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Role of EphA/ephrin-A signaling in the development of topographic maps in mouse corticothalamic projections

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Abstract

Corticothalamic (CT) feedback outnumbers thalamocortical projections, and regulates sensory information processing at the level of the thalamus. It is well established that EphA7, a member of EphA receptor family, is involved in the topographic mapping of CT projections. The aim of the present study is to dissect the precise impact of EphA7 on each step of CT growth. We used *in utero* electroporation-mediated EphA7 overexpression in developing somatosensory CT axons to dissect EphA7/ephrin-A-dependent mechanisms involved in regulating both initial targeting and postnatal growth of the CT projections. Our data revealed that topographic maps of cortical afferents in the ventrobasal complex and medial part of the posterior complex in the thalamus become discernible shortly after birth and are fully established by the second postnatal week. This process starts with the direct ingrowth of the CT axons to the designated areas within target thalamic nuclei and by progressive increase of axonal processes in the terminal zones. Large-scale overproduction and elimination of exuberant widespread axonal branches outside the target zone was not observed. Each developmental event was coordinated by spatially and temporally different responsiveness of CT axons to the ephrin-A gradient in thalamic nuclei, as well as by the matching levels of EphA7 in CT axons and ephrin-As in thalamic nuclei. These results support the concept that the topographic connections between the maps in the cerebral cortex and corresponding thalamic nuclei are genetically pre-specified to a large extent, and established by precise spatio-temporal molecular mechanisms that involve the Eph family of genes.

Keywords

axon branching; axon guidance; somatosensory system; topographic map

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Introduction

The thalamus plays an essential role in relaying sensory information to the cerebral cortex via thalamocortical (TC) projections. However, an increasing number of studies in different sensory systems and species have revealed that the thalamus is not just a simple relay center, but performs complex information processing and integration that underlie complex mammalian behaviors through the function of CT feedback input (Briggs and Usrey, 2008). CT projections comprise almost 50% of the synaptic input onto thalamic sensory neurons and are approximately 10-fold more abundant than TC projections (Jones, 2002). This massive CT feedback dynamically influences thalamic processing of sensory information by sharpening the receptive fields and/or shifting the tuning of thalamic neurons, as well as enhancing or suppressing the transmission of sensory signals from the periphery to the cortex (Briggs and Usrey, 2008; Briggs and Usrey, 2009; Kondo and Kashino, 2009; Lee et al., 2008; Li et al., 2011; Olsen et al., 2012; Paz et al., 2011). Such intimate functional interconnection largely depends upon the high degree of reciprocity in neural circuits that connect the thalamus and cerebral cortex (Temereanca and Simons, 2004). Each functional area of the cerebral cortex has a reciprocal connection with a unique set of thalamic nuclei, composing TC and CT projections (Carvell and Simons, 1987; Nothias et al., 1988; Torii and Levitt, 2005). TC and CT projections also map topographically within their target cortical areas and thalamic nuclei (Alloway et al., 2003; Fabri and Burton, 1991; Lozsadi et al., 1996; Nothias et al., 1988).

While a greater understanding of CT functions is emerging, relatively little is known about the timing and molecular mechanisms involved in the establishment of topographic CT projections. In contrast, much attention has focused on understanding the mechanisms of guiding and patterning TC axons to their cortical target (Bielle et al., 2011; Bonnin et al., 2007; Demyanenko et al., 2011; Lopez-Bendito et al., 2006; Lopez-Bendito and Molnar, 2003; Price et al., 2006; Uemura et al., 2007; Vanderhaeghen and Polleux, 2004; Wright et al., 2007), which are directed in a highly specific fashion.

