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Genetic analysis of EphA-dependent signaling mechanisms controlling topographic mapping in vivo

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Ephrin/Eph ligands and receptors are best known for their prominent role in topographic mapping of neural connectivity. Despite the large amount of work centered on ephrin/Eph-dependent signaling pathways in various cellular contexts, the molecular mechanisms of action of Eph receptors in neural mapping, requiring dynamic interactions between complementary gradients of ephrins and Eph receptors, remain largely unknown. Here, we investigated in vivo the signaling mechanisms of neural mapping mediated by the EphA4 receptor, previously shown to control topographic specificity of thalamocortical axons in the mouse somatosensory system. Using axon tracing analyses of knock-in mouse lines displaying selective mutations for the *Epha4* gene, we determined for the first time which intracellular domains of an Eph receptor are required for topographic mapping. We provide direct in vivo evidence that the tyrosine kinase domain of EphA4, as well as a tight regulation of its activity, are required for topographic mapping of thalamocortical axons, whereas non-catalytic functional modules, such as the PDZ-binding motif (PBM) and the Sterile-α motif (SAM) domain, are dispensable. These data provide a novel insight into the molecular mechanisms of topographic mapping, and constitute a physiological framework for the dissection of the downstream signaling cascades involved.

KEY WORDS: Topographic mapping, Ephrin, Eph, Thalamocortical

INTRODUCTION

Ephrin/Eph genes have been shown to play a prominent role in topographic mapping of neural connectivity in several sensory and motor systems (reviewed by Flanagan, 2006; Flanagan and Vanderhaeghen, 1998; McLaughlin and O'Leary, 2005).

Most of what is known about topographic mapping mediated by ephrin/Eph genes was learnt from studies on the retinotectal system; however, recently it has also been studied in other contexts, in particular in neuronal networks from higher brain structures, such as the thalamus and cerebral cortex. For example, several ephrin/Eph genes have been shown to control the patterning of thalamocortical (TC) maps in the primary somatosensory and visual areas of the neocortex (Cang et al., 2005; Dufour et al., 2003; Prakash et al., 2000; Vanderhaeghen et al., 2000; Vanderhaeghen and Polleux, 2004), as well as the mapping of somatosensory cortico-thalamic efferents (Torii and Levitt, 2005).

The molecular mechanisms of signaling downstream of Eph receptors have been intensively investigated over the past few years (reviewed by Klein, 2004; Kullander and Klein, 2002; Pasquale, 2005). Eph receptors are receptor tyrosine kinases (RTKs) that show ligand-induced autophosphorylation and kinase activation, and many in vivo functions mediated by Eph signaling are thought to require an intact kinase activity (Egea et al., 2005; Kullander et al., 2001; Kullander and Klein, 2002). Like other RTKs, they are kept in an autoinhibited state by their juxtamembrane region, which interacts intramolecularly with the kinase domain, thereby maintaining it in an inactive conformation. Phosphorylation of specific tyrosine residues within the juxtamembrane region relieves this autoinhibition (Kullander et al., 2001; Kullander and Klein, 2002). Recent work also demonstrated the paramount importance of receptor clustering in triggering ephrin-dependent responses, aside of tyrosine kinase activation (Egea et al., 2005).

In addition to juxtamembrane tyrosines and the kinase domain, the cytoplasmic region of Eph receptors contains other functional modules, including a Sterile-α motif (SAM) and a PDZ-binding motif (PBM). So far no obvious role has been assigned to the SAM domain, either in vitro or in vivo (Kullander et al., 2001). Similarly, although the PBM of ephrin-B ligands is thought to be important for neural migration and lymphangiogenesis (Lu et al., 2001; Makinen et al., 2005), the functional relevance of this domain in Eph receptors remains poorly understood.

By contrast, a number of potential intracellular interactors for Eph receptors have been identified, including MAP kinases, Src family kinases (SFK), PI 3-kinase, phopholipase C and small G-protein regulators (Kullander and Klein, 2002; Pasquale, 2005). Among the G-protein regulators, ephexins (Shamah et al., 2001) have been shown to be phosphorylated by SFKs upon ephrin stimulation (Knoll and Drescher, 2004), and to play a key role in Eph-mediated growth cone collapse, although their function in vivo remains partially unknown as a result of genetic redundancy (Sahin et al., 2005).

