

# Multiple Roles of Ephrins during the Formation of Thalamocortical Projections: Maps and More

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**ABSTRACT:** The functional architecture of the cerebral cortex is based on intrinsic connections that precisely link neurons from distinct cortical laminae as well as layer-specific afferent and efferent projections. Experimental strategies using *in vitro* assays originally developed by Friedrich Bonhoeffer have suggested that positional cues confined to individual layers regulate the assembly of local cortical circuits and the formation of thalamocortical projections. One of these wiring molecules is ephrinA5, a ligand for Eph receptor tyrosine kinases. EphrinA5 and Eph receptors exhibit highly dynamic expression patterns in distinct regions of the cortex and thalamus during early and late stages of thalamocortical and cortical circuit formation. *In vitro* assays suggest that ephrinA5 is a multi-functional wiring molecule for different populations of cor-

tical and thalamic axons. Additionally, the expression patterns of ephrinA5 during cortical development are consistent with this molecule regulating, in alternative ways, specific components of thalamic and cortical connectivity. To test this directly, the organization of thalamocortical projections was examined in mice lacking ephrinA5 gene expression. The anatomical studies in ephrinA5 knockout animals revealed a miswiring of limbic thalamic projections and changes in neocortical circuits that were predicted from the expression pattern and the *in vitro* analysis of ephrinA5 function. © 2004 Wiley Periodicals, Inc. *J Neurobiol* 59: 82–94, 2004

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## INTRODUCTION

In his “neuron doctrine”, Ramon y Cajal (1894) was the first neuroscientist who postulated that the brain is

composed of an enormous number of individual cells, the neurons, which have two different kinds of processes. According to Cajal, neurons are polarized, possessing axons that can diverge to form terminal arbors that make precise contacts with the dendrites of

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many other neurons. Because axons often project over long distances to their target cells, Cajal was also intrigued by the question of how such specific connections are established during embryogenesis. In his neurodevelopmental studies, Ramon y Cajal discovered that an individual, growing axon has a specialized structure at its tip, which he named “growth cone”. He suggested that the growth cone can explore the local environment and is attracted by substances released by distant target cells. Among the first experiments to test this “chemotropic theory” were those by Roger Sperry. He demonstrated that regenerating axons from retinal ganglion cells consistently form precise and stereotyped connections with their target cells in the tectum, independent of the route by which the axons enter their target field. In his original “chemoaffinity hypothesis”, Sperry proposed that each cell in the tectum has a specific molecular label that is recognized by the axons that elect to terminate on a particular cell (Sperry, 1963). An obvious problem with this theory, however, is that such a targeting mechanism would require a complex number of different molecules beyond what seemed possible, particularly in the context of the assembly of circuitry throughout the brain. Therefore, Sperry, and later Alfred Gierer and others, suggested different gradients models, where the targeting of growth cones is critically dependent on a given concentration of a small number of chemoattractive substances (Gierer and Meinhard, 1972; Willshaw and van der Malsburg, 1976, 1979; Gierer, 1981, 1983). Thus, if growth cones were sensitive to small changes in the amount of guidance factors, then many fewer molecules would be required for the formation of an ordered topographic retinotectal projection.

A major issue in the field then became defining the biochemical nature of the postulated chemoattractant factors. Many attempts to isolate and identify attractants from different brain regions failed. One reason for these failures was the extreme difficulty in isolating substances of very low abundance from the highly complex molecular milieu in the brain. Moreover, it was not clear how to examine the effects of putative chemoattractive molecules.

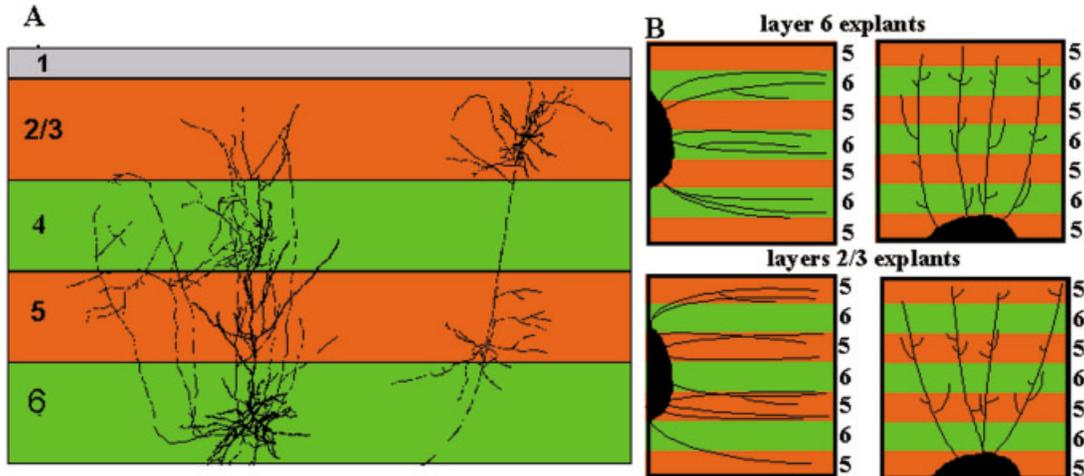
It was the pioneering work of Friedrich Bonhoeffer, who developed elegant and efficient assays to study the targeting of growing axons *in vitro*, that transformed the field. In one of these test systems—now called the Bonhoeffer assay—axons from defined regions of the retina grow on alternating stripes of cell membranes from the tectum. When one set of membrane stripes is prepared from the target and the other set from the nontarget region of the tectum, retinal axons show a preference for growing on target mem-