EphA receptors and their ligands, ephrin-As, serve as graded topographic labels in many circuits by either positively or negatively regulating axon growth (Cang et al., 2005; Dufour et al., 2003; Feldheim et al., 1998; Flanagan, 2006; Hansen et al., 2004; Miller et al., 2006; Rashid et al., 2005; Torii and Levitt, 2005; Uziel et al., 2002; Vanderhaeghen et al., 2000). One of the best characterized models of EphA/ephrin-A-mediated topographic map formation is retinotopic mapping of retinal ganglion cell (RGC) axons to the optic tectum or superior colliculus (O'Leary and McLaughlin, 2005). During embryonic and early postnatal development, RGC axons initially overshoot, rather than directly targeting and arborizing at topographically correct termination zone. The topographic map is therefore established by subsequent formation of interstitial branches and arbors at the terminal zone, as well as large-scale elimination of exuberant axonal branches and arbors (Nakamura and O'Leary, 1989; Simon and O'Leary, 1992). From the results of *in vitro* studies and end point analyses of mature retinotectal/retinocollicular projection using *ephrin-A* and *EphA* mutants, it was suggested that inhibitory interaction of EphAs and ephrin-As is involved in restricting interstitial axon branching/arborization only at the terminal zones and eliminating overshooting axon segments (Sakurai et al., 2002; Yates et al., 2001). However, the time course of the change in axon branching and arborization has not been demonstrated in these studies, and it is yet unknown whether EphA/ephrin-A-mediated topographic mapping in other parts of the central nervous system, including the thalamus, undergo a similar pattern of development.

It has been established that EphA/ephrin-A-mediated signaling is involved in the formation of CT projections within specific thalamic targets in the somatosensory, visual (Sestan et al.,

2001; Torii and Levitt, 2005), and auditory (Torii et al., 2012) systems. By overexpression and RNAi-based knockdown of EphA7 using *in utero* electroporation, we demonstrated that topographic targeting of somatosensory CT axons along the dorsomedial-ventrolateral axis within the ventrobasal complex (VB) and medial part of the posterior complex (POm) is determined by matching levels of EphA7 in the CT axons and ephrin-As within thalamic nuclei through their potential inhibitory interaction. CT axons with higher levels of EphA7 therefore terminate closer to the VB/POm border region, in which the expression level of ephrin-As is the lowest, whereas CT axons with lower levels of EphA7 terminate in the region with higher levels of ephrin-As in these nuclei (Torii and Levitt, 2005). The mode of initial ingrowth into thalamic nuclei, and the pattern of following growth that mediate specific CT axon targeting, however, have not been investigated. The overexpression of EphA7 in CT projections provides a unique opportunity to highlight CT axon behavior under conditions in which there is a strong response to EphA/ephrin-A signaling. This strong response consistently causes confined accumulation of CT axons at the VB/POm border region in their final mapping (Torii and Levitt, 2005). In the present study, we have taken advantage of this strong, consistent effect of EphA7 overexpression, to examine the temporal and spatial contribution of EphA/ephrin-A signaling in the postnatal development of somatosensory CT projections.

Materials and Methods

Animals

All experiments using animals were in accordance with the protocols approved by Institutional Animal Care and Use Committee of Yale University. CD-1 wild-type mice (Charles River) were used for all analyses. Noon of the day on which a vaginal plug was observed and the day of birth is designated as embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively.

Immunohistochemistry

Brains were immersion-fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.2) at 4 °C overnight, and coronal slices (75 µm) were produced with a vibratome (Leica). Slices were incubated with rabbit polyclonal anti-green fluorescent protein (GFP) antibody (1:1000; #A11122; Invitrogen) at 4 °C overnight. After washing in PBS, the slices were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (1:1000; #711-035-152; Jackson ImmunoResearch) and visualized with TSA Plus Fluorescence System (PerkinElmer). Slices were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen) to reveal cell nuclei. Images were captured using a confocal LSM 510 NLO system or an Axioplan2 microscope (Carl Zeiss) equipped with epifluorescence. Images were not modified other than to balance brightness and contrast using Photoshop.

Antibody Characterization

The anti-GFP antibody (#A11122; Invitrogen) is IgG fraction from rabbit serum raised against full-length GFP isolated from *Aequorea victoria*. In control experiments, we confirmed the lack of staining with this antibody on sections of mouse brains that were not electroporated with enhanced GFP (EGFP) expression plasmid (data not shown).

