Published in final edited form as:

Gene Expr Patterns. 2013 December ; 13(8): . doi:10.1016/j.gep.2013.08.002.

Parcellation of the Thalamus into Distinct Nuclei reflects EphA Expression and Function

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Abstract

Intercellular signaling via the Eph receptor tyrosine kinases and their ligands, the ephrins, acts to shape many regions of the developing brain. One intriguing consequence of Eph signaling is the control of mixing between discrete cell populations in the developing hindbrain, contributing to the formation of segregated rhombomeres. Since the thalamus is also a parcellated structure comprised of discrete nuclei, might Eph signaling play a parallel role in cell segregation in this brain structure? Analyses of expression reveal that several Eph family members are expressed in the forming thalamus and that cells expressing particular receptors form cellular groupings as development proceeds. Specifically, expression of receptors EphA4 or EphA7 and ligand ephrin-A5 is localized to distinct thalamic domains. EphA4 and EphA7 are often coexpressed in regions of the forming thalamus, with each receptor marking discrete thalamic domains. In contrast, ephrin-A5 is expressed by a limited group of thalamic cells. Within the ventral thalamus, EphA4 is present broadly, occasionally overlapping with ephrin-A5 expression. EphA7 is more restricted in its expression and is largely nonoverlapping with ephrin-A5. In mutant mice lacking one or both receptors or ephrin-A5, the appearance of the ventroposterolateral (VPL) and ventroposteromedial (VPM) nuclear complex is altered compared to wild type mice. These *in vivo* results support a role for Eph family members in the definition of the thalamic nuclei. In parallel, *in vitro* analysis reveals a hierarchy of mixing among cells expressing ephrin-A5 with cells expressing EphA4 alone, EphA4 and EphA7 together, or EphA7 alone. Together, these data support a model in which EphA molecules promote the parcellation of discrete thalamic nuclei by limiting the extent of cell mixing.

Keywords

EphA4; EphA7; Ephrin-A5; Thalamus; Development; Segregation; Nuclei

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1. Introduction

The thalamus, a structure of the diencephalic forebrain, serves as a sophisticated sensorimotor processor. Sensory input is received from the periphery, integrated, and sent onto other brain regions. At the same time, descending, efferent information also moves through the thalamus (Sherman and Guillery, 2002; Sillito and Jones, 2002). Distinct groupings of cells, called nuclei, provide the structural basis for precise thalamic function, such that each nucleus receives and processes unique afferent and/or efferent information (Sherman and Guillery, 2002). Given the striking functional organization of the thalamus, understanding the mechanisms by which cells become grouped into particular nuclei is an important question.

Patterns of gene expression support a developmental model in which the forebrain, including the diencephalon, is divided along anteroposterior and dorsoventral axes into transient embryonic structures called prosomeres (Chen et al., 2009; Puellas and Rubenstein, 2003; Vue et al., 2009). While some of the patterned molecules that delineate prosomeres also mark portions of the developing thalamus (Bluske et al., 2012; Mackarehtschian et al., 1999; Nakagawa and O'Leary, 2001; Nakagawa and O'Leary, 2003; Nakagawa and Shimogori, 2012; Sestan et al., 2001; Wang et al., 2011), the cellular mechanism by which cells are further compartmentalized into thalamic nuclei remains unclear. Here, we ask whether a family of surface-bound receptors and ligands plays a role in this process.

The Eph family of intercellular signaling molecules consists of a variety of plasma membrane bound Eph receptor tyrosine kinases and ephrin ligands (Committee, 1997). Engagement of receptors on one cell with ligands on a neighboring cell can initiate activation of the receptor's kinase domain thus promoting a "forward" signal in the receptor-containing cell. At the same time, a "reverse" signal may be initiated in the ligand-containing cell. A cell's signaling context determines whether receptor-ligand engagement initiates uni- or bi-directional activation of signaling cascades. (Eberhart et al., 2004; Holland et al., 1996; Kullander and Klein, 2002; Kullander et al., 2001b; Martinez et al., 2004; Pasquale, 2008). Additional studies have demonstrated that receptors and ligands coexpressed on a single cell can interact in *cis* (Carvalho et al., 2006) and that receptors can be shed from a cell and act solubly in *trans* (Oricchio et al., 2011). Furthermore, variability in the level of engagement or identity of receptors can lead to diverse and sometimes opposing consequences (Brown et al., 2000; Hansen et al., 2004; Holmberg et al., 2000). Thus, remarkable diversity can be generated by interactions between Eph receptors and ephrin ligands.