branes, avoiding the nontarget membranes. Using this reductionist approach to study growth cone steering, Bonhoeffer and colleagues could easily manipulate the membrane substrates. In one of the most surprising findings in the axon guidance field, they demonstrated that retinotectal axons are not attracted by membranes from their target regions, as initially proposed by Sperry, but rather they are repelled by membrane preparations from the nontarget region (Bonhoeffer and Huf, 1985; Walter et al., 1987a,b). After many years of intensive work, the Bonhoeffer laboratory identified one of these chemorepellent molecules in the tectum. Originally, it was called repulsive axonal guidance signal (RAGS) and later renamed ephrinA5 (Drescher et al., 1995). EphrinA5 is one of the ligands from the large family of Eph receptor tyrosine kinases discovered by Yankopolous and colleagues (Davis et al., 1994). As postulated by the theoretical work on topographic mapping in the brain, ephrinA5 (as well as other ephrins) was expressed in a graded distribution in the tectum, and several Eph receptors displayed a countergradient in the retina.

From the initial discovery of the function of ephrins in the formation of retinotectal projections by Bonhoeffer, Drescher, Flanagan, and colleagues, many other groups demonstrated that ephrins also play important roles in the development of neuronal connections in several regions of the brain (for recent reviews see Flanagan and Vanderhaeghen, 1998; Melitzer et al., 2000; Cowan and Hekemeyer, 2002; Kullander and Klein, 2002). In this report, we focus on the development of axonal projections between the thalamus and the cerebral cortex during embryogenesis and early postnatal stages. We also will consider possible functions of ephrins during the formation of local cortical circuits. We will emphasize our findings of the role played by ephrinA5, demonstrating that this molecule plays many different roles during early and late stages of thalamocortical and cortical circuit formation.

### **IN VITRO EVIDENCE FOR MULTIPLE ROLES OF EPHRINA5 DURING CORTICAL DEVELOPMENT**

The mammalian cerebral cortex can be broadly subdivided into the limbic cortex and the neocortex. The limbic cortex contains three to five cell layers, whereas the neocortex is structurally more homogeneous and always contains six layers. During evolution, the neocortex increased in size markedly, both in

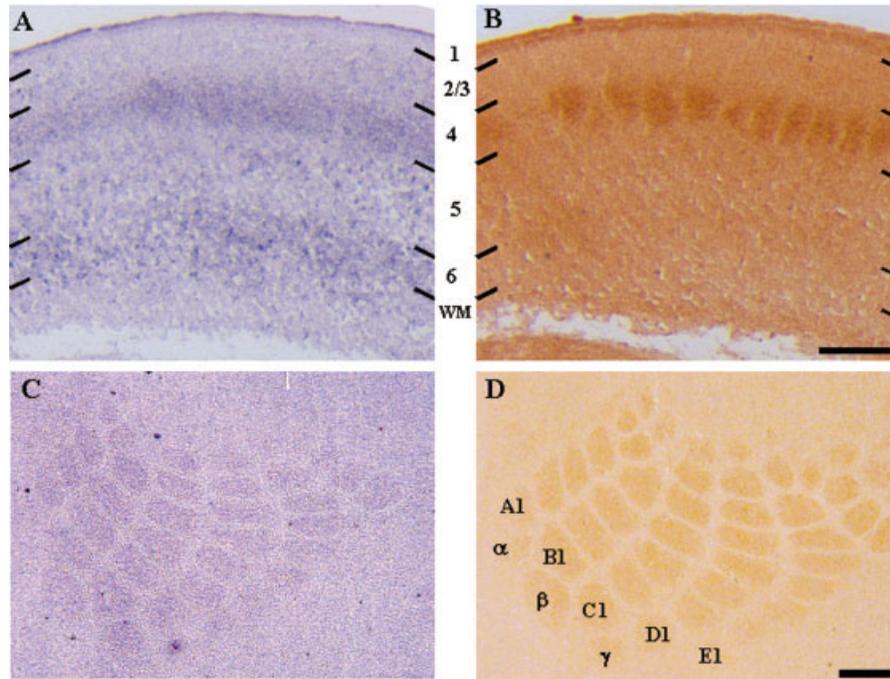


**Figure 1** (A) Interlaminar connections in the neocortex. Pyramidal neurons in layer 6 form axon collaterals in lamina 6 and layer 4 (thin processes, the thick processes are dendrites), but not in the intervening layer 5. Conversely, pyramidal neurons in layers 2/3 extend axon collaterals in the same layer and in layer 5, but not in layer 4 and 6. Thus the cortical layers marked in green are target laminae for layer 6 cells and nontarget laminae for layers 2/3 cells. On the other hand, the layers marked in red are target laminae for layers 2/3 neurons and nontarget laminae for layer 6 neurons. (B) *In vitro* evidence for positional cues confined to individual cortical layers that regulate the laminar specificity of axon collateral formation. Left: in the Bonhoeffer assay, with axons growing parallel to alternating stripes of membranes from target and nontarget laminae, fibers grow preferentially on membranes from their target lamina (lamina 6 for layer 6 cells and lamina 5 for layers 2/3 cells) and avoid the stripes of membranes prepared from the nontarget lamina. Right: in an *in vitro* assay designed to examine axonal branching, fibers extend perpendicular to membrane stripes from target and nontarget lamina. As fibers cross the membrane stripes from the target lamina they emit more branches than on the stripes from nontarget lamina. Additional experiments described in Castellani and Bolz (1997) indicated that both attractive cues in the target laminae and repulsive cues in the nontarget laminae regulate the formation of local cortical circuits.