Receptor binding histochemistry

Receptor binding histochemistry on cryosections (20 µm) was performed as described (Torii and Levitt, 2005). Briefly, the sections were incubated with recombinant mouse EphA7-Fc chimeric protein (2 µg/ml; #608-A7, R&D Systems) for 90 min at room temperature. After

rinse in PBS, the sections were fixed in 4% PFA in PBS for 30 min, and incubated with HRP-conjugated secondary antibody (1:1000; #715-035-150, Jackson ImmunoResearch) and visualized with TSA Plus Fluorescence System (PerkinElmer). Images were captured using an Axioplan2 microscope (Carl Zeiss) equipped with epifluorescence. Images were not modified other than to balance brightness and contrast using Photoshop.

***In utero* electroporation**

EphA7 expression plasmid (pCAGGS-rEphA7-IRES-Venus) containing full-length cDNA of rat EphA7, control plasmid (pCAGGS-IRES-EGFP), and pCAGGS-EGFP were described previously (Hashimoto-Torii et al., 2008; Torii et al., 2009; Torii and Levitt, 2005). *In utero* electroporation was performed as previously described (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Briefly, at E12.5, the dam was anesthetized with ketamine/xylazine (100/10 mg/kg) and the uterine horns were exposed. EphA7 expression plasmid (2mg/ml) or control plasmid (2 mg/ml) with pCAGGS-EGFP (0.5 mg/ml) was injected into the lateral ventricle of each embryo using pulled glass capillaries, and electrical current (five 50 ms pulses of 30 V with 950 ms intervals) was passed to transfer the plasmids into the cortex using a ECM 830 Square Wave Electroporator (BTX-Harvard Apparatus). The uterine horns were replaced and the dam was allowed to recover and give birth normally. As a technical limitation for *in utero* electroporation, the expression pattern of exogenous genes in the targeted cortical area inevitably varied across animals. For the quantitative analyses (Fig. 5), we carefully selected the brains with EGFP expression located centrally in the somatosensory cortex. The selection of brains was done prior to examination and analysis of CT axons.

Quantification of histological data

Gradient of EphA7-Fc binding is presented as pixel intensity profiles of the staining along the mediodorsal to ventrolateral axis through POM and VB (arrow in Fig. 1G) using ImageJ. The distribution of EGFP⁺ CT projections within POM and VB was measured as described previously (Torii and Levitt, 2005). Briefly, images were collected from brain sections through the dorsal thalamus at 3 different postnatal ages. POM and VB were divided into 30 spatial bins vertical to the mediodorsal-to-ventrolateral axis of the nuclei (Fig. 5A). In order to obtain the relative distribution along the designated axis through the nuclei, the area occupied by EGFP⁺ axons in each bin was measured using ImageJ and normalized to the total area occupied by EGFP⁺ axons in the entire POM and VB. The slides were coded so that the investigator was blind to the electroporation condition. Mixed design ANOVA and the Kolmogorov-Smirnov (K-S) test were performed using SPSS (IBM) and SAS (SAS Institute Inc.) software, respectively.

Results

Topographic mapping of somatosensory CT projections is regulated by the level of EphA7 in cortical neurons

By using *in utero* electroporation-mediated gene transfer, we introduced the EGFP expression plasmid in CT projection neurons at E12.5 in the mouse somatosensory cortex. The labeling allows subpopulations of CT axonal projections to be traced along their path from the cortex to thalamic targets, the VB and POM nuclei. This labeling strategy eliminates the problem associated with conventional tracing methods that unavoidably label axons or cell bodies of reciprocal TC projections (Fig. 1). EGFP⁺ CT axons were directed downward from the cortex (Fig. 1A) through the internal capsule (Fig. 1B), where they turned medially and caudally, and then extended medially and dorsally towards VB and POM in the thalamus (Fig. 1C, D). At later ages, the expected topographic organization of labeled terminals in discrete areas of VB and POM was evident (Alloway et al., 2003;