In contrast to the knowledge accumulated on ephrin/Eph-dependent signaling, the molecular mechanisms of action of Eph receptors in the context of neural topographic mapping remain much less characterized, although their major role as axon guidance factors has been uncovered in this biological context. The main reason for this discrepancy is that ephrin/Eph-dependent neural mapping is a complex system. In particular it relies on dynamic interactions between complementary gradients of ephrins and Eph receptors that influence several aspects of axon guidance, including repulsion and differential adhesion/attraction of the growth cone, as well as collateral branching (Flanagan, 2006; McLaughlin and O'Leary, 2005). Because of this complexity, it has been exceedingly difficult, if not impossible, to recapitulate mapping by using in vitro systems that are amenable to signal transduction pathway dissection. For the same reason, it

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is conceivable that distinct ephrin/Eph signaling mechanisms may be used specifically to achieve topographic mapping, but nothing is known about these.

We have previously shown that EphA4, which is expressed in a graded fashion in the somatosensory thalamic ventrobasal nucleus (VB), is required to generate proper topography of TC axons within the somatosensory cortex S1, which expresses a complementary gradient of ephrin A5 (Dufour et al., 2003). The somatosensory TC system thus constitutes an attractive system of topographic mapping that is dependent upon EphA4 signaling.

To try to gain insight into the signaling mechanisms of Eph-dependent mapping, we analyzed EphA4-dependent TC mapping in several mouse knock-in lines displaying distinct targeted mutations for EphA4 (Egea et al., 2005; Grunwald et al., 2004; Kullander et al., 2001). This set of mouse models had previously enabled the demonstration of distinct signaling mechanisms involved in EphA4-dependent midline pathfinding (Kullander et al., 2001), and suggested a differential requirement of EphA4 kinase regulation for repulsion at the midline and TC mapping (Egea et al., 2005). Here, we extended the analysis of TC mapping to all EphA4 alleles available and determined for the first time which intracellular domains of an Eph receptor are required for topographic mapping in vivo. We provide direct in vivo evidence that the tyrosine kinase domain of EphA4, as well as dynamic regulation of its activity, are required for the mapping of TC axons, whereas non-catalytic modules, such as the PBM and the SAM domain, are dispensable. Our data provide an in vivo framework of the signaling mechanisms downstream of Eph receptors that mediate topographic mapping in vivo.

MATERIALS AND METHODS

Generation of *EphA4* mutant mice

Mutant *EphA4*^{KO}, *EphA4*$^{\Delta SAM}$, *EphA4*^{KD}, *EphA4*^{GFP} and *EphA4*^{EE} mice have been described previously (Egea et al., 2005; Grunwald et al., 2004; Kullander et al., 2001). The mutant *EphA4*$^{\Delta PBM}$ allele was generated in the same way and encoded EphA4 lacking the last 12 amino acids, including the PDZ-binding domain. The *loxP* flanked *neo* cassette was removed in vivo by crossbreeding to a Cre recombinase-expressing transgenic mouse strain. All mutant phenotypes were analyzed in littermates from a comparable mixed 129/Svev×C57Bl/6 background. Western blot analyses of EphA4 protein content in neonatal thalamic extracts of wild-type and mutant mice were performed as described previously (Egea et al., 2005).

Axon tracing

Axon tracing was performed by focally injecting a carbocyanin dye [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 3% in dimethyl-formamide, Molecular Probes] into the cortex of living mice anesthetized by avertin (aged P13-P18, the time where all aspects of thalamocortical connectivity have been well established), using capillary micropipettes hooked up to a PicospritzerII (Dufour et al., 2003). Animals were sacrificed 1 to 2 days later, fixed by perfusion, and forebrain was vibratome sectioned at 200 μm. After examination of the sections using fluorescence microscopy, only cases in which the injections were restricted to the cortex without encompassing the white matter were further analyzed, to preclude artefacts due to dye uptake by fibers ‘en passant’. We focused our analysis to the topography of the VPM (ventro-postero-medial) part of the VB to the barrel cortex, which corresponds to the upper levels of the trigeminal representation. The position of the VB nucleus and its boundaries with respect to neighbouring nuclei in the thalamus was determined by inspection of the bright-field image, as well as by nuclear stain and a comparison of atlas data, and by cytochrome oxidase staining in some cases (Dufour et al., 2003). The phenotype was considered to be abnormal only when more than 10 cells were found outside the normal cluster of retrogradely labeled cells. Indeed, in a few cases, a few (less than 10) cells were found occasionally outside of the normal cluster irrespective of their genotype (including in wild type).