In the forebrain, Eph signaling underlies a variety of processes, including cell division, neuronal migration, network formation, and synaptic function (North et al., 2013). In considering the parcellation of thalamic cells into nuclei, parallels with the organization of cells in the transient rhombomeres of the hindbrain are especially intriguing, since Eph signaling appears to both aggregate cells within a rhombomere and segregate cells between rhombomeres (Cooke et al., 2005; Mellitzer et al., 1999; Xu et al., 1999; Xu et al., 2000). Previous studies demonstrate that the thalamus expresses Eph family members in discrete patterns (Mackarehtschian et al., 1999; Sestan et al., 2001). The current study advances that work by demonstrating that receptors EphA4 and EphA7 and ligand ephrinA5 are expressed in the developing thalamus as nuclei are emerging. Analysis of loss-of-function *in vivo* models demonstrates that EphA family members impact the segregation of the ventroposteromedial and ventroposterolateral nuclei. *In vitro* data reveals that the aggregation or segregation of cells corresponds to particular Eph, ephrin pairings.

2. Results

2.1 EphA4, EphA7, and ephrin-A5 are expressed in the early thalamus

A variety of genes, including EphA receptors and ephrin-A ligands, are expressed in the developing mouse thalamus as progenitors divide, neurons differentiate, and cytoarchitectonically distinct nuclei emerge (Bluske et al., 2012; Chen et al., 2009; Mackarehtschian et al., 1999; Nakagawa and O'Leary, 2001; Nakagawa and Shimogori, 2012; Sestan et al., 2001; Wang et al., 2011; Yun et al., 2001) (Fig. 1). *In situ* hybridization within the embryonic day (E)12.5 mouse thalamus, a stage characterized by histological simplicity (Fig. 1A), reveals that EphA4 is expressed within prosomere 2 (P2) (Puelles and Rubenstein, 2003), including both the epithalamus (ET) and dorsal thalamus (DTh) (Fig. 1B); and within prosomere 3 (P3), in the prethalamus (PTh) and hypothalamus (HTh) (Fig. 1B). In contrast, EphA7 expression at E12.5 is confined to the P2 region (Fig. 1C). Further analyses demonstrate that EphA4 and EphA7 are separately (red or green, respectively, in Fig. 1D) and coordinately (yellow in Fig. 1D) expressed. Consistent with results from other studies (Mackarehtschian et al., 1999; Sestan et al., 2001), there was no detectable expression of any A-class ephrin ligand in the E12.5 mouse thalamus.

Later in thalamic development, at E14.5, when proliferation is decreasing and cytoarchitectonic demarcation of nuclei is becoming apparent (Fig. 1E), EphA4 is strongly expressed in a lateral-medial band within the DTh (Fig. 1F) and EphA7 is present in discrete portions of that EphA4-positive domain (Fig. 1G, I). One A-class ligand, ephrin-A5, was detectable at this stage, present at the dorsal and ventral edges of the DTh (Fig. 1H). Comparison of receptor and ligand expression patterns at E14.5 demonstrates that cells expressing EphA4 or EphA7 are largely distinct from cells expressing ephrin-A5 (Fig. 1J).

2.2 EphA4, EphA7, and ephrin-A5 expression marks emerging thalamic nuclei

At E15.5, the thalamus is composed primarily of differentiated neurons and nuclei can be discerned (Fig. 2A). At this stage, strong, patterned expression of Eph receptors is apparent: EphA4 expression is present throughout the DTh in an anterior-high, posterior-low gradient, present in the dorsal PTh and the zona incerta (ZI) (Fig. 2B, E) (Chen et al., 2009; Nakagawa and O'Leary, 2001). EphA7 is more diffusely expressed within the E15.5 thalamus in a posterior-high gradient in the DTh, PTh, and ZI (Fig. 2C, E). Ephrin-A5, weakly expressed within the DTh, is present throughout the reticular nucleus (Rt), PTh, and ZI (Fig. 2D). Ephrin-A5 colocalizes with EphA4 in the anterior region of the DTh and in ventral structures (Fig. 2F).