Deschenes et al., 1998; Fabri and Burton, 1991; Nothias et al., 1988); the specific location of labeled axons in VB depended upon the site of electroporation in the somatosensory cortex (Fig. 1E). Consistent with our previous report (Torii and Levitt, 2005), increasing the sensitivity to EphA/ephrin-A signaling in CT axons by the co-expression of EphA7 caused a shift of CT terminal zones toward the VB/POM border region (Fig. 1F), associated with the lowest expression level of ephrin-As in this region (Fig. 1G, H). Taking advantage of specific labeling of CT projections and highlighting the contribution of EphA/ephrin-A signaling in CT axon development, we further explored the normal process of CT topographic map formation over time in the postnatal brain through the observation of the growth pattern of control (EGFP⁺ only) axons, and the role of EphA/ephrin-A signaling by mapping EphA7-overexpressing (EphA7/EGFP⁺) CT axons postnatally.

Initial growth of CT axons into the VB and POM is differently affected by thalamic ephrin-As

In the mouse, the earliest axons from the cerebral cortex reach the thalamus at E18.5 (Jacobs et al., 2007). In control-electroporated brains, many EGFP⁺ CT axons have entered VB at P0 from its ventrolateral side (Fig. 2A), which shows the highest expression of ephrin-As including *ephrin-A5*, during late embryonic through early postnatal stages (Bolz et al., 2004; Torii and Levitt, 2005)(Fig. 1G, H). Surprisingly, we found that EGFP⁺ CT axons in EphA7-electroporated brains have similarly entered VB from its ventrolateral side without exhibiting any apparent obstacles (Fig. 2B), indicating that these axons are not repelled by the ephrin-A cues during the initial entry into VB. This finding is consistent with our previous observation that the ephrin-A expression in the subcortical telencephalon does not disrupt the guidance of CT axons (Torii and Levitt, 2005). In both control- and EphA7-electroporated brains, CT axons showed beaded and mostly unbranched morphology (Fig. 2C), coursing dorsomedially toward POM. At this early age, some axons had already crossed the VB/POM boundary (Fig. 2A, B), frequently ending with growth cones (Fig. 2C).

After P1, the difference in the distribution of control and EphA7-overexpressing CT axons became apparent (Fig. 3). In control brains at P1, EGFP⁺ CT axons showed further growth into the body of POM, in which they start to form branches (Fig. 3A). In contrast, EphA7-overexpressing CT axons were stalled around the VB/POM border region, and the accumulation of EGFP⁺ axonal puncta at this region becomes evident (Fig. 3D). Thus, CT axons appeared to directly target their terminal zones by responding to the ephrin-A gradient in POM, in contrast to their earlier unresponsiveness to ephrin-A cues during the initial ingrowth into VB. At P2, the number of labeled axonal puncta was increased in both control- and EphA7-overexpressing brains (Fig. 3B, E), but their overall distribution patterns were similar to those observed at P1. The increase of labeled puncta was observed in the body of VB in control animals (Fig. 3B, C). In contrast, labeled axonal puncta was concentrated at the VB/POM border region in EphA7-overexpressed brains (Fig. 3E, F).

Topographic map of CT projections develops through a progressive increase of axonal processes at their targets

During the first and second postnatal week, a marked quantitative increase in the number of punctuate labeling of CT axons was evident in VB and POM, restricted to specific target areas within each nucleus of the dorsal thalamus (Fig. 4)(Torii and Levitt, 2005). At P12, a topographic organization of labeled puncta was evident in discrete areas of VB and POM in controls (Fig. 4A), matching the location of the electroporated site in the cortex. Less evident but similar topographic organization was observed as early as P4 (Torii and Levitt, 2005). In EphA7-electroporated brains, the labeling was always heavily coalesced at the VB/POM border region, the site of the lowest ephrin-A expression (Fig. 4F). In control brains, labeled puncta also were evident in a diffuse area surrounding the clusters of CT

terminals (Fig. 4B–D), whereas such diffuse labeling was minimal in EphA7-electroporated brains (Fig. 4G–I). The distributions of normal and EphA7-perturbed CT axons were stable in brains examined after P12 through puberty (Fig. 4E, J).