absolute terms and also in proportion to the limbic cortex. The laminar architecture of the neocortex reflects the organization of projections both to and from the cortex and intracortically. Pyramidal cells, which account for about 80% of all cortical neurons, send efferent axons to distant subcortical targets or to other cortical regions. In addition, collaterals of pyramidal cell axons form stereotyped connections within and between cortical laminae. For instance, as illustrated in Figure 1, pyramidal cells in layers 2/3 elaborate axon collaterals in layers 2/3 and layer 5, but not in layer 4 or layer 6. In contrast, layer 6 pyramidal neurons extend axon collaterals in layer 6, which ascend and branch in layer 4, but not in the intervening layer 5. Such specific and precise inter- and intralaminar connections are major components of local cortical circuits (Gilbert and Wiesel, 1979; Bolz et al., 1989; Katz and Callaway, 1992).

What are the cellular and molecular mechanisms that lead to the formation of such highly specific branching patterns in the cortical layers? We and others have hypothesized that there are factors con-

fining to distinct laminae that regulate the targeting and branching of specific sets of axons. Alternatively, it has been suggested that afferent fibers, or activity patterns relayed by these afferent fibers, are responsible for the laminar specification of cortical circuits. Inspired by the work of Bonhoeffer, Castellani and Bolz (1997) used stripe assays with membranes from single cortical layers, prepared at the time at which intrinsic cortical circuits are established in the brain, in order to identify potential attractive and repulsive cues that could differentially regulate axon growth. These membrane stripes were offered as substrates for explants of different populations of cortical neurons. As illustrated in Figure 1, the results of these experiments provided the first evidence for the existence of positional cues restricted to individual layers that may contribute to the laminar specificity of local cortical circuits. Some of these signals induce or inhibit collateral branch formation in different cortical layers, and other factors act as attractive or repellent axonal guidance cues. Together, these membrane-associated signals regulate the formation of axonal branches in



**Figure 2** (A,C) *In situ* hybridization with an ephrinA5 antisense cRNA probe and (B,D) cytochrome oxidase staining of the somatosensory cortex (S1). (A,B) Coronal section of a P6 mouse brain shows a strong hybridization signal in layer 4 and a weaker signal in layer 6. (C,D) Flat-mounted tangential sections of a P8 brain reveal the whisker pattern in S1. The first barrel in the five rows (A–E) and the additional whiskers  $\alpha$ – $\gamma$  are indicated in the cytochrome oxidase stained section. Scale bars: 200  $\mu$ m.

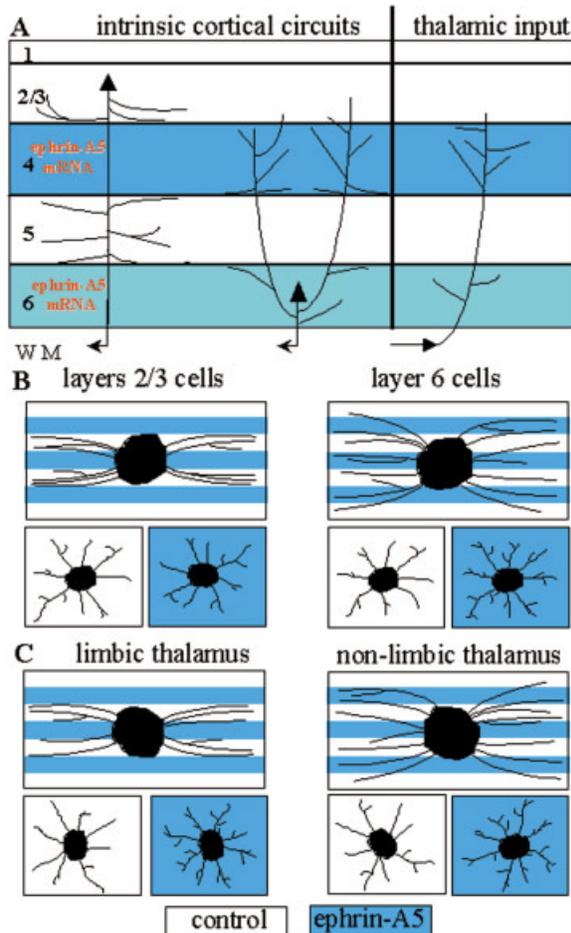
the cortical target layers, preventing axon collaterals from growing into nontarget layers.

While we had identified the existence of laminar cues, the molecular nature of the signals that regulate the formation of layer-specific cortical circuits remained a mystery. Many different classes of molecules are expressed in a lamina-specific pattern at early postnatal developmental stages, including neurotrophic factors, growth factors, cell adhesion molecules, and semaphorins (e.g., Allendoerfer et al., 1990; Ringstedt et al., 1993; Götz et al., 1997; Skaliara et al., 1998; Lein et al., 2000; Yamamoto et al., 2000a,b). There is increasing evidence that several members of these molecular families influence the growth, branching, and targeting of cortical axons. In this report, we discuss the effects of ephrinA5 on guidance and branching of cortical and thalamic fibers.