Nuclei are morphologically apparent in the E17.5 thalamus (Fig. 3A). EphA4 is expressed throughout the E17.5 thalamus: highest dorsolaterally in the lateral posterior (LP), dorsal lateral geniculate (dLG), and posterior (Po) nuclei and lowest ventromedially in the ventroposterior (VP) nuclei and excluded from the ventral lateral geniculate nucleus (vLG), Rt and ZI (Fig. 3B). EphA7 is predominantly medioventrally expressed, present a medial portion of the Po and VP as well as the vLG (Fig. 3C). At E17.5, ephrin-A5 is present in the vLG and at the ventral edge of the VP in the Rt and ZI (Fig. 3D). Strikingly, while EphA4 and EphA7 expression domains overlap (Fig. 3E), cells expressing EphA4 and/or EphA7 and cells expressing ephrin-A5 appear to be exclusively localized (Fig. 3F). Interestingly, VP has a medial domain consisting of cells expressing both EphA4 and EphA7 but no ephrin-A5 and a lateral portion containing cells that express EphA4 and ephrin-A5 but no EphA7 (Fig. 3E, F). Such patterning of Eph expression suggests that the VP can be subdivided based upon Eph family expression. As in other parts of the nervous system, Eph expression levels in the thalamus are greatly reduced postnatally (North et al., 2009; Sestan et al., 2001; Yun et al., 2003), consistent with intercellular signaling via Eph receptors and

ligands playing a role in the establishment of cellular groupings rather than in their maintenance.

2.3 Loss of EphA4, EphA7, and ephrin-A5 alters thalamic nuclear organization

To identify potential roles for Eph-ephrin signaling in early thalamic development, the consequences of disrupting EphA7 and EphA4/EphA7 expression were examined. Comparisons of thalamic organization in control mice versus *EphA7*^{-/-} or *EphA4*^{-/-}*EphA7*^{-/-} were undertaken (Helmbacher et al., 2000; Rashid et al., 2005). In these analyses, characteristics of the VP nuclei were assessed since these cell groupings expressed both EphA4 and EphA7 and exhibited the most distinctive differential Eph-ephrin expression (Fig. 3). Given the dearth of VP markers, gene products produced by expression of the EphA4 locus were used to identify the VP complex: immunohistochemical detection of the native EphA4 protein (Fig. 4A, C, E, G) (North et al., 2010; North et al., 2009) or galactosidase activity encoded by a *lacZ* gene that had been inserted or “knocked-in” to replace the coding sequence in the EphA4 locus (Fig. 4B, D, F, H) (Helmbacher et al., 2000). Differences in staining between native EphA4 and EphA4-driven *lacZ* are due to direct staining of the former membrane bound protein, versus enzyme activity of the latter cytoplasmic marker.

In control animals at an anterior location within the newborn (P0) thalamus, EphA4 is expressed throughout the dorsal thalamus, including the posterior, ventrolateral, ventroposterolateral, ventroposteromedial, reticular and dorsal lateral geniculate nuclei (Fig. 4A, B). Similarly, again in control animals but at a more posterior location within the P0 thalamus, EphA4 again marks dorsal thalamic structures, including the parafascicular, ventroposterolateral, ventroposteromedial, reticular and dorsal lateral geniculate nuclei (Fig. 4E, F). Importantly, in control thalamus, discrete nuclei are apparent, including VPL and VPM (Fig. 4A, B, E, F). While the thalamus of *EphA4*^{-/-} mice appear normal (data not shown), the thalamus of *EphA7*^{-/-} mice have less clear definition of nuclei, particularly in the posterior VP region (Fig. 4C, G). Indeed, in *EphA4*^{-/-}*EphA7*^{-/-} double knock-out mice, a melding of VP thalamic nuclei is apparent (Fig. 4D, H). Together, these data support a role for EphA4 and EphA7 in the segregation of cells in developing nuclei of the thalamus.

2.4 Cell segregation is affected by Eph signaling

To understand the interactions of EphA4 and EphA7, individually and together, with ephrin-A5 in the context of cell segregation, *in vitro* cell mixing experiments were undertaken. To this end, HEK293 cells were transfected with green fluorescent protein GFP and either an inert vector as control, or vectors expressing EphA4, both EphA4 & EphA7, or EphA7. In parallel, another pool of HEK293 cells were transfected with red fluorescent protein (RFP) and either an inert vector as control or a vector expressing ephrin-A5. One day after transfection, GFP- and RFP-expressing cells were mixed. Drops of media containing cell mixtures were pipetted and then inverted, grown in suspension as a “hanging drop” (Heintzelman et al., 1978; Thomas and Yancey, 1988). This approach allows cells to assemble based upon attractive or repulsive forces between cells rather than due to substrate interactions.

