Thalamus & Related Systems, 2005, 3(3), 235–244. ©2007 Cambridge University Press
doi:10.1017/S1472928807000234 Printed in the United Kingdom

REVIEW ARTICLE

Grading the thalamus: how can an 'Eph' be excellent?

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The Eph family of receptor tyrosine kinases and their partner ligands, the ephrins, mediate cell–cell interactions in the developing nervous system. Signaling events between Eph receptors and ephrin ligands on interacting cells affect the growth, maturation, migration and connectivity of individual neurons and neural networks. Here we review the known roles of Eph–ephrin signaling in the development of the thalamus and its connections, and pose new questions for experimental study.

Keywords: Eph, ephrin, thalamocortical, corticothalamic, compartmentalization

INTRODUCTION

Since their discovery over a decade ago the Eph family of receptor tyrosine kinases and their partner ligands the ephrins, have excited molecular neurobiologists with the range and variety of their activities during neural development. Originally implicated as the molecular tags that Roger Sperry envisioned were necessary for the development of precise topographic mapping (Sperry, 1963), these remarkable proteins mediate a host of effects including axon guidance, neurite outgrowth, cell migration and proliferation, and cell–cell communication leading to boundary formation (Reviewed in Flanagan and Vanderhaeghen, 1998; McLaughlin and O'Leary, 2005; Pasquale, 2005). In recent years much has been learned regarding the timing and pattern of expression of Ephs and ephrins in the developing brain, in addition to the molecular mechanisms of signaling by members of the Eph family of receptor tyrosine kinases. This knowledge has led to the attribution of ever more specialized functions to these molecules at different stages of neural development. Here, we review the roles of Eph–ephrin signaling in the establishment of axonal connectivity to and from the thalamus and speculate on the possible contributions of Eph–ephrin interactions in the development and compartmentalization of thalamic nuclei.

Inputs to the thalamus

Perhaps the most conserved and immediately notable feature of the Eph family of receptor tyrosine kinases and their ligands is their characteristic expression pattern in smooth gradients from high to low concentrations across developing nuclei. This pattern drew attention to these molecules as possible guidance cues for the development of precise topography in sensory maps. Originally, it was proposed that the graded expression of guidance receptors in axons of projection, coupled with complementary ligand gradients in targets, might route axons to their final location while preserving the spatial order of the projecting fibers. This attractive hypothesis has been tested extensively in the development of retinotectal projections and its most salient predictions have largely been confirmed (McLaughlin and O'Leary, 2005). Early *in vitro* studies demonstrated that the interaction between Eph receptors on axons and ephrin ligands in the environment results in growth-cone collapse and repulsion, demonstrating that this receptor–ligand interaction is a true candidate for axon guidance in the CNS. Subsequently it was shown that there are opposing and complementary gradients of Eph receptors and ephrin ligands in the retina and the optic tectum of the chick; Eph receptors are present in a high temporal to low nasal gradient in the retina whereas ephrin ligands are highest in the posterior tectum and decrease smoothly towards the anterior tectal pole. In light of previous knowledge of the anatomy of retinotectal projections, these patterns, and evidence for Eph–ephrin-induced repulsion, led to the development of models for axon guidance in which fibers that contain high levels of Eph are repelled from high concentrations of their ligands whereas axons in which Eph expression is lower are able to enter ephrin-rich territories and form mature connections therein. It was only a short time from the inception of these models that their principle predictions were examined via genetic manipulations. The results, in large part, confirm the central hypothesis that axon-termination zones are patterned via the graded expression of molecular guidance factors.

A natural extension of this pioneering research in retinotectal axon guidance was the investigation of expression patterns of Eph–ephrin in other developing sensory projections, particularly in the regions of thalamus where the preservation of topography in sensory maps is crucial. Beginning at embryonic day fourteen (E14) in the mouse, which is before the innervation of the thalamus by incoming retinal afferents, there are distinct gradients of ephrin-A2 and ephrin-A5 in a high ventral–anterior–lateral to low dorsal–medial–posterior gradient in both the dorsal and

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ventral lateral geniculate nuclei (dLGN and vLGN) (Feldheim *et al.*, 1998). There is a complementary gradient of EphA5 across the ganglion cell layer of the retina, with high levels of expression in the temporal pole and low levels of expression in the nasal pole. In accordance with models of axon repulsion via Eph–ephrin interactions that were originally born out in retinotectal mapping experiments, the topography of retinogeniculate afferents is perturbed in ephrin-A5-knockouts. Ipsilateral retinal projections arising from the temporal retinal pole contain high levels of EphA receptors; normally, these would be repelled by high concentrations of ligand, thereby mapping to their appropriate location in the dLGN. In ephrin-A2/ephrin-A3/ephrin-A5-mutant mice however, ectopic ipsilateral fibers extend further into the LGN, into zones that would contain high concentrations of ephrins in wild-type mice (Pfeiffenberger *et al.*, 2005). This expansion of ipsilateral axonal arbors in the dLGN of ephrin mutants is qualitatively similar to defects observed in previous retinotectal mapping studies and is consistent with a model of topographic-map formation in which opposing and complementary gradients between afferents and their targets impart positional information to ingrowing axons in a smooth, continuous fashion. In the absence of repulsive signals in the thalamus, incoming afferents fail to restrict themselves to their proper domains and the topography of retinogeniculate projections is distorted.