RESULTS AND DISCUSSION

To determine the distinct requirements of the signaling modules of the intracellular domain of EphA4 involved in mediating mapping function in vivo, we focused on the thalamocortical (TC) system (Fig. 1A) and performed axon-tracing analyses of mice with targeted mutations of EphA4 (Fig. 1E). These included mice displaying a full disruption of the gene (EphA4^{KO}) (Kullander et al., 2001), mice lacking the whole intracellular domain (EphA4^{GFP} mice, where GFP replaced the whole intracellular domain) (Grunwald et al., 2004) or mutated within the kinase catalytic domain (EphA4^{KD}) (Kullander et al., 2001), and mice lacking the SAM domain (EphA4$^{\Delta SAM}$) (Kullander et al., 2001) or the PBM (EphA4$^{\Delta PBM}$; this study). Phenotypes observed in these loss-of-function mutants were also compared with the ones observed in a previously described mutant, in which the juxtamembrane tyrosines are replaced by two glutamic acid residues (EphA4^{EE}), thereby resulting in constitutive activation of the tyrosine kinase domain (Egea et al., 2005). Importantly, none of these mutations altered the level of expression of EphA4 in the thalamus at relevant perinatal stages (Fig. 1F) (see also Egea et al., 2005).

In wild-type (EphA4$^{WT/WT}$) animals (n=17 in this study), a single injection of DiI in the S1 cortex (Fig. 1B,C) systematically results in a robust retrograde labeling of a single cluster of cells in the ventro-posterior-medial (VPM) part of the VB that corresponds to barreloids, the thalamic counterparts of cortical barrels (Fig. 1A-C) (see also Dufour et al., 2003). We have previously shown that in EphA4$^{KO/KO}$ mutants, in spite of a grossly normal thalamocortical barreloid to barrel topography, some animals (43%, n=7/16) (Dufour et al., 2003) have retrogradely labeled cells located at ectopic positions with respect to the normal barreloid cluster (arrows in Fig. 1D).

Importantly, the same phenotype was observed in EphA4$^{GFP/GFP}$ mutants (penetrance 83%, n=5/6), providing direct evidence that TC mapping requires the intracellular domain of EphA4 (Fig. 2A) (Egea et al., 2005). This indicates that forward signaling is the most important component required for EphA4 in this system, although EphA-dependent reverse signaling is known to be involved in mapping of the visual or olfactory systems (Cutforth et al., 2003; Knoll et al., 2001; Rashid et al., 2005).

Given the involvement of the EphA4 intracellular domain, we next analyzed EphA4$^{KD/KD}$ mutants, in which the kinase catalytic domain was disrupted (Fig. 2C). Strikingly, these mutants displayed a similar loss-of-function phenotype (penetrance 55%, n=5/9; Fig. 2C). This phenotype was also reminiscent of the phenotype observed for the EphA4$^{EE/EE}$ mutants in which EphA4 kinase is constitutively activated [penetrance 61%, n=8/13; Fig. 2B) (see also Egea et al., 2005). In all cases, the phenotypes appeared to be grossly the same in severity (Fig. 2A-C), but, interestingly, the penetrance tended to be higher for mutants in which the EphA4 variants are still capable of interacting with other Eph receptors (Fig. 2F).

Overall, these data indicate that regulation of kinase activity is crucial for the ability of EphA4 to mediate mapping of TC axons, as either a disruption (this study), or a constitutive activation [this study and Egea et al. (Egea et al., 2005)], of the kinase domain result in a similar disruption of TC mapping.

This situation is different from EphA4-dependent repulsion of cortical axons at the midline, which is dependent on an intact tyrosine kinase activity (Kullander et al., 2001), but is seemingly normal in EphA4$^{EE/EE}$ mutants (Egea et al., 2005). This interesting dissociation somehow suggests that Eph-dependent mapping is particularly sensitive to the regulation of kinase activity. Indeed, it is likely that EphA4 kinase activity acts as a

