Regional Differences in the Developing Cerebral Cortex Revealed by *Ephrin-A5* Expression

Katrin Mackarehtschian, Camilla K. Lau, Ingrid Caras[1] and Susan K. McConnell

Department of Biological Sciences, Stanford University, Stanford, California and [1]Department of Neuroscience, Genentech, Inc., South San Francisco, California, USA

The development of axonal connections between thalamic nuclei and their cortical target areas occurs in a highly specific manner. To explore the mechanisms of thalamocortical axon pathfinding, we investigated the expression of several members of the ephrin and Eph gene families in the forebrain. The Eph ligand *ephrin-A5* was expressed in three distinct gradients during the development of the telencephalon. The first gradient occurred in the cortical ventricular zone and established *ephrin-A5* as one of the earliest markers distinguishing cells residing in the anterior versus posterior cortical neuroepithelium. The second gradient was apparent in the subplate and occurred in spatial opposition to a distinct gradient for the low-affinity NGF receptor *p75*. This finding reveals that different regions of the early subplate are molecularly heterogeneous. Third, we confirmed that *ephrin-A5* is expressed in a bi-directional gradient in the cortical plate, with highest levels in the somatomotor cortex. Three putative receptors for *ephrin-A5* – *EphA3*, *EphA4* and *EphA5* – showed distinct expression patterns in the developing thalamus. The graded distributions of *ephrin-A5* in the developing subplate and cortex and the expression of its receptors in the thalamus are consistent with the notion that the Eph ligands and their receptors may function in the topographic mapping of thalamic axons to specific cortical areas.

Introduction

During the development of the nervous system, axonal growth is regulated by molecular cues that are distributed along the pathways through which the axons grow and within their target destinations (Cook *et al.*, 1998). Interactions between growth cones and both attractive (permissive) and repulsive (inhibitory) molecules enable axons to navigate through complex cellular environments and innervate the appropriate target cells. Despite substantial progress in understanding the nature of these interactions in several regions of the nervous system (e.g. Serafini *et al.*, 1994, 1996; Frisen *et al.*, 1998), little is known about the molecules that guide the formation of the axonal connections in the cerebral cortex, including its major inputs from, and outputs to, the thalamus.

It has long been appreciated that the cortex is organized along its tangential dimension into distinct areas that perform specialized functions (Brodmann, 1909). Neurons in different areas acquire thalamic inputs that determine their functional modality: for example, neurons in the auditory cortex are normally innervated by inputs from the medial geniculate nucleus (MGN) of the thalamus (Kreig, 1946), which conveys auditory information via the midbrain from the cochlea. If retinal axons are misrouted experimentally into the MGN, the auditory cortex in these 'miswired' animals now responds to visual stimulation (Sur *et al.*, 1988). Thus the specificity of thalamocortical connections is crucial for the normal processing of sensory information. How this specificity is achieved during development remains unclear. It seems likely that thalamic axons somehow detect differences between possible target areas, but it is not known whether these differences emerge within the progenitor cells of the cortical ventricular zone (Rakic, 1988) well before thalamic axon invasion, or within the cortex itself around the time of axon ingrowth (O'Leary, 1989). To date, the strongest candidate for playing a role in thalamocortical axon targeting is the limbic-associated membrane protein (LAMP), which appears to help guide the axons from the limbic thalamus to the perirhinal (limbic) cortex (Barbe and Levitt, 1992). The molecules that orchestrate the development of sensory and motor projections remain unknown, although a number of possibilities, such as the cadherins (Suzuki *et al.*, 1997) and ephrins (Gao *et al.*, 1998), have been suggested.

Recent studies have identified a large family of candidate molecules that may coordinate the development of axonal connections in both the central and peripheral nervous systems. The ephrins comprise a family of putative axon guidance ligands, and their cognate receptors are known as the Eph receptor tyrosine kinases (RTKs) (reviewed by Flanagan and Vanderhaeghen, 1998). The Eph family is the largest known subfamily of receptor tyrosine kinases, with over a dozen members identified to date. Similar to other RTKs, the Eph-type receptors possess an extracellular ligand binding domain, a hydrophobic segment than anchors the proteins in the plasma membrane, and an intracellular domain that displays catalytic activity. The identified ligands of Eph-type RTKs are all membrane-anchored. This membrane anchorage appears to be essential for efficient receptor activation and allows the ligands to be deployed with the spatial resolution that is necessary to encode positional information. The ligands for Eph-type receptors fall into two classes. Members of the ephrin-A class are anchored to the cell membrane by a GPI-link, whereas members of the ephrin-B class span the plasma membrane with their transmembrane domain. The ligands of each class appear to interact with a separate set of Eph-like receptors, but within each class there appears to be a considerable amount of promiscuity in receptor–ligand binding (Brambilla *et al.*, 1995; Gale *et al.*, 1996).

Members of the Eph-receptor and ephrin gene families play key roles in directing retinal axon terminals to the proper target areas in the tectum (Cheng *et al.*, 1995; Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Monschau *et al.*, 1997; Frisen *et al.*, 1998) and the thalamus (Feldheim *et al.*, 1998). Two GPI-linked ligands, ephrin-A5 and ephrin-A2, are expressed in a decreasing posterior-to-anterior gradient within the chicken tectum (Cheng *et al.*, 1995; Drescher *et al.*, 1995) and in multiple gradients within the pretectal nuclei and thalamus (Feldheim *et al.*, 1998). A cognate Eph-receptor, EphA3, is expressed in a temporal-to-nasal gradient in the retina (Cheng *et al.*, 1995). *In vitro* and *in vivo* experiments suggest a role for ephrin-A2 and ephrin-A5 in regulating the growth and branching of retinal ganglion cell axons. Both function as repulsive factors for temporal retinal axons (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Monschau

et al., 1997; Feldheim *et al.*, 1998), and by virtue of their expression in the most posterior region of the tectum, probably promote the formation of temporal axon terminals in more anterior regions of tectum. Gene targeting experiments in mice have confirmed that *ephrin-A5* is required for the normal development of the retinotectal map (Frisen *et al.*, 1998) and the topographic organization of visual inputs to the thalamus (Feldheim *et al.*, 1998). Members of the Eph family have been implicated in the topographic mapping of hippocampal–septal projections (Gao *et al.*, 1996) and the development of intracortical axonal connections (Castellani *et al.*, 1998). Finally, ephrin-A5 inhibits axon growth by limbic neurons in both the thalamus and cortex, which may prevent the inappropriate innervation of primary sensorimotor areas by these neurons (Gao *et al.*, 1998).

We were interested in further exploring the possibility that Eph-type receptors and their ligands might function in the development of axonal connections between thalamic nuclei and their cortical target areas. A candidate cellular structure that may express axon guidance cues for innervating thalamic axons is the cortical subplate, a transient zone of neurons in the primitive white matter, and one of the first layers generated during cortical development (Luskin and Shatz, 1985). When thalamic axons first reach the cortex (at embryonic day 16–18 in the rat: Catalano *et al.*, 1996) their target cells of layer 4 are still migrating into the cortical plate. The thalamic axons pause in the subplate and form temporary synaptic connections with subplate neurons (Shatz and Luskin, 1986; Herrmann *et al.*, 1994); these transient connections are maintained until layer 4 neurons have migrated into place. Besides serving as a 'waiting' station, subplate cells also play an important role in targeting thalamic axons to the proper cortical area. Thalamic axons growing through the intermediate zone extend branches into the overlying subplate, as if sampling different regions of subplate until they identify the appropriate target area (Ghosh and Shatz, 1992; Catalano *et al.*, 1996). The ablation of a small region of subplate results in the failure of thalamic axons normally destined for that region to stop and invade the appropriate cortical area (Ghosh *et al.*, 1990; Ghosh and Shatz, 1993). These observations suggest that subplate neurons express area-specific or regionally distinct markers that are recognized by thalamic axons and required for target selection.

