

Pathfinding of Corticothalamic Axons Relies on a Rendezvous with Thalamic Projections

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SUMMARY

Major outputs of the neocortex are conveyed by corticothalamic axons (CTAs), which form reciprocal connections with thalamocortical axons, and corticosubcerebral axons (CSAs) headed to more caudal parts of the nervous system. Previous findings establish that transcriptional programs define cortical neuron identity and suggest that CTAs and thalamic axons may guide each other, but the mechanisms governing CTA versus CSA pathfinding remain elusive. Here, we show that thalamocortical axons are required to guide pioneer CTAs away from a default CSA-like trajectory. This process relies on a hold in the progression of cortical axons, or waiting period, during which thalamic projections navigate toward cortical axons. At the molecular level, Sema3E/PlexinD1 signaling in pioneer cortical neurons mediates a “waiting signal” required to orchestrate the mandatory meeting with reciprocal thalamic axons. Our study reveals that temporal control of axonal progression contributes to spatial pathfinding of cortical projections and opens perspectives on brain wiring.

INTRODUCTION

Reciprocal connections between brain structures provide critical feedback and feedforward loops in major neural circuits. However, how they are established during development remains largely to be determined. Reciprocal connections between the thalamus and the neocortex are formed by thalamocortical axons (TCAs) and corticothalamic axons (CTAs) that convey sensory and motor information essential for cortical functioning. TCAs and CTAs contribute to the internal capsule, a large axonal highway navigating through the basal ganglia or subpallium,

which also comprises output corticosubcerebral axons (CSAs) en route toward the cerebral peduncle and pyramidal tract (Auladell et al., 2000; Price et al., 2006). The internal capsule is thus a major gateway to and from the neocortex. Studies over the past decades have focused on the mechanisms governing its development, particularly on the role of transcription factors in CTA and CSA specification and on the function of subpallial guidepost cells in TCA pathfinding.

CTAs and CSAs are generated by corticofugal pyramidal neurons located in distinct layers: subplate and deep layer VI generate pioneer CTAs (pCTAs) neurons, layer VI contains CTA neurons that grow in a second step, and layer V produces CSA neurons (McConnell et al., 1989, 1994; De Carlos and O'Leary, 1992; Auladell et al., 2000; Del Rio et al., 2000; Molyneaux et al., 2007). Recent experiments have demonstrated that this laminar specificity is controlled by distinct transcriptional programs that define the identity of cortical pyramidal neurons, including their axonal trajectory (Arlotta et al., 2005; Chen et al., 2008; Kwan et al., 2008; Lai et al., 2008; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011; Shim et al., 2012). In parallel, the subpallium has been shown to constitute an essential intermediate target for axons of the internal capsule (Métin and Godement, 1996). During embryogenesis, pCTAs and TCAs reach the subpallium and progress in opposite directions toward their reciprocal targets while staying in close vicinity (Molnár et al., 1998; Auladell et al., 2000; Bellion et al., 2003; Jacobs et al., 2007; Grant et al., 2012). Analyses of mutant mice have shown that the subpallium controls the navigation of CTAs, CSAs, and TCAs by secreting guidance factors and generating guidepost cells (Métin et al., 1997; Richards et al., 1997; Braisted et al., 1999; Tuttle et al., 1999; Hevner et al., 2002; Tissir et al., 2005; Uemura et al., 2007; Zhou et al., 2008; Magnani et al., 2010; Molnár et al., 2012).

The subpallium comprises the lateral ganglionic eminence (LGE) and the medial ganglionic eminence (MGE), which participate in the guidance of distinct axonal populations. In particular, LGE-derived corridor neurons have been shown to guide TCAs along their route toward the neocortex (López-Bendito et al., 2006; Bielle et al., 2011a, 2011b). These guidepost neurons

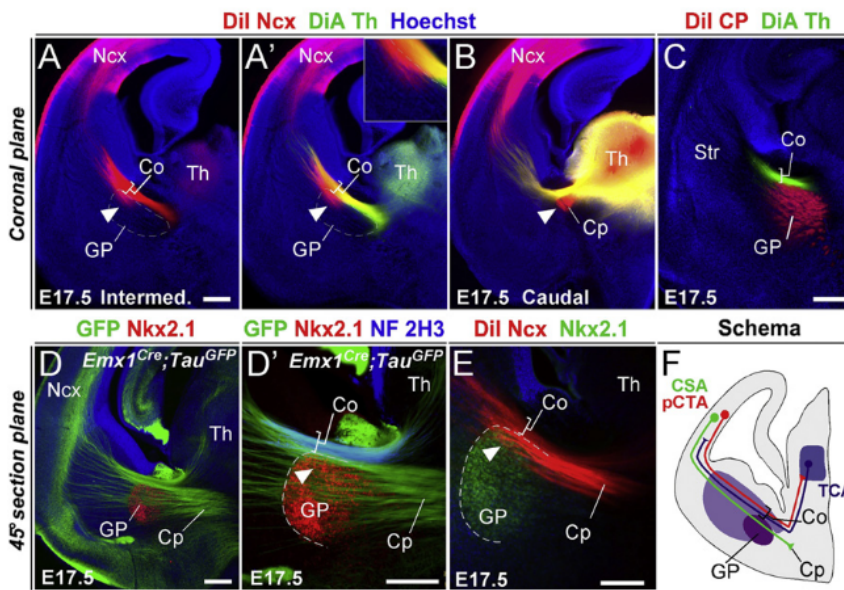


Figure 1. pCTAs and CSAs Follow Distinct Trajectories in the Subpallium

(A, A', and B) Serial coronal sections of a wild-type brain at E17.5 after Dil crystal insertions in the somatosensory neocortex and DiA crystal insertions in the thalamus showing distinct pathways for TCAs, pCTAs on the one hand (A', yellow), and CSAs on the other hand (A and A', red, arrowhead). While TCAs and pCTAs navigate in the corridor, CSAs grow in the GP and join the cerebral peduncle (B, arrowhead).

(C) Coronal section of a wild-type brain at E17.5 after insertions of Dil and DiA crystals into the cerebral peduncle and thalamus, respectively, confirm that CSAs navigate through the GP (red) in contrast to TCAs and pCTAs (green).

(D and D') Forty-five degree angle sections (between coronal and sagittal planes) of E17.5 *Emx1^{Cre}; Tau^{GFP}* embryos show GFP-positive corticofugal axons passing through either the corridor together with TCAs immunostained by neurofilament 2H3 or GP immunostained with Nkx2.1 (arrowhead in D').

