Ephrin-A5 promotes the formation of terminal thalamocortical arbors

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Received 28 January 2008; accepted 21 February 2008

Ephrins-A5 are expressed in the cortical target layer of thalamic afferents at the time when these axons form terminal arbors. Previous in-vitro studies provided evidence that ephrin-A5 supports the branching of thalamic axons, but there is no direct in-vivo evidence for such a growth-promoting effect. Here we examined thalamocortical projections in ephrins-A5 deficient mice. Our results demonstrate that the laminar specificity of thalamic afferents in ephrin-A5$^{-/-}$ mutants remains preserved, but axonal arbor formation is greatly reduced. Thus, ephrin-A5 specifically regulates branch formation of thalamic axons, but does not affect target layer selection. Ephrin-A5-mutant mice are, therefore, a unique model to study the effects of reduced thalamic innervation on the assembly of cortical circuits and sensory processing. *NeuroReport* 19:877–881 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: axonal branching, axonal guidance, cortical development, ephrin, laminar specificity, thalamocortical projections, wiring molecules

Introduction

Ephrin receptor tyrosine kinases and their ligands, the ephrins, serve as topographic labels in many neuronal circuits (reviewed in Refs [1–5]). In the thalamocortical projection system, for instance, ephrin-A5 can act as a repulsive cue for axons from the ventrobasal (VB) complex of the thalamus, which project to the somatosensory cortex (S1). Complementary gradients of ephrin-A5 in S1 and EphA4 in VB then lead to a topographic mapping within S1, because thalamic axons with low and high expression levels of EphA4 project to regions in S1 with high and low levels of ephrin-A5, respectively [6,7]. In addition to this intra-areal topography, an ephrin-A5 gradient within the subcortical telencephalon also seems to contribute to the interareal topography, the projections of specific thalamic nuclei to individual cortical regions [8]. Moreover, ephrin-A5 also serves as a local repulsive cue for limbic thalamic axons, as in ephrin-A5-knockout mice a portion of limbic axons from the laterodorsal nucleus, which normally project to the cingulate cortex and bypass S1, form inappropriate projections to S1 [9]. Finally, a recent study provided evidence that counter gradients of EphA7 in S1 and EphAs in VB regulate the topography of feedback projections from the cortex to the thalamus [10].

In addition to its role as a repulsive guidance signal, there is now increasing evidence from in-vitro studies for attractive effects of ephrin-A5. Initially it was shown that ephrin-A5 induces axon collateral formation in cortical neurons from layer 6, which in-vivo target layer 4, where this ligand is expressed [11]. Later, similar effects were also found for thalamic axons, which also arborize in layer 4 [12]. Furthermore, in-vitro assays with membranes purified from individual cortical layers demonstrated that native membranes from layer 4 contain a branch-promoting activity for thalamic fibers that was abolished when endogenous ephrin-As were blocked [12]. Here we examined thalamocortical projections in transgenic mice carrying a null mutation of the ephrin-A5 gene. First, we demonstrate that the branch-promoting activity of layer 4 membranes from ephrin-A5-knockout animals is greatly diminished. Tracing studies of thalamocortical connections in these mutant animals then revealed that terminal axonal arbors are strongly reduced in layer 4 of S1, whereas the laminar specificity of the thalamic input remains preserved. These results provide the first in-vivo evidence for EphA/ephrin-A interactions resulting in a growth promoting activity.

Methods

All animals were treated, maintained, and killed in accordance with Society for Neuroscience resolutions on the use of animals in research and National Institutes of Health guidelines as well as institutional protocols. Ephrin-A5 knockouts are the same as previously described [13].

