary mapping labels would presumably require an attractant interaction (Braisted et al 1997), in contrast to the repellent axon guidance effects of ephrin-B1 observed thus far (Wang & Anderson 1997). Ephrin-B1 and ephrin-B2 are also expressed in a dorsal high to ventral low gradient within the retina (Marcus et al 1996, Holash et al 1997). The retinal RNA expression for these ligands is in the inner nuclear layer rather than the ganglion cell layer, suggesting an intraretinal function. They might be involved in regulating axon projections within the retina (Catsicas et al 1987). Alternatively, the complementary ligand and receptor gradients seen within the retina might be involved in patterning retinal cell position (Marcus et al 1996, Braisted et al 1997, Holash et al 1997).

The Topographic Hippocamposeptal Projection

Topographic maps are found throughout the vertebrate nervous system and are typically the way that projecting axons are organized. The involvement of ephrins in retinotectal mapping clearly raises the question of whether they may serve as topographic labels in other maps. Already there is at least one likely example, the hippocamposeptal system, a projection involved in learning and memory. Complementary gradients of receptor and ligand expression and binding were found, with ephrin-A2, -A3, and -A5 in the septum and EphA5 in the hippocampus (Gao et al 1996, Zhang et al 1996). In vitro, ephrin-A2 can inhibit neurite outgrowth from explants of topographically appropriate medial but not lateral hippocampus (Gao et al 1996). Although this is not necessarily an axon guidance assay, at the molecular level control of axon guidance and axon growth may not be different. Ephrin-A2 could therefore function as a topographic mapping molecule in the hippocamposeptal map, as in the retinotectal map.

Motor Axon Guidance

Motor output, like sensory input, is organized topographically (Sperry 1963, Hunt & Cowan 1990, Jacobson 1991). The organization is somewhat different, however. Sensory maps such as visual maps are smooth and continuous, obviously lending themselves to mapping by gradients. Motor maps in some cases also appear continuous, for example the map of muscles in the body wall. However, in other cases, for example in the limb, the problem may be one of innervating discrete muscle targets. In principle, motor topography might be specified either by gradients, by discrete labels, or by both. In addition to final
target recognition, motor axons also have to follow distinct pathways to reach their target areas guided by pathway-derived cues (Tosney 1991).

Several of the Eph receptors are expressed in motor neurons, and potential roles for the ephrins have been identified in both target recognition and pathway selection.

MOTOR COLUMN SPECIFICITY Motor neurons in the spinal cord are divided into columns that run along the anteroposterior axis, express different sets of nuclear factors, and innervate distinct muscle targets (Tosney 1991, Landmesser 1992, Tsuchida et al 1994). At least two receptors, EphA3 and EphA4, appear to be expressed in the chick within specific spinal motor columns. EphA4 is expressed at brachial and lumbar levels in lateral motor column neurons, which innervate the limb buds (Ohta et al 1996). Ligands that bind this receptor are found in the limb bud (Cheng & Flanagan 1994a; Flenniken et al 1996; Gale et al 1996b), suggesting the possibility of a mechanism for guidance within the limb. EphA3, on the other hand, is expressed in a subset of medial motor column neurons, which innervate the body wall but not the limb (Kilpatrick et al 1996). EphA3 is also found in axial, but not limb, musculature. In view of the limb expression of ephrin-A ligands, it has been proposed that these ligands might keep EphA3 bearing axons and muscle precursors out of the limb by a repulsion mechanism (Kilpatrick et al 1996). Although this might initially seem at variance with the finding that EphA4-bearing axons can enter the limb, EphA3 has a higher binding affinity for at least one of the ligands (Table 2), so it might mediate a more powerful repellent effect and keep axons out of the limb altogether.