(E) Forty-five degree angle section at E17.5 showing corticofugal axons labeled by Dil tracing from the neocortex passing through the corridor and Nkx2.1-positive GP (arrowhead).

(F) Schematic drawing showing the distinct paths followed by TCAs (blue), pCTAs (red), and CSAs (green) within the subpallium.

Co, corridor; Cp, cerebral peduncle; CSAs, corticosubcerebral axons; GP, globus pallidus; Ncx, neocortex; pCTAs, pioneer corticothalamic axons; TCAs, thalamocortical axons; Th, thalamus.

Scale bars represent 250 μ m in (C)–(E) and 500 μ m in (A).

migrate from the LGE into the MGE and form a permissive corridor for TCAs deep to the globus pallidus (GP). In addition, the MGE-derived GP has been associated with CSA pathfinding, as CSA navigation is specifically affected in *Nkx2.1* mutant mice that impair MGE development (Marín et al., 2002). In contrast, the structures or mechanisms governing pCTA pathfinding have remained elusive. The close vicinity of CTAs and TCAs together with analyses of mutant mice affecting one of the two axonal populations have suggested that these reciprocal projections might generally guide each other (Molnár and Blakemore, 1995; Molnár et al., 1998; Hevner et al., 2002; Jones et al., 2002; López-Bendito et al., 2002; Wu et al., 2010; Chen et al., 2012). However, this hypothesis has remained controversial (Bagnard et al., 2001; Torii and Levitt, 2005), particularly since TCAs and cortical axons collapse each other in vitro (Bagnard et al., 2001).

Here, we investigated the mechanisms governing the pathfinding of somatosensory pCTAs. We found that pCTAs and CSAs follow distinct trajectories within the subpallium: pCTAs navigate together with TCAs in the permissive corridor, whereas CSAs grow in the GP before joining the cerebral peduncle. Using a combination of in vivo genetic ablation of the thalamus and ex vivo experiments, we demonstrated that TCAs are required to guide pCTAs into the corridor. In absence of TCAs, pCTAs follow a default CSA-like trajectory. This guidance function of TCAs relies on a pause, or waiting period, in the progression of corticofugal axons, given that pCTAs reached the lateral subpallium at least a day before TCAs. At the molecular level, PlexinD1/Semaphorin3E signaling is required to prevent premature subpallial progression of pCTAs before TCAs have reached the proper position, thereby ensuring that pCTAs follow their normal

trajectory. Taken together, these results show that a waiting period controls the pathfinding of pCTAs, by allowing their interaction with incoming reciprocal TCAs. Our study reveals how temporal regulation of axonal progression regulates the pathfinding of pCTAs and opens perspectives on the role of timing in the formation of brain circuits.

RESULTS

pCTAs and CSAs Follow Distinct Trajectories within the Subpallium

To investigate the mechanisms controlling pCTA pathfinding, we first examined the precise localization of pCTAs and CSAs in the subpallium using axonal tracing experiments with carbocyanine dyes (Figure 1 and see Figure S1 available online). Axonal paths were characterized at embryonic day (E) 17.5, when TCAs have reached the neocortical subplate, pCTAs the thalamus, and CSAs the cerebral peduncle (McConnell et al., 1989, 1994; Molnár et al., 1998; Auladell et al., 2000; Jacobs et al., 2007). Hence, Dil injections in the presumptive somatosensory cortex and DiA injections in the thalamus labeled axons exiting and entering each of these structures (Figures 1A and 1B). While TCAs and pCTAs costained by DiA and Dil formed a compact tract in the corridor (López-Bendito et al., 2006), CSAs only labeled with Dil were positioned in the deep GP, visualized by Nkx2.1 immunostaining, and joined the cerebral peduncle ($n = 7$) (Figures 1A, 1B, and 1E). By performing additional tracing experiments from the thalamus and cerebral peduncle as well as from the internal capsule, we similarly found that pCTAs and CSAs have segregated trajectories in the corridor and GP, respectively (Figures 1C and S1). We furthermore examined globally the trajectory of

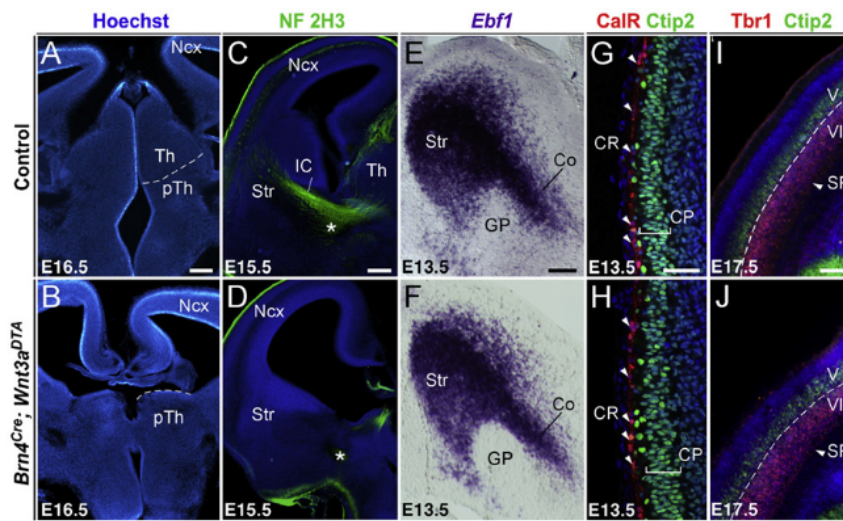


Figure 2. Genetic Ablation of the Thalamus in *Brm4^{Cre};Wnt3a^{DTA}* Embryos

(A and B) Hoechst counterstaining of E16.5 coronal sections showing the morphology of a wild-type thalamus (A) and its absence in *Brm4^{Cre};Wnt3a^{DTA}* embryos (B). Dotted line indicates the boundary between the thalamus and prethalamus.

(C and D) E15.5 coronal sections showing TCAs immunostained by Neurofilament 2H3 in control embryos (C) and TCA absence in *Brm4^{Cre};Wnt3a^{DTA}* embryos (D). Some low level 2H3-positive axons are detected superficially to TCAs (asterisks).

(E and F) *Ebf1* expression on E13.5 coronal sections is unaffected in the striatum and the corridor of thalamus-ablated embryos (F) compared to the controls (E).

(G and H) E13.5 sections of control (G) and thalamus-ablated (H) embryos showing Calretinin-positive Cajal-Retzius cells (white arrowheads) and Ctip2-positive cortical neurons.

(I and J) Tbr1 and Ctip2 immunohistochemistry at E17.5 showing neocortical layering in control (I) and thalamus-ablated (J) embryos. Dotted lines mark the border between layer V and VI.