In-vitro experiments

Cortical membranes were prepared from brains of postnatal day 8 (P8) wild-type and knockout mice as described before [12]. Thalamic explants were obtained from normal E14 (E1=day of conception) mice after caesarian section of pregnant female mice. Thalamic blocks were dissected and cut in a McIlwain tissue chopper (Campden Instruments, Loughborough, London) in 200 μm^2 explants. Explants were transferred to cover slips previously treated with laminin/poly-L-lysin and cortical substrate. Cultures were kept for

2 days at 37°C under 5% CO_2 in air atmosphere and then fixed with 4% paraformaldehyde (PFA) for microscopic analysis. Petri dishes were analyzed using a Zeiss Axiovert microscope (Carl Zeiss, Jena, Germany). Number of side branches was determined along individual axons excluding crossing fibers and fascicles. Axonal branching was defined as the ratio between the total number of axonal branches and the total segment length analyzed. Statistical analysis was performed using *t*-test for number and length and the Fisher's permutation test for branching.

In-vivo axonal tracing

P8 control (n=8) and knockout animals (n=10) were perfused with 4% PFA. Brains were removed from the skull and immersed in 4% PFA before implantation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate (Invitrogen, Molecular Probes, Carlsbad, California, USA) crystals. Brains were embedded in agarose (4%) and cut in 400 μm slices in an angle that preserves the thalamocortical pathway [14]. The VB complex of the thalamus was localized by counter-illumination and then crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate were implanted in the nucleus. The slices were kept in phosphate-buffered saline (PBS) containing 0.2% sodium azide and after 2 weeks they were analyzed using a confocal microscope (ZEISS LSM 510, Zander IVF). Retrograde-labeled cells were observed in deep cortical layers in some of the slices, but their dendritic