SEGMENTAL GUIDANCE THROUGH THE SOMITE The sensory and motor connections that the spinal cord makes with the periphery form a series of segmentally arranged spinal nerves. This segmental arrangement is not intrinsic to the spinal cord but is imposed by the segmentation of the somites that flank it. Specifically, the spinal nerves pass through the anterior somite and avoid the posterior somite (Keynes & Stern 1984, Bronner-Fraser & Stern 1991, Tosney 1991). The evidence for a role of the ephrin-B family in this segmental patterning will be described more fully in the section on segmental patterning of neural crest migration. Briefly, the idea of the ephrin-B subfamily playing a role in repulsion of motor axons from inappropriate parts of the somite is consistent with the expression patterns of ephrin-B ligands and EphB receptors (Bergemann et al 1995, Wang & Anderson 1997). Furthermore, in vitro stripe assay experiments demonstrated that axons growing from spinal cord explants avoid stripes containing ephrin-B1 or ephrin-B2. Ephrin-B ligands, like ephrin-A ligands, can thus have repellent effects on axon growth (Wang & Anderson 1997).
ANTEROPosterior PATTERning OF Motor AND SOMatosensory CONneCtions

Motor neurons in the spinal cord, as well as sensory neurons in the dorsal root ganglia, selectively synapse on targets from corresponding positions along the anteroposterior body axis. This phenomenon might, in principle, be explained by recognition labels in gradients (Sanes 1993). In a subtractive hybridization screen to identify candidate labels, ephrin-A5 was identified as a cDNA expressed more highly in anterior than posterior muscles (Donoghue et al 1996). It was further shown that ephrin-A5 inhibits the outgrowth of spinal cord and sensory ganglion neurites in vitro, with a preferential inhibition of posterior over anterior explants. Low expression of ephrin-A5 in muscles was seen compared to other parts of the embryo, and no evidence could be found for gradient expression (Donoghue et al 1996). Nevertheless, these observations do suggest a potential role for the ephrin family in the topographic patterning of axon connections along the anteroposterior body axis.

Pathway Selection in the Central Nervous System:
Targeted Gene Disruptions of Eph Receptors

In addition to guidance in the periphery and topographic mapping in the central nervous system, the ephrins have been implicated in pathway selection within the central nervous system. The main evidence for such functions has come from targeted gene disruptions of Eph receptors. Indeed, defects in central nervous system pathway selection are the main phenotype observed thus far from receptor gene targeting.

Targeted disruption of the EphB2 gene results in loss of the posterior part of the anterior commissure, one of the connections that run between the two halves of the cerebral cortex (Henkemeyer et al 1996). In homozygous mutant mice, rather than following the usual pathway across the midline, axons penetrate into a more ventral region. Surprisingly, deletion of only the kinase domain of the receptor does not produce this phenotype (though this depends on the mouse strain background). Moreover, the affected axons do not express the EphB2 receptor, but instead express ephrin-B ligands, while the receptor is found in the ventral region that the axons normally avoid. These observations suggest a model in which the transmembrane ephrin-B ligands might transduce a repellent guidance signal into the axons that carry them, in response to receptors along the pathway. It also remains possible that the defect in axon guidance could be explained by other mechanisms; for example, it might be secondary to changes along the pathway (Henkemeyer et al 1996). In the context of these experiments, it is very intriguing that ephrin-B ligands become tyrosine phosphorylated in response to stimulation with the EphB2 extracellular domain (Holland et al 1996, Bruckner et al 1997). Taken together, these studies suggest
the interesting possibility that phosphorylation of the ligand cytoplasmic tails may trigger guidance within the ligand-bearing axons.

Targeted disruption of EphB3 also results in abnormal connections between the two cortical hemispheres, affecting formation of the corpus callosum (Orioli et al 1996). Again, the affected axons express ephrin-B ligands, whereas receptors are expressed in the vicinity of the pathway. In this case, however, rather than appearing to follow an abnormal trajectory, the axons reach the midline normally but then fail to cross it. These results are less obviously explicable in terms of the loss of a repellent axon guidance signal, and the abnormal axon pathfinding could instead be secondary to changes along the pathway. This study also showed that double homozygotes with disruptions of both EphB2 and EphB3 show synergistic midline defects in both the anterior commissure and corpus callosum, providing direct evidence for functional redundancy among the Eph receptors (Orioli et al 1996).