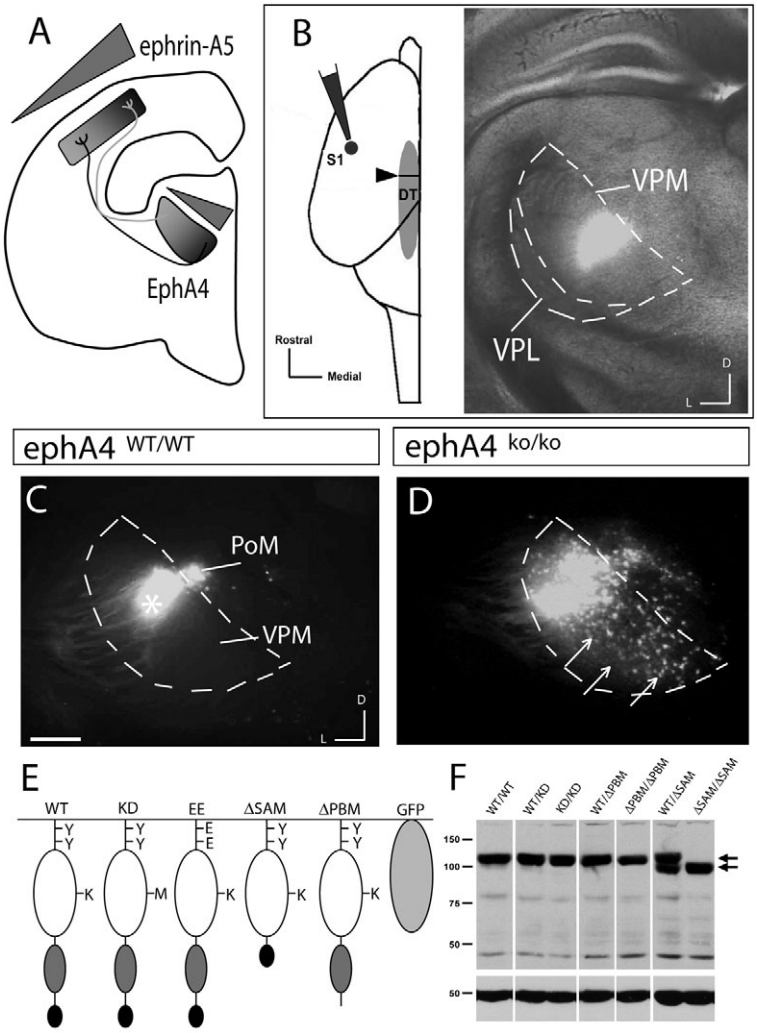


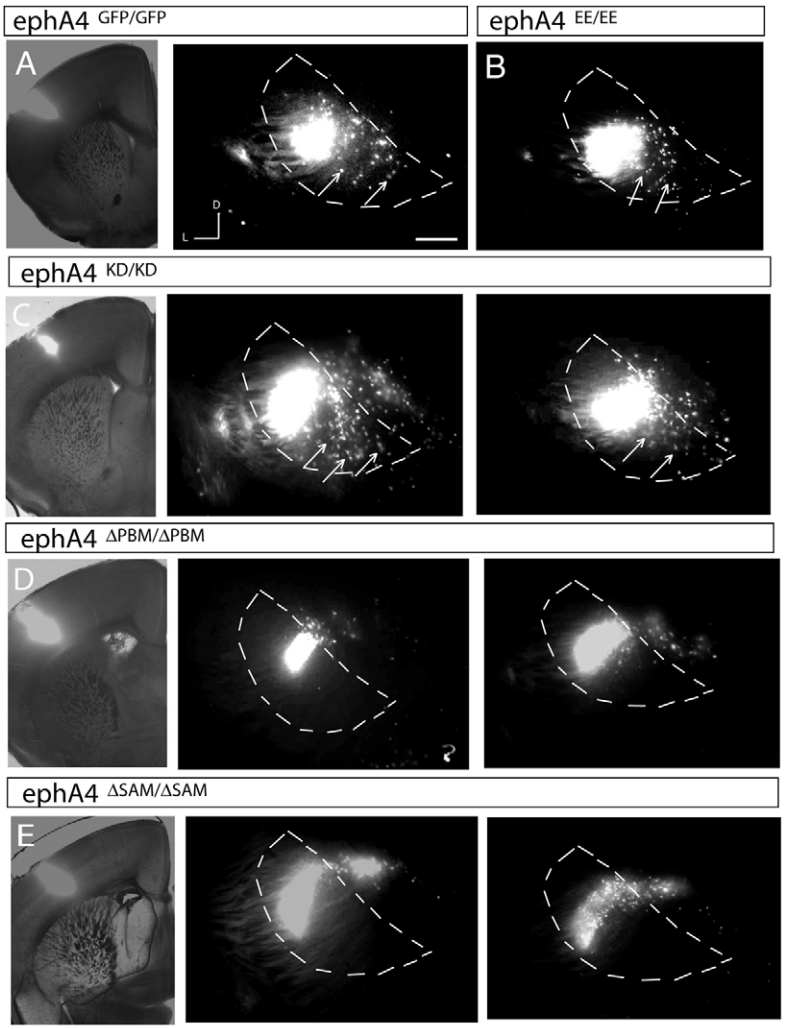
Fig. 1. EphA4 mutants and the thalamocortical somatosensory map. (**A**,**B**) The point-to-point topography of projections between the thalamic primay somatosensory nucleus VB [subdivided into a medial and a lateral part, ventro-postero-medial (VPM) and ventro-postero-lateral (VPL), respectively) and the primary somatosensory area S1, and its relation to ephrin/Eph gradients. Schematic drawings and a low-power view are shown. In control wild-type (WT) mice (B,**C**), focal injection of DiI in S1 resulted in the retrograde labeling of a single cluster of cells in the thalamic nucleus VPM (asterisk in C). In addition, labeling of the postero-medial nucleus (PoM), a secondary somatosensory nucleus, can be detected in some cases outside the VB, but its diffuse and variable pattern was not analyzed in this study. (**D**) By contrast, the EphA4$^{KO/KO}$ mutants show, in addition to the normal cluster, ectopic cells that are located more medially than their normal location (arrows), indicating that thalamocortical projections are not properly organized. Scale bar in C: 400 μm for B-D. (**E**) Schematic model of the intracellular domains of wild-type and mutant forms of EphA4 encoded by the mutant alleles examined in this study. In KD, EphA4 lysine residue K653 (K) in the kinase catalytic domain was replaced by a methionine (M), rendering the receptor kinase domain completely inactive. In EE mutants, the two juxtamembrane residues (Y) are replaced by two glutamic acid residues (E), rendering the receptor kinase activity constitutively active. In ΔSAM mutants, the entire SAM domain (gray oval) is deleted. In ΔPBM mutants, the entire PBM (black oval) is deleted. In GFP alleles, the entire intracellular domain is replaced by GFP (large gray oval). (**F**) Expression of wild-type and mutant EphA4 protein in the thalamus of the indicated mutant mice. Protein (50 μg) from thalamic extracts of the indicated mutant mice was resolved by SDS-PAGE and analyzed by western blot using an anti-EphA4 antibody raised against the extracellular domain of the protein (upper panels); the blot was reprobed with an anti-tubulin antibody for loading control (lower panels). EphA4 expression was analyzed in at least two independent animals per genotype. The lanes are representative expression examples of the different genotypes, and all of them belong to the same gel and the same western blot analysis.