### EPHRINA5 IS A MULTIFUNCTIONAL GUIDANCE CUE IN THE CORTEX

In the primary somatosensory cortex (S1) of mice, at postnatal stages during which pyramidal cell axons extend collateral branches within the cortex, ephrinA5

is expressed robustly in layer 4 and, to a lesser extent, in layer 6 (Fig. 2). Thus, ephrinA5 is present in the target laminae of layer 6 neurons and in the nontarget laminae of layer 2/3 neurons. EphrinA5 was characterized as a repulsive axonal guidance signal in the retinotectal system, and so we suggested that ephrinA5 might have similar effects on layers 2/3 axons. To test this hypothesis, Castellani et al. (1998) repeated the Bonhoeffer assays, but rather than attempting to isolate membranes from specific cortical layers, membrane stripes were prepared from an ephrinA5 transfected and a vector transfected control cell line. As predicted, ephrinA5 acts as a repulsive guidance signal for layers 2/3 axons, but not for layer 6 axons, which target the ephrinA5-expressing layers 4 and 6. However, in an *in vitro* assay designed to test for axonal branching, ephrinA5 was found to selectively increase collateral formation of layer 6 axons, without affecting layer 2/3 axons (Fig. 3). It appears, therefore, that ephrinA5 has a dual action on cortical neurons: acting selectively as a “repulsive axonal guidance signal” for layers 2/3 axons, without affecting axonal arborization, and as a branch-promoting signal for layer 6 pyramidal cell axons. EphrinA5 has



**Figure 3** (A) Schematic drawings of layer-specific local circuits and thalamic projections in the neocortex and the expression pattern of ephrinA5 mRNA during postnatal stages. Layer 6 pyramidal cells and thalamic axons arborize in the ephrinA5 expressing layers 4 and 6, whereas axon collaterals of layers 2/3 pyramidal cells avoid these layers. (B,C) *In vitro* assays with membranes from ephrinA5 transfected NIH3T3 cells (blue) and control transfected cells (white). (B) EphrinA5 acts as a repulsive axonal branching cue for layers 2/3 cells, but has no effect on axonal guidance. Conversely, for layer 6 cells, ephrinA5 promotes collateral formation, but has no effect on axon guidance. (C) EphrinA5 also induces branching for thalamic axons. It also acts as a repulsive axonal guidance cue for limbic thalamic axons, whereas most nonlimbic thalamic axons are not repelled by ephrinA5. [Color scheme can be viewed in the online issue, which is available at <http://www.interscience.wiley.com>]

no influence on axonal guidance for this class of cortical neurons.

The observations by Castellani et al. (1998) of an “attractive” effect of ephrinA5 were novel at that time. Several other groups have recently supported this dual biological activity concept for Eph signaling, reporting that the repulsive effects of ephrins can

become, under some circumstances, attractive (Holmberg et al., 2000; Davy and Robins, 2000; Huai and Drescher, 2001; Knöll et al., 2001; Mann et al., 2002b; Moreno-Flores et al., 2002). In the thalamocortical system, for example, Mann et al. (2002a) showed that ephrinA5 also acts as a branch-promoting factor for thalamic fibers. In addition, ephrinA5 serves as a repellent guidance cue for limbic thalamic fibers, whereas most thalamic fibers that project to the neocortex do not respond to ephrinA5 in the Bonhoeffer assay [Mann et al., 2002a; see also Fig. 3(C)]. Together with many other studies, these results indicate that ephrins can have a wide and complex range of actions.

### THALAMOCORTICAL PROJECTIONS AND CORTICAL CIRCUITS IN THE ABSENCE OF EPHRINA5

The results from the *in vitro* studies discussed so far indicate that ephrinA5 has diverse effects on different populations of thalamic and cortical axons and therefore might potentially regulate the formation of thalamocortical projections and cortical circuits in multiple ways. To test directly whether ephrinA5 participates in the wiring of the cortical connections *in vivo*, several groups examined thalamocortical connections and intrinsic cortical circuits in genetically manipulated mice, in which the gene encoding for ephrinA5 has been disrupted.

The initial axonal projections from different thalamic nuclei into the cortex develop in a highly stereotypical, specific fashion. Thalamocortical axons grow through the internal capsule prenatally, reaching the cerebral wall beneath the cortical plate even before their cortical target cells, the layer 4 neurons, have been generated (Rakic, 1977; Ghosh et al., 1990; de Carlos and O’Leary, 1992; Erzurumlu and Jhaveri, 1992). It has been shown that thalamic axons use a temporary scaffold, the earliest generated neurons of the subplate, as an intermediate target. The subplate neurons have been suspected of carrying positional cues for the targeting of thalamocortical projections (for review see Goodman and Shatz, 1993). Vanderhaeghen et al. (2000) observed that at the time thalamic axons reach the subplate zone, which in mice is between embryonic day (E) 16.5 and 17.5, there is a gradient of ephrinA5 mRNA in the subplate and cortical plate of the primary somatosensory cortex (S1) (Yun et al., 2003; see Fig. 6). S1 contains a map of the body surface, where the body representation is scaled according to the extent of peripheral sensory innervation. In rodents, there is a prominent representation of the whiskers within S1. Thalamic input representing