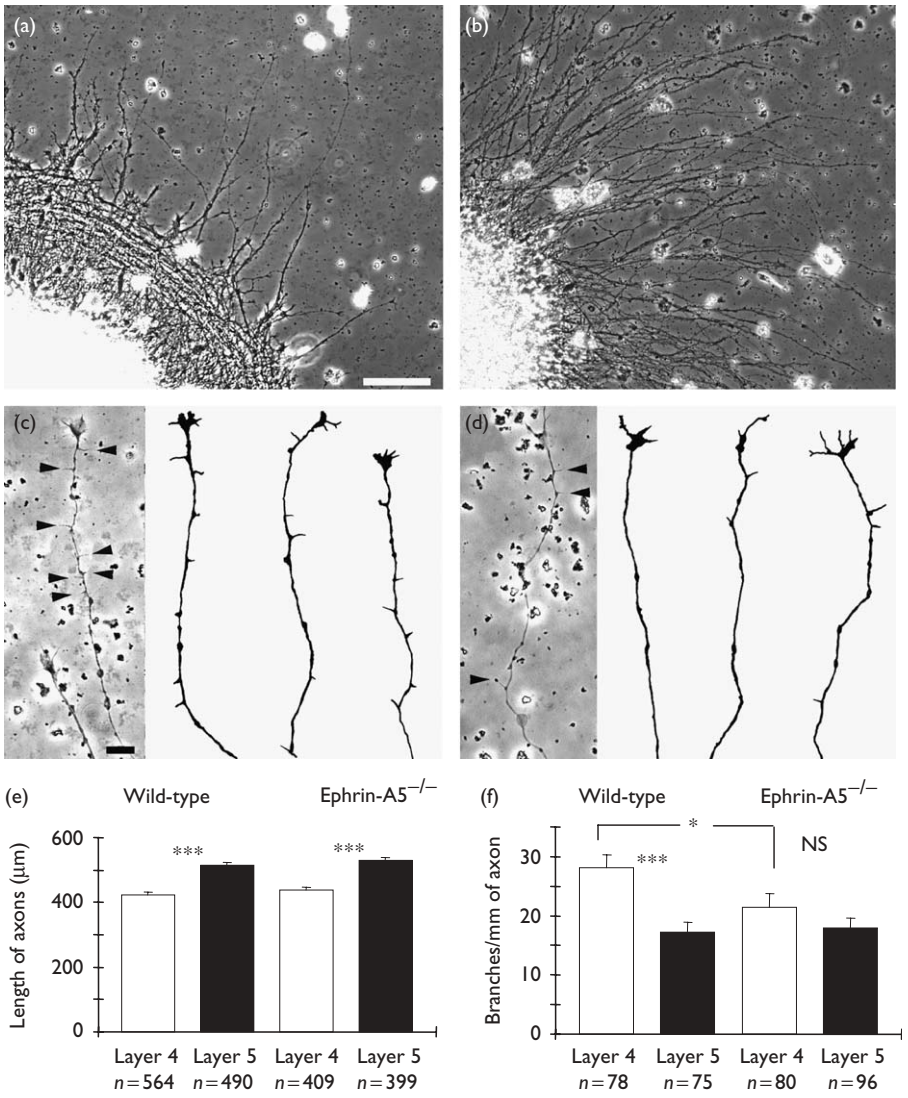

Fig. 1 (a, b) Low-power phase-contrast photomicrographs of thalamic explants on (a) layer 4 and (b) layer 5 membranes prepared from ephrin-A5$^{-/-}$ animals. Fewer and shorter axons are observed on layer 4 membranes, the target layer of thalamic axons, than on layer 5 membranes, a nontarget layer. (c, d) High power micrographs and drawings of individual axons growing on layer 4 membranes from (c) wild-type and (d) mutant animals. Arrowheads indicate collaterals along the axons. Note that layer 4 membranes from wild-type animals possess a branch-promoting activity for thalamic afferents that is absent in layer 4 membranes from ephrin-A5-knockout animals. (e, f) Quantification of (e) axonal length and (f) number of collateral branches for wild-type thalamic fibers growing on cortical membranes from laminae 4 and 5 prepared from wild-type (left) and ephrin-A5-deficient (right) animals. (e) Compared with layer 5 membranes, axons on layer 4 membranes are shorter, both on membranes from wild-type and mutant animals. (f) Thalamic axons on layer 4 membranes prepared from normal animals exhibit many more branches than on layer 5 membranes. This layer-specific branching signal is not present in membranes prepared from ephrin-A5-deficient animals. Scale bars: 100 μm for (a, b) and 10 μm for (c, d). *$P<0.05$; ***$P<0.001$.

arbors could be easily distinguished from thalamic axonal branches. Neighboring axons frequently formed overlapping arbors making identification of individual terminations difficult. Therefore, only well-isolated fibers were used for analysis. Thalamic fibers in the cortex were identified and photographed in different *z*-axis planes and then reconstructed. After microscopic scanning of the whole arbors, drawings were done based on the series of scans and then images were stacked together for illustration. The number and length of branches of each individual fiber were quantified and a *t*-test was used to compare arborizations of wild-type and ephrin-A5-null mice.

Results

In-vitro assays

Previous work indicated that thalamic axons growing on membrane substrates prepared from their target layer (lamina 4 of S1) exhibit a reduced growth rate and increased branching density compared with thalamic axons growing on membrane substrates from a nontarget layer (lamina 5 of S1), suggesting that membrane-associated molecules in the target layer provide both a stop signal and a branching signal for thalamic afferents [12]. We, therefore, first characterized the growth behavior of wild-type thalamic axons on membranes purified from cortical layers 4 and 5 of S1 from wild-type and ephrin-A5$^{-/-}$ mice. Figure 1 illustrates representative examples of axonal outgrowth from thalamic explants on membranes prepared from target and nontarget layers. A quantitative analysis of the fiber growth indicated that on target layer membranes there were fewer (data not shown) and shorter (Fig. 1e) axons than on nontarget layer membranes, irrespective of whether the membrane substrates were prepared from wild-type or mutant mice. However, the analysis of branch formation revealed significant differences between wild-type and homozygous mutant animals. In accordance with previous results [12], there was almost two-fold increase in the number of branches on the target layer compared with nontarget layer membranes prepared from wild-type mice (Fig. 1f). In contrast, collateral formation of thalamic axons on ephrin-A5$^{-/-}$ layer 4 membranes was significantly reduced when compared with wild-type layer 4 membranes (Fig. 1f). Thus, there was no branch-promoting activity for thalamic axons on target layer membranes from ephrin-A5-null mice.