sensor for ephrin gradients during mapping, which requires a normal basal level and a normal capacity to be activated or inhibited. Such a hypothesis would be compatible with current models of topographic mapping in the retinotectal system, where different concentrations of ephrins have qualitatively different effects on retinal axons, depending on their relative expression of Eph receptors (Flanagan, 2006; Hansen et al., 2004). It is also in line with a recent report describing the crucial role of tyrosine phosphatase receptor O in controlling the level of phosphorylation of EphA receptors and, hence, the sensitivity of retinal axons to ephrin ligands, both in vitro and in vivo (Shintani et al., 2006). This could help to explain how the molecular mechanisms of EphA4 receptor-dependent mapping may be distinct from other guidance decisions, such as midline repulsion (Egea et al., 2005): the ability to sense a smooth gradient, as for TC mapping, may require a tighter or more dynamic range of regulation of Eph kinase-dependent signaling than repulsion at the midline, for instance, which is thought to rely on sensing a single-step boundary of cues (Egea et al., 2005; Kullander et al., 2001). In this context it would be interesting to test the qualitative responsiveness of TC axons from the various EphA4 mutants to graded concentrations of ephrin ligands using dedicated in vitro systems (Rosoff et al., 2004).

These data are also of interest in the context of recent reports demonstrating a crucial role for ephexins, a family of small G protein regulators, in the transduction of ephrin-dependent axon guidance. Ephexins have been shown to be phosphorylated following ephrin activation of EphA receptors (Knoll and Drescher, 2004; Sahin et al., 2005), and this phosphorylation is required to mediate growth cone collapse (Sahin et al., 2005), suggesting that they could constitute important mediators of EphA4 kinase-dependent TC mapping. In vivo analysis of compound ephexin mutants should help to address this question.