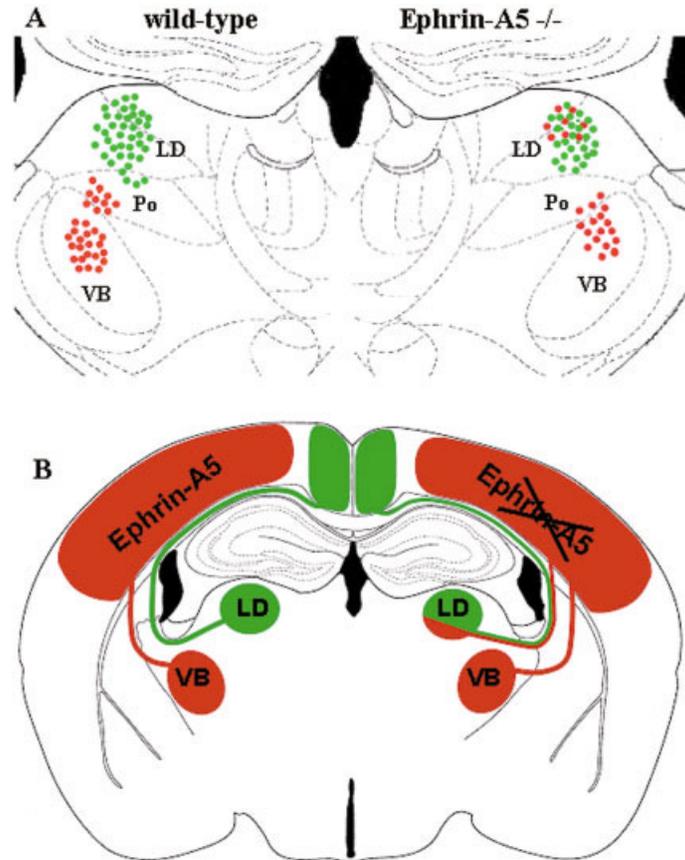
the neuronal activity of a single whisker converges to a restricted zone in layer 4 of S1, called a barrel. The barrel field within S1 forms an ordered topographic map, that is, a point to point representation of the contralateral whisker pad (Woolsey and Van der Loos, 1970; Welker and Van der Loos, 1986). The medial part of the barrel field has a high concentration of ephrinA5 mRNA, whereas in the lateral part, the concentration is low. In the ventrobasal complex of the thalamus (VB), which projects to the barrel field, there is a countergradient of EphA4, one of the receptors of ephrinA5. Thus, EphA4 expression is poor in the medial part of VB, which projects to the medial part of the barrel field in S1, a region rich in ephrinA5. Conversely, EphA4 expression is high in the lateral part of VB, which innervates the ephrinA5 poor lateral regions of S1. In ephrinA5 knockout mice, there is a distortion of the barrel field representation, with medial barrels compressed and lateral barrels expanded (Vanderhagen et al., 2000). This suggests that ephrinA5 participates in the precise topographic mapping of thalamic afferents into S1, as has been suggested previously for the retinotectal (Cheng et al., 1995; Drescher et al., 1995) and hippocamposeptal projections (Gao et al., 1996).

Several Eph receptors as well as ephrin ligands are differently expressed in various nuclei of the thalamus, and these expression patterns change during development. For example, at the embryonic time period when thalamic fibers invade the cortical subplate, EphA5 is expressed in the medial group of thalamic nuclei that normally project to the limbic cortex (Gao et al., 1998). Thalamic axons that project to medial limbic areas (prefrontal, cingulate, and retrosplenial cortices) bypass the neocortical area S1, where ephrinA5 is expressed. *In vitro*, ephrinA5 inhibits outgrowth of neurites from medial (limbic) thalamic neurons but has no effect on lateral (nonlimbic) thalamic neurons (Gao et al., 1998). Moreover, as described in the previous section, ephrinA5 acts as a repulsive guidance signal for limbic thalamic axons, whereas for most nonlimbic thalamic fibers it exhibits no guidance activity (Mann et al., 2002a). The expression patterns and the results from the *in vitro* assays suggested to us that ephrinA5 acts as a signal that restricts limbic thalamic axons from entering inappropriate neocortical regions. To test this idea directly, Uziel et al. (2002) examined thalamic projections to limbic and neocortical areas in ephrinA5 knockout mice. As illustrated in Figure 4, in the absence of ephrinA5, there is a misrouting of limbic thalamocortical projections. Although most cells of the laterodorsal nucleus of the thalamus (LD) from ephrinA5 deficient animals innervate correctly the medial limbic

cortex, a nonoverlapping subpopulation of LD neurons forms (or maintains) misrouted axons, reaching the neocortical S1 area. These data provided the first direct evidence *in vivo* that Eph-ephrin signaling participates in thalamocortical axon patterning. Here, cortical expression of ephrinA5 is essential for limbic thalamic axons to avoid targeting errors. The functional consequences of such mistargeting, where LD maintains an appropriate and an inappropriate cortical projection, are unknown.

There also is *in vivo* evidence for attractive effects of ephrinA5 in the thalamocortical system. Tracing studies with fluorescent dyes of individual thalamocortical arbors in layer 4 of S1, the cortical target layer of thalamic afferents, revealed a significant reduction of the terminal arbors of VB axons in ephrinA5 deficient mice (Mühlfriedel et al., 2000). The diminished branching of thalamic input in lamina 4 of S1 might have repercussions on structural and functional features of spiny stellate cells in this layer. Using diolistic labeling techniques to visualize the complete morphology of individual spiny stellate cells in layer 4, work in progress indicates that in ephrinA5 mutant mice there are distinct alterations in the number and morphology of dendritic filopodia and spines (Fig. 5; Güllmar et al., 2003).