Scale bars represent 250 μ m in (A)–(D), 100 μ m in (E), (F), (I), and (J), and 50 μ m in (G) and (H).

cortical axons by taking advantage of the cortex-specific *Emx1^{Cre}* mouse line (Gorski et al., 2002) backcrossed to the reporter line *Tau^{GFP}* (Bielle et al., 2005). Using a 45° plane of section that encompasses the entire projection, we consistently observed that: (1) subsets of cortical axons navigate in the corridor with TCAs, labeled by 2H3 neurofilament immunostaining, and (2) others form a less compact bundle in the Nkx2.1-expressing GP and extend into the cerebral peduncle (n = 5) (Figures 1D and 1D'). Taken together, our results show that somatosensory pCTAs and CSAs have distinct trajectories within the subpallium, with pCTAs navigating along the same route as TCAs in the corridor and CSAs growing in the deep GP (Figure 1F).

pCTAs Switch to a CSA-like Trajectory in the Absence of Thalamic Axons In Vivo

pCTAs navigate within the corridor, raising the possibility that corridor neurons may directly guide pCTAs or that TCAs may orient pCTA pathfinding into the corridor. To discriminate between these possibilities, we first performed a genetic ablation of the thalamus to assess its long-distance impact on the pathfinding of pCTAs within the subpallium. To this aim, we backcrossed *Wnt3a^{DTA}* mice (Yoshida et al., 2006) that conditionally express the subunit A of the diphtheria toxin (DTA) in the cortical hem and thalamus (Yoshida et al., 2006; Louvi et al., 2007) with *Brm4^{Cre}* mice that drive the expression of Cre recombinase in the embryonic neuroepithelium, albeit not efficiently in the cortical hem (Ahn et al., 2001; Heydemann et al., 2001). In *Brm4^{Cre};Wnt3a^{DTA}* embryos, complete thalamus ablation was detected by tissue loss and lack of thalamic molecular markers (Figures 2A, 2B, and S2 and data not shown), with a subsequent absence of TCAs (Figures 2C and 2D). Using specific markers, we found that the patterning of the prethalamus and the subpallium (*Ebf1*, *Islet1*, *Nkx2.1*, *Ctip2*, and *Foxp2*), as well as cortical lamination (Calretinin, *Tbr1*, *Ctip2*, and *ZFPM2*), were

not affected in thalamus-ablated embryos (n = 8) (Figures 2E–2J and S2 and data not shown). We next examined the subpallial trajectory of pCTAs in the absence of TCAs, using Dil labeling in the presumptive somatosensory neocortex and L1 immunostaining, which labels both cortical and thalamic axons (Figures 3A–3F and S3). We observed that corticofugal axons grew normally into the subpallium at E14.5 (n = 6) (Figures 3A, 3B, and S3), indicating that this event is independent of incoming TCAs. However, in E16.5 and E18.5 thalamus-ablated embryos, all the labeled corticofugal axons passed through the Nkx2.1-expressing GP and reached the cerebral peduncle (n_{E16.5} = 5 and n_{E18.5} = 4) (Figures 3D, 3D', 3F, and S3). This is in sharp contrast with the control situation in which pCTAs and TCAs navigate in the corridor and CSAs in the GP (Figures 3C, 3C', 3E, and S3). Our observations indicated that in thalamus-ablated embryos, pCTAs either do not grow into the subpallium or adopt an abnormal CSA-like trajectory. To discriminate between these possibilities, we backlabeled cortical neurons that had extended axons in the corridor or GP at E17.5 and E18.5 (Figures 3G and 3H and data not shown). In both controls and thalamus-ablated embryos, we labeled a large majority of neurons in the subplate and layer VI, as well as in layer V (n_{E17.5} = 4 and n_{E18.5} = 4) (Figures 3G and 3H and data not shown). Taken together, these observations showed that, in thalamus-ablated embryos, subplate and deep layer VI pCTAs are misguided along an alternative CSA-like trajectory (Figures 3I and 3J), in spite of their CTA-specific molecular identity (Figures 3H and S2). Our results thus reveal that corridor cells and subpallial structures are not sufficient to guide pCTAs and that the ablation of the thalamus has a distal impact on the trajectory of pCTAs in the subpallium.

TCAs Are Necessary to Guide pCTAs into the Corridor

Since our in vivo analysis strongly suggested that TCAs might guide pCTAs, we set up an ex vivo assay to directly test this hypothesis. We took advantage of the fact that TCAs grow along

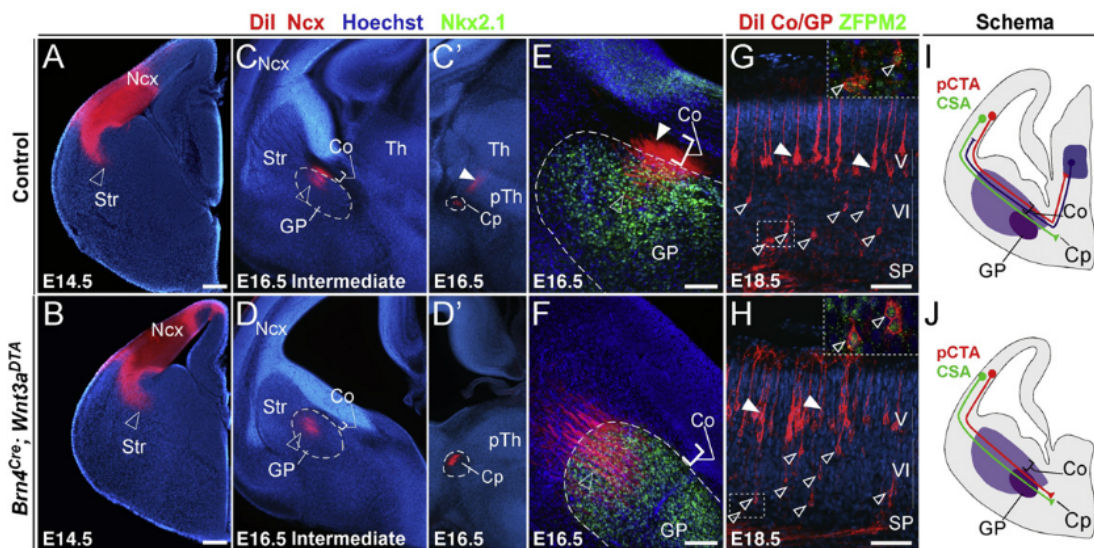


Figure 3. Pathfinding Defects of pCTAs in the Subpallium of Thalamus-Ablated Embryos

(A and B) Dil tracings from the presumptive somatosensory neocortex at E14.5 in control (A) and thalamus-ablated (B) embryos reveal a similar trajectory of corticofugal axons (arrowheads).