Although these data highlight the paramount importance of Eph tyrosine kinase activity in neural mapping, they do not exclude the possibility that other signaling mechanisms may be involved. To address this issue, we therefore turned to mouse lines displaying mutations in other signaling modules of the cytoplasmic domain of EphA4 to evaluate their importance in TC mapping. Interestingly, mutants for either the PBM (EphA4$^{\Delta PBM/\Delta PBM}$; n=7) or the SAM domain (EphA4$^{\Delta SAM/\Delta SAM}$, n=5) displayed normal TC projections from VPM to S1 (Fig. 2D-F).

These data thus suggest that these domains, although largely conserved among Eph receptor subtypes between and within species, do not seem to be important for topographic mapping. A subtle regulation of neural mapping by these domains cannot be



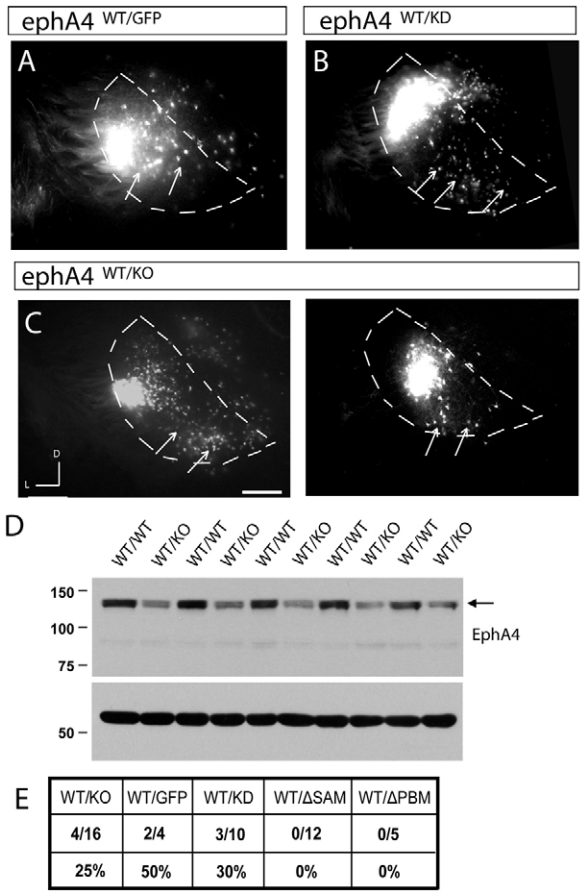
F

WT/WT	KO/KO	GFP/GFP	KD/KD	EE/EE	ΔSAM/ΔSAM	ΔPBM/ΔPBM
0/17	7/16	5/6	5/9	8/13	0/5	0/7
0%	43%	81%	55%	61%	0%	0%

Fig. 2. Analysis of somatosensory TC projections in EphA4 mutants. (**A-C**) Analysis of somatosensory TC projections in mutants displaying abnormal EphA4 kinase activity. In EphA4$^{GFP/GFP}$ (A), EphA4$^{EE/EE}$ (B) and EphA4$^{KD/KD}$ (C) mutants, similar arrays of ectopic cells can be found in the medial part of the VPM (arrows). (**D**,**E**) Analysis of somatosensory TC projections in mutants displaying EphA4 lacking non-catalytic functional domains. EphA4$^{\Delta PBM}$ (D) and EphA4$^{\Delta SAM}$ (E) mutants display a normal pattern of TC projections. Scale bar in A: 400 μm for A-E. In A,C,D,E, left panels show corresponding DiI injection sites in S1. (**F**) Summary of the penetrance of TC topographic defects found in the various EphA4 mutants analyzed. The first line shows the number of affected animals/number of tested animals. The second line shows the percentage of animals displaying defective TC mapping in the VB.

excluded at this stage, however, and could be revealed in a sensitized background. This could be achieved, for instance, by crossing these mutants with other mutants of the ephrin/Eph genes involved in TC mapping, such as ephrin-A5 or other EphA receptors, or by looking at compound mutants for different domains of EphA4. In any case, although the PBM of ephrin Bs is known to be crucial for lymphangiogenesis and neural migration (Lu et al., 2001; Makinen et al., 2005), these results illustrate the modular organization of ephrin/Eph factors, where unique sets of functional modules are required for distinct biological processes, depending on the cellular context or the ephrin/Eph factor considered.