A recent study also examined axon collateral formation of layers 2/3 pyramidal cells in the barrel cortex of ephrinA5 knockout mice (Yabuta et al., 2000). The authors report that the laminar specificity of layers 2/3 cells is normal in mutant animals, the axons collaterals preferentially locate in layers 2/3 and 5, and they avoid layer 4. This is perhaps not surprising, because the *in vitro* work of Castellani et al. (1998) described above indicated that ephrinA5 induces branch formation of layer 6 cells, but has no effect on axon collateral formation of layer 2/3 pyramidal neurons. Instead, the *in vitro* assays suggested that ephrinA5 acts as a repulsive axonal guidance cue for layer 2/3 axons and thereby might prevent these axon collaterals, once being formed, from entering inappropriate layers. Unfortunately, it is not clear from the study of Yabuta et al. (2000) whether there are targeting errors of axon collaterals to inappropriate layers in ephrinA5 knockout animals. However, previous work indicated that multifunctional wiring molecules act in concert to specify local cortical circuits. For example, neurotrophin 3 (NT-3) is expressed in the cortical layers 4 and 6, that is, the same layers where ephrinA5 is present (Lein et al., 2000). *In vitro* assays indicated that NT-3, like ephrinA5, acts as a repulsive axonal guidance signal for layers 2/3 neurons. Unlike ephrinA5, however, NT-3 has an effect on collateral formation; it inhibits the branching



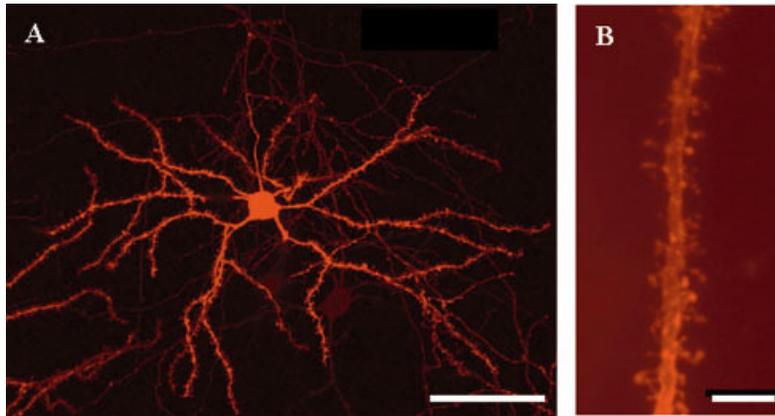
**Figure 4** Corticothalamic projections in ephrinA5 knockout animals. (A) In wild-type animals, tracer injections in the cingulate cortex back label cells in medial thalamic nuclei, including the LD, the laterodorsal nucleus (shown in green). A second tracer injection in the primary somatosensory cortex S1 (shown in red) back labels cells in lateral thalamic nuclei including VB, the ventrobasal complex. In ephrinA5 deficient mice there is a miswiring of a portion of limbic thalamic axons to the neocortical area S1. (B) Schematic representation of thalamic projections in wild-type (left) and ephrinA5  $-/-$  mice (right). In knockout mice limbic thalamic axons form additional projections to S1, suggesting that ephrinA5 is a repulsive cue that restricts limbic thalamic axons from innervating inappropriate cortical regions.

of layers 2/3 axons (Castellani and Bolz, 1999). Thus, in wild-type as well as in ephrinA5 knockout animals, NT-3 might prevent collateral formation and collateral growth in the nontarget laminae of layers 2/3 cells. Because of the overlapping actions and partial redundancy of cortical wiring molecules, eliminating only one of these cues might lead to very subtle, if any, alterations in local cortical circuits.

#### AREAL SPECIFIC EXPRESSION PATTERNS OF EPHRIN LIGANDS AND RECEPTORS: EFFECTOR MOLECULES OF SPECIFIED CORTICAL AREAS

The protomap hypothesis of Rakic (1988) suggested that there was a point to point transformation of

progenitor cells arising from distinct spatial locations in the pallial ventricular zone to the overlying developing cortical plate. The inference from this hypothesis included an early molecular specification of cortical domains that may reflect the early expression of molecular features that distinguish neuronal populations. Barbe and Levitt (1991, 1992, 1995) suggested that the early molecular diversity would be best reflected in guidance cues that facilitated the early and accurate targeting of thalamocortical projections from distinct nuclei. The distribution of the limbic system-associated membrane protein (LAMP) highlighted meso- and allocortical regions (Horton and Levitt, 1988). While it was thought that a later differentiation of neocortical domains allowed for specification to occur via afferent influences (O'Leary, 1989; Schlaggar and



**Figure 5** Spiny stellate cells in layer 4, the target layer for thalamic projections to the neocortex. The cells were visualized with a modified diolotic labeling technique (Gan et al., 2000). (A) Low power and (B) high power photomicrographs showing dendrites with spines. Scale bars: (A) 50  $\mu\text{m}$ ; (B) 5  $\mu\text{m}$ .