In order to validate these qualitative observations, the innervation pattern of CT projections was quantitated during postnatal development by analyzing the relative distribution of labeled axon densities along a dorsomedial-to-ventrolateral trajectory in VB and POm (Fig. 5A). The analysis revealed a strong shift of CT innervation toward the VB/POm boundary at all ages by EphA7 overexpression, at the expense of innervation in the body of VB and POm (Fig. 5B) (Mixed design ANOVA group $\times$ location interaction term: P2, $F(29, 174) = 3.49$, $p < 0.0001$; P12, $F(29, 261) = 5.13$, $p < 0.0001$; P40, $F(29, 232) = 10.74$, $p < 0.0001$; K-S test: P2, $p = 0.0001$; P12, $p < 0.0001$; P40, $p < 0.0001$). The shift was evident from the beginning of increasing axonal puncta, as early as P2. The same shifted pattern was maintained through maturation up to P40 (Fig. 5). These results indicate that the topographic mapping of CT axons achieve an adult configuration via a progressive increase in number and/or length of axonal processes (primary axons and their branches) within topographically appropriate terminal zones in thalamic nuclei, through the interaction between EphAs in CT axons and ephrin-A gradients in the nuclei.

Discussion

The long standing issue of whether TC projections induce specification of the initially equipotential cortex [tabula rasa hypothesis (Creutzfeldt, 1977)] or TC projections are attracted to genetically pre-specified cortical areas [protomap hypothesis (Rakic, 1988)] has been resolved in favor of the latter (Cholfin and Rubenstein, 2008; Grove and Fukuchi-Shimogori, 2003; Levitt et al., 1997; O'Leary and Borngasser, 2006; O'Leary and Sahara, 2008; Rakic et al., 2009), but the mechanism through which formation of the reciprocal CT projections occur remains an open question. In our previous study, we showed that EphA/ephrin-A signaling plays an essential role in establishment of a mature pattern of somatosensory CT projections (Torii and Levitt, 2005). In the present study, we revealed that the formation of this mature pattern of CT projections includes multiple developmental events, to each of which EphA/ephrin-A signaling appears to make specific contributions (Fig. 6). CT axons with high expression levels of EphA7 enter the dorsal thalamus from the ventrolateral part of VB around birth, without showing an apparent repulsion response to the highest level of ephrin-As. The mechanism through which this refractory period occurs is unknown, but we reported a similar non-responsiveness of EphA7-expressing CT axons prenatally, when they grow through a dense ephrin-A5 zone of the ventral telencephalon (Torii and Levitt, 2005).

In contrast to this lack of repulsion in VB, CT axons that enter POm stop growing at the presumptive terminal zone. Thus, the axons do not overshoot, instead respond to the increasing inhibitory effect of ephrin-A. Through the first and second postnatal week, CT axons progressively increase the number and/or length of axonal processes at the terminal zones in VB and POm, complementary to the matching level of EphAs in CT axons and graded ephrin-As in these nuclei.

Spatial and temporal difference in the responsiveness of CT axons to ephrin-As

We found that control and EphA7-overexpressing CT axons similarly enter VB at the zone of highest ephrin-A expression. Lack of repulsion of CT axons to ephrin-As during their initial entry into VB appears to be a crucial mechanism for enabling CT axons to enter the nucleus through a high level of ephrin-As, which generally exerts inhibitory effects on axon growth. Moreover, the main axon shafts, which are continuously exposed to a high level of ephrin-As maintained at the ventrolateral part of VB, were maintained without being

eliminated or pruned. CT axons, thus, appear 1) to change their responsiveness to ephrin-As temporally before and after the period of their entrance into VB, and 2) to have different responsiveness spatially between the main axon shafts and their branches. A number of possibilities exist for molecular regulation of the change in responsiveness, including post-transcriptional and post-translational changes such as local protein synthesis, receptor trafficking, or activity modifications by co-receptors and downstream signal mediators. These transient changes in response to guidance cues are similarly seen in the regulation of commissural axons in both vertebrates and invertebrates (Brittis et al., 2002; Chen et al., 2008; Evans and Bashaw, 2010; Hong et al., 1999; Nawabi et al., 2010; Parra and Zou, 2010; Sabatier et al., 2004; Shirasaki et al., 1998; Stein and Tessier-Lavigne, 2001).