Additionally, ephrin-A2 and ephrin-A5 ligands have been implicated in the mapping of retinal projections to the diencephalon in avian model systems. At E9 in chicks (when topography is developing in retinorecipient nuclei of the thalamus) ephrin-A2 and ephrin-A5 form distinct spatial gradients in all thalamic areas that develop topographic retinal maps (Marin *et al.*, 2001). Interestingly, retinorecipient areas of the developing diencephalon that fail to develop refined retinotopy do not contain these ephrin gradients during retinal afferent innervation, which, again, implies that at least one function of spatial gradients in developing relay nuclei might be to provide positional information to incoming afferents that preserve and maintain topographic mapping.

This hypothesis has been explored in a series of miswiring studies in which retinotopic maps were established in thalamic nuclei and cortical areas that are normally reserved for auditory representations (Sur *et al.*, 1988; Roe *et al.*, 2000). This mapping appears to rely on positional cues that are inherent in the medial geniculate nucleus (MGN) in the form of ephrin gradients because ephrin-A2/ephrin-A5-knockout animals have deficits in the formation of these ectopic maps (Ellsworth *et al.*, 2005). Specifically, retinal projections to the MGN of wild-type rewired animals exhibit axonal refinement into segregated eye-specific patches in the nucleus. In ephrin-A2/ephrin-A5-mutant rewired animals the refinement process is altered such that retinal ganglion cells (RGCs) that project from the temporal retinal pole with high EphA levels extend further into the MGN than in wild-type mice. This aberrant expansion occurs through territory that would normally be highly repulsive for these axons. A similar phenotype occurs in the dLGN of the double mutant, which confirms previous results from ephrin-A5-knockout mice (Feldheim *et al.*, 1998). These experiments demonstrate that graded Eph–ephrin repulsive signaling contributes to the refinement of retinal projections to the thalamus, both to the LGN and in rewired animals the MGN, and implicates the ephrin gradients in the MGN in the guidance of fibers to this target.

The principle thrust of this early research on Eph–ephrin signaling in axon guidance to the thalamus focused on the development of topographic maps in the vertebrate species that were used classically in the pioneering studies of retinotectal mapping. However, it remained of interest to investigate the role of these signaling molecules in the development of the visual system in higher-order species such as carnivores and primates where complex features of developmental patterning accompany the evolution of binocular vision. This line of inquiry has revealed an interesting difference between mice and humans. Unlike the single gradient in expression of EphA receptors from high temporal to low nasal in mouse and chick retinae, EphA5 and EphA6 receptors are expressed in the human eye in two gradients from high central to low peripheral concentrations whereas in the dLGN there is a gradient of ephrin-A5 along a high latero-ventral to low mediodorsal axis (Lambot *et al.*, 2005). The graded presence of these axon-guidance molecules in the developing human retina and its thalamic target indicate their potential role in establishing retinotopic mapping but leave unresolved the question of whether Eph–ephrin signaling contributes to the development of the highly ordered, eye-specific laminae that characterize retinogeniculate projections in primates and carnivores.

This question was addressed recently in the ferret, a carnivore whose visual system development approaches that of primates and humans in terms of biological complexity. Similar to humans, EphA5 receptors are expressed in a high central to low peripheral gradient in the ferret retinae during the period of eye-specific segregation, and ephrin-A5 protein is located in a high lateral to low medial gradient along the length of the developing LGN (Huberman *et al.*, 2005). Overexpression of EphA5 in retinal ganglion cells by *in vivo* electroporation shifts the termination zone of the ipsilateral projecting temporal RGCs toward the medial aspect of the nucleus away from the regions of highest expression of ephrin-A5. Again, consistent with a repulsive interaction between cells that express the ligand–receptor partners, this study indicates that Eph–ephrin repulsive signaling contributes to the development of eye-specific termination zones by specifying positional information for afferent fibers from the two eyes via the actions of opposing and complementary gradients in the retinae and thalamus. Consistent with this hypothesis, *in vivo* siRNA knockdown of EphA3 and EphA5 receptors in the eye yields an alternative phenotype in which ipsilateral RGC termination zones are elongated along the retinotopic axis, perhaps via the reduction of sensitivity of these axons to a repulsive gradient of ephrin ligand that runs lateroanterior to mediposterior (Speer and Chapman, unpublished observations). Together these experiments indicate a role for Eph–ephrin gradients in constraining eye-specific termination zones in retinorecipient nuclei of the thalamus, although it is likely that other molecular cues are also crucial for this patterning.

The picture that has emerged for Eph–ephrin-mediated axon guidance indicates that axons of projection express Eph receptors and map to appropriate areas of their targets by interaction with ephrin ligands, both of which are present in graded fashion. Repulsive interactions along smooth gradients serve to impart positional information to incoming fibers, thereby preserving afferent topography as well as constraining the final

termination zones of separate fiber tracts to the same nucleus. However, it should be noted that interpretations of loss-of-function experiments in knockout mice are potentially confounded. Eph–ephrin signaling occurs bidirectionally in many instances, which makes it difficult to localize the true effect of a single gene knockout to either axons or their targets. Additionally, gradients of Eph and ephrin are numerous and might often act in redundant fashion thereby masking effects of gene deletion. To add greater complexity, Eph receptors and ephrin ligands are coexpressed in a mirror complementary fashion wherever they are found and this expression pattern is crucial for the development of other aspects of thalamic connectivity.