(C–F) E16.5 serial coronal sections of a control (C and C') and a thalamus-ablated (D and D') embryo after Dil crystal insertions in the presumptive somatosensory neocortex showing corticofugal axon pathway in the subpallium. In controls, corticofugal axons navigate in the corridor and GP (arrowhead in C) and grow toward the thalamus (solid arrowhead in C') or cerebral peduncle (Cp). In contrast, they all navigate through the GP (arrowhead in D) and into the Cp in mutant embryos (D'). Coimmunostaining with Nkx2.1 (E and F) ascertained the location of axons in the corridor (solid arrowhead) or in the GP (open arrowhead).

(G and H) Dil retrograde labeling from the corridor and GP at E18.5 similarly labeled neurons in layer V (solid arrowhead), layer VI, and subplate (open arrowheads) in both control (G) and thalamus-ablated (H) embryos. Insets show that backlabeled cells in layer VI are positive for ZFPM2 immunostaining (open arrowheads). (I and J) Schematic drawing representing the subpallial trajectory of pCTAs and CSAs in WT (I) and thalamus-ablated (J) embryos.

Scale bars represent 250 μ m in (A)–(D'), 100 μ m in (E) and (F), and 50 μ m in (G) and (H).

a 45° angle trajectory and are thus present in E13.5 caudal coronal slices but not yet present in E13.5 intermediate coronal slices (Figure 4A and data not shown). In such TCA-free intermediate slices, we grafted cortical explants from transgenic embryos ubiquitously expressing the GFP and found that *Gfp*-expressing corticofugal axons did not enter the corridor but massively grew into the GP ($n = 60/60$) (Figure 4B), as in thalamus-ablated embryos. Using Dil retrograde labeling of GFP-positive axons that had extended into the GP ($n = 4$) (Figure 4C), we found that these axons are mainly pCTAs, as they are generated by subplate and deep layer VI neurons (Figure 4D). Thus, ex vivo as in vivo, pCTAs adopt an alternate CSA-like trajectory in the absence of TCAs. To exclude that this behavior was due to ex vivo culture conditions, we performed 45° angle slices that contained TCAs and found that GFP-positive cortical axons grew normally both into the corridor to the thalamus and into the GP to the cerebral peduncle ($n = 12/12$) (Figures 4E and 4F).

To next determine whether TCAs are essential to guide pCTAs into the corridor, we performed grafts of thalamic explants harvested from *tdTomato*-expressing embryos or from control embryos and labeled with Dil (Figure 4G). When thalamic explants were grafted at the ventral tip of the corridor and generated TCAs, we found GFP-positive pCTAs growing in the Islet1-positive corridor, in vicinity of TCAs ($n = 29/37$) (Figures 4I and 4J). This is in striking contrast to control incisions in which pCTAs did not grow in the corridor and directly entered the GP ($n = 37/37$) (Figure 4H). This difference in cortical axonal trajectory was

very robust, since we found that pCTAs enter the Islet1-expressing corridor in 78% of slices in which thalamic explants were grafted and extended TCAs ($n = 29/37$) (Figure 4L). Finally, when thalamic explants were grafted in the lateral cortex, they did not induce a deviation of pCTA trajectory ($n = 10$) (data not shown). This observation indicates that thalamic explants do not produce a diffusible long-range signal but rather act on cortical axon pathfinding via a local activity of thalamic axons. Consistent with this finding, high magnification of rescue experiments showed that TCAs and CTAs, although not tightly fasciculated, grow in close vicinity within the corridor (Figure 4K). Overall, our data show that the presence of TCAs inside the corridor is both necessary to guide pCTAs and sufficient to prevent them from undertaking an alternate CSA-like trajectory.

Waiting Period of Cortical Axons Coordinates Their Progression with TCAs

Our results raised the intriguing question of how pCTA and TCA progression is coordinated to ensure the proper pathfinding of pCTAs. To determine the underlying mechanisms, we re-examined the precise timing of cortical and thalamic axon progression by implantation of Dil and DiA crystals in the presumptive somatosensory cortex or thalamus, respectively (Figure 5). Consistent with previous studies (McConnell et al., 1989, 1994; Mélin and Godement, 1996; Molnár et al., 1998; Auladell et al., 2000; Bellion et al., 2003; Jacobs et al., 2007), we found that pioneer corticofugal axons exited the cortex and, between E13.5 and