After two days of growth, hanging drops were imaged. Examination of hanging drops consisting of control transfected cells revealed intermingling of transfected cells within a hanging drop; merged images of cells transfected with green or red fluorescent proteins and inert vectors produced hanging drops that are primarily yellow, indicating mixing of transfected populations (Fig. 5A). Similarly, there was little segregation when cells expressing a receptor or ligand were in a hanging drop with control cells (Supplemental Figure 1), indicating that receptor- and ligand-independent autoactivation is minimal in this

paradigm. Cells transfected with EphA4 or EphA4 & EphA7 mingle with cells transfected with ephrin-A5, but seemingly to a lesser extent than those cell populations with control cells (Fig. 5B and C). In contrast, hanging drops consisting of cells transfected with EphA7 and ephrin-A5 tended to segregate, such that merged images reveal green-only and red-only domains within a hanging drop and little overlap of cell populations (Fig. 5D).

Quantitation of hanging drop characteristics on a “segregation scale”, with 1 denoting full intermingling and 5 indicating full segregation revealed that control cells in a hanging drop mixed freely (1.40, Fig. 5E left column, n=50) while EphA7/ephrin-A5 cells in a hanging drop were highly segregated (4.27, Fig. 5E right column, n=45). Hanging drops containing cells corresponding to EphA4/ephrin-A5 (2.07, Fig. 5E second column from left, n=36) and EphA4 & EphA7/ephrin-A5 (3.03, Fig. 5E second column from right, n=32) mixed at intermediate levels. Interestingly, these *in vitro* cell segregation experiments correspond to the degree of coincidence of EphA4, EphA7 and ephrin-A5 expression patterns; EphA4 alone or with EphA7 often colocalize with ephrin-A5 whereas EphA7 and ephrin-A5 are fully segregated as the thalamus forms (Fig. 1–3).

3. Discussion

Signaling via the Eph family of receptor tyrosine kinases and ligands affects a multitude of neural processes during development and in maturity (North et al., 2013; Pasquale, 2005; Pasquale, 2008). In the forming hindbrain, Eph interactions promote the formation of rhombomeres (Mellitzer et al., 1999; Xu et al., 1999; Xu et al., 2000). In many ways thalamic development parallels hindbrain organization; discrete thalamic nuclei are segregated from one another while cells within a nucleus are aggregated. Thus, our study asked whether Eph signaling influences the emergence of thalamic nuclei during development.

First, gene expression was examined. Analyses revealed that EphA4, EphA7, and ephrin-A5 are dynamically expressed during thalamic development, eventually marking populations of cells that comprise particular nuclei. Further, loss-of-function analyses revealed that the definition of thalamic nuclei is perturbed when Eph signaling is absent. Specifically, the VPM and VPL, which normally express both EphA4 and EphA7, failed to segregate when these receptors or their cognate ligand were absent. Finally, *in vitro* analyses demonstrated hierarchical interactions between cells expressing EphA4, EphA4 & EphA7 together, and EphA7 in combination with cells expressing ephrin-A5. Together, these data support a model in which Eph receptors and an ephrin ligand guide cellular groupings within the thalamus.

Proper formation of thalamic nuclei is essential for relaying and integrating both ascending sensory input and descending motor, limbic, and cognitive output. It is well-known that Eph signaling plays a role in the guidance of axons and the formation of synapses throughout the nervous system, including the thalamus (Dufour et al., 2003; Gao et al., 1998; Hruska and Dalva, 2012; Torii and Levitt, 2005; Torii et al., 2013; Uziel et al., 2006). Our data suggest that Eph-ephrin signaling may play an additional role in assuring proper connectivity by arranging cellular groupings within the forming thalamus so that afferent and efferent connections target defined locations.

The somatosensory system is particularly well marked by Eph signaling molecules. Eph signaling impacts signal transmission from the periphery (North et al., 2010) as well as central processing (Miller et al., 2006; Polley et al., 2004; Prakash et al., 2000; Vanderhaeghen et al., 2000). Our data extend this role by implicating Eph signaling in the organization of the somatosensory thalamus: EphA4 and EphA7, in combination with ephrin-A5, appear to act to sort VPM and VPL. Similar to other sensory systems, such as

vision, we suspect that after the formation of specific thalamic nuclei, Eph signaling facilitates topographic maps of connectivity (Cang et al., 2005; Cheng et al., 1995; Feldheim et al., 2000; Feldheim et al., 2004; Huberman et al., 2005). Together, these data demonstrate that Eph signaling acts in supporting somatosensation throughout the neuraxis.