Development of thalamocortical connections

INTER-AREAL PATHFINDING

A major milestone in the timeline of neural development is the innervation of the cortical subplate and cortex by outgrowing axons of the developing thalamus. The subplate is crucial for the normal development of thalamocortical pathways and the mechanisms that regulate this process are beginning to be understood (Ghosh *et al.*, 1990; Vanderhaeghen and Polleux, 2004). Several Eph family receptor tyrosine kinases and their ligands have been implicated in the inter-areal and intra-areal patterning of thalamocortical projections, first at the level of the subplate and then during later innervation of the cortical layers, respectively.

A model system employed to study thalamocortical connectivity is the principle relay of somatosensory information from the ventroposterior nuclei (VPm/VPl) of the thalamus to the primary somatosensory region of the cortex (S1). Early studies of the expression of Eph and ephrin molecules demonstrate a spatial gradient of ephrin-A5 ligand in the ventricular zone, subplate and cortical plate, which is particularly pronounced in the deep layers of S1 at early time points when thalamocortical innervation is initiating (Mackarehtschian *et al.*, 1999; Gao *et al.*, 1998; Vanderhaeghen *et al.*, 2000; Dufour *et al.*, 2003). Beginning at E16 in mice, expression of EphA5 receptor is high in medial and anterior thalamic nuclei that are destined to innervate targets outside of somatomotor cortical areas (Gao *et al.*, 1998), whereas expression of this receptor is conspicuously reduced in the ventroposterior nucleus of the thalamus, the projection nucleus to S1 (Mackarehtschian *et al.*, 1998). Additionally, EphA receptors are localized on axons and growth cones of developing thalamocortical afferents, which is consistent with their involvement in the detection of repulsive gradients in the cortex (Greferath *et al.*, 2002; Kudo *et al.*, 2005).

These expression studies indicate that the high concentration of ephrin-A5 in the regions of subplate underlying S1 might repel thalamocortical afferents that originate from medial thalamic nuclei but allow the passage of fibers from lateral nuclei, thereby establishing an early level of inter-areal thalamocortical axon guidance. Culture assays designed to test these predictions by mimicking the gradients found *in vivo* reveal that neurite growth from medial thalamic explants is inhibited in the presence of ephrin-A5-expressing cells whereas the imposed gradient did not affect the growth of lateral thalamic neurons that express much lower levels of Eph receptor (Gao *et al.*, 1998). This finding is consistent with the hypothesis that the selective spatial gradients of ephrin-A5 in the developing somatosensory subplate/cortex and EphA receptors in thalamus serve to pattern the gross inter-areal connectivity of afferents that emanate from distinct regions of the thalamus. Recent *in vivo* analysis of ephrin-A5-mutant mice confirms that thalamocortical axons arising from laterodorsal nuclei are misrouted to the somatomotor areas of cortex in the absence of the strong repulsive gradient that is normally present in S1 (Uziel *et al.*, 2002).

Subsequent *in vitro* studies have been designed to reproduce more faithfully the spatial relationships of thalamic projection nuclei and their cortical targets. Coculture assays using thalamic explants and telencephalic vesicles reveal a characteristic repulsive effect of ephrin-A5 in the subcortical telencephalon on outgrowing axons of the rostral thalamus (Dufour *et al.*, 2003). This repulsive effect on axon guidance is disrupted either by exogenous application of binding partners for the ligands or, in culture assays, using explants from ephrin-A5-knockout animals. The result of perturbing ephrin-A5 expression is the abnormal development of inter-areal pathfinding in which rostral thalamocortical axons miswire to more caudal regions of the cortex.

In vivo analysis of thalamocortical pathways in ephrin-A5–EphA4-double knockouts shows abnormally caudalized termination zones consistent with results from *in vitro* studies (Dufour *et al.*, 2003). Retrograde labeling of thalamocortical axons in the barrel cortex of mutants reveals a number of ectopically labeled cells in the ventrolateral nucleus (VL) of the thalamus. Normally, the VL nucleus projects exclusively to primary motor cortex without projecting into the neighboring somatosensory regions. The abnormal projections in Eph–ephrin mutants implicate Eph–ephrin signaling in the development and/or maintenance of normal inter-areal corticothalamic connectivity *in vivo*, and show that the precision of normal inter-areal mapping is such that thalamocortical afferents are directed to appropriate targets even when they might be adjacent and expressing grossly similar molecular gradients. Together, these explant and labeling studies

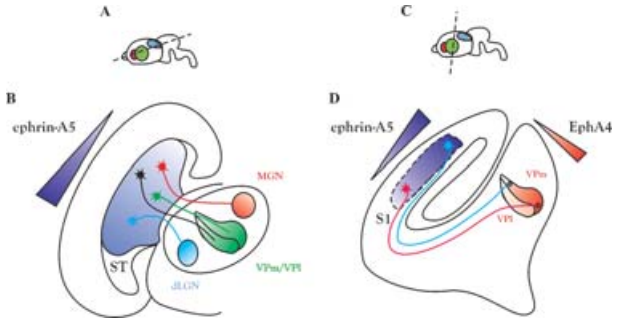

Fig. 1. EphA/ephrin-A signaling contributes to the development of thalamocortical projections. (A) Schematic of mouse brain demonstrating plane of section for (B). (B) Developing thalamocortical afferents encounter an ephrin-A5 gradient in the subcortical telencephalon (ST). Interactions between this gradient and EphA receptors on migrating growth cones generate repulsive signals that direct early inter-areal pathfinding. (C) Schematic of mouse brain demonstrating plane of section for (D). (D) Thalamocortical afferents from sensory relay neurons map topographically to distinct primary cortical areas. In this example, fibers projecting from the medial ventroposterior nucleus (VPm) of the thalamus project to the primary somatosensory cortex (S1). Axons expressing high levels of EphA4 are repelled from the peak of ephrin-A5 expression in S1, while axons with lower expression levels are able to establish termination zones in ephrin-A5 rich cortical territory. (Adapted from Garel and Rubenstein, 2004; Vanderhaeghen and Polleux, 2004)