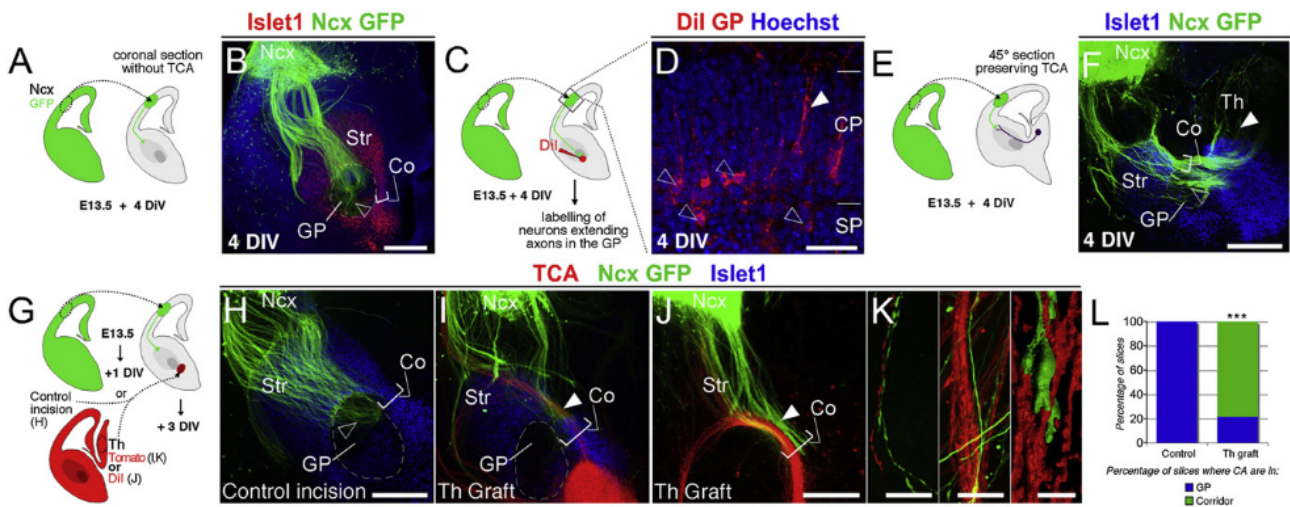


Figure 4. TCAs Are Necessary to Guide pCTAs in the Subpallium

(A) Experimental paradigm to follow ex vivo the behavior of corticofugal axons in the absence of TCAs. (B) *Gfp*-expressing cortical explants were homotopically grafted in coronal wild-type slices. After 4 days in vitro (DIV), corticofugal axons massively invaded the GP (open arrowhead), visualized by lack of Islet1-immunostaining (dotted lines). (C) Experimental paradigm to test the neuronal origin of axons reaching the GP after 4 DIV. (D) Dil injection in the GP of slices cultured for 4 DIV backlabeled within the GFP-positive neocortex grafted a vast majority of subplate and deep layer VI neurons that give rise to pCTAs (open arrowheads) and very few layer V neurons (solid arrowhead). (E) Experimental paradigm to follow ex vivo the behavior of corticofugal axons into 45° angle slices (between coronal and sagittal planes), which contain the thalamus and TCAs. (F) *Gfp*-expressing cortical explants were homotopically grafted into 45° angle slices (between coronal and sagittal planes) and cultured for 4 DIV. Some corticofugal axons entered the Islet1-negative GP (open arrowhead) and some reached the thalamus (solid arrowhead). (G) Experimental paradigm to test the role of TCAs in pCTA pathfinding. (H–J) Coronal slices grafted with *Gfp*-expressing cortical explants were cultured for 1 DIV and processed for a control incision at the ventral tip of the corridor (H) or grafts of thalamic explants either expressing *tdTomato* (I) or labeled with Dil (J). After 2 additional DIV, GFP-positive axons entered the GP (open arrowhead) in incised controls, whereas their trajectory was rescued into the Islet1-positive corridor (solid arrowhead) in the presence of TCAs. (K) High magnifications of rescue experiments showing that axons are close but not in full contact. Right: a three-dimensional reconstruction of axonal surfaces (Bitplane Imapris software) shows an example of physical interactions between TCAs and the growth cone of a corticofugal axon. (L) Quantification of the cases in which pCTAs were located in the GP or in the Islet1-positive corridor in TCA-free controls or in the presence of TCAs. Chi-square test, $p < 0.0001$. Scale bars represent 250 μm in (B)–(D) and (H)–(J), 50 μm in (F) and (K), and 5 μm in the right panel in (K).

E14.5, paused in the adjacent lateral part of the subpallium or lateral striatum (Figures 5A and 5B). After this waiting period, corticofugal axons progressed into the subpallium and Dil backlabeling from the internal capsule indicated that subplate pCTAs progressed by E15, followed by layer V CSAs at E17.5 (Figure S4). In contrast to the waiting behavior of cortical axons, between E13.5 and E14.5, TCAs progressed through the subpallium to reach the lateral striatum (Figures 5C–5F). Thus, pCTAs enter the subpallium at least a day before TCAs and halt their progression during a waiting period that allows TCAs to reach their location. Since TCAs guide pCTAs, these findings reveal a major role for the waiting period in pioneer cortical axon pathfinding.

To determine whether this waiting period is regulated by local signals, we performed ex vivo experiments in coronal slices and found that *Gfp*-expressing cortical axons pause for a day similarly as they do in vivo (Figures 5G–5I). The waiting period is thus probably controlled by dynamic changes in either corticofugal axons or subpallial cells. Dynamic changes in cortical neurons between E13.5 and E15.5 were revealed by a refinement of their molecular identity as well as by a modification of their

axon guidance properties. Indeed, we found that the expression of the transcription factors *Tbr1* and *Ctip2*, which are essential for defining the identity and pathfinding of CTAs and CSAs, respectively (Hevner et al., 2002; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011), are initially coexpressed at high levels at E13.5 and become progressively restricted to each neuronal cell type (Figure S4). More importantly, when we performed heterochronic grafts of E13.5 to E15.5 cortical explants into E13.5 wild-type host slices, we observed that cortical axons from older explants do not pause when confronted with a younger subpallium ($n = 10/11$) (Figures 5J and 5K' and data not shown). Taken together, these results indicate that the waiting period of pioneer corticofugal axons, which is required for their "encounter" with TCAs, is regulated by temporal modifications in cortical neuron properties.

PlexinD1 and Sema3E Are Candidate Factors for the Waiting Period

To characterize the molecular cues controlling this mandatory waiting period, we searched for candidate receptors transiently expressed by corticofugal neurons. We particularly examined

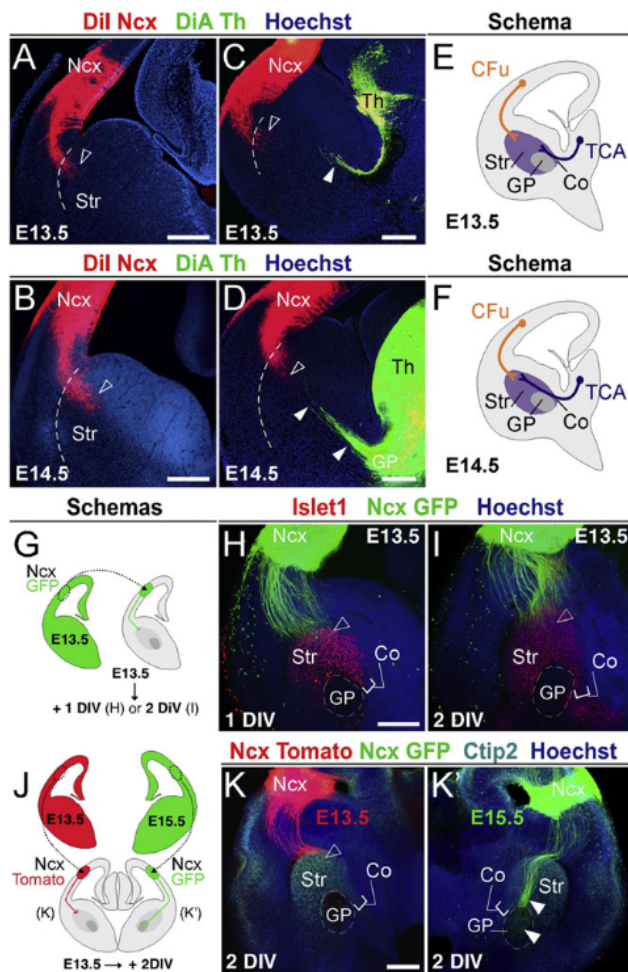


Figure 5. Waiting Period of Cortical Axons Coordinates pCTAs and TCA Progression

(A and B) Dil tracings from the presumptive somatosensory neocortex show that corticofugal axons (CFu) (open arrowheads) stall in the lateral striatum between E13.5 (A) and E14.5 (B). Dotted lines mark the boundary between the cerebral cortex or pallium and the subpallium.