Our findings suggest that EphA4 and EphA7 engage with ephrin-A5, collaborating to sculpt the thalamus into discrete nuclei. How might this collaboration be accomplished and the hierarchical interactions of EphA4 and EphA7 with ephrin-A5 be explained? One consideration is the two orders of magnitude difference in affinity with which ephrin-A5 binds to EphA4 versus EphA7 (Kd for EphA4= 3.1×10^{-8} , Kd for EphA7= 3.1×10^{-10}) (Himanen et al., 2004). Since ephrin-A5 engages EphA7 more intensely than EphA4 it is possible that differences in signal strength and activation time lead to distinct cellular responses according to the receptor that has been engaged. Moreover, distinct cellular responses are likely to result from each receptor's activation; previous studies demonstrate that kinase active EphA7 is consistently repulsive (Depaepe et al., 2005; Oricchio et al., 2011; Rashid et al., 2005; Torii and Levitt, 2005) while kinase active EphA4 can act to promote or inhibit cellular function (Helmbacher et al., 2000; Kullander et al., 2003; Kullander et al., 2001a; North et al., 2010; North et al., 2009). Such discrete cellular consequences of receptor engagement may reflect either intrinsic receptor differences or discrete receptor signaling partner proteins.

Furthermore, while it is well known that Eph receptors and their ligands dimerize upon engagement and that receptor/ligand interactions are promiscuous (Himanen et al., 2004; Himanen and Nikolov, 2003; Himanen et al., 2001), it is unknown whether Eph heterodimers exist and whether they are functional. Given the discrete consequences of EphA4 and EphA7 activation documented in the literature, the intermediate results obtained when EphA4 & EphA7 were cotransfected in our hanging drop assay may correspond to either 1) cells expressing homodimers of EphA4 and EphA7 with each receptor's signaling summed intracellularly and since there is less of the most repulsive receptor there is less segregation or 2) the formation of EphA4/EphA7 heterodimers with signaling parameters separate from either homodimer. Other interesting possibilities include possible *trans*- and dominant negative effects (Holmberg et al., 2000; Oricchio et al., 2011). Whatever the explanation, *in vivo* and *in vitro* data demonstrate that signaling via EphA4, EphA7, or a combination of EphA4 and EphA7 is discrete.

Finally, although downstream effectors of EphA signaling have been identified (Gale et al., 1996; Knoll and Drescher, 2004; Margolis et al., 2010; Murai et al., 2003; Nie et al.; Noren and Pasquale, 2004; Richter et al., 2007; Sahin et al., 2005), the molecules responsible for Eph-mediated segregation in the thalamus remain a mystery. Nevertheless, our results implicate Eph signaling as one factor in the segregation of thalamic nuclei during development.

4. Experimental Procedures

4.1 Animal Husbandry

All animal use and care was in accordance with institutional, Georgetown's GUACUC protocol #09-020, and federal guidelines. Wild type mice were either CD-1, purchased from Charles River Laboratories, or C57/BL6, bred within our facility. Mice mutant for EphA4, provided by P. Charney (INSERM, Paris, France), Ephrin-A5, obtained from J. Frisen (Karolinska Institute, Stockholm, Sweden), and EphA7, made available by U. Drescher (King's College, London, UK) were backcrossed onto the C57Bl/6 strain (5–15 generations), and bred either as heterozygotes (EphA4) or homozygotes (ephrin-A5 and EphA7), as previously described (Feldheim et al., 2004; Helmbacher et al., 2000; Miller et

al., 2006; North et al., 2010; North et al., 2009; Rashid et al., 2005). The day of the vaginal plug was considered embryonic day 0.5 (E0.5) and the day of birth, postnatal day 0 (P0). Mouse genotypes were obtained with standard tail PCR.

4.2 Tissue Preparation

Timed pregnant or newly born mice, control or mutant, were euthanized and embryonic or postnatal brains were dissected. Brains were rinsed in phosphate-buffered saline (PBS, pH 7.4) and then incubated in 4% paraformaldehyde for 1–1.5 hours at 4°C, washed with PBS, and submerged in 30% sucrose/PBS overnight at 4°C. Brains were frozen in OCT compound on dry ice and stored at –80°C. Cryostat sections of 14–18 micrometers were collected, mounted on slides, and dried on a slide warmer for ten minutes before being used or frozen prior to use.