provide strong evidence that Eph–ephrin interactions during thalamocortical ingrowth contribute to inter-areal specificity of these fibers by differentially directing axons according to their relative expression levels of EphA receptors (Fig. 1). Axons from thalamic nuclei that express high concentrations of EphA receptors are repelled away from the ephrin-A5 gradient in S1, which prevents innervation of this region by thalamic afferents destined to target other cortical areas.

Similarly, the development of inter-areal topography is perturbed for the optic radiations of ephrin-A2/A3/A5 triple mutant mice. Projections from the LGN to the primary visual cortex in these animals are more widespread along the mediolateral axis and, as a group, are shifted medially relative to normal. Misexpression of ephrin-A5 in the posterolateral visual cortex of normal mice leads to a medial shift in the location of visual cortex (Cang *et al.*, 2005). These experiments demonstrate a role for cortical EphA–ephrin-A signaling in shaping the inter-areal topography of cortical maps, perhaps by setting a limiting boundary on the lateral positioning of thalamocortical afferents.

INTRA-AREAL PATHFINDING

The established roles of Eph-ephrin signaling in the generation of topography in the projections of peripheral afferents to the tectum and thalamus prompted hypotheses that these same molecules are also involved in the generation of intra-areal topography in thalamic projections to cortical areas. As in the visual system, expression studies of Eph and ephrin have set the groundwork for interpretations of function in the development of thalamocortical somatosensory projections. EphA4 receptor is expressed across the ventrobasal nucleus in a ventromedial > dorsolateral gradient whereas ephrin-A5 ligand is expressed in a gradient in S1, the orientation of which relative to the known anatomical connectivity is consistent with a role for the generation of topography via repulsive interactions (Vanderhaeghen *et al.*, 2000).

Stripe assay experiments *in vitro* confirm that outgrowing axons of the ventrobasal complex are affected differentially by ephrin-A5 ligands, which is consistent with a role for these molecules in the establishment of topographic connectivity within S1 (Vanderhaeghen *et al.*, 2000). Indeed, analysis of the barrel field in S1 of ephrin-A5-knockout mice reveals a graded perturbation of the normal arrangement of whisker-barrels that is in direct register with the normal alignment of the ephrin-A5 gradient. *In vivo* functional imaging of mutant barrel fields shows abnormal spacing and distribution of whisker functional representations in the cortex that are consistent with a loss of patterning via the ephrin gradient (Prakash *et al.*, 2000). Additionally, the size of the individual barrels are affected with some smaller than normal and others unusually enlarged. This functional deficit has its counterpart in visual system development where expansion of axon terminals of retinal ganglion cells in the LGN of ephrin mutant mice has been proposed to underlie the aberrant expansion of the binocular cortical field (Ellsworth *et al.*, 2005). Similarly to the development of aberrant retinogeniculate afferents in ephrin-A5 knockouts, the distortions of the somatosensory cortical map in these animals can be accounted for by an expansion of EphA-positive thalamocortical afferents into territories that normally contain highly repulsive ephrin-A5 concentrations. Retrograde labeling of these expanded afferents to the barrel cortex of ephrin-A5/EphA4 double-knockout mice results in several ectopically labeled cells in medial positions of VPm compared to the normally tight termination zones characteristic of wild-type controls (Dufour *et al.*, 2003). Each of these phenotypes reflects abnormalities in guidance in the target area (S1) and demonstrates a role for Eph–ephrin repulsion in the normal development of intra-areal patterning that is consistent with models of topographic map formation via opposing and complementary gradients.

Similar findings have been demonstrated in the developing visual system. Ephrin triple-knockout mice have degraded retinotopic maps in the visual cortex and thalamocortical projections are more diffuse in these animals than in controls. Misexpression of either ephrin-A5 or ephrin-A2 within V1 leads to dramatic disruptions in the structure of retinotopic maps determined by intrinsic signal optical imaging (Cang *et al.*, 2005). These experiments offer further compelling evidence for the conclusion that EphA–ephrin-A signaling is essential for the development of normal intra-areal topography, both structurally and functionally.

INTRA-AREAL AND INTER-AREAL: CAN ONE GRADIENT DO IT ALL?

A recent analysis of EphA7-knockout mice contributes unexpected information to the debate about the development of intra-areal and inter-areal specificity of thalamocortical projections. EphA7-mutant mice have similar inter-areal deficits in thalamocortical pathfinding to those previously mentioned for ephrin-A5 and ephrin-A4–EphA5-knockout animals. Interestingly, the intra-areal topography of projections from VPM to S1 is unaffected by this mutation, which indicates that inter-areal and intra-areal mapping of corticothalamic afferents are distinct processes that might be differentially regulated by specific interactions between Ephs and ephrins. The precise mechanisms by which this occurs are unknown.