To address the question of whether Eph-type receptors and their ligands may be involved in the guidance of thalamic axons or the formation of area-specific targeting cues, we investigated the expression of several of these molecules in the forebrain. We report here on the in-situ hybridization expression patterns of *ephrin-A5* and three Eph-like receptors.

Materials and Methods

Animals

We used timed-pregnant Long-Evans rats (Simonsen). The day of vaginal plug detection was considered embryonic day 0 (E0). Several embryonic and postnatal rats were analysed at each of the following ages: E11 (n = 8 animals; three litters), E13 (n = 9; three litters), E15 (n = 8; three litters), E17 (n = 16; five litters), E19 (n = 12; four litters), E21 (n = 4; two litters), P0 (n = 2; two litters), P1 (n = 2; two litters), and P6 (n = 2; two litters).

Preparation of Brain Sections

Postnatal rats or pregnant mothers were anesthetized with an overdose of Nembutal (100 mg/kg body weight, i.p.). Brains were dissected from pups or fetuses, placed in OCT embedding compound, and immediately frozen on dry ice. The brains were stored at –80°C until use. 15 µm sections were collected onto polylysine-coated slides. The sections were postfixed in 4% paraformaldehyde (pH 7.0), washed in PBS, dehydrated in ethanol, and finally stored at –80°C.

Preparation of Riboprobes

Probe templates were either generated by linearization of plasmids containing a segment of the gene or by PCR amplification using primers that contained the T3 or the T7 RNA polymerase promoter sequences (see details below). All templates were gel-purified before being included in the in-vitro transcription reaction.

The sense and antisense probes of human *ephrin-A5* were transcribed from a construct that contained approximately 0.7 kb of the open reading frame as a *Xho*I/*Not*I fragment cloned into pBluescript. The plasmid was linearized with either *Xho*I (antisense) or *Not*I (sense) and transcribed in the presence of [^{35}S]UTP with either T3 polymerase (antisense) or T7 (sense) polymerase to generate the riboprobes.

The template for the 3′-terminal probe of *ephrin-A5* was generated by PCR amplification of a 294 bp fragment (bp 724–977 of the *ephrin-A5* gene) using the primers 5′-nnnnnnn gtaatacgactcactatagggc TGCAAT0 CCCAGATAATGGAA-3′ and 5′-nnnnnnn aattaaccctcactaaaggg TGTGAC-AAGTGATGGGAGGA-3′. The antisense probe was generated from this template using T3 RNA polymerase.

The antisense probe of human *ephrin-A3* was transcribed from a construct that contained ~1.0 kb of the open reading frame as a *Bgl*II fragment cloned into the *Bam*HI site of pBluescript. The plasmid was linearized with Xho I and transcribed in the presence of S^{35}-UTP with T3 polymerase to generate antisense riboprobes.

The antisense probe of mouse *ephrin-A4* was transcribed from a construct that contained ~0.7 kb of the open reading frame as a *Bgl*II fragment cloned into the *Bam*HI site of pBluescript. The plasmid was linearized with *Xho*I and transcribed in the presence of S^{35}-UTP with T3 polymerase to generate the antisense riboprobes.

The template for the murine *p75* probe was generated by PCR amplification of a 647 bp fragment (bp 188–794; the template for amplification was obtained from L. Reichard's laboratory and contained the *p75* ORF plus 60 bp of the upstream and 300 bp of the downstream sequences cloned into a pGEMHE vector) using the primers 5′-AGCGCGC aattaaccctcactaaaggg TGCTGATTCTAGGGGTGTCC-3′ and 5′-AGCGCGC gtaatacgactcactatagggc TCACCATATCCGCCACTGTA-3′. The antisense probe was generated from this template using T7 RNA polymerase. The sense probe was generated using T3 RNA polymerase.

The template for the *EphA3* probe was generated by PCR amplification of a 541 bp fragment (bp 52–555 of the *EphA3* gene) using the primers 5′-nnnnnnn aattaaccctcactaaaggg AAGAGCTCAGCTCTG-ACACCCC-3′ and 5′-nnnnnnn atacgactcactatag TGCCAAGCTTGTCGACC-AGG-3′. The antisense probe was generated from this template using T3 RNA polymerase. The sense probe was generated using T7 RNA polymerase.

The template for the *EphA4* probe was generated by PCR amplification of a 533 bp fragment (bp 215–709 of the *EphA4* gene) using the primers 5′-nnnnnnn aattaaccctcactaaaggg GAGGAAGTGAGCATTAT-GGA-3′ and 5′-nnnnnnn gtaatacgactcactatagggc GCCTCGAACTTCCAC-CAG-3′. The antisense probe was generated from this template using T3 RNA polymerase. The sense probe was generated using T7 RNA polymerase.

The template for the *EphA5* probe was generated by PCR amplification of a 524 bp fragment (bp 130–617 of the *EphA5* gene) using the primers 5′-nnnnnnn aattaaccctcactaaaggg GCTATTCCGCACCTC-TAA-3′ and 5′-nnnnnnn gtaatacgactcactatagggc GAGGTCCTACATCTC-TGA-3′. The antisense probe was generated from this template using T7 RNA polymerase. The sense probe was generated using T3 RNA polymerase.

In Situ *Hybridization*

The protocol for *in situ* hybridization was modified from that of Simmons *et al.* (Simmons *et al.*, 1989), as described more fully in Frantz *et al.* (Frantz *et al.*, 1994). Sections were pretreated with proteinase K (0.9 µg/ml) and acetic anhydride, and dehydrated in a series of ethanol baths. Hybridization occurred overnight at 65°C. Following hybridization, the sections were treated with ribonuclease A (50 µm/ml) at 37°C for 30 min before being washed at high stringency in 0.1 × SSC at 60°C.

Autoradiography and Photography

Sections were once again dehydrated in a series of alcohols and xylenes and air-dried before being dipped into Kodak NTB2 nuclear track emulsion and stored for 4–7 weeks in the dark at 4°C. Finally, the sections were developed with Kodak D-19, fixed with Kodak Rapid Fix, and counterstained with cresyl violet. Images of selected sections were captured through a CCD camera and processed using Adobe Photoshop. None of the sense control probes generated signal above background levels (data not shown).

Results

Ephrin-A5 Expression in the Telencephalic Ventricular Zone

The expression pattern of *ephrin-A5* in the developing rat forebrain was analysed by in-situ hybridization. Sagittal and coronal sections of rat brains from embryonic day 11 (E11) to postnatal day 1 (P1) were hybridized with an *ephrin-A5* antisense riboprobe. This developmental period encompasses the onset of neurogenesis, neuronal migration, and the growth of axonal and dendritic processes in the forebrain (McConnell, 1995). The first positive hybridization signal was detected at E11 (Fig. 1*A*). As previously reported by others, *ephrin-A5* expression was graded in the dorsal mesencephalon, being more highly expressed in the neuroepithelium of the inferior colliculus and decreasing sharply towards the anterior superior colliculus (data not shown) (Cheng and Flanagan, 1994). *Ephrin-A5* mRNA expression was also graded in the telencephalon at E11: mRNA was most abundant in the anterior telencephalon, and expression decreased from this anterior tip towards posterior regions, both in the ventral half of the telencephalon (the neuroepithelium of the developing basal ganglia) and in the dorsal half of the telencephalon (the developing neocortex) (Fig. 1*A*). This anterior to posterior expression gradient in the forebrain is in the orientation opposite to that observed in the midbrain.