Targeting of the EphA8 receptor results in an abnormality in the projection of axons leaving the superior colliculus of the midbrain. EphA8 is normally expressed in a subset of neurons in the superior colliculus. In mice with a disrupted EphA8 gene, dye labeling of axons shows that most of the projections from the superior colliculus are normal, but there is a reduction in axons projecting posteriorly to the contralateral inferior colliculus. Instead, a new projection appears in the ipsilateral spinal cord, implying an effect on axon guidance (Park et al 1997). Candidate ligands for EphA8 that might be involved in this guidance include ephrin-A2 and ephrin-A5 (Cheng et al 1995, Drescher et al 1995, Donoghue et al 1996, Zhang et al 1996). For example, ephrin-A5, which is expressed prominently in the inferior colliculus, might provide a posterior barrier that could normally prevent axons with EphA8 from growing as far as the spinal cord, and might divert them to the contralateral inferior colliculus (Park et al 1997).

Additional EphA receptor genes have been targeted, including EphA2 and EphA4, though obvious phenotypes have not yet been reported (Skarnes et al 1995, Chen et al 1996). Altogether, the phenotypes resulting from receptor gene deletions have been relatively mild thus far, suggesting some degree of functional redundancy within the family, an idea confirmed by the synergistic effects of disrupting EphB2 and EphB3 (Orioli et al 1996). The idea of partial redundancy could also clearly be consistent with the high degree of binding promiscuity in the family, as well as the tendency for multiple receptors or ligands to be expressed in overlapping patterns. Thus, rather than a particular function being controlled by individual ligands or individual receptors, overlapping groups of receptors and groups of ligands may work together to produce a composite of effects. Although such overlap may cause problems
for functional analysis, it could be an important feature of the family, perhaps allowing a greater precision and complexity in developmental guidance.

REGIONAL PATTERNING OF THE NEURAL TUBE AND NEURAL CREST

As well as guiding axonal connections, the Eph family has been strongly implicated in regional patterning of the nervous system.

*Segmental Patterning in the Hindbrain Region*

Regularly repeated, or metameric, segmentation of structures along the main body axis is widely seen in metazoans and is presumably an efficient and economical way to organize development: The same developmental mechanisms and molecules can be used repeatedly in each segment, with modifications superimposed at different axial levels. In the developing hindbrain, a series of bulges known as rhombomeres show the classic features of metameric segments. First, they are regularly repeated, developing into a series of hindbrain nuclei and cranial nerves with a two-segment repeat. Second, cell movement between rhombomeres is restricted, resulting in a partial lineage compartmentation, which presumably helps in maintaining distinct rhombomere identities. Finally, regulatory genes, notably the Hox genes, are expressed in specific rhombomeres and can modify the repetitive plan to produce segment-specific properties (reviewed by Lumsden & Krumlauf 1996).

Although a great deal has been learned about nuclear factors that program rhombomere identity, little has been known about the roles of cell-cell signaling molecules. It was therefore very intriguing to find rhombomere-specific expression patterns for EphA4 (Nieto et al 1992), and subsequently many other Eph receptors (Figure 3) (Becker et al 1994, Ganju et al 1994, Henkemeyer et al 1994, Ruiz & Robertson 1994, Winning & Sargent 1994, Ellis et al 1995, Taneja et al 1995, Irving et al 1996, Cooke et al 1997). Segment-specific patterns have also been found for some of the ephrins (Figure 3) (Cheng & Flanagan 1994a, Bergemann et al 1995, Flenniken et al 1996, Gale et al 1996a). These patterns suggest two interesting possibilities. The ephrins and Eph receptors could be upstream of nuclear regulators such as Krox-20 or the Hox genes and could help establish segmentation or segment-specific properties. Alternatively, they could be downstream of these nuclear regulators and could be involved in translating the code of nuclear factors into cell properties such as lineage compartmentation, cell migration, or axon connectivity.