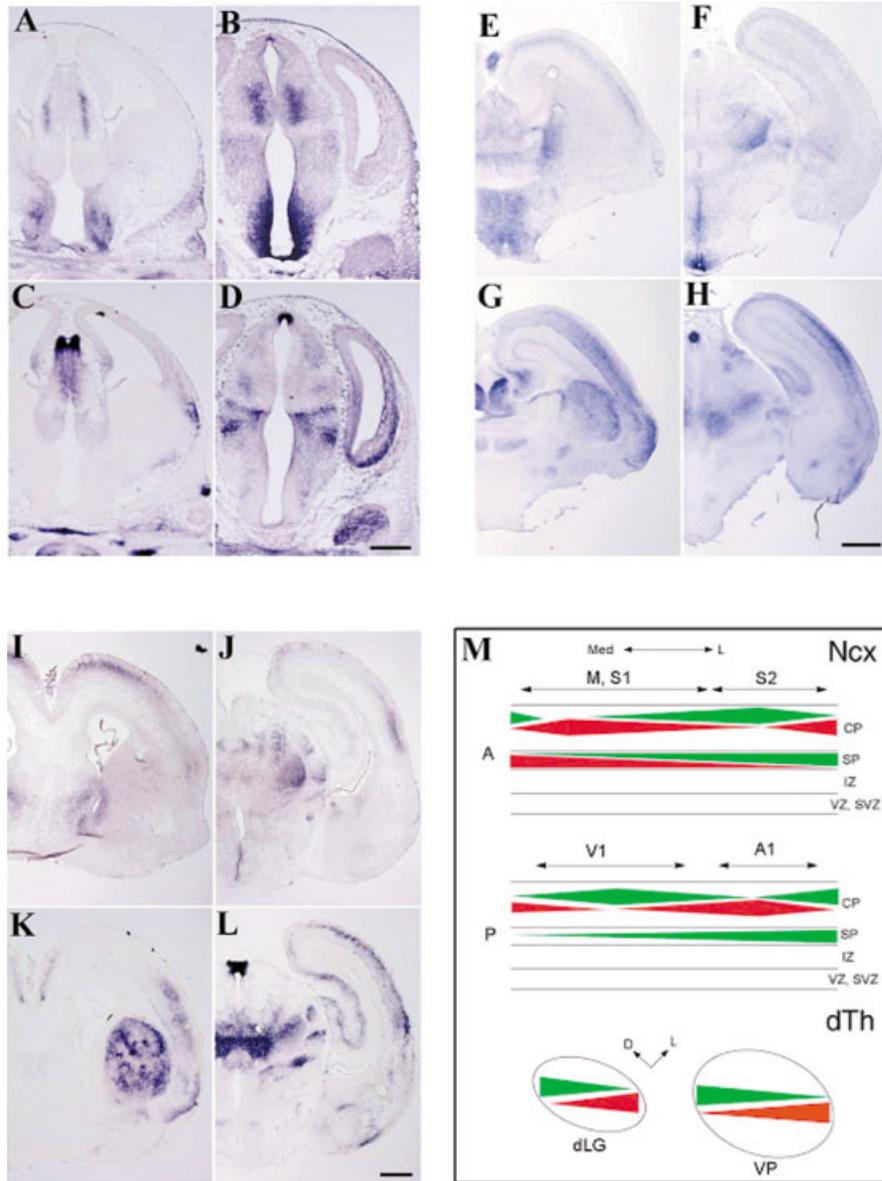
O'Leary, 1991), additional descriptive studies of cadherins (Redies and Takahashi, 1993; Inoue et al., 1998), neurotransmitter receptors (Paysan et al., 1994, 1997; Gurevich et al., 2001), Ephs, and ephrins (Gao et al., 1998; Donghue and Rakic, 1999a,b; Sestan et al., 2001; Yun et al., 2003) revealed parcelation of molecular differences prior to or at the time of the influx of thalamic axons. The most convincing evidence for independent patterning of the cortex, prior to thalamic input, came from analysis of the *Gbx2* (Miyashita-Lin et al., 1999; Hevner et al., 2002) and *Mash1* (Tuttle et al., 1999) mutant mice, both of which fail to develop thalamocortical afferents, yet the classic patterns of transcription factor expression are unchanged.

The most appealing concept of early specification comes from recent studies of transcription factor mutants *emx-2* (Mallamaci et al., 2000; Bishop et al., 2000; Lopez-Bendito et al., 2002) and *Pax-6* (Bishop et al., 2000, 2002), and the soluble growth factor FGF-8 (Fukuchi-Shimogori and Grove, 2001). Elimination of either transcription factor results in dramatic shifts in the expression of downstream genes such as cadherins, LAMP, and other areal markers. The molecular changes are paralleled by altered thalamocortical projections. Disruption of the normal anterior FGF-8 signaling center causes molecular disruptions that also result in changes in thalamocortical projections. In all instances, the downstream guidance molecules appear to be the most likely candidates for disruption of normal thalamocortical patterning.

We were intrigued that in most developing brain regions, gradients of molecules, rather than sharp zones, are responsible for the development of topographical projections. The retino-tectal system is a

good example of this, with Eph and ephrins participating in the process. We expected, therefore, to observe candidates in the same family to have similar complementary patterns of distribution. Cortical expression patterns of Eph and ephrin transcripts have been described at some developmental stages (Yun et al., 2003), but thalamic patterns were not studied in depth. In focusing on two complementary ligand-receptor molecules, ephrinA5 and EphA7, respectively, we determined that there was indeed an early and highly complementary pattern of expression in both thalamus and cortex.