The gradient of *ephrin-A5* expression in the telencephalic ventricular zone was also apparent at E13 (Fig. 1*B*, 2*A*), E15 (Fig. 1*C*), and E17 (Fig. 1*D*, *E*). At these ages, *ephrin-A5* mRNA was most abundant in the anterior tip of the telencephalon, which gives rise to the olfactory bulb. Expression levels decreased sharply towards the posterior neocortical neuroepithelium and more gradually towards the posterior ventral telencephalic neuroepithelium. The far posterior ends of the developing neocortex and basal ganglia showed very little or no *ephrin*-A5 hybridization, but a low level of expression was seen in the hippocampal anlage (Fig. 1*C–F*). The gradient of *ephrin-A5* expression was quantified at both E13 and E17 by counting the number of silver grains overlying a fixed area of the ventricular zone and normalizing these to the background levels (Fig. 2*C*, *D*). At these ages, the number of grains overlying the anterior ventricular zone was roughly 2.5–3.5 greater than that over posterior regions, although the expression of *ephrin-A5* was less intense at E17.

The graded expression of *ephrin-A5* in the dorsal and ventral telencephalic ventricular zones persisted until E19, an age at which cortical neurogenesis is coming to a close (Frantz *et al.*, 1994). Beginning at about E16, cells in the neocortical ventricular zone began to decrease their expression of *ephrin-A5*, whereas progenitor cells of the subventricular zone still expressed *ephrin-A5* mRNA at high levels (cf. Fig. 1*D*). In the basal ganglia, in contrast, *ephrin-A5* message persisted in the ventricular zone but was not present in the subventricular zone (Fig. 1*D–F*). Between E15 and E19, *ephrin-A5* was not detectable in the intermediate zone, but mRNA was expressed in the cortical plate of the developing neocortex.

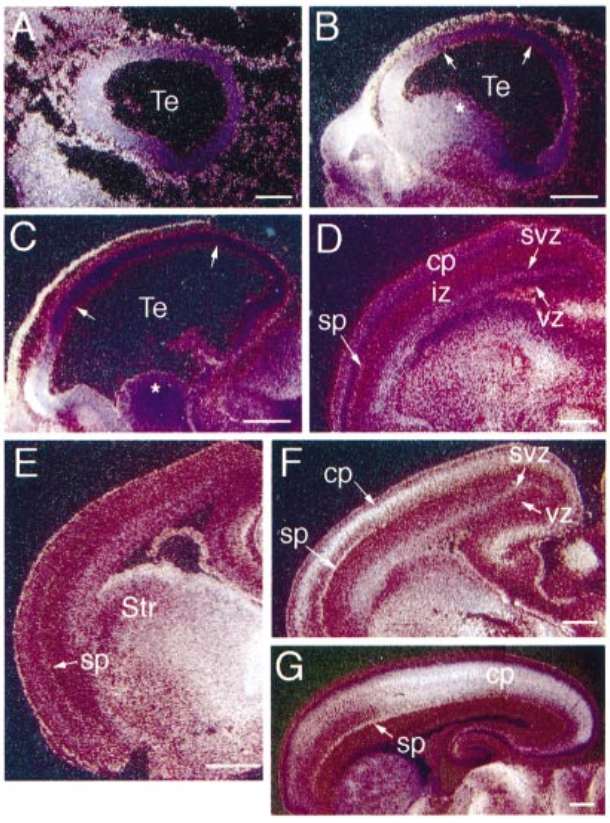

Figure 1. *Ephrin-A5* is expressed in distinct gradients in three regions of the developing telencephalon. Dark-field views of sagittal (*A–D*, *F–G*) and coronal (*E*) sections of rat brains isolated at (*A*) E11, (*B*) E13, (*C*) E15, (*D*, *E*) E17, (*F*) E19, and (*G*) P1, each hybridized with an antisense *ephrin-A5* probe. For the sagittal sections anterior is to the left and dorsal is to the top; for the coronal section medial is to the right. In the dorsal telencephalon, hybridization was apparent from E11 to E19 in the ventricular zone of the telencephalon (marked by arrows in *B*, *C*), from E17 to P1 in the subplate, and from E19 to P1 in the cortical plate. *Ephrin-A5* message was present in an anterior to posterior gradient in the neuroepithelium and the subplate, and was more concentrated in mid-dorsal regions of the cortical plate. In the ventral telencephalon, *ephrin-A5* was expressed in the ventricular zone of the ganglionic eminence (asterisks in *B*, *C*). Abbreviations: Te, telencephalon; cp, cortical plate; sp, subplate; svz, cortical subventricular zone; vz, cortical ventricular zone; iz, intermediate zone; Str, striatum. Each scale bar represents 0.5 mm.

Collectively, these results suggest that *ephrin-A5* forms a true spatial gradient rather than a maturational or temporal gradient. It has long been appreciated that anterior and temporal regions of cortex are developmentally more advanced than are posterior and medial areas; however, at their most extreme these differences reflect no more than 2 days in maturational stage (Uylings *et al.*, 1990). Our evidence from ages ranging from E11 to at least E17 indicates that *ephrin-A5* is always expressed at higher levels in the anterior ventricular zone than in posterior regions, and thus forms a temporally stable spatial gradient of gene expression.

Ephrin-A5 and p75 mRNAs Form Opposing Gradients in the Subplate

Because the subplate is likely to play an important role in the topographic mapping of thalamic axons to the cerebral cortex,