Thalamocortical projections *in vivo*

To determine whether ephrin-A5 is directly responsible for the formation of terminal arbors *in vivo*, we examined thalamocortical afferents in wild-type and ephrin-A5-deficient animals (Fig. 2a and b). At P8, all fibers have reached cortical layer 4 and elaborate branches in this layer. In several cases, the main axon overshoots the target layer and extends into the superficial layers, but there are very few branches outside layer 4. As adjacent thalamic fibers formed closely overlapping arbors, we examined only individually labeled axons clearly separated by other labeled axons (Fig. 2c and d). Examination of individual fibers revealed that axonal arbors from wild-type animals are more complex than those from mutant animals (Fig. 3a and c). After scanning the whole terminal arbor, drawings were made from a series of scans, which were then superimposed for statistical analysis. We reconstructed 32 thalamocortical axons from mutant mice and the same number

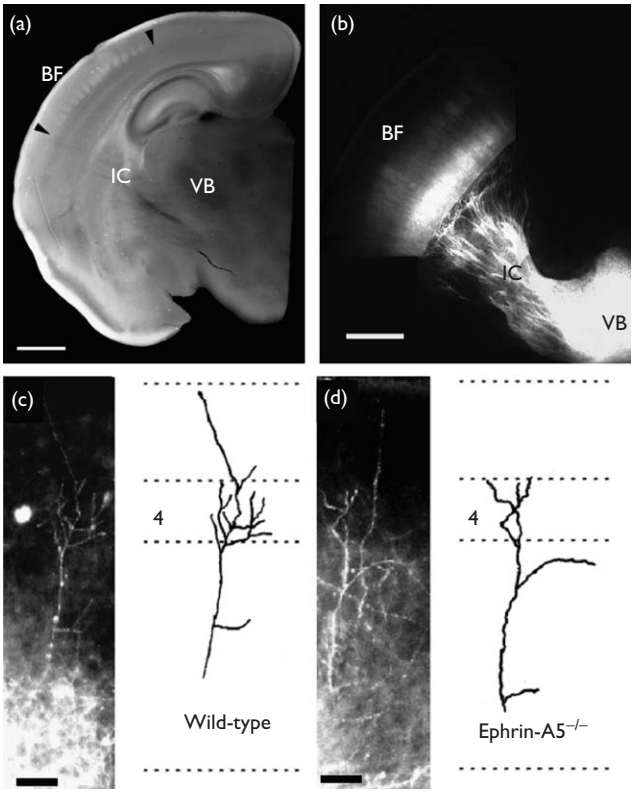

Fig. 2 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate tracing of thalamocortical projections *in vivo*. (a) Low-power photomicrograph with transillumination of a thalamocortical slice preparation from the barrel field [(BF) (indicated by arrowheads)] of somatosensory cortex used for fiber tracing. (b) Fluorescent micrograph at higher magnification shows the injection side in ventrobasal (VB) complex of the thalamus and the thalamocortical projection through the internal capsule (IC) to the BF. (c, d) Confocal images and drawings of individual thalamocortical arbors in layer 4 of SI from (c) wild-type and (d) mutant animals. Thalamic axons arborize predominantly in layer 4, but the number and length of axon collaterals in ephrins-A5$^{-/-}$ mice are reduced compared with wild-type animals. Scale bars: 1 mm in (a and b); 0.5 mm in (c); and 100 μm in (d).

of fibers from wild-type mice. In mutant animals, thalamic axons exhibit less and shorter branches than in wild-type animals (Fig. 3b and c). As a result, the cortical innervation density of thalamic afferents, estimated by the total length of axon collaterals within layer 4, was more than two times higher in normal than in ephrin-A5-deficient mice (Fig. 3d). Retrogradely labeled cells were occasionally observed in the deep cortical layers, but the dendritic and axonal branches of these corticothalamic neurons could be clearly distinguished from thalamocortical afferents (data not shown).