Topographic mapping of CT projections

Theoretically, two models can explain the formation of topographically ordered axonal projections. According to the first model, the initial targeting of axons and their arborizations are imprecise; topographic precision is achieved through selective elimination or pruning at inappropriate regions, as well as elaboration of axonal branches at appropriate targets. According to the second model, axons exhibit limited branching only within the final target territories, and a mature branching is subsequently refined at the appropriate termination zone. There are many examples for both models. For example, topography of TC axons in layer IV (Agmon et al., 1993; Catalano et al., 1996) and callosal axons in the visual cortex (Olavarria and Safaeian, 2006) are established via specific initial ingrowth and elaboration of axonal branches at topographically correct targets, rather than corrective pruning of exuberant axonal branches. As an example for the first model, RGC axons initially overshoot their topographically correct termination zone in the tectum/superior colliculus, and achieve topographic innervation by subsequent large-scale elimination of axons that overshoot and their exuberant branches/arbores, as well as formation of interstitial branches and arbors at the termination zone (Nakamura and O'Leary, 1989; Simon and O'Leary, 1992). The same mechanism appears to occur for cortico-pontine projections (O'Leary and Terashima, 1988; Stanfield et al., 1982). For RGC axons, EphA/ephrin-A signaling has been suggested to be involved in both inhibiting axon branching/arborization outside of the terminal zones and eliminating overshooting axon segments (Sakurai et al., 2002; Yates et al., 2001).

Because CT projections from somatosensory cortex have two major target nuclei (VB and POm), interpretation of our data is somewhat complex. For example, it has been shown in the rat that there are three groups of somatosensory CT axons that innervate VB and POm. These are axons that exclusively project to VB, that exclusively project to POm, and that project to the POm with collaterals in VB (Bourassa et al., 1995; Levesque et al., 1996). Our data indicate that the innervation of the latter two groups in POm can be included in the second mechanistic model. Axons of the third group collateralize also in VB. Our observations of mostly unbranched axons in VB at P0, and the emergence of many branches after P1, strongly suggest that these are formed by interstitial budding from the axon shafts under the control of EphA/ephrin-A signaling, similar to RGC axons in their topographic mapping. Axons with interstitial branches in developing somatosensory CT projections also have been described in the rat (Frasson et al., 1995). For the first group of CT axons that only innervate VB, the methodology used here does not have the resolution to identify individual CT axons that either accurately target or overshoot terminal zones. Nevertheless, the results show that initial exuberant branching/arborization or subsequent elimination, if any, does not occur on a large scale, and the progressive increase of axonal processes at specific terminal zones controlled by EphA and ephrin-A interaction makes a major contribution. Differences in the particular EphA/ephrin-A mechanism used for establishing topographic mapping by either RGC axons or CT axons are consistent with the idea that

different intrinsic programs control both the spatiotemporal presentation and activation of EphA/ephrin-A signaling in developing axons.

Targeted increase of axonal processes in topographic innervation of CT projections

After initial targeting, our results revealed that progressive increase of axonal processes at correct terminal zones plays a major role in topographic innervation of CT projections within target thalamic nuclei. The robust CT axon labeling obtained after P1, however, prevented us from clear visualization of individual axonal branches to address precise mechanisms such as the relative contributions of length and numbers of axonal branches in the increase of axonal processes. Although the literature about the sequence of the postnatal development of CT projections is limited, our results corroborate a previous report obtained by anterograde tracer biotinylated dextran amine (BDA) in the rat (Frassoni et al., 1995). The rat study showed that somatosensory CT projections achieve the adult innervation pattern to specific thalamic nuclei through progressive increase of axonal branches, without showing exuberant branches/arbores in inappropriate thalamic nuclei at any developmental period (Frassoni et al., 1995). It should be noted, however, that while our analysis of fixed tissues provides information about the fine topography of emerging connections, it does not have temporal resolution to address detailed description of local pruning via retraction or degeneration of small axonal branches that can occur simultaneously with their growth. Such pruning mechanisms have been observed using two-photon time-lapse imaging both *in vitro* and *in vivo* in other systems (O'Rourke et al., 1994; Portera-Cailliau et al., 2005; Uesaka et al., 2005).