Clues to the solution of this problem might lie in the temporal regulation of Eph–ephrin signaling. Eph and ephrin gradients display altered spatial expression patterns at different stages of development and shift relatively rapidly during the development of thalamocortical connectivity (Gao *et al.*, 1998; Marin *et al.*, 2001; Greferath *et al.*, 2002; Yun *et al.*, 2003; Dufour *et al.*, 2003; Kudo *et al.*, 2005; Torii and Levitt, 2005). Additionally, *in vitro* experiments have shown that Eph receptor-containing axons have multiple responses to ephrin gradients encountered along a trajectory depending, in part, on the molecular history of Eph–ephrin signaling within the axon (Weinl *et al.*, 2005). Therefore it is possible that Eph–ephrin signaling between thalamocortical afferents and subplate neurons might direct gross inter-areal mapping while imprinting axons with a record of the interaction. In principle, this early interaction might differentially affect the responses of axons as they encounter subsequent gradients in the deep layers of the cortical plate. Further experiments are needed to clarify the precise mechanisms by which inter-areal and intra-areal thalamocortical connectivity is established and to further elucidate the relationship, if any, between these processes.

Layer-specific thalamocortical targeting

In addition to targeting appropriate cortical regions and establishing topographic maps, thalamocortical axons have a layer-specific pattern of connectivity within the cortical plate.

The expression of ephrin-A5 ligand is highest in layers 4 and 6 of the cortical plate and is reduced notably in layer 5, perhaps implicating the differential expression of these factors in the development of layer-specific connections during thalamocortical ingrowth (Vanderhaeghen *et al.*, 2000). *In vitro* experiments using cortical layer-specific cell cultures have implicated Eph signaling in the elaboration of terminal arbors in layer 4 of the cortex via induction of back-branching in axons (Mann *et al.*, 2002). Thalamocortical axons exhibit increased outgrowth on layer 5 preparations in culture, and outgrowth slows and branching accelerates when these thalamic processes are exposed to layer 4 substrates. Eph–ephrin signaling can cause transition from growth promotion to growth inhibition in a concentration-dependent fashion, so it is likely that layer-specific gradients of these molecules contribute to the growth and branching of thalamocortical afferents within the cortical plate *in vivo* (Hansen *et al.*, 2004).

Development of corticothalamic projections

It has been shown that reciprocal projections from the cortex to the thalamus are ten-fold more numerous than their thalamocortical counterparts and might contribute the majority of synaptic inputs to any given sensory relay neuron (Jones, 2002). The function of this feedback projection is well studied but the molecular mechanisms of its developmental specificity have remained elusive (Reviewed by Alitto and Usrey, 2003). Expression studies implicate Eph receptors and ephrin ligands as potential candidates for this corticothalamic mapping based on the expression of Eph receptor gradients in cortical areas and ephrin ligand gradients in their thalamic targets (Gao *et al.*, 1998; Marin *et al.*, 2001; Greferath *et al.*, 2002; Yun *et al.*, 2003; Dufour *et al.*, 2003; Kudo *et al.*, 2005; Torii and Levitt, 2005). However, it is only recently that the role of Eph–ephrin signaling in the patterning of corticothalamic afferents has been examined directly.

Overexpression of EphA7 receptors on corticothalamic axons was induced by *in vivo* electroporation in mice (Torii and Levitt, 2005). It was found that successfully transfected axons consistently mistarget regions of lowest ephrin-A5 expression in their thalamic targets, which is consistent with enhanced repulsive interactions between Eph receptors and ephrin ligands (Fig. 2). Additionally, siRNA-mediated knockdown of EphA7 shifted corticothalamic projections to regions of higher ephrin-A5 concentration. It was determined that these shifts in response to manipulations of receptor levels on corticothalamic axons did not arise at the level of the subcortical telencephalon where a pronounced gradient of ephrin-A5 is expressed when the corticothalamic pathway develops. Instead, corticothalamic axons in manipulated animals pass through the subcortical telencephalon at normal locations and intermingle normally with thalamocortical fibers ascending to the cortex. It is only on reaching their target destinations that the overexpression/knockdown of EphA7 yields an abnormal determination of positional information and the establishment of ectopic termination zones.

Strikingly, the manipulation of receptor levels in cortical domains does not affect the normal targeting of appropriate nuclei, which further supports a dissociation of intra-areal and inter-areal targeting mechanisms. Additionally, thalamocortical projections are unaffected in EphA7-manipulated mice as assessed by retrograde fiber labeling from the cortex. No defects are seen in the commingling of thalamocortical and corticothalamic axons within the internal capsule, which leaves open the possibility that positional information for thalamocortical patterning is determined at the level of the ventral telencephalon (subplate) whereas corticothalamic patterning is determined by gradients of ligand in target thalamic nuclei rather than by selective sorting along the afferent fiber path. However, it has been shown previously that ablation of the subplate region of the developing cortex results in targeting errors in corticothalamic projections in 50% of treatments (McConnell *et al.*, 1994). Whether this result reflects a true role of the subplate in sorting corticothalamic afferents or a limitation of experimental technique remains to be assessed.