The idea of ephrins being upstream of the Hox code could be consistent with timing and patterns of expression. For example, EphA4 is expressed early in
Figure 3  Expression of ephrins and Eph receptors in relation to segmental patterning of the hindbrain and spinal cord. The hindbrain can be divided into segments known as rhombomeres (r1–r6 shown here). Several Eph ligands and receptors are expressed in segment-specific patterns in the hindbrain, suggesting roles either in creating the segmented pattern or in determining segment specific properties. In the spinal region, segmentation of the migrating neural crest cells and spinal nerves is imposed by the flanking somites. Ephrin-B ligands are present in the somites; EphB receptors are present on motor axons and neural crest cells and may play a role in guiding the segmented pathways of both axons and migrating cells.

rhombomeres r3 and r5 (Nieto et al 1992) at about the same time as Krox-20, which is known to act as a transcriptional activator of Hox genes (Sham et al 1993, Schneider-Maunoury et al 1993, Swiatek & Gridley 1993). However, in mice with a targeted disruption of the Krox-20 gene, EphA4 expression fails to be up-regulated in r3 and r5 (Swiatek & Gridley 1993, Seitanidou et al 1997), suggesting that EphA4 is downstream rather than upstream of Krox-20, though it is also possible that the two could be in a regulatory loop.

An approach that has been taken to understand Eph receptor functions in regional patterning has been to test the effects of dominant negative receptors in embryos. The basis of the dominant-negative approach, first applied to receptor tyrosine kinases in other subfamilies (Amaya et al 1991, Ueno et al 1991), is that these receptors signal as dimers. Therefore, a modified receptor with a deletion or mutation of the kinase domain is expected to heterodimerize with, and dominantly inactivate, the endogenous normal receptor. This approach has been applied to the EphA4 receptor in Xenopus and zebrafish (Xu et al 1995). RNA for a dominant negative version was injected into early embryos, where it is presumed to inactivate the endogenous EphA4 receptor and may also interfere with other Eph receptors by heterodimerization, or ligands by
sequestration. The result was that groups of cells from rhombomeres r3 and r5, as identified by Krox-20 expression, were found ectopically in the territory of adjacent rhombomeres. The ectopic cells were not scattered but seemed to take the form of coherent streams or bulges from r3 and r5. These results suggest a model where Eph function may normally prevent mixing of cells from adjacent rhombomeres and implicate the ephrins in sharpening of the rhombomere boundaries (Xu et al 1995). Recent experiments have also indicated that the ephrins may be involved in segregating the streams of neural crest cells that migrate out from the rhombomeres. In *Xenopus*, EphA4 and EphB1 are normally expressed in cells migrating at the level of r5 into the third branchial arch. Dominant negative versions of these receptors cause the cells to migrate abnormally into adjacent territories (Smith et al 1997). Ephrin-B2, a ligand that can bind these receptors (Table 2), is normally expressed in the adjacent stream of cells migrating into the second branchial arch, and ectopic expression of this ligand also causes abnormal dispersal of third arch crest cells. These findings are consistent with EphA4, EphB1, and ephrin-B2 normally being involved in keeping the third arch crest cells within their correct territory (Smith et al 1997).

Regarding the potential cellular mechanisms for these hindbrain effects, in view of the repellent effects of ephrins in other systems, perhaps the most straightforward explanation is that the ephrins and Eph receptors might normally mediate a cell-cell repulsion that prevents the mixing of cells from adjacent rhombomeres or from adjacent streams of neural crest cells (Xu et al 1995). Since numerous ephrins and Eph receptors are expressed in the hindbrain (see Figure 3), it remains to be determined exactly which ones are affected by the dominant negative EphA4 and precisely what their respective roles might be in segmentation. In any case, these experiments strongly implicate ephrins in formation or maintenance of developmental boundaries. Boundary formation is an important process that can allow continuously graded positional information, of the type expected to pattern early embryonic fields, to be translated into the discrete structures found later. It will be interesting to see if ephrins may also be involved in boundary formation in other parts of the embryo.