At E13.5, ephrinA5 expression is not apparent in the neocortex. At E16.5, weak ephrinA5 expression is detected in the cortical plate, with a medial to lateral gradient in the presumptive primary motor (M), somatosensory (S1), and primary visual (V1) cortices. Expression in secondary somatosensory cortex (S2) exhibits a reciprocal lateral to medial. The overall expression pattern at E18.5 is similar to that at E16.5. At E13.5, EphA7 is expressed in the emerging cortical plate of the neocortex in a lateral to medial gradient, with weak expression in the ventricular zone. Hybridization signal greatly intensifies by E16.5, with EphA7 strongly expressed in the subplate and cortical plate. Weak expression is detected in the subventricular zone, but not in the ventricular zone. The gradient of expression of EphA7 becomes more complex at E16.5. In general, there is a lateral to medial gradient similar to that at E13.5, in the subplate and deep cortical layer. However, EphA7 expression in the most superficial layer of the cortical plate at E16.5 shows a different pattern. At this stage, there is uniformly intense EphA7 hybridization signal in posterior S1,



**Figure 6** Complementary expression of ephrinA5 and EphA7 in the embryonic mouse brain. (A–L) *In situ* hybridization using antisense cRNA probes specific to ephrinA5 (A,B,E,F,I,J) and EphA7 (C,D,G,H,K,L) on 25  $\mu\text{m}$  thick coronal sections at E13.5 (A–D), E16.5 (E–H), and E18.5 (I–L). (M) Schematic representation of ephrinA5 and EphA7 expression in the neocortex (Ncx) and the dorsal thalamus (dTh) based mostly on expression at E18.5. Representative expression patterns within the cortical plate (CP) and the subplate (SP) in the neocortex, and within the dorsal lateral geniculate (dLG) and ventroposterior nucleus (VP) in the dorsal thalamus are shown. Red and green polygonal bars indicate expression gradients of ephrinA5 and EphA7, respectively, along the medial (Med) to lateral (L) axis within the neocortex at rostral (R) and caudal (C) levels, and within dLG and VP in the thalamus. D, dorsal; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 500  $\mu\text{m}$ .

S2, V1, and A1, with the pattern complementary to that of ephrinA5 expression. At E18.5, overall EphA7 expression pattern is similar to that at E16.5, but expression in S1 is decreased and the

superficial layer of the medial-most portion of the neocortex begins to express EphA7.

Individual nuclei of the dorsal thalamus (dTh) cannot be distinguished at E13.5. However, expression of

ephrinA5 and EphA7 already show distinct patterns. The ventricular zone of the dTh expresses ephrinA5 weakly at the caudal level, with no detectable expression in the mid- or rostral levels of the dTh. In contrast, EphA7 is heavily expressed at the rostral level and more modestly at caudal levels of the dTh. A band of high expression of ephrinA5 is located in the medial part of the mantle zone of the dTh. A band with low level of EphA7 expression is located lateral to this zone of high ephrinA5 expression at the caudal level. At E16.5, ephrinA5 and EphA7 are expressed in nearly a perfect complementary manner. EphrinA5 is strongly expressed in the nuclei located in the lateral part of the dTh, whereas EphA7 is expressed more strongly in the more medially located nuclei, with a gradient of weaker expression extending laterally. In nuclei in which expression of ephrinA5 and EphA7 overlap, such as VP, there is also complementary expression. EphA7 expression occurs in a ventral to dorsal gradient, whereas ephrinA5 is in a dorsal to ventral gradient. Expression patterns in the dTh of each at E18.5 are similar to those at E16.5.

## CONCLUSIONS

Here we showed that ephrinA5, a molecule previously called “repulsive axonal guidance signal” by Bonhoeffer and colleagues, can also act as a “branch promoting signal” for some specific sets of cortical and thalamic axons. There are now many examples of diffusible or membrane-associated molecules that have a wide and complex range of actions on growing axons. For example, semaphorins have been originally described as mediators of growth cone collapse and repulsive axonal guidance, but at least one member of the semaphorin family acts as an attractive signal for cortical axons (Bagnard et al., 1998). Moreover, the same signal acting on the same class of neuron can have different effects, depending on the substrate upon which axons grow (Höpker et al., 1999), the spatial distribution of the signal (Bagnard et al., 2000), or the internal state of the neuron (Song et al., 1997). Because of this biological variation, we proposed to call such signals “wiring molecules”; they function in many alternative ways, but collectively they serve as signals for the assembly of the complex array of neurons that exhibit highly specific patterns of connections (Bolz and Castellani, 1997).

Understanding the role of a given wiring molecule for the assembly of the brain relies on knowing its spatial and temporal expression pattern and its effects on defined populations of neurons. The key technical advance from the Bonhoeffer group afforded the field

a timely and accurate means to assess putative wiring molecules. As illustrated here for ephrinA5, the functional characterization with *in vitro* assays, together with data about its expression, led to specific predictions about its putative roles in wiring cortical circuits and thalamic projections. These could then be tested directly in ephrinA5 knockout mice. Such information made it possible to identify even subtle, but consistent miswiring abnormalities in limbic and neocortical connectivity in ephrinA5 deficient brains. We believe that the functional characterization of brain wiring molecules, in combination with transgenic techniques, will lead to new mouse models in which developmental origins of structure–function relationships in the mature brain can be directly investigated, and which leads to insights into how abnormal gene expression during fetal development can lead to permanent defects in brain circuits involved in higher functions.

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