4.3 Nomenclature

Coronal and sagittal sections allow visualization of the three diencephalic domains, according to the prosomeric model, located along the anteroposterior axis (Puelles and Rubenstein, 2003). The most anterior domain, prosomere one, corresponds to the dorsal portion of the section. Diencephalon regions and thalamic nuclei were identified based on previous studies (Chen et al., 2009; Puelles and Rubenstein, 2003; Vue et al., 2007; 2009; (Mackarehtschian et al., 1999; Nakagawa et al., 1999; Sestan et al., 2001) and standard mouse brain atlases (Jacobowitz and Abbott, 1998; Schambra et al., 1992).

4.4 In situ hybridization

The radioactive *in situ* hybridization (ISH) protocol and the specifics of each probe have been previously described (Miller et al., 2006; Yun et al., 2003). All EphA receptors and ephrin-A ligands were examined using high specific activity probes. The sensitivity of these analyses was high, judged from signal corresponding to an abundantly expressed gene (*Tbr1*) and a low abundance gene (*RXR*). Thus, the ISH results faithfully reflect levels of gene expression. To assess coordinate expression, images of adjacent sections were pseudocolored and then overlaid to obtain two-colored images in Figure 1–3.

4.5 Beta-galactosidase staining

Coronal sections of mutant and control mice were fixed in 4% paraformaldehyde (PFA), 0.2% glutaraldehyde in PBS, pH 7.4, then rinsed in PBS three times for five minutes each. Sections were incubated in a solution consisting of 4 mM Potassium Ferrocyanide, 4 mM Potassium Ferricyanide, 0.8 mg/mL Xgal in DMSO, 2 mM MgCl₂ in PBS, pH 7.4 for 10–20 hours at 30°C. Following three PBS washes of 5 minutes each, the sections were fixed for 20 minutes in 4% PFA, washed again with PBS, and mounted.

4.6 Immunohistochemistry

Sections were incubated with blocking solution (BB⁺: 2.5% goat serum, 2.5% donkey serum, 1% BSA, 1% glycine, 0.1% lysine, 0.4% Triton X-100 in PBS) for one hour at room temperature and then primary antibody, diluted in BB⁺, at 4°C overnight (North et al., 2010; North et al., 2009). Following washes, sections were incubated with a species-specific secondary antibody linked to HRP for 2 hours at room temperature, washed again, and a standard HRP reaction was performed. After a final wash, sections were mounted using an aqueous hardening mountant.

4.7 Hanging Drop Assay and Analysis

HEK293 cells were grown under standard conditions (10% FBS in DME at 37°C and 5% CO₂) and transfected using FuGene reagent according to the manufacturer's instructions

(Roche Biochemical). One group of HEK293 cells was transfected with an inert vector (pSK⁺), or expression vectors for EphA4, EphA7, or EphA4&EphA7 (the EphA4 expression vector was obtained from Nancy Ip and Mustafa Sahin (Fu et al., 2007); the EphA7 expression vector was created in our laboratory via RT-PCR from P0 cortex (Miller et al., 2006), each combined with a GFP-expressing vector. For receptor expressing GFP⁺ cells, the total amount of plasmid DNA (9 µg) encoding an Eph receptor- either EphA4 or EphA7 alone or EphA4 & EphA7 together, was held constant. Thus, when receptor signaling was derived from one or another or a mixed population of cells, all transfected with the same total amount of Eph receptors. Another group of HEK293 cells was transfected with an inert vector (pSK⁺) or an expression vector for ephrin-A5 (ephrin-A5 expression vector was obtained from Pierre Vanderhaeghen (Depaepe et al., 2005), each combined with a dsRed-expressing vector. Twelve to twenty-four hours after transfection, when transfected cells were visibly fluorescent but expression levels were still low, cells were trypsinized, triturated, and counted. One hundred and fifty GFP-transfected cells and one hundred and fifty dsRed- transfected cells were mixed together in 20µ and pipetted onto the top of a petri dish which was then inverted over DME to prevent evaporation and incubated for 48 hours, the time point that showed the most dynamic range in cell aggregation/segregation while all cells appeared healthy, at 37°C in 5% CO₂. Live imaging was performed on an inverted stereomicroscope, with images obtained on both green and red channels. Merged images were created for 40–50 hanging drops per condition from at least 3 separate experiments. Quantification of coincidence of green and red cells was performed by blinded investigators using a “segregation scale” in which full mixing of red and green fluorescent cells was assigned a value of 1 and full segregation of red and green cells was assigned a value of 5. Assignment of a segregation value was, of course, subjective. Before beginning, evaluators examined the range of possible hanging drops to ensure that the full scale was used.