**Organization of the Forebrain**

Regional patterning in the forebrain remains poorly understood. A number of early region-specific markers have been identified, and there is evidence to support the idea of metameric segmentation, at least in the posterior part of the forebrain, the diencephalon (Figdor & Stern 1993, Rubenstein et al 1994, Lumsden & Krumlauf 1996). However, little is known of the molecular mechanisms that underlie pattern formation in this region.

Many ephrins and Eph receptors are expressed in the forebrain, where they might in principle be involved in either axon guidance or regional patterning.
To address potential functions, a dominant negative approach in the zebrafish has been taken with EphA4, as in the hindbrain (Xu et al 1996). In a normal embryo, the retina is formed by protrusion from the diencephalon. Injection of a dominant negative EphA4 resulted in expansion of the retinal territory to include cells that would normally be fated to become diencephalon. As in the hindbrain, wide overexpression of normal EphA4 had no obvious effect, and in many ways the results in the forebrain and hindbrain appear similar. Surprisingly, however, the results differ in that in the diencephalon the expanded territory encroaches on the region with high EphA4 expression, whereas in the hindbrain the result is the opposite. Perhaps these findings could be interpreted in terms of a mutual repulsion of cell populations, or alternatively they may suggest that further endogenous receptors other than EphA4 may be involved. The cellular mechanism for the change in forebrain cell fate could, in principle, be an effect on cell differentiation, proliferation, or migration. Whatever the specific mechanism, these experiments clearly implicate the ephrins in the poorly understood processes of forebrain regional patterning.

Segmental Patterning of Spinal Neural Crest Migration

The neural crest is a transient population of progenitor cells that migrate out from the dorsal neural tube and form a wide variety of structures, including most cells of the peripheral nervous system. As they migrate, the neural crest cells follow defined pathways and reach specific targets, in a process presumably similar to axon guidance. One aspect of this guidance is that the cells migrating from the hindbrain and spinal cord follow segment-specific pathways. In the hindbrain region, this segmentation appears to be partly specified along the migration pathways and partly determined by properties intrinsic to the neural tube (Graham et al 1993, Sechrist et al 1993). In the spinal region, however, the pathways seem to be determined entirely by the flanking somites, which impose their segmental organization on the neural tube and neural crest (Keynes & Stern 1984, Bronner-Fraser & Stern 1991, Tosney 1991). Specifically, the migrating neural crest cells follow pathways through the anterior part of each somite and avoid the posterior part.

The ephrin-B family has been implicated in this segmental guidance through the somite (Figure 3). The receptors EphB2 and EphB3 are expressed on migrating neural crest cells and spinal motor neurons, as well as on cells of the anterior somite itself (Henkemeyer et al 1994, et al 1997, Wang & Anderson 1997). Matching ligands are expressed in the somites (Bergemann et al 1995), with ligand expression within the somite localized to the dermatome—the part of the somite near the body wall—as well as the posterior part of the underlying sclerotome (Krull et al 1997, Wang & Anderson 1997). Both of these somite regions are avoided by migrating neural crest cells and spinal nerves. At least
two ligands, ephrin-B1 and ephrin-B2, are present in the somite, and surpris-
ingly the patterns differ between species: The ligand restricted to the caudal
sclerotome is ephrin-B1 in chick but ephrin-B2 in rat (Wang & Anderson 1997).
The effects of these ligands have been tested on migrating neural crest cells in
several assays. Soluble ephrin-B1 disrupts the normal metameric pattern of
migration in tissue explants, while ephrin-B1 or ephrin-B2 can act as cell repel-
lants in a transfilter assay or in a stripe assay, as tests of in vitro cell migration

Surprisingly, no obvious abnormalities of the spinal ganglia or spinal nerves
have yet been detected in mice with targeted gene deletions of EphB2 or EphB3,
including doubly homozygous mice with both receptors missing (Henkemeyer
some form of redundancy. Other EphB receptors might be present. Moreover,
other molecules, such as T-cadherin and collagen IX, are also known to be
expressed in posterior somite and to display inhibitory effects in vitro (Fredette
et al 1996, Ring et al 1996). Guidance in the somite might therefore result from
a composite of the effects of several molecules. The existence of multiple cues
might make the system more robust or might give it a greater subtlety. It would
not be surprising if multiple overlapping guidance signals were needed in the
somite, considering the multiple types of migrating cells (both neural crest and
somite derived) as well as axons (sensory and motor), all following different
pathways.