(C and D) DiI/DiA tracings from the presumptive somatosensory cortex and the thalamus in 45° slices at E13.5 (C) and E14.5 (D) indicate that during this time period, TCAs (solid arrowheads) progress up to the pallium/subpallium boundary (dotted lines).

(E and F) Schematic drawings showing the position of CFu at the pallium/subpallial boundary and TCAs crossing the subpallium between E13.5 (E) and E14.5 (F).

(G) Experimental paradigm to follow ex vivo the behavior of E13.5 corticofugal axons.

(H and I) Grafts of *Gfp*-expressing cortical explants into coronal wild-type slices indicate that corticofugal axons (open arrowheads) stall in the Islet1-positive lateral striatum between 1 and 2 DIV.

(J) Experimental paradigm to compare ex vivo the behavior of E13.5 and E15.5 corticofugal axons in E13.5 host slices.

(K and K') Homochronic and heterochronic grafts of E13.5 *Tomato*-expressing and E15.5 *Gfp*-expressing cortical explants into an E13.5 host slice show that E13.5 corticofugal axons pause (open arrowhead), whereas E15.5 axons directly progress into the GP (solid arrowheads).

Scale bars represent 250 μm.

Plexin and Neuropilin receptors, as they have been involved in the regulation of waiting periods in other systems (Huettl et al., 2011). Using this strategy, we focused on PlexinD1, a member of the plexin family that has been shown to bind directly to its ligands, including the secreted semaphorin 3E (Sema3E) (Torres-Vázquez et al., 2004; Gu et al., 2005). *PlexinD1* expression was detected from E15.5 in the striatum and the lateral cortex (including piriform and insular cortex) but not in the dorsal neocortex (Chauvet et al., 2007). In addition, while *PlexinD1* and *Sema3E* inactivation have been reported to perturb the late development of some *PlexinD1*-expressing axons (Chauvet et al., 2007), their early expression or phenotype had not been described. We found that *PlexinD1* is transiently highly expressed in the preplate and subplate during the waiting period at E13.5 and E14.5 (Figures 6A–6D) and *PlexinD1* protein is detected on corticofugal neurons labeled by *Tbr1* and *Ctip2* coimmunostainings (Figures 6E). From E15.5 onward, *PlexinD1* expression is maintained in the lateral cortex while it is downregulated in the neocortex (Figures 6C and 6D). Consistent with this observation, *PlexinD1* protein could not be detected on corticofugal axons after E15.5 (Figure 6F). Since *PlexinD1* was shown to mediate either repulsion alone or attraction when coexpressed with Neuropilin1 and VEGFR2 (Chauvet et al., 2007; Bellon et al., 2010), we examined the expression patterns of these putative coreceptors. We found that neither Neuropilin1 nor VEGFR2 is present on corticofugal axons during the waiting period (Figures 6G and 6H), suggesting that *PlexinD1* could mediate a repulsive or growth-inhibiting signal in early corticofugal axons. Consistent with this finding, we found that *Sema3E* is expressed in the radial glia of the striatum and in the GP (Figure 6I) and could thus act on cortical axons as they enter the subpallium. To characterize the activity of *Sema3E* on cortical axons, we performed collapse assays on dissociated neurons prepared at E13.5 or E15.5 from either the dorsal neocortex, which downregulates *PlexinD1* expression, or from the lateral cortex, which maintains *PlexinD1* expression (Figure 6J). While lateral cortical neurons collapsed at E13.5 and E15.5 when exposed to *Sema3E*, neocortical neurons massively collapsed at E13.5 but showed no significant response at E15.5 (Figures 6K–6N). These results indicate that *PlexinD1*/*Sema3E* signaling mediated by transient expression of *PlexinD1* is a prime candidate for the regulation of the waiting period of corticofugal axons.

Sema3E/PlexinD1 Signaling Controls the Waiting Period and pCTA Pathfinding

To investigate the role of *Sema3E*/*PlexinD1* signaling in the waiting period, we examined both *Sema3E*^{-/-} mutant embryos (Gu et al., 2005) and *Emx1*^{Cre};*PlexinD1*^{D/flox} mouse embryos (Gorski et al., 2002; Pecho-Vrieseling et al., 2009; Zhang et al., 2009), which carry a cortex-specific deletion of the *PlexinD1* gene (Figure S5). We first checked using Dil labeling, immunohistochemistry, and in situ hybridization that the growth and pathfinding of TCAs, the patterning of the subpallium (*Ctip2*, *Nkx2.1*, *Ebf1*, and *Islet1*), as well as the layering and maturation of the neocortex (Calretinin, *Ctip2*, *Foxp2*, *Tbr1*, and *ZFPM2*) were not affected by either *Sema3E* or *PlexinD1* cortical inactivation (n = 9 for each genotype) (Figure S6 and data not shown). We next