Discussion

Molecular determinants of layer-specific cortical circuits

The laminar architecture of the mammalian neocortex is reflected by stereotyped interlaminar and intralaminar connections, local cortical circuits, and by long-distance projections to and from the cortex. Previous in-vitro studies provided evidence for molecular cues confined to individual cortical laminae that regulate the layer-specific targeting of cortical and thalamic axons [3,15–17]. So far, however, very little is known about the molecular nature of these signals. Ephrin-A5 is strongly expressed in layer 4 at the

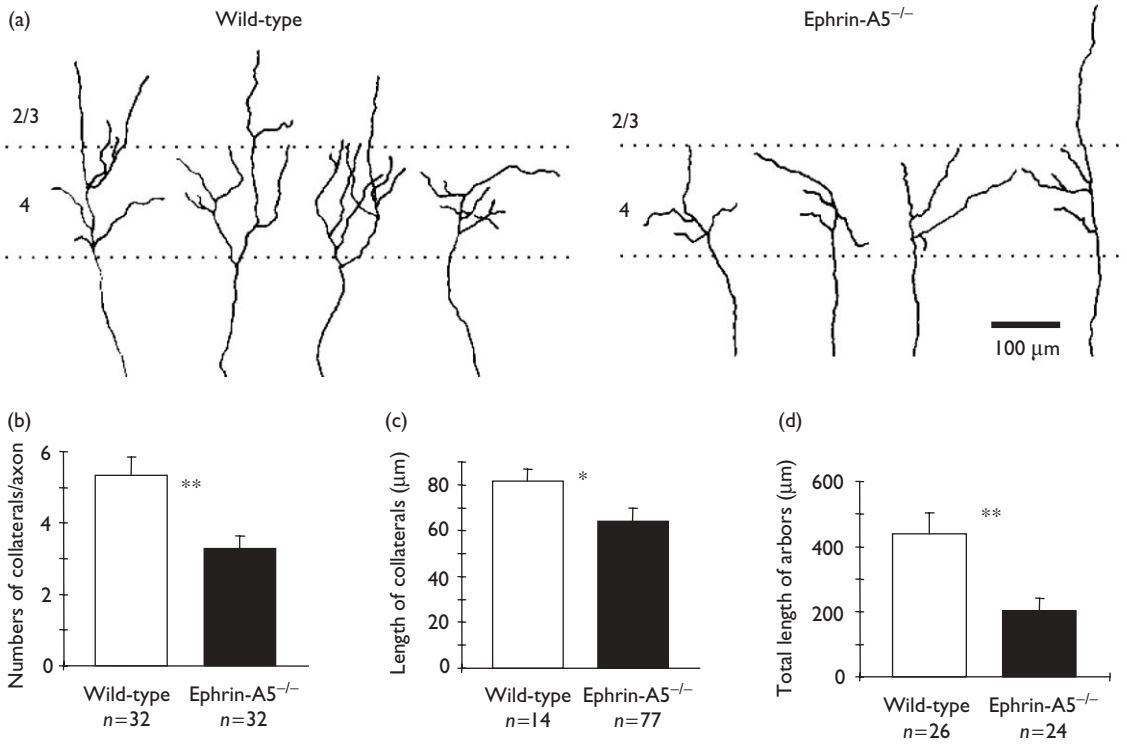

Fig. 3 (a) Drawings of representative confocal scans of thalamocortical arbors from wild-type (left) and ephrin-A5-deficient mice (right). Both in wild-type and in mutant animals, most axon collaterals are confined to layer 4. However, terminal arbors from control mice are much denser than the axonal arbors from knockout mice. (b, c, and d) Quantitative analysis of thalamocortical arbors in layer 4 of the somatosensory cortex of wild-type and ephrin-A5-knockout mice. (b) Number of axon collaterals per axon, (c) length of axon collaterals, and (d) total length of the terminal thalamic arbor. In ephrin-A5-mutant animals, the number and length of axon collaterals is greatly reduced. Scale bar: 100 μm in (a). $*P < 0.05$; $**P < 0.01$.