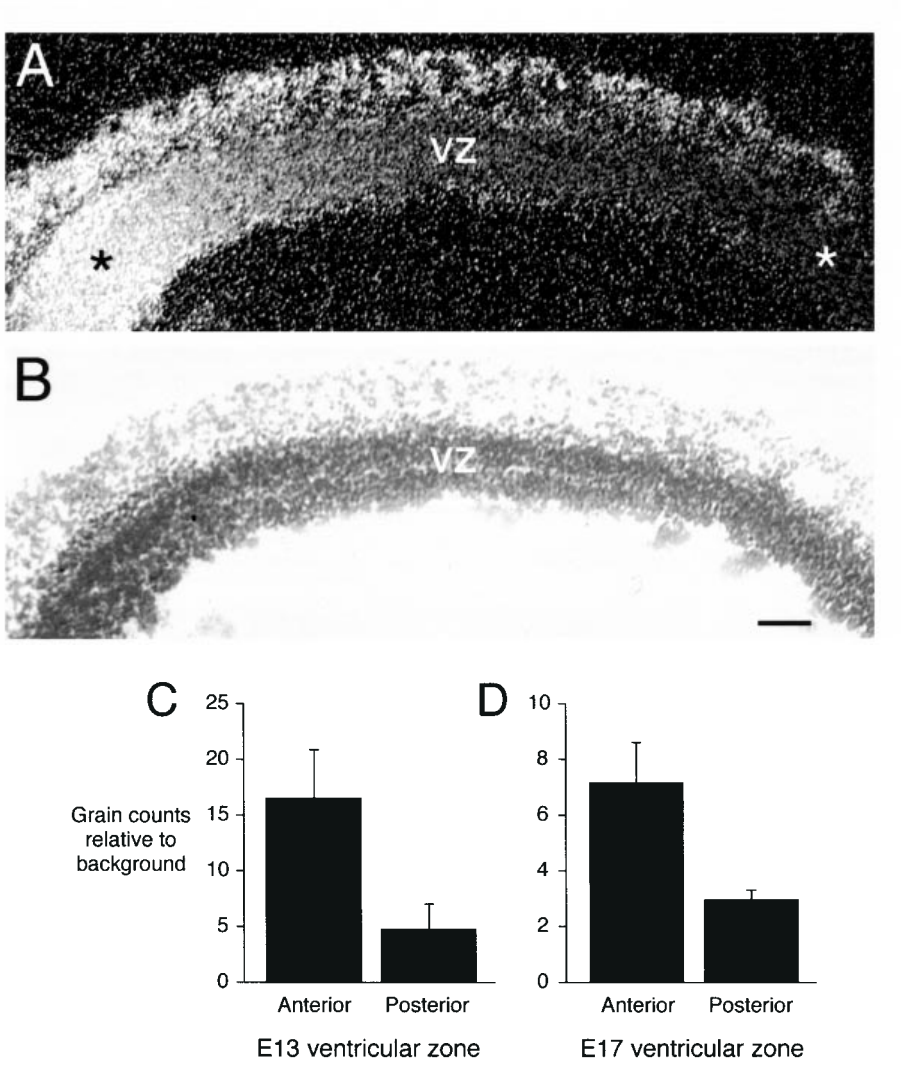

Figure 2. *Ephrin-A5* is expressed in an anterior to posterior gradient within the ventricular zone. (*A*) Darkfield view of a sagittal section through the E13 rat telencephalon showing *ephrin-A5* expression in the ventricular zone (vz). High expression is seen in the anterior ventricular zone (black *) whereas expression in the posterior ventricular zone (white *) is markedly lower. Anterior is to the left; dorsal is to the top. (*B*) Brightfield view of cresyl violet staining; same section as in *A*. Scale bar, 0.1 mm. (*C*, *D*) Comparison of the relative number of silver grains overlying the anterior versus posterior ventricular zone in (*C*) E13 embryos and (*D*) E17 embryos. The total number of silver grains in a ×100 field was counted for each of three different fields in the anterior and posterior ventricular zone at each age. Background counts of silver grains were obtained over four fields and averaged. The expression level of *ephrin-A5* relative to background was obtained by dividing the number of grains overlying the ventricular zone by the number of background silver grains. Each bar thus represents the average normalized grain count for each probe; error bars represent standard deviations.

we were particularly interested in the expression pattern of *ephrin-A5* in this cellular structure. Upon reaching the cortex, thalamic axons form their first synapses in the subplate (Shatz and Luskin, 1986; Herrmann *et al.*, 1994), and the axons appear to be incapable of identifying their proper cortical target area in the absence of the subplate (Ghosh *et al.*, 1990; Ghosh and Shatz, 1993). It is thus likely that the subplate encodes guidance cues that are used by ingrowing thalamic axons to map to the correct area of the cortex.

In-situ hybridization revealed that *ephrin-A5* was indeed expressed in the embryonic subplate, and moreover that it was expressed in a graded pattern, similar to that observed at earlier ages in the dorsal mesencephalon and cortical ventricular zone. At E17, *ephrin-A5* expression in the newly formed subplate was only visible in the most anterior cortical regions and was absent from middle and posterior areas (Fig. 1*D*). Analysis of coronal sections revealed that *ephrin-A5* mRNA was also expressed in a lateral to medial gradient in the subplate (Fig. 1*E*). The graded distribution of *ephrin-A5* message in the subplate was even more apparent at E19 (Figs 1*F*, 3*A*) and during early postnatal life (Figs 1*G*, 3*C*).

To identify cortical subplate cells more accurately, we used the low-affinity NGF receptor *p75* as a marker. p75 has previously been reported to be expressed in embryonic subplate cells (Allendoerfer *et al.*, 1990; Koh and Higgins, 1991). Neighboring sagittal sections were hybridized with antisense probes to either *ephrin-A5* or *p75* (Fig. 3). This side-by-side comparison revealed that at E19 and P1, both *p75* and *ephrin-A5* were localized to the subplate (although expression within the cortical plate differed between the two probes). In contrast to the expression of *p75*, which was mostly concentrated to the posterior half of the subplate (Figs 3*B*, *D* and 4*A*), *ephrin-A5* was primarily expressed in the anterior subplate (Figs 3*A*, *C* and 4*A*). Quantification of these images revealed that for *p75*, grain

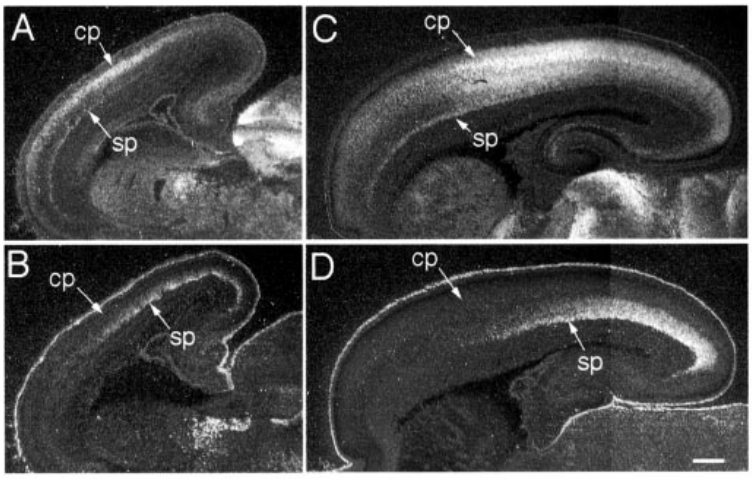

Figure 3. *Ephrin-A5* and *p75* have complementary expression profiles in the developing subplate. Shown are side-by-side comparisons of in-situ hybridization for (*A*, *C*) *ephrin-A5* mRNA and (*B*), *D p75* mRNA in sagittal sections of the developing rat cerebral cortex at (*A*, *B*) E19 and (*C*, *D*) P1. Anterior is to the left. *Ephrin-A5* is expressed in the anterior segment of the subplate, whereas *p75* expression is restricted to the posterior subplate. Abbreviations: cp, cortical plate; sp, subplate. Scale bar, 0.5 mm.