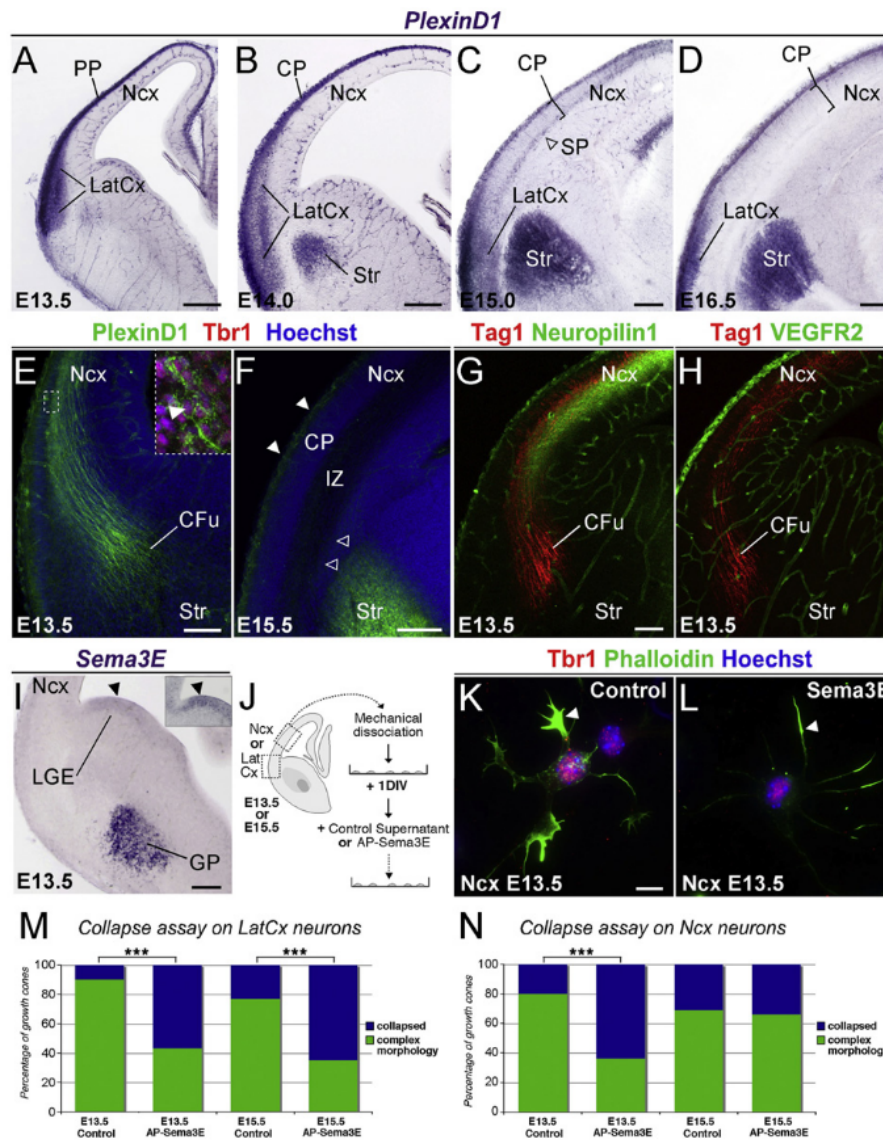


Figure 6. Transient Cortical Expression of PlexinD1 Induces Sema3E-Dependent Collapse

(A–D) Coronal sections of wild-type brains showing transient mRNA expression of *PlexinD1* in the dorsal neocortex preplate at E13.5 (A), cortical plate at E14.0 (B), subplate at E15.0 (arrowhead) (C), and only marginal zone at E16.5 (D). In contrast, *PlexinD1* expression is observed at all stages in the lateral cortex (insular piriform and perirhinal).

(E and F) *PlexinD1* immunostainings on E13.5 (E) and E15.5 (F) coronal sections show that corticofugal axons are transiently labeled. Inset in (E) indicates that E13.5 *Tbr1*-positive preplate neurons coexpress *PlexinD1*. At E15.5, *PlexinD1* immunolabeling is detected in the marginal zone (solid arrowheads) and striatum but not in corticofugal axons (open arrowheads).

(G and H) E13.5 coronal sections indicating that *Tag1*-positive corticofugal axons do not coexpress *Neuropilin1* (G) or *VEGFR2* (H).

(I) E13.5 coronal section showing *Sema3E* expression in the ventricular zone of the LGE (solid arrowhead and inset) and in the GP.

(J) Experimental procedure to test the activity of *Sema3E* on E13.5 and E15.5 cortical neurons from either the dorsal neocortex or the lateral cortex.

(K and L) Collapse assays on E13.5 dissociated dorsal neocortical neurons identified by *Tbr1* immunostaining in the presence of the control supernatant (K) and AP-*Sema3E* (L). Phalloidin staining shows the complex morphology of growth cones (K) in the control situation or the collapsed morphology (L) in the presence of *Sema3E* (solid arrowheads).

(M–N) Quantification indicating the percentage of growth cones collapsing in response to control supernatant or AP-*Sema3E*. Neurons coming from the lateral cortex (M) at E13.5 and E15.5 collapse in the presence of AP-*Sema3E*, whereas neurons from the dorsal neocortex (N) collapse at E13.5 in response to AP-*Sema3E* and lose their ability to respond at E15.5. Chi-square test, *** $p < 0.0001$ (E13.5 lateral cortex: $n_{\text{control}} = 209$ and 10% of collapsed growth cones/ $n_{\text{Sema3E}} = 146$ and 57% of collapsed growth cones; E15.5 lateral cortex: $n_{\text{control}} = 143$ and 23% of collapsed growth cones/ $n_{\text{Sema3E}} = 179$ and 65% of collapsed growth cones; E13.5 neocortical neurons: $n_{\text{control}} = 168$ and 20% of collapsed growth cones/ $n_{\text{Sema3E}} = 162$ and 64% of collapsed growth cones; E15.5 neocortical neurons $n_{\text{control}} = 214$ and 31% of collapsed growth cones/ $n_{\text{Sema3E}} = 189$ and 34% of collapsed growth cones).

Scale bars represent 100 μm in (A)–(I) and 10 μm in (K) and (L).