time when thalamocortical arbors are being formed in this layer and in-vitro experiments with recombinant ephrin-A5 demonstrated that it is able to elicit branching of these fibers [12]. The present in-vivo results demonstrate that the formation of terminal arbors of thalamic axons in S1 is greatly reduced in ephrin-A5-deficient mice, whereas the layer-specific targeting was as precise as in wild-type animals. This is consistent with the present in-vitro results, which demonstrated that membrane-associated molecules in layer 4 of ephrin-A5$^{-/-}$ mice, like in wild-type animals, arrest the growth of thalamic fibers. Thus, the 'stop-signal' for thalamic afferents in their target layer is not affected in ephrin-A5 mutants. This, then, suggests that target layer recognition and the formation of terminal arbors in this layer are controlled by independent molecular cues, and that in ephrin-A5 mutants only the latter process is affected.

The role of ephrin-A5 as a growth-promoting molecule

Previous studies with ephrins-A5-knockout mice provided evidence for subtle changes in the extent of S1 and in limbic thalamic projections [6,9,18]. These phenotypical alterations have been explained by repulsive effects of ephrins-A5 on thalamic axons. Therefore, the observation reported here that ephrin-A5 provides a branch-promoting activity to thalamic axons might seem surprising. Several different, but not mutually exclusive, possible explanations are available for this apparent discrepancy. First, there are previous studies that already demonstrated that different concentrations of ephrins can regulate axonal growth, either positively or negatively [19,20]. Thus, a relatively high concentration of ephrin-A5 in the subplate might have repulsive effects for thalamic axons, which express high levels of EphA receptors, whereas a relatively low expression of ephrin-A5 in layer 4 might evoke axonal branching. Second, different receptor complexes and cofactors associated with Eph receptor complexes can convert repulsive effects to attraction [21]. Third, changes in the internal state of the neuron can also switch the response from repulsion to attraction. For instance, ephrin-A5 induces repulsion of migrating cortical neurons, an effect that is mediated at least in part by Src family kinases (SFK). In contrast, when SFK activity was reduced, ephrin-A5 acted as an adhesive signal for this population of neurons [22]. As SFK activity is under the control of several ligands other than ephrins, a cross talk, for instance with semaphorins, might change the response of thalamic afferents to ephrin-A5 once they arrive in their target layer. Finally, the response of growth cones to a given brain-wiring signal can also depend on the spatial context in which it is presented. The molecule distributed either as a uniform substrate, a sharp border, or as a gradient can determine the behavior of the axons. For example, in the stripe assay, when given a choice, limbic thalamic axons avoid ephrin-A5-containing substrates, but on a uniform substrate ephrin-A5 induces axonal branching [12]. At the time when axons from the VB first reach the subplate of S1, the graded expression of ephrin-A5 in this region might regulate topographic mapping through repulsive effects [6]. At later stages, when thalamic afferents invade the cortical layers and ephrin-A5 is uniformly

expressed in layer 4, this molecule might then elicit the branch-promoting effects described here.

Conclusion

The present results demonstrate that thalamocortical arbors in S1 of ephrin-A5$^{-/-}$ mice are strongly reduced, whereas the laminar specificity remained preserved. This indicates that the stop-signal in the target layer of thalamic afferents is maintained in the absence of ephrin-A5. Thus, ephrin-A5 specifically mediates terminal arbor formation of thalamic fibers and, thereby, plays a critical role in the establishment of thalamocortical connections. Ephrin-A5-mutant mice are, therefore, a unique model to study the effects of thalamic innervation density on the organization of cortical circuits and sensory processing.

Acknowledgements

The authors thank Wolfgang Wurst and Konstantinos Zarbalis for providing the ephrins-A5-knockout mice and Erika Krause and Christine Raue for excellent technical assistance. Financial support came from IZKF Jena (J.B.), PRONEX-CNPq (D.U.). D.U. was a fellow from CAPES.

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