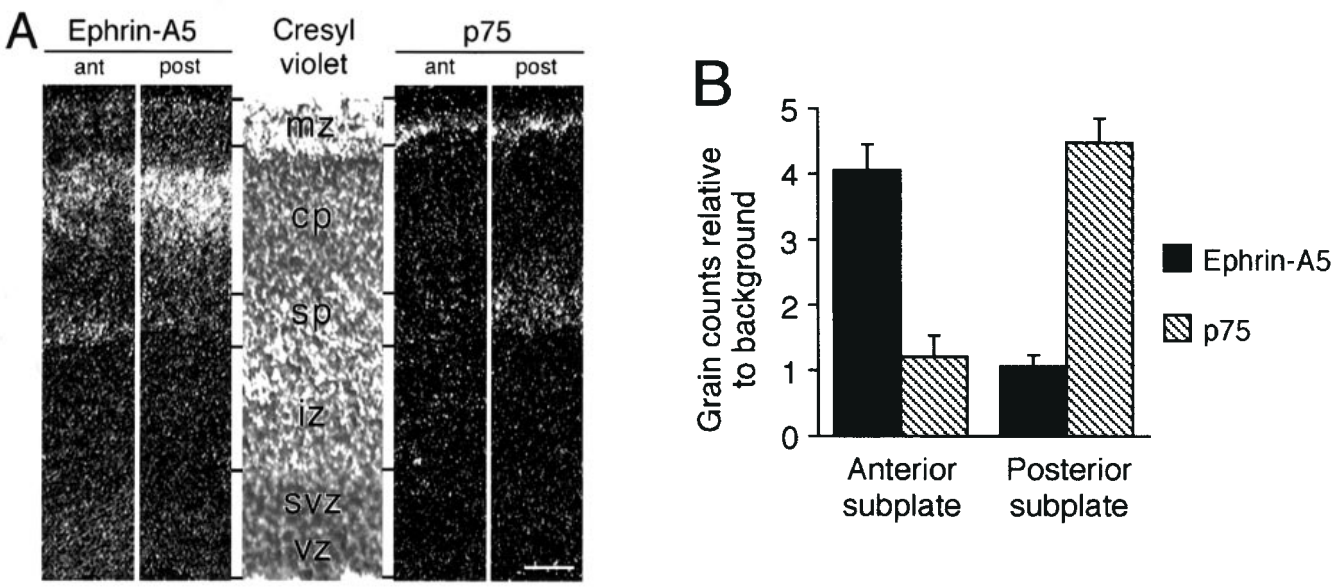

Figure 4. (*A*) High-magnification views of patterns of gene expression in anterior and posterior neocortex of E19 rats comparing *ephrin-A5* (left panels) and *p75* (right panels). Subplate cells in anterior cortex express high levels of *ephrin-A5* and low levels of *p75*, whereas the converse is seen in subplate cells from posterior cortex. Abbreviations: mz, marginal zone; cp, cortical plate; sp, subplate; iz, intermediate zone; svz, subventricular zone; vz, ventricular zone. Scale bar, 0.1 mm. (*B*) Comparison of expression levels of *p75* and *ephrin-A5* in the anterior and posterior subplate of E19 rats as measured by silver grain counts. The silver grains overlying five subplate neurons in a $\times 100$ microscopic field were counted for each of three different fields in both anterior and posterior cortex. Background counts of silver grains were also obtained over three fields and averaged for each probe separately. The expression levels of *ephrin-A5* and *p75* relative to background were obtained by dividing the number of grains overlying the subplate cells by the number of background silver grains for each probe. Each bar thus represents the average normalized grain count for each probe; error bars represent standard deviations.

counts per area in the anterior subplate were no greater than background levels, whereas the posterior subplate contained roughly 4.5-fold higher levels (Fig. 4*B*). In contrast, *ephrin-A5* counts were at background levels in the posterior subplate and approximately 4 times higher than background in the anterior subplate (Fig. 4*B*). It thus appears that from early times in development, anterior and posterior subplate cells have complementary expression profiles of *ephrin-A5* and *p75* mRNAs and therefore differ from each other in their presentation of at least two types of cell-surface molecules. These studies reveal that an anterior–posterior and temporal–medial gradient of *ephrin-A5* expression in the subplate was detected as early as E17 and maintained into postnatal life. Although anterior and temporal regions of cortex are developmentally more advanced than posterior and medial areas by 1–2 days (Uylings *et al.*, 1990), the maintenance of graded expression of *ephrin-A5* over

at least a week's time and the presence of an opposing gradient of *p75* indicate that *ephrin-A5* forms a true spatial gradient within the subplate.

Ephrin-A5 Expression in the Cortical Plate

A third gradient of *ephrin-A5* expression in the developing neocortex became apparent with the emergence of the cortical plate and was quite evident by E19 (Fig. 1*F*). *Ephrin-A5* message in the cortical plate was most abundant in the mid-dorsal area of sagittal sections. Its abundance tapered off gradually towards anterior regions and more sharply towards posterior regions. This graded expression was even more pronounced at later ages (see P1 in Fig. 1*G*). Three-dimensional reconstructions of sagittal and coronal sections of P1 rat brains (not shown) revealed that the highest levels *of ephrin-A5* hybridization in the cortical plate were present in a central region in each cortical hemisphere, corresponding to presumptive somatosensory cortex. At P1, *ephrin-A5* mRNA was distributed across all cortical layers that are present in the cortical plate at this age, with the exception of the marginal zone (layer 1) (Fig. 1*G*). Particularly high levels of expression were apparent in layer 5 at P1, and in layers 4 and 5 at P6 (not shown; this contrasts somewhat with the results Castellani *et al.* (Castellani *et al.*, 1998), who saw expression limited to layer 4). In more anterior regions at P1, *ephrin-A5* expression was markedly diminished in all layers and almost absent in layer 6 (Fig. 1*G*). Expression in the posterior cortical plate was also reduced, but much less severely. The pattern of *ephrin-A5* expression in the somatosensory cortex is similar to that reported recently (Gao *et al.*, 1998).

Expression of Ephrin-A3 and Ephrin-A4 in the Forebrain

Because we utilized a full-length human *ephrin-A5* probe for these studies, we needed to verify that the hybridization signal observed was indeed due to the presence of *ephrin-A5* mRNA rather than due to cross-hybridization with related molecules. In-situ hybridization experiments were performed on neighboring brain sections from E19 rats using probes directed against the coding regions of two other members of the ephrin-A class of ligands, human *ephrin-A3* and mouse *ephrin-A4*. We also used a probe directed against the 3′-end of human *ephrin-A5*, the region that shows the least amount of homology to other ephrins. As shown in Figure 5*A*, only very low levels of *ephrin-A4* message were present in the developing rat forebrain. *Ephrin-A3*, on the other hand, was expressed widely and at high levels in the cortical plate and subplate (Fig. 5*B*), but in a pattern distinct from that seen for *ephrin-A5*. Most notable, *ephrin-A3* is expressed uniformly throughout the subplate. Thus hybridization with neither the *ephrin-A3* nor the *ephrin-A4* antisense probe resulted in the specific and graded hybridization pattern obtained with the *ephrin-A5* probe (Fig. 5*D*). Only hybridization with the 3′-end probe of *ephrin-A5* (Fig. 5*C*) reproduced the graded expression profiles in the ventricular zone, subplate, and cortical plate that were seen using the full length probe (Fig. 5*D*). These results indicate that the full-length *ephrin-A5* probe used for these studies did not cross-hybridize with related ephrins, and that the observed graded expression pattern in the forebrain was specific to *ephrin-A5*.

Distribution of Putative Receptors for Ephrin-A Ligands

To interpret the positional information encoded in the graded distribution of *ephrin-A5*, responding cells or axons must express a cognate receptor. We therefore analysed the expression profile of several *ephrin-A5* receptors in the forebrain. There is a considerable degree of promiscuity in the interaction between Eph-like receptors and their ligands. *In vitro*, *ephrin-A5* interacts with any of the eight known members of the EphA receptor family (Gale *et al.*, 1996). We analysed the expression pattern of three of these receptors, *EphA3*, *EphA4*, and *EphA5*. To enable the accurate localization of receptor mRNA in various nuclei of the developing thalamus, hybridization profiles were obtained from coronal sections at E17 (an age at which thalamic neurons are extending axons to the cortex in the rat: Catalano *et al.*, 1996), E19, and P1.