Supplementary Material

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Acknowledgments

Sponsor information: This work was funded by R01 NS039979 (NINDS) and start-up funds from Georgetown University (MJD) and Howard Hughes Medical Institute University program grant support (KML)

We thank Paul Sampognaro for his endless support in regards to this project. We appreciate the work of Randall Johnson, Ana Efthymiou and Annabelle Williams on projects related to this work. We thank Yasushi Nakagawa for consultation on thalamic nuclear structure.

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- Eph receptors EphA4 and EphA7 and ligand ephrin A5 pattern the forming thalamus during mouse embryogenesis
- Mice mutant for EphA4 and/or EphA7 have altered thalamic organization.
- Cells expressing EphA4 and/or EphA7 sort discretely with cells expressing ephrin A5
- Distinct combinations of Eph signaling appear to act to parcellate the thalamus into discrete nuclei

Figure 1. EphA4, EphA7, and ephrin-a5 expression in the developing thalamus
 (A–D) E12.5 coronal sections of wild type mouse brain stained for Nissl substance (A) or subjected to *in situ* hybridization using probes for EphA4 (B and red in D) or EphA7 (C and green in D). (B) EphA4 is expressed within the DTh as well as within the ET, PTh, and HTh. (C) EphA7 is expressed mainly in the DTh and ET. (D) A merged image of EphA4 (red) and EphA7 (green) demonstrates that each receptor has a unique expression pattern (red or green alone) with some colocalization (yellow). (E–J) E14.5 coronal sections of wild type mouse brain stained for Nissl substance (E) or subjected to *in situ* hybridization using probes for EphA4 (F, red in I, part of red in J), EphA7 (G, green in I, part of red in J), or ephrin-A5 (H and green in J). (F) EphA4 expression is restricted to the DTh and ET as well as ventral domains of the HTh. (G) EphA7 is expressed in defined domains of the DTh and ET and the PT. (H) Ephrin-A5 is expressed along the dorsal and ventral border of the DTh

and in the most ventral HTh. (I) EphA4 (red) and EphA7 (green) are colocalized in DTh (yellow). (J) EphA4 and EphA7 (red) are not coexpressed with ephrin-A5 (green). [Anatomical axes: D=dorsal, L=lateral; Abbreviations: III-third ventricle, DTh-dorsal thalamus, EML-external medullary lamina, ET-epithalamus HTh-hypothalamus, P1-prosomere 1, P2- prosomere 2, P3- prosomere 3, PT-pretectum, PTh-prethalamus (previously known as ventral thalamus)].

Figure 2. EphA4, EphA7, and ephrin-A5 mark discrete thalamic nuclei

(A–F) E15.5 sagittal sections of wild type mouse brain stained for Nissl substance (A) or subjected to *in situ* hybridization using probes for EphA4 (B, red in E, part of red in F), EphA7 (C, green in E, part of red in F), or ephrin-A5 (D, green in F). (B) EphA4 is expressed in the dorsal thalamus (DTh) with strongest expression in more anterior regions, as well as in the dorsal aspects of the Rt, PTh, and ZI. (C) EphA7 is expressed in the posterior portion of the DTh and in the more posterior border between DTh and PTh. (D) There is weak ephrin-A5 expression in the Rt, PTh, and ZI. (E) In the DTh and PTh, there is little colocalization of EphA4 (red) and EphA7 (green), except for a stripe just ventral to the EML. (F) There is little overlap between EphA4 and EphA7 (red) and ephrin-A5 (green) in the E15.5 thalamus. [Anatomical axes: D=dorsal, P=posterior; Abbreviations: DTh-dorsal thalamus, EML-external medullary lamina, Hb-habenula, Hp-Hippocampus, Hth-hypothalamus, PTh-prethalamus (previously known as ventral thalamus), Rt-reticular nucleus, ZI-zona incerta]