In the course of this analysis, we also noticed to our surprise that some heterozygote mutants displayed abnormal TC mapping (Fig. 3). In particular, 30% of EphA4$^{WT/KD}$ (n=10) and 50% of EphA4$^{WT/GFP}$ (n=4) animals displayed defects that were qualitatively similar to the ones found in the corresponding homozygous mutants but never observed in EphA4$^{WT/\Delta SAM}$ or EphA4$^{WT/\Delta PBM}$ heterozygotes (Fig. 3A,B,E; data not shown). When analyzing EphA4$^{WT/KO}$ animals, we found that they too displayed a similar phenotype in 25% cases (n=16; Fig. 3C,E). Interestingly, defects in heterozygotes were previously described in EphA4 mutants for the development of the spinal cord pattern generator (Kullander et al., 2003). Given the nature of the mutations and the known multimerization of Eph receptors that occurs upon ephrin binding, such a phenotype could be due to either a dominant-negative effect or haploinsufficiency. We also checked for the amount of receptor found in the thalamus of heterozygote mutants and found that it was half of the amount found in EphA4$^{WT/WT}$ thalamus (Fig. 3D). This observation implies that the phenotype observed in heterozygotes is at least compatible with haploinsufficiency. Conversely, work on the retinotectal system has shown the paramount importance of relative versus absolute concentration of ephrin/Eph factors to generate topographic maps, which makes this hypothesis less likely (Brown et al., 2000; Feldheim et al., 2000; Feldheim et al., 2004). Besides, it is of interest to note that the penetrance of the defect observed in heterozygotes and homozygotes is highest in the mutants in which the mutated EphA4 is still capable of interacting with other Eph receptors (Fig. 3D,E). This strongly suggests that a part of the phenotypes observed in heterozygote and even homozygote mutants is due to dominant-negative



E

WT/KO	WT/GFP	WT/KD	WT/ΔSAM	WT/ΔPBM
4/16	2/4	3/10	0/12	0/5
25%	50%	30%	0%	0%

Fig. 3. Analysis of somatosensory TC projections in EphA4 heterozygote animals. (**A-C**) In some EphA4$^{WT/GFP}$ (A), EphA4$^{WT/KD}$ (B) and EphA4$^{WT/KO}$ (C) mutants, arrays of ectopic cells can be found in the medial part of the VPM (arrows), as in corresponding homozygous mutants. Scale bar in C: 400 μm for A-C. (**D**) Expression of EphA4 in the thalamus of heterozygous EphA4 mutant mice. Protein (50 μg) from thalamic extracts of the indicated mutant mice was resolved by SDS-PAGE and analyzed by western blot using an anti-EphA4 antibody raised against the extracellular domain of the protein (upper panels); the blot was reprobed with an anti-tubulin antibody for loading control (lower panels). (**E**) Summary of the penetrance of TC topographic defects found in the various EphA4 heterozygote mutants analyzed. The first line shows the number of affected animals/number of tested animals. The second line shows the percentage of animals displaying defective TC mapping in the VB.

effects. It may also suggest that the right balance of EphA4 concentration relative to other Eph receptors with which it could conceivably heterodimerize (such as EphA3 and EphA7. which are also expressed in the VB) (Dufour et al., 2003) is important for controlling mapping in vivo. It will be interesting to test this hypothesis genetically and biochemically, not only in this system, but also in the better-characterized retinotectal system.

As topographic mapping is known to constitute a multi-step process, it will be also interesting in the future to dissect further the stage and process in which EphA4 is actually required for TC mapping (i.e. earlier directed axon outgrowth in the target, later axonal branching, or pruning). A time-course analysis of the defects observed in the various EphA4 mutants would help to determine this issue.

In conclusion, we demonstrate that neural mapping is particularly dependent on the tight regulation of EphA4 kinase activity, whereas the other signaling modules of this receptor appear to be dispensable for the same function. The level of EphA4-dependent signaling in thalamic axons, which perhaps allows the right signal combination with other thalamic EphA receptors of the system, also seems to be important. These data provide a physiological framework for further dissection of the intracellular signaling cascades, downstream of Eph receptors, that mediate the graded responsiveness of axons to ephrin labels, thereby controlling neural topographic mapping.

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