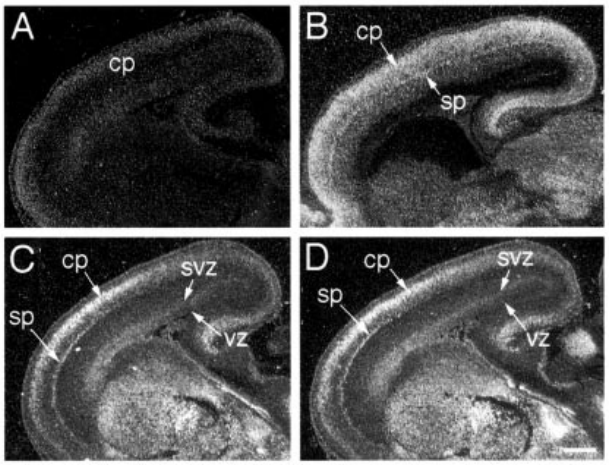

Figure 5. The expression patterns of *ephrin-A3* and *ephrin-A4* in the developing telencephalon differ from that of *ephrin-A5*. Hybridization patterns were compared on E19 sagittal rat brain sections; anterior is to the left. (*A*) *Ephrin-A4* probes revealed no hybridization above background levels in the telencephalon. (*B*) *Ephrin-A3* is expressed in a pattern distinct from that seen for *ephrin-A5*. Expression is apparent in the cortical plate, subplate, hippocampus, and thalamus; however, there is no evidence for gradients of expression within the cortex. (*C*) A probe to the 3′-terminal region of *ephrin-A5* probe produced a hybridization pattern comparable to that obtained with the full-length *ephrin-A5* probe (cf. *D* and Figs 1, 2). (*D*) In situ hybridization using the full-length *ephrin-A5* antisense probe on a section adjacent to those shown in (*A–C*). Abbreviations: cp, cortical plate; sp, subplate; svz, subventricular zone; vz, ventricular zone. Scale bar, 0.5 mm.

At E17 all three receptors were expressed in the developing brain (Fig. 6*A*, *D*, *G*). *EphA3* mRNA was expressed at high levels in both the telencephalon and dorsal thalamus (Fig. 6*A*). In the developing neocortex, *EphA3* was expressed in the ventricular zone and cortical plate, and at much higher levels in the subventricular zone and the subplate. *EphA3* was also expressed in the ventricular zone of the ventral telencephalon and at lower levels in the subventricular zone and the differentiating field of the basal ganglia and amygdala. In the diencephalon, expression of *EphA3* was concentrated in the dorsal thalamus. Specific thalamic nuclei in which *EphA3* was expressed at high levels included the ventral posterior (VP) and laterodorsal (LD) nuclei, as well as more medial dorsal nuclei (Fig. 6*A*); expression was notably absent from the ventral lateral geniculate nucleus (vLGN), but was strong in the dorsal lateral geniculate nucleus (dLGN; not shown). At E19, high expression was still observed in VP and LD, as well as dLGN (Fig. 6*B*). At early postnatal ages the same subset of thalamic nuclei continued to express *EphA3*, although expression levels in the thalamus appeared lower compared to earlier ages (Fig. 6*C*). Within the neocortex at later ages, the *EphA3* probe labeled the cortical plate, subplate, and subventricular zone. Expression in the subplate was quite distinctive; notably, no gradient of expression was observed in