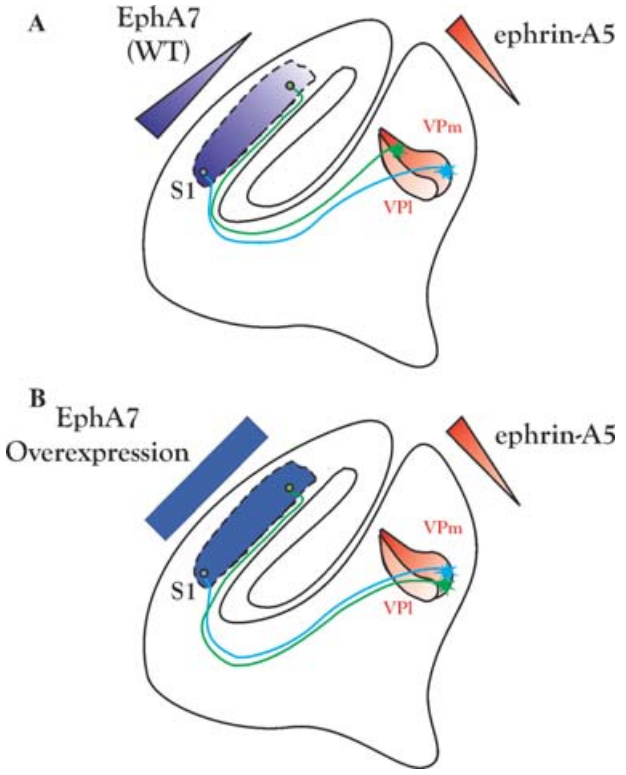

Fig. 2. EphA–ephrin-A signaling contributes to the development of corticothalamic projections. (A) Corticothalamic fibers that originate from the deep layers of the somatosensory cortex (S1) map topographically to the VPm of the thalamus. Axons with a high concentrations of EphA7 receptors are repelled by high concentrations of ephrin-A5 whereas axons with less EphA7 receptors terminate in ephrin-A5-rich territories. (B) Overexpression of EphA7 in the cortical plate of S1 disrupts the normal receptor gradient, leading to enhancement of repulsive signaling and perturbed topographic mapping of corticothalamic projections to VPm. (Adapted from Torii and Levitt, 2005)

For technical reasons the dissociation of the molecular events underlying corticothalamic and thalamocortical development is challenging. As mentioned previously, systemic knockouts might be confounded by the complexities of bidirectional Eph–ephrin signaling. Furthermore, Eph and ephrin are coexpressed at the level of single cells and their axons, and it appears that this coexpression might lead to masking of receptor–ligand interactions between opposing cells, thereby affecting the shape and efficacy of gradients *in vivo* (Hornberger *et al.*, 1999; Carvalho *et al.*, 2006; but see Marquardt *et al.*, 2005). These complexities of interaction and signaling due to coexpression are difficult to assess with

traditional genetic manipulation. *In vivo* manipulation of receptor levels marks the beginning of the studies needed to tease apart the contributions of Eph and ephrin to the connectivity of thalamic nuclei and their targets. The generation of spatially selective and conditional mutations/manipulations is important in elucidating the mechanisms by which receptor–ligand signaling imparts positional information in axonal connections of the developing thalamus.

Compartmentalization of thalamic nuclei

Although most research in Eph–ephrin signaling has focused on the processes of axon guidance and the formation and maintenance of topographic maps, there is much to indicate that these proteins mediate other aspects of development, ranging from cell proliferation and migration to synapse formation and stabilization (Reviewed in Pasquale, 2005). Soon after the discovery of the first ephrin ligands it was appreciated that repulsive interactions between Eph and ephrins might contribute to the segmentation of discrete body components and compartmentalization of neural nuclei (Gale *et al.*, 1996; Flenniken *et al.*, 1996). Further research has implicated Eph–ephrin repulsion in the development of rhombomeres (Reviewed in Cooke and Moens, 2002), somites (Barrios *et al.*, 2003), and compartmentalization of the striatum (Janis *et al.*, 1999) and cerebellum (Hashimoto and Mikoshiba, 2003).

The patterns of expression of Eph receptors and ephrin ligands in the developing cortex and thalamus of all species investigated have led to predictions of their function in the generation of areal specificity within these regions via repulsive interactions (Gao *et al.*, 1998; Donoghue and Rakic, 1999; Sestan *et al.*, 2001; Marin *et al.*, 2001; Greferath *et al.*, 2002; Yun *et al.*, 2003; Dufour *et al.*, 2003; Kudo *et al.*, 2005; Torii and Levitt, 2005). Analysis of areal development in cortex has been investigated more thoroughly than in the thalamus and EphA7- and ephrin-A5-knockout mice reveal several phenotypes. In EphA7 mutants, the spatial extent of ephrin-A5 expression in S1 is shifted to more posterior regions of the cortical plate and the somatosensory cortex is reduced in size compared to wild-type controls (Miller *et al.*, 2006). Despite the redistribution of the ligand gradient in cortex, the total amount of ephrin-A5 expressed is unchanged, a finding consistent with the suggestion that the expression of EphA7 in posterior regions of the cortex serves as a repulsive buttress against domains of ephrin-A5 expression in adjacent somatomotor regions. In the absence of the receptor, ephrin-A5-positive neurons might undergo aberrant migration into the now permissive areas of mutant cortex. This hypothesis is supported by the finding that ventral migration of developing cortical neurons in the subventricular zone is dependent on normal Eph–ephrin signaling, which indicates that containment of cells within particular spatial domains is, in part, determined by Eph–ephrin signaling between developing cortical areas (Conover *et al.*, 2000; Nomura *et al.*, 2006).