Figure 3. EphA4, EphA7, and ephrin-A5 expression distinguishes thalamic nuclei

(A–F) E17.5 coronal sections of wild type mouse brain stained for Nissl substance (A) or subjected to *in situ* hybridization using probes for EphA4 (B, red in E, part of red in F), EphA7 (C, green in E, part of red in F), or ephrin-A5 (D, green in F). (B) EphA4 is expressed throughout the dorsal thalamus, including the dLG and Po. Ventrally, EphA4 is present in the medial aspect of VP. (C) EphA7 is strongly expressed in the vLG and medial portion of the VP. (D) Ephrin-A5 is ventrally expressed in the vLG and Rt. (E) EphA4 (red) and EphA7 (green) are colocalized (yellow) within the Po and VP. (F) Ephrin-A5 (green) is coexpressed (yellow) with EphA4 or EphA7 (red) in the lateral VP, vLG, and Rt.

[Anatomical axes: D=dorsal, L=lateral; Abbreviations: dLG-dorsal lateral geniculate nucleus, EML-external medullary lamina, LP-lateral posterior nucleus, Po-posterior nucleus, Rt=reticular nucleus, vLG- ventral lateral geniculate nucleus, VP- venteroposterior nucleus, ZI-zona incerta]

Figure 4. Separation of thalamic nuclei varies with EphA4 and EphA7 genotype

P0 coronal sections from anterior (A–D) and posterior (E–H) thalamic positions stained with an antibody for EphA4 (A, C, E, G) or for β -galactosidase activity driven by the EphA4 locus (B, D, F, H) in control (A, B, E, F), *EphA7*^{-/-} (C, G) or *EphA4*^{-/-}*EphA7*^{-/-} (D, H) mice with anatomical axes for A, C, E, and G shown in A, and for B, D, F, and H shown in B. (A, E) In control mice, EphA4 is present throughout the dorsal thalamus, marking discrete thalamic nuclei. (B, F) In control mice, EphA4-driven LacZ staining marks the same dorsal nuclei as EphA4 immunohistochemistry (compare A to B and E to F). (C, G) In *EphA7*^{-/-} mice, the distribution of EphA4 is similar to control animals but the limits of thalamic nuclei are less well-defined. (D, H) In *EphA4*^{-/-}*EphA7*^{-/-} mice, dorsal nuclei express LacZ in a continuous pattern with unclear limits. [Anatomical axes: D=dorsal, L=lateral, M=medial; Abbreviations: CP=cerebral peduncle, dLG=dorsal lateral geniculate nucleus, fr=fasciculus retroflexus, MHb=medial habenular nucleus, nsp=nigrostriatal pathway, Pf=parafascicular nucleus, Po=posterior nucleus, PT=pretectum, Rt=reticular nucleus, vLG=ventral lateral geniculate nucleus, Vpl=ventroposterolateral nucleus, Vpm=ventroposteromedial nucleus)

Figure 5. EphA4 and/or EphA7 with ephrin-A5 mediate cell segregation *in vitro*

(A–D) Representative images of HEK cells transfected with DNA constructs encoding either green (GFP) or Red (dsRed) fluorescent proteins and then mixed and cultured to form a hanging drop. Hanging drops consisted of: (A) cells transfected with I) biologically inert DNA (pSK) and an expression vector for GFP and II) biologically inert DNA and an expression vector for dsRed (Control/Control; C/C), (B) cells transfected with I) expression vectors encoding EphA4 and GFP or II) expression vectors encoding ephrin-A5 and dsRed (EphA4/ephrin-A5; A4/fa5), (C) cells transfected with I) expression vectors encoding EphA4, EphA7, and GFP or II) expression vectors encoding ephrin-A5 and dsRed (EphA4&7/ephrin-A5; A4&7/fa5), or (D) cells transfected with I) expression vectors encoding EphA7 and GFP or II) ephrin-A5 and dsRed (EphA7/ephrin-A5; A7/fa5). (E) Quantification of the degree of aggregation (score=1) or segregation (score=5) in each condition using a scale in which the higher the value, the less green/red cell mixing. Control/Control cells intermingle (1.40, left bar), whereas EphA7/ephrin-A5 cells segregate strongly (4.27, right bar), EphA4/ephrin-A5 segregate weakly (2.07, second bar from left), and cells expressing both EphA4 & EphA7 mixed with ephrin-A5 segregate intermediately (3.03, second bar from right).