Complementary Ligand and Receptor Patterns
in Other Parts of the Embryo: Positional Labels
of Cell Location?

Using tagged fusions of ephrins and Eph receptors in the affinity probe in situ
approach, as well as by in situ RNA hybridization, it has been found that the Eph
receptors and ephrins are expressed in complementary patterns in several addi-
tional regions of the embryo. Examples include the developing spinal cord, limb
bud, and branchial arches, where binding sites for soluble receptor and ligand
probes are in patterns that appear strikingly complementary and nonoverlapping
(Gale et al 1996b). Another example is the developing retina, where A sub-
family receptors and ligands form opposing gradients along the anteroposterior
axis, and B subfamily receptors and ligands form them along the dorsoventral
mediate repellent effects in these contexts, it seems plausible that they might
keep adjacent cell populations apart. More speculatively, they might even keep
cells in the correct position within a gradient across a developmental field by
a process perhaps analogous to topographic mapping. Additional experiments
are needed to further address these observations, but they raise the intriguing possibility that the ephrins might act as labels specifying cell position in many parts of the embryo.

ROLES OUTSIDE THE NERVOUS SYSTEM

Most studies on function of the ephrins have concentrated on the nervous system. However, there is evidence for functions in other parts of the embryo. For example, targeted disruption of the EphB3 receptor gene results in cleft palate, as well as nervous system abnormalities (Orioli et al 1996), and ephrin-A1 has been implicated in the angiogenic response to TNFα (Pandey et al 1995b). In view of the expression of several of the receptors and ligands in nonneural tissues, future studies are likely to uncover additional roles.

CONCLUSIONS

The ephrins and Eph receptors are large molecular families, most of which have been identified only in the past few years. What features can be identified at this early stage that might be unifying characteristics of the family?

To date, most reports on ephrin function can be interpreted in terms of control of cell movement: guidance of axons, guidance of cell migration, or maintenance of boundaries between groups of cells. Evidence for support of neuronal survival in vitro has also been reported (Magal et al 1996). However, more remarkable is the paucity of evidence so far for roles in proliferation or survival. This provides a striking contrast with other families of receptor tyrosine kinases, which are generally best known for such functions.

Another notable feature is that all the known ligands are anchored to cell surfaces. This may serve to localize their signaling potential. Moreover, since the ligands seem to be inactive in soluble forms, their actions may be even more tightly localized than other classes of cell-associated signaling molecules. This may be a key feature allowing the family to serve as precise labels for axon guidance, the definition of boundaries, and other spatially precise signaling functions.

The potential to specify positional information in gradients is another unusual feature of the family. In particular, the existence of complementary gradients in both ligand and receptor is unique. This complementarity in positional information is essential for topographic mapping, where the positional labels must map one developmental field onto another. It will be interesting to see whether similar principles may operate in other types of developmental patterning.

The ability of the ephrins to act as repellents is another notable feature, shared with two other recently identified classes of guidance molecules, the netrins and
the semaphorins/collapsins (Kolodkin et al 1993, Luo et al 1993, Colamarino & Tessier-Lavigne 1995, Tessier-Lavigne & Goodman 1996). As proposed in the mass action model for topographic mapping, this feature of the ephrins may allow a stable map to be generated in response to matched countergradients of ligand and receptor. In other developmental systems, a repellent action might be an efficient way to define boundaries for axon growth or cell migration and might provide an effective way to keep cell populations apart at compartment borders. It will be interesting to see whether some of the guidance functions of the ephrins are attractant in the nervous system, as proposed in angiogenesis, or whether they are all repellent.

Although much remains to be learned about the ephrins and Eph receptors, it is already clear that these signaling molecules have widespread and unique roles in specification of the precise and complex spatial order that characterizes the nervous system.

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