Knockouts for EphA7 and ephrin-A5 have an overall reduction in the size of their barrel fields (Miller *et al.*, 2006). Although the mechanisms responsible for these effects on cortical field size are unclear, one possibility lies in the finding that Eph–ephrin signaling triggers apoptosis of cortical progenitor neurons during corticogenesis, and this cell death might contribute directly to the final size of the mature cortical sheet (Depaepe *et al.*, 2005). Disruption of Eph–ephrin signaling leads to an increase in cell proliferation within the subventricular zone (Conover *et al.*, 2000). These findings demonstrate a role for Eph-family signaling in the determination of the final size of different cortical zones, and highlight the possibility that cell number in addition to the overall area of axonal termination zones might affect sensory map size.

Within the thalamus itself, several pieces of evidence indicate a role for Eph–ephrin signaling in the differentiation/compartmentalization of developing nuclei. In the primate thalamus at E65, before compartmentalization of relay nuclei, ephrin-A5 is expressed predominantly in the ventrolateral nucleus whereas EphA3, EphA6 and EphA7 are found in overlapping gradients in the pulvinar and geniculate nuclei that are destined to innervate the visual cortical plate. Although these regions of expression abut, they do not overlap, which leaves open the possibility that repulsive signaling between these domains might contribute to the developing specification of the border between the nuclei (Donoghue and Rakic, 1999; Sestan *et al.*, 2001). Studies of the expression of ephrin-A2 and ephrin-A5 show that a high concentration of these ligands marks the border of the MGN and LGN (Lyckman *et al.*, 2001). In rewiring experiments of double-knockout mice in which retinal axons are redirected to the auditory nucleus of the thalamus, axons from the retina extend further and with greater spatial volume in the MGN compared to wild-type, rewired controls. These experiments indicate that the gradient of ephrin ligands in the auditory thalamus might establish a boundary for migrating axons of the optic tract that overlays the border between the LGN and MGN. This boundary, in turn, coincides with the cellular boundary of the MGN, which is consistent with the possibility that the initial compartmentalization of afferents via Eph–ephrin signaling might contribute to the final cytoarchitectonic structure of the target itself. Further experiments are needed to investigate the contributions made by Eph–ephrin signaling to the specification and refinement of thalamic territories during development.

Induction of Eph–ephrin expression in the thalamus

Studies of the molecular mechanisms of regional specification in the developing cortex have revealed that individual areal domains can be visualized based on the expression patterns of transcription factors and axon guidance cues in the cortical plate at early times before the arrival of thalamocortical afferents (Miyashita-Lin *et al.*, 1999; Donoghue and Rakic, 1999; Nakagawa *et al.*, 1999; Yun *et al.*, 2003). These findings demonstrate that the mechanisms by which the cortex is initially segregated into functional areas do not depend on signaling from the thalamus. It is likely that similar mechanisms might pattern early thalamic development independently of input from the periphery, but the question of how the nuclei of the developing diencephalon establish boundaries remains unresolved. Several pieces of evidence indicate that the selective expression of transcription factors in thalamic nuclei generates early patterning of the thalamus, in part, via regulation of expression of the genes that encode Ephs and ephrins.

One of the key regulators of neural differentiation in the thalamus and elsewhere is Pax6, a paired-box domain transcription factor that is believed to generate areal specialization (O'Leary *et al.*, 1994). In the cortex Pax6 is expressed in a high rostrolateral to low caudomedial gradient, which is consistent with a role in the specialization of somatosensory and motor regions of the developing cortical sheet (Stoykova and Gruss, 1994). In the thalamus, Pax6 is expressed in the alar plate of the developing diencephalon as early as E10.5, which, in mice, marks the initiation of neurogenesis in the thalamus, and it is differentially regulated within the structure as thalamocortical afferents begin to form (Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Stoykova *et al.*, 1996; Mastick *et al.*, 1997; Warren and Price, 1997; Kawano *et al.*, 1999; Auladell *et al.*, 2000; Mastick and Andrews, 2001).

Pax6-mutant mice have perturbed patterns of transcription factor expression in the thalamus, which leads to the disruption of cell migration and axon guidance (Mastick *et al.*, 1997; Kawano *et al.*, 1999; Pratt *et al.*, 2000; Vitalis *et al.*, 2000). Additionally, Pax6 mutants have deficits in the cortical expression of EphA7 and ephrin-A5, two axon guidance molecules that are required for the mapping of thalamocortical and corticothalamic afferents (Bishop *et al.*, 2000; Bishop *et al.*, 2002). The combined effect of these deficits is an absence of the thalamocortical pathways in Pax6 mutants and molecular disorganization of the diencephalon. This disorganization is constituted by abnormal expression of several molecules in the thalamus and abnormalities in cell migration, proliferation and differentiation.