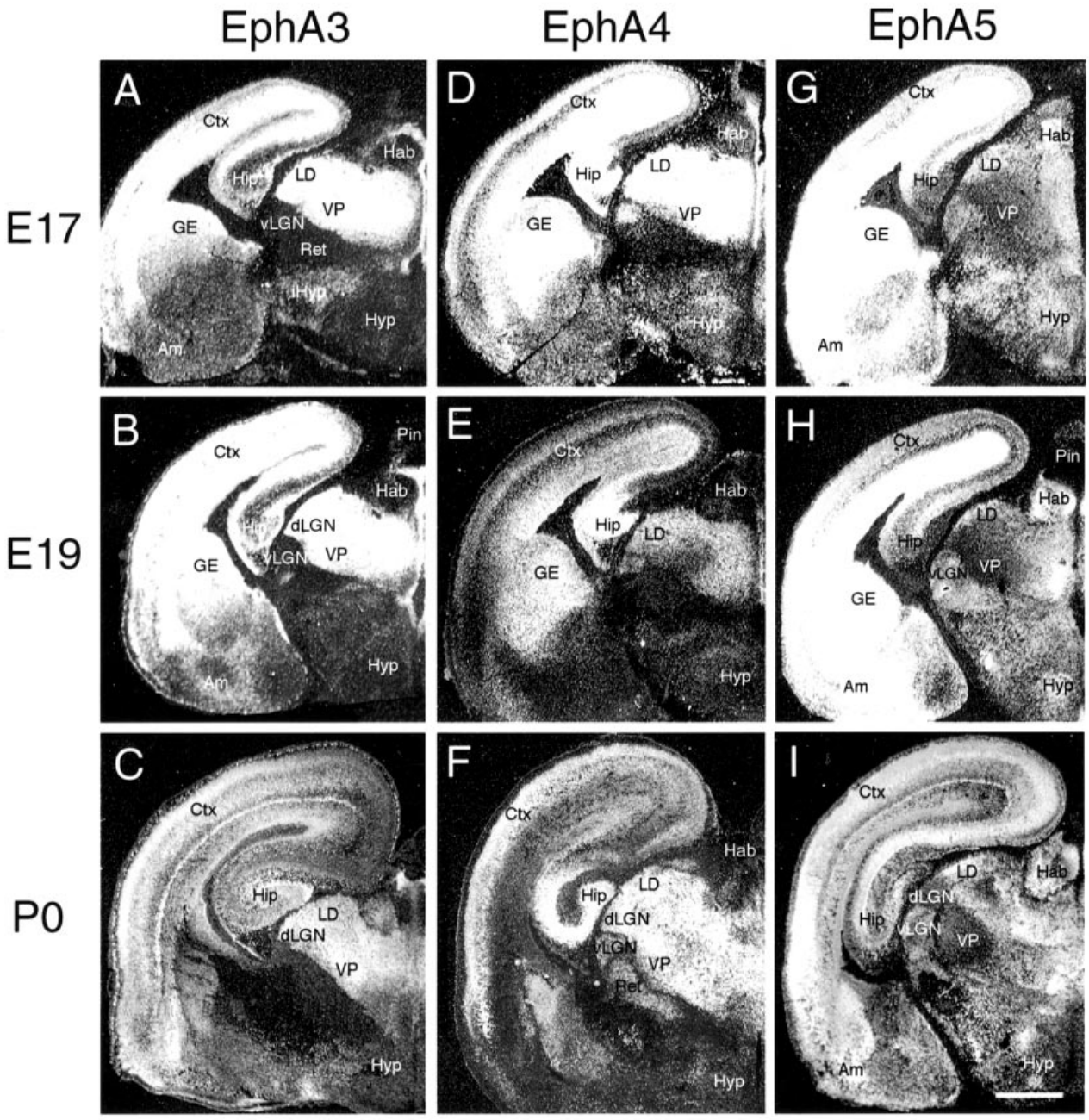

Figure 6. Three Eph receptors known to bind *ephrin-A5 in vitro* are expressed in various thalamic and cortical structures. Dark-field view of in-situ hybridization for (*A–C*) *EphA3*, (*D–F*) *EphA4*, and (*G–I*) *EphA5* mRNAs, performed on coronal sections of (*A*, *D*, *G*) E17, (*B*, *E*, *H*) E19 and (*C*, *F*, *I*) P0 rat brains. All three receptors are expressed in the dorsal thalamus. (*A–C*) At all three ages, *EphA3* expression is high in the telencephalon (particularly in the cortical subventricular zone and subplate), ganglionic eminence (GE), and dorsal thalamus. While expression of *EphA3* is obvious in the dorsal lateral geniculate nucleus (dLGN), it appears absent in another visual region, the ventral lateral geniculate nucleus (vLGN). Expression of *EphA3* is also high in the laterodorsal (LD) and ventroposterior (VP) nuclei, as well as in more medial thalamic nuclei at all three ages. At P0 a band of staining is visible in the subplate. (*D–F*) *EphA4* is expressed at high levels in the developing hippocampus (Hip), GE, and dorsal thalamic nuclei including dLGN, LD, VP,and the medial nuclei. In comparison to *EphA3*, expression extends slightly more ventrally in the thalamus to encompass vLGN and the reticular nuclei (Ret). Interestingly, there is no obvious expression of *EphA4* in the subplate at P0. *G–I* Expression of *EphA5* is high in the ventricular zones of the cortex and GE, and is particularly strong in the amygdala (Am) at the younger ages. Like *EphA3*, there does appear to be expression in the cortical subplate at P0. *EphA5* is more broadly expressed within the thalamus than are *EphA3* and *EphA4*, with some of the highest levels of expression in the medial nuclei. Most noticeable is the low level of *EphA5* staining in the somatosensory region VP,whereas staining is apparent in the visual nucleus vLGN. Another visual nucleus, dLGN, does not appear to express *EphA5* at high levels. (*H*) provides some hint that expression of *EphA5* within the posterior VP may be graded with the highest levels seen dorsomedially and the lowest ventrolaterally. In contrast to the other receptors, *EphA5* does appear to be expressed in the habenulum (Hab). Abbreviations: Ctx, cerebral cortex; Hip, hippocampus; Am, amygdala; GE, ganglionic eminence; Hab, habenular nucleus; Pin, pineal; dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus; VP,ventral posterior nucleus; LD, laterodorsal nucleus; Ret, thalamic reticular nucleus; iHyp, inferior hypothalamus; Hyp, anterior and ventral hypothalamus. Scale bar, 0.5 mm.

either the mediolateral plane (Fig. 6*C*) or anteroposterior plane (not shown).

EphA4 was expressed at high levels within the E17 telencephalon throughout the ventricular zone, the subventricular zone, and the cortical plate of the neocortex (Fig. 6*D*), with expression extending ventrally into the rhinencephalon. Expression was also high throughout the ganglionic eminence in the ventral telencephalon. *EphA4* was expressed at high levels in the dorsal thalamus and at lower levels in the fields of Forel in the hypothalamus. High levels of expression in the thalamus were observed in VP, LD, and the medially-located dorsal nuclei (Fig. 6*D*), as well as in dLGN and vLGN (not shown). Expression levels appeared to decrease in the thalamus at E19, but expression was maintained in each of the above-mentioned nuclei (Fig. 6*E*). At postnatal ages, *EphA4* continued to be expressed in VP, LD, the medial nuclei, dLGN, and vLGN (Fig. 6*F*). *EphA4* mRNA was also expressed in the hippocampus and basal ganglia at all ages. As observed at embryonic ages, *EphA4* was expressed in the cortical plate at high levels postnatally, with no obvious regional differences between different areas. Compared to *EphA3* and *EphA5*, however, expression levels were high in the outer layers of the cortical plate and low or absent in the deep layers and subplate (Fig. 6*F*).

The expression of *EphA5* differed markedly from that seen for *EphA3* or *EphA4*. High levels of *EphA5* staining were observed throughout the embryonic telencephalon, particularly in the hippocampus, the neocortical ventricular and subventricular zone of the developing neocortex and in the basal ganglia, and extended past the rhinencephalon into the amygdala (Fig. 6*G*, *H*). *EphA5* expression was far lower in the cortical plate than in the proliferative zones. In the diencephalon of embryonic brains, *EphA5* mRNA was highly expressed in the habenular nucleus, dorsal thalamus, and hypothalamus (Fig. 6*G*, *H*), with the strongest expression in the medial thalamus and epithalamus. The levels of expression in dLGN and VP were markedly lower, at roughly background levels, compared to structures such as vLGN and the medial thalamic nuclei, in which expression levels were high (Fig. 6*G–I*). At postnatal ages, *EphA5* continued to be expressed at high levels throughout the hippocampus and the cortex (Fig. 6*I*). As observed for *EphA3*, the subplate was distinctly labeled by the *EphA5* probe. Expression was also present in a number of thalamic nuclei, with expression notably absent from dLGN and VP, but present in vLGN.

Discussion

Axonal connections in the cerebral cortex are organized in specific patterns in both the radial dimension (between the neurons of different layers) and the tangential dimension (between neurons in different cortical areas and between cortical neurons and subcortical targets) (Gilbert, 1983; Gilbert and Wiesel, 1985). The molecular mechanisms by which these connections are established in the forebrain are not well understood. In recent years a number of proteins have been implicated in axon pathfinding. Members of two gene families, the Eph-like receptor tyrosine kinases and their ligands, the ephrins, have been shown to function in the topographic mapping of retinal ganglion axons to the proper regions of the tectum (Cheng *et al.*, 1995; Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Monschau *et al.*, 1997; Frisen *et al.*, 1998). We show here by in-situ hybridization that a member of the ephrin family, *ephrin-A5*, is expressed in the developing telencephalon in three distinct gradients: a bi-directional gradient within the cortical plate, an anterior to posterior gradient within the ventricular zone, and an anterior to posterior gradient within the subplate.

Ephrin-A5 in the Cortical Plate

One of the three gradients of *ephrin-A5* expression emerged in the cortical plate at around E19. Three-dimensional reconstructions of sagittal and coronal sections revealed that the highest level of *ephrin-A5* message was present in the somatosensory area of the cortex. Within this region, expression of *ephrin-A5* was highest in layer 5 and by P6 was also quite high in layer 4. These results differ somewhat from those of Castellani *et al.* (1998), who showed that *ephrin-A5* is expressed only in layer 4 of the cortical plate at P7, an age at which *ephrin-A5* and *EphA5* appear to play a role in the establishment of interlaminar connections within the cortex. At earlier times in development, ingrowing thalamic axons must traverse layers 6 and 5 of the cortex to reach their target neurons in layer 4. It is of interest in this context that there was a distinctive lack of expression of one of the putative receptors of *ephrin-A5*, *EphA5*, in the ventral posterior nucleus (VP) of the thalamus, because neurons from this thalamic structure innervate the somatosensory cortex in which *ephrin-A5* levels are highest. Our findings differ somewhat from the results of Gao *et al.* (Gao *et al.*, 1998). Both our study and that of Gao *et al.* observed high levels of *ephrin-A5* message in somatosensory cortex. However, while they reported little or no *EphA5* expression outside the medial thalamic nuclei, our experiments revealed that *EphA5* is also expressed, albeit differentially and at overall lower levels, in the lateral and ventral (sensorimotor) nuclei of the thalamus. Since ephrin-A5 has been shown to repulse axons that can detect the presence of this ligand (Drescher *et al.*, 1995; Gale *et al.*, 1996), the lack of EphA5 expression in VP cells may enable their axons to invade the cortical plate in the area of high ephrin-A5 expression, as suggested previously (Gao *et al.*, 1998). This hypothesis remains to be tested by analysing thalamocortical projections in *ephrin-A5* or *EphA5* mutant mice.