We also found the interaction between EphAs in the CT axons and ephrin-As in VB and POm contribute to the targeted increase of axonal processes at specific terminal zones by analyzing the growth of CT axons that overexpress EphA7. The results are consistent with our previous observation that the knockdown of EphA7 using RNAi also shifts CT axon mapping, in the expected opposite direction to EphA7 overexpression; this occurs through a progressive increase of axonal processes within inappropriate terminal zones between P4 and P12 (Torii and Levitt, 2005).

In the topographic mapping of the RGC axons, the interaction of EphA and ephrin-A is considered to be mostly inhibitory for branching/arborization outside the correct terminal zone (O'Leary and McLaughlin, 2005; Sakurai et al., 2002; Yates et al., 2001). However, *in vitro* studies have reported that ephrin-As exert not only inhibitory but also growth-promoting effects on hippocampal (Gao et al., 1999), cortical (Castellani et al., 1998), thalamocortical (Uziel et al., 2008) and RGC (Hansen et al., 2004) axons. The transition from positive to negative effects has been proposed to depend on the level of ephrin-As expression, which provides a potential mechanism for each axon forming a termination zone at the neutral point of these effects (Flanagan, 2006; Hansen et al., 2004). A similar mechanism may be involved in promoting CT axon branching/growth at specific terminal zone. Alternatively, the balancing of axon repulsion, mediated by EphA/ephrin-A forward and reverse signaling, may display strict point-to-point specificity of CT axon branching/growth (Gebhardt et al., 2012).

Finally, neural activity also appears to be a regulator of axon branching/growth in various neural circuits (Gibson and Ma, 2011). Ephrin-A-induced axon retraction and EphA expression are modified by activity in RGC projections to the tectum/superior colliculus and the projections of spinal motor axons, respectively (Hansen and Landmesser, 2004; Nicol et al., 2007). Future studies should incorporate experiments to examine the role of neural activity and its potential modification of EphA/ephrin-A signaling on the progressive increase of CT axonal processes at specific terminal zones to establish their final topographic innervation.

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Figure 1.

Shift of CT projections in VB by the overexpression of EphA7 using *in utero* electroporation.

A–D: Immunohistochemistry for EGFP (green, counterstained with DAPI [magenta]) at P1 in a brain that was electroporated with the EGFP-expression plasmid at E12.5. CT projections are labeled by EGFP from the somatosensory (SS) cortex, through the internal capsule (ic) to POm and VB in the thalamus. E, F: Images at POm and VB of control- (E) and EphA7-electroporated (F) brains at P12. CT axons in the control brain (E) distribute in discrete areas of VB and POm (arrows), whereas CT axons that overexpress EphA7 accumulate densely at the VB/POm boundary region (F). G: Fc-tagged EphA7 receptor binding at POm and VB at P4, revealing minimal binding of EphA7 at the POm/VB border region¹. H: Plot profile of the gray value quantified in POm and VB along the axis indicated by the arrow in G. Scale bars = 500 μm . ¹The image was taken from the same slide that was previously shown in Torii and Levitt, (2005).

Figure 2.

Ingrowth of CT axons into VB and POM at P0.

A, B: Immunohistochemistry for EGFP (counterstained with DAPI) at VB and POM in control-(A) and EphA7-electroporated (B) brains. Several EGFP-labeled CT axons with few side branches cross the VB/POM border (broken line) in both cases (arrows). Scale bar = 500 μ m. C: High magnification view of the EGFP labeling in the area boxed in B, showing little branching from the main shaft of these axons. Arrows point the growth cones at the tip of growing axons. Scale bar = 50 μ m.

Figure 3.

Increase of CT axonal processes in VB and POM at P1–P2.