In the thalamus Pax6 might contribute to the organization of developing nuclei by regulating the expression of transcription factors and cell surface proteins in order to compartmentalize groups of cells. In support of this, Pax6 establishes the borders of cell migration by inducing repulsive ephrin-A5 expression that delineates the boundary of a nucleus (Nomura *et al.*, 2006). Furthermore, Pax6 regulates the normal expression patterns of DLX transcription factors in the thalamus (Warren and Price, 1997), and DLX transcription factors regulate cell migration, proliferation and differentiation (Panganiban and Rubenstein, 2002). Each of these developmental processes is abnormal in the diencephalons of Pax6-knockout animals (Warren and Price, 1997; Vitalis *et al.*, 2000). DLX transcription factors have also been linked to ephrin-A5 expression via the DLX activator Mash-1 (Porteus *et al.*, 1994; Eisenstat *et al.*, 1999; Fode *et al.*, 2000) and in Mash-1 mutants the expression of repulsive ligands in the somatosensory cortex is expanded and the thalamocortical pathway is disorganized (Nakagawa *et al.*, 1999; Tuttle *et al.*, 1999; Yun *et al.*, 2003).

Pax6 also regulates the expression of cell adhesion molecules of the cadherin family. The normal complement of cadherins is absent from the diencephalon in Pax6 mutants and this defect is accompanied by altered boundaries and abnormal cell segregation in the thalamus of Pax6 mutants (Stoykova *et al.*, 1997). Given that E-cadherin regulates expression and subcellular localization of EphA2, it is possible that this pathway is another means by which Pax6 directs thalamic development via modulation of Eph–ephrin signaling (Orsulic and Kemler, 2000).

An additional pathway of Eph–ephrin regulation that is affected in Pax6 mutants is the activity of the LIM family homeodomain transcription factors (Mastick *et al.*, 1997; Pratt *et al.*, 2000; Mastick and Andrews, 2001). LIM homeodomain transcription factors are crucial for the regulation of expression levels and patterns of EphA4 receptors and ephrin-A5 ligands in the developing motor system (Kania and Jessell, 2003). Expressed in motor neuron populations of the lateral motor column and limb mesenchyme, these transcription factors are necessary and sufficient to coordinate axon guidance and targeting of motor axons via the induction of Eph–ephrin expression and signaling. In the thalamus, several LIM homeodomain transcription factors are expressed in subsets of cells as early as E10.5 as neurogenesis proceeds. These expression patterns are largely maintained through early postnatal development and appear to correlate with the boundaries of individual thalamic nuclei at several embryonic timepoints (Nakagawa and O'Leary, 2001). Given the direct role that LIM homeodomain transcription factors have on Eph–ephrin expression it will be interesting to investigate the connection if any, between the specific expression patterns seen in the developing thalamus and the subsequent development of Eph–ephrin gradients in thalamic nuclei. It remains a possibility that these transcription factors might control the parcellation of the thalamus via the selective, differential induction of Ephs and ephrins in adjacent regions that are destined to become independent thalamic structures. Via bidirectional repulsive interactions, originally intermingled cell populations expressing either EphA receptors or ephrin-A ligands might be differentially sorted to establish a border between emerging nuclei (Fig. 3). The degree to which the Ephs and their ligands contribute to the compartmentalization of the thalamus is an interesting and, as yet, open question.

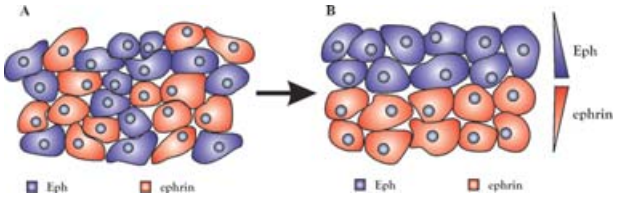

Fig. 3. Eph–ephrin signaling establishes cytoarchitectonic boundaries. (A) In an initially mixed population of cells that contain Eph receptors and ephrin ligands, bidirectional repulsive signaling leads to cell migration and the establishment of distinct nuclear boundaries. (B) The final boundary between the two cell populations occurs at the peak of Eph–ephrin gradients. (Adapted from Pasquale, 2005)

CONCLUSIONS

Over a decade of intensive research centered on the Eph family of receptor tyrosine kinases and their ligands, the ephrins, has led to great insight regarding the spatial and temporal expression patterns of these molecules, the molecular interactions between multiple family members of this class, and the mechanisms of intracellular signaling by which these proteins mediate a wide range of developmental processes. These processes, which include cell proliferation, migration, apoptosis, axon outgrowth and guidance, and synaptogenesis, place the Eph family members in a unique class of proteins that exert varied influence at many stages of neural growth.

It is clear that the Eph–ephrin interaction is one of the molecular mechanisms by which thalamic nuclei differentiate and connect to their cortical targets while receiving many reciprocal feedback connections from these cortical areas. Eph and ephrin proteins exist in mirror complementary gradients in multiple areas of the developing diencephalon in all species examined thus far. These gradients are crucial for the development of topographic connectivity from peripheral afferents to the thalamus and for establishing the topography of feedback projections from the cortex to the thalamus. Additionally, it appears that the gradients of these receptor tyrosine kinases in both the thalamus and cortex are involved in establishing the inter- and intra-areal selectivity of the thalamocortical projections, although the precise mechanisms that govern these independent processes remain to be identified.

Experiments to investigate the functions of Eph–ephrin signaling in establishing cellular compartments have shown that repulsive signaling between discrete and opposing spatial domains of these factors regulates boundary formation between nuclei, cellular migration to and within nuclei, and the overall size of these areas. In light of the early, graded segregation of these molecules in the thalamus, it will be interesting to evaluate further the specific roles of Eph–ephrin signaling in the regulation of cell proliferation, neural migration and compartmentalization during the development of thalamic nuclei.

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