Ephrin-A5 in the Ventricular Zone

At earlier times in development we also observed a gradient of *ephrin-A5* message in telencephalic progenitor cells. The expression gradient was already apparent within the ventricular zone at E11, and it persisted until E19, a time when neurogenesis ceases and the neuroepithelium disappears (Frantz *et al.*, 1994). *Ephrin-A5* was most abundant in the anterior tip of the telencephalon (the developing olfactory bulb) with levels sharply decreasing toward more posterior regions of the cortical and the septal neuroepithelium. Beginning at E16, *ephrin-A5* expression shifted from the ventricular zone to progenitors of the subventricular zone.

This observation suggests that there is spatial information present within the ventricular zone at a time when cortical neurons are being generated. The question of how regional differences arise in distinct cortical areas has long fascinated those who study cortical development (Rakic, 1988; O'Leary, 1989). Several investigators have suggested that at least some aspects of areal fate determination may be specified in the ventricular zone. Well before thalamic axons innervate the cortex, the paired-box gene *Pax-6* and the homeobox gene *Emx2* are expressed in gradients along the anterior–posterior axis of the cortical neuroepithelium (Walther and Gruss, 1991; Simeone *et al.*, 1992). *Ephrin-A5* constitutes another example of a gene that displays a graded expression pattern in the cortical neuroepithelium, further supporting the notion that some level

of intrinsic specification may exist in the cortical primordium, independent of the extrinsic influences of thalamic input.

It is not clear how or whether *ephrin-A5* might function at such early stages of cortical development. Indeed, all three of the putative receptors for *ephrin-A5* we studied, *EphA3*, *EphA4*, and *EphA5*, were expressed widely in the developing cortex, including the ventricular and subventricular zones, implying that individual progenitor cells might co-express both ligand and receptor. It is conceivable that interactions between Eph receptors and ephrin-A5 could mediate or regulate a variety of developmental processes in the early cortex, including the differentiation and migration patterns of cortical neurons, as has been observed for migrating neural crest cells (Wang and Anderson, 1997).

Ephrin-A5 in the Subplate

In contrast to the cortical ventricular zone, to date there have been no reports of positional differences among subplate neurons, despite the hypothesis that such differences might play a key role in the targeting of thalamic axons to appropriate cortical areas (Ghosh and Shatz, 1993). The graded expression of *ephrin-A5* and the low-affinity NGF receptor *p75* reported here provide a first indication that this cell layer is not regionally homogeneous. From E17 onwards, anterior and posterior subplate cells differ in their expression of *ephrin-A5* and *p75*: anterior subplate cells express high levels of *ephrin-A5*, whereas posterior subplate cells express *p75* mRNA. The stability of these gradients over time, and the uniform expression of *ephrin-A3* in the subplate at the same time (Fig. 5*b*), suggest that *ephrin-A5* is expressed in a true spatial gradient and is not simply reflecting maturational differences between subplate neurons (Uylings *et al.*, 1990). Our observations raise the possibility that thalamic axons might identify their target area by using the graded expression in the subplate of an axon pathfinding cue such as *ephrin-A5*. A simple prediction is that for such a mechanism to function successfully, thalamic neurons innervating more frontal areas of cortex, which express high ligand levels, should show low levels of receptor expression. Conversely, visual thalamic neurons innervating the low-ligand-expressing occipital cortex could afford to express high levels of Eph receptors. However, the expression patterns of three putative receptors for *ephrin-A5*, *EphA3*, *EphA4*, and *EphA5*, are more complicated than predicted by such a simple notion. All three receptors are indeed expressed in the thalamus. Two of the receptors, *EphA3* and *EphA4*, are expressed at high levels in most nuclei of the dorsal thalamus, including visual nuclei (dLGN and vLGN), somatosensory nuclei (VP), and the medial thalamic nuclei (which project to frontal regions). *EphA5* expression showed a more complex pattern in the dorsal thalamus, being most robust in medial nuclei with generally lower expression in the more laterally situated nuclei. However, relatively high levels of expression were detected in vLGN and LD compared to dLGN and VP, in which expression was negligible. It is not clear whether these complex spatial patterns of Eph receptor expression could confer areal specificity onto ingrowing thalamic axons.

If most or all neurons in the dorsal thalamus express some combination of Eph receptors, how might thalamic axons be differentially sensitive to the graded expression of ephrin-A5 in the cortical subplate? One possibility is that the timing of axon arrival may correlate with different levels of ephrin expression in the subplate. Axons from the thalamus first reach the cortex at E16 in the rat (Catalano *et al.*, 1996), an age just before the onset of *ephrin-A5* expression in the subplate. It is thus unlikely that ephrin-A5 guides the initial innervation of more anterior and lateral regions of the cortex. It is possible, however, that ephrin-A5 expression at slightly later times could repel later-arriving thalamic axons from the already innervated areas and steer them to progressively more posterior and medial regions of the cortex. At E17, the earliest age at which we detect *ephrin-A5* expression in the subplate, thalamic axons are just innervating the subplate area below the future somatosensory area (Catalano *et al.*, 1996), which at this age is devoid of *ephrin-A5* message. Later-arriving axons from the visual thalamus may encounter increased levels of ephrin-A5 in temporal and frontal regions of cortex, which might then repel the axons into more posterior (visual) cortical regions. This notion is consistent with the observation that thalamic innervation of the cortex occurs in an anterior to posterior and lateral to medial gradient, the same gradient in which *ephrin-A5* message is distributed. However, given the overlap in both neurogenesis and axon ingrowth from different thalamic nuclei, this model cannot fully explain the precision with which thalamic axons identify and innervate their cortical targets.

In summary, we have shown that *ephrin-A5* message is distributed in distinct gradients during the development of the telencephalon. Our studies identified *ephrin-A5* as one of the earliest markers of anterior-to-posterior specification of cells in the cortical neuroepithelium. In addition, the graded expression of *ephrin-A5* in the cortical plate, combined with the lack of expression of the putative *ephrin-A5* receptor *EphA5* in VP of the thalamus, supports the suggestion by Gao *et al.* (Gao *et al.*, 1998) that this ligand/receptor pair may function in the innervation by thalamic axons of somatosensory cortex. Lastly, we have shown that *ephrin-A5* and the low-affinity NGF receptor *p75* are expressed in the subplate in opposing gradients, revealing that the early subplate is not a molecularly homogeneous structure. Most neurons in the dorsal thalamus express the Eph receptors *EphA3* and *EphA4*, and the graded expression of *ephrin-A5* in the subplate mirrors the spatiotemporal gradient of cortical innervation by thalamic axons. These observations thus raise the possibility that *ephrin-A5* may participate in the topographic mapping of thalamic axons to specific cortical areas, a hypothesis that remains to be tested directly.

Notes

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Please address correspondence to: Dr Susan K. McConnell, Department of Biological Sciences, Stanford University, 385 Serra Mall, Stanford, CA 94305–5020, USA.E-mail: suemcc@ stanford.edu.

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