A–F: Immunohistochemistry for EGFP (counterstained with DAPI in A, B, D, E) at VB and POM in control- (A–C) and EphA7-electroporated (D–F) brains at P1 (A, D) and P2 (B, C, E, F). C and F are higher magnification views of the yellow-boxed areas in B and E, respectively. At P1, in contrast to further growth of CT axons into the body of POM in the control-electroporated brain (A, arrow), EphA7-overexpressing axons appear to be stalled in close proximity of the POM/VB border region (D, arrow). The insets in A and D are higher magnification views of the square areas in each panel, showing the branching of CT axons. At P2, some EGFP⁺ CT axons in control-electroporated brains reach the dorsomedial border of POM (arrow in B), and the punctuate labeling in the body of POM and VB is increased (B, C). The inset in B is a higher magnification view of the square area, showing the puncta of labeled CT axons in the body of VB. In the EphA7-electroporated brain, EGFP⁺ axonal puncta were significantly increased in the vicinity of the POM/VB border (arrow in E and F), but not in the body of the nuclei (arrowheads in F). The bundles of main axon shafts in VB are similarly observed in both control- (C, brackets) and EphA7-electroporated brains (F, brackets). Scale bars = 500 μ m (A, B, D, E), 100 μ m (C, F).

Figure 4.

Robust increase of CT axonal processes at the terminal zones in VB and POM at P12–P40. A–J: Immunohistochemistry for EGFP (counterstained with DAPI in E, J) at VB and POM in control- (A–E) and EphA7-electroporated (F–J) brains at P12 (A–D, F–I) and P40 (E, J). B, C and G, H are higher magnification views of the red- and yellow-boxed areas in A and F, respectively. D and I are higher magnification views of the boxed areas in C and H, respectively. At P12, labeling of EGFP⁺ axons is extensive at their specific terminal zones in VB and POM in the control-electroporated brain (arrows in A), whereas it is restricted to the VB/POM border region in the EphA7-electroporated brain (F). Punctate labeling of CT axonal processes accumulates densely around the VB/POM border region (arrow in G), with much less labeling in the body of VB and POM (arrowheads in G, and H) in the EphA7-electroporated brain. A wider distribution of CT axonal processes is observed in the control-electroporated brains (B, C). In the EphA7-electroporated brain, bundles of main axonal shafts appeared similar to control (brackets in D and I). However many fewer axonal branches and terminals surrounded the shafts (I, compare with D). Similar patterns of CT innervation in discrete areas of VB and POM in the control (E, arrows) and at the VB/POM border region in the EphA7-electroporated brain (J) were observed in more mature brains at P40. Scale bars = 500 μ m (A, E, F, J), 100 μ m (B, C, G, H), 20 μ m (D, I).

Figure 5.

Continuous increase of CT axonal processes at the terminal zones in VB and POm.

A: Schema to quantify the distribution of CT axons in VB and POm. B: The percentage distribution of area occupied by EGFP⁺ CT axons (y axis) is represented against relative position across POm and VB (x axis, as indicated by the arrow in A). In contrast to the distribution in control-electroporated brains, the accumulation of EGFP⁺ axons at the POm/VB border region is evident as early as P2. This restricted distribution is maintained during the increasing of CT axonal processes through the postnatal period. $n = 4$ and 4 (P2), 5 and 6 (P12), and 4 and 6 (P40) for control- and EphA7-electroporated brains, respectively. Error bars represent SEM.

Figure 6.

Models of CT axon mapping in VB and POM.

CT axons (green) in both control- and EphA7-electroporated brains invade VB around P0 from the ventrolateral margin without showing the expected repulsion to the high level of ephrin-As (gradients in orange). Within POM, initial targeting of CT axons is regulated by the interaction of EphAs in CT axons and the ephrin-A gradient in the nucleus. After P1, CT axonal processes (primary axons and their branches) are progressively increased in their number and/or length at specific terminal zones in VB and POM, determined by the levels of EphA expression in the CT axons and ephrin-As within these nuclei. The main axon shafts are not eliminated at any time of CT development despite being exposed to ephrin-As at a high level.