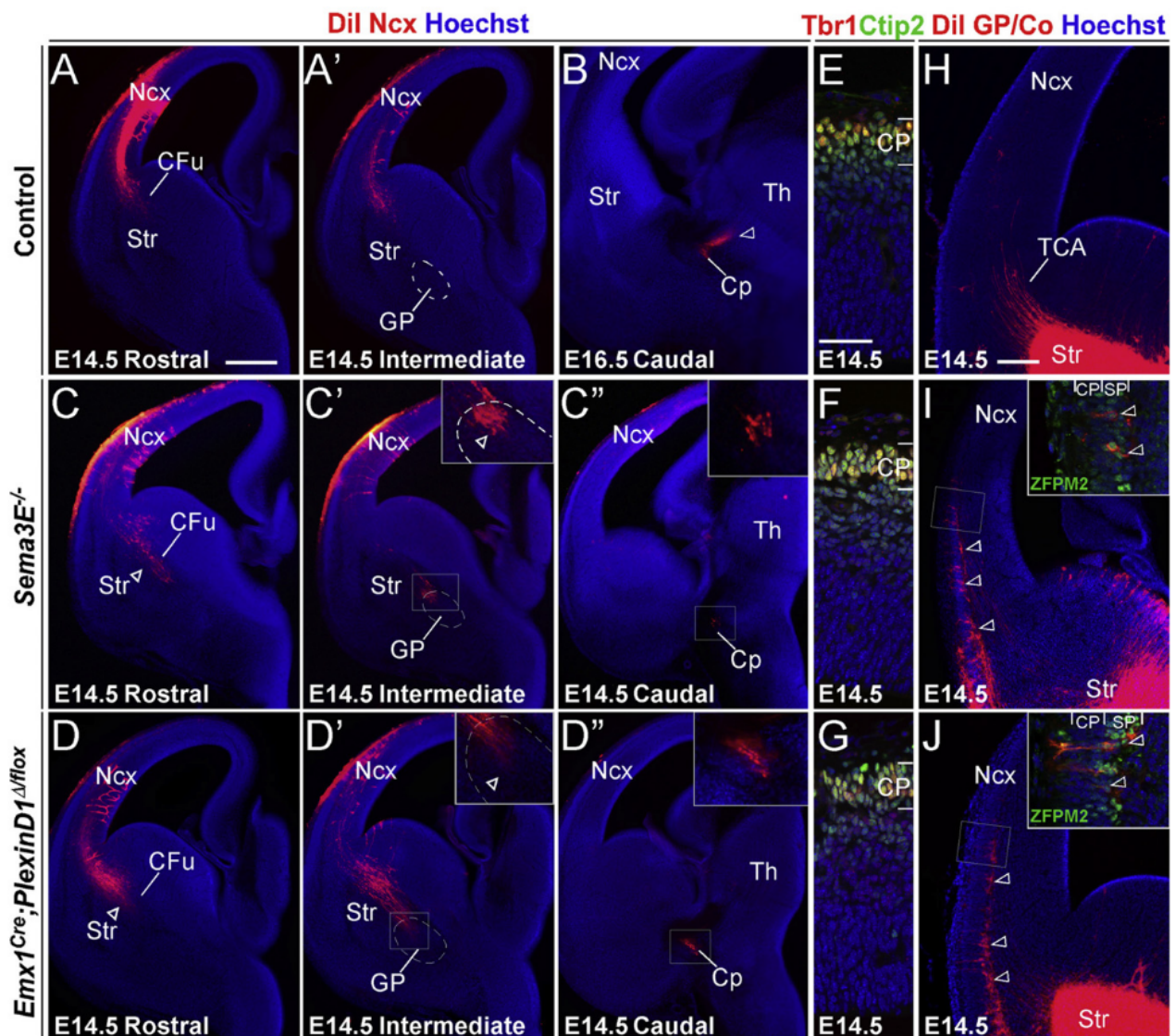


Figure 7. Premature and Abnormal Growth of pCTAs in *Sema3E*^{-/-} and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} Embryos

(A–D'') Serial coronal sections at E14.5 or E16.5 (B) after Dil crystal insertions in the presumptive somatosensory neocortex of wild-type (A, A', and B), *Sema3E*^{-/-} (C–C''), and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} (D–D'') embryos. In wild-type brains, corticofugal axons paused at E14.5 and reached the cerebral peduncle (Cp) and thalamus (Th) (open arrowhead in B) at E16.5. However, in E14.5 *Sema3E*^{-/-} and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} embryos, corticofugal axons have crossed the striatum (open arrowheads in C and D) and GP (open arrowheads in the insets in C' and D') and already reached the Cp (insets in C'' and D'').

(E–G) Double immunocytochemistry for Tbr1 and Ctip2 on E14.5 coronal sections showing similar coexpression in controls (E), *Sema3E*^{-/-} (F), and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} (G) embryos.

(H–J) E14.5 coronal sections showing retrogradely labeled cells in the neocortex after Dil crystal insertions in the globus pallidus and the corridor of wild-type (H), *Sema3E*^{-/-} (I), and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} (J) embryos. Open arrowheads show the presence of backlabeled subplate neurons identified by ZFPM2 coimmunolabeling (insets) only in *Sema3E*^{-/-} (I) and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} (J) embryos.

Scale bars represent 500 μm in (A)–(D''), 50 μm in (E)–(G), and 250 μm in (I)–(J).

examined the early pathfinding of corticofugal axons using Dil injections in the presumptive somatosensory neocortex (Figures 7A–7D''). In contrast to control embryos in which corticofugal axons paused at E13.5 and E14.5 in the lateral striatum (Figures 5A–5F, 7A, and 7A'), corticofugal axons were detected as early as E14.5 in the GP and cerebral peduncle of both *Sema3E*^{-/-}

and *Emx1*^{Cre};*PlexinD1*^{Δ/flox} embryos (n = 10 for *Sema3E*^{-/-} and n = 6 for *Emx1*^{Cre};*PlexinD1*^{Δ/flox}) (Figures 7C–7D''). Using immunohistochemistry and Dil backlabeling from the GP, we found that these axons belonged to neurons located in the subplate and layer VI, which expressed the corticothalamic-specific gene ZFPM2 (n = 3 for *Sema3E*^{-/-} and n = 6 for *Emx1*^{Cre};

Thalamic Axons Guide Pioneer Corticothalamic Projections

From the early observation that TCAs grow in the cortex along a scaffold of subplate axons, Molnár and Blackmore proposed the handshake hypothesis (Molnár and Blackmore, 1995). Reciprocal TCAs and CTAs appeared closely intermingled throughout the internal capsule (Molnár et al., 1998), raising the possibility that these two sets of axons guide each other. Several analyses of mutants affecting TCA pathfinding, including *Gbx2* and *Mash1* mutants (Tuttle et al., 1999; Hevner et al., 2002), showed defects in corticofugal tracts, thereby bringing support to the handshake hypothesis. Furthermore, it has been recently reported that mutants perturbing axonal fasciculation in the internal capsule generally affect corticofugal pathfinding (Wu et al., 2010). However, these analyses remain inconclusive as the observed defects could be due to subpallial abnormalities. In addition, in vitro studies revealed that thalamic and cortical axons repel each other (Bagnard et al., 2001). Since the navigation of TCAs through the subpallium is known to be independent of CTAs and relies on the presence of guidepost corridor neurons (López-Bendito et al., 2006), it has been proposed that corticofugal axons might be directly guided by subpallial cues. To directly investigate the mechanisms governing CTA navigation, we have focused on pioneer axons of the somatosensory cortex and revealed that pCTAs and CSAs have distinct trajectories in the subpallium: they grow predominantly through the corridor and the GP, respectively. Phenotypic analysis of *Bra4^{Cre};Wnt3a^{DTA}* embryos showed that in absence of the thalamus, pCTAs adopt a CSA-like trajectory at a long distance from the thalamus, within the subpallium. These observations revealed that subpallial guideposts, such as corridor cells, are not sufficient to guide pCTAs. They furthermore suggested that either the thalamus or thalamic axons guide pCTAs. Using an ex vivo slice assay, we have consistently found that thalamic explants do not attract cortical axons when grafted in the lateral cortex (data not shown) or when confronted with cortical explants (data not shown). Similarly, when grafted thalamic explants did not extend axons into the corridor of host slices, they were unable to rescue the trajectory of cortical axons (data not shown). Thus, our findings are not supportive of a long-range trophic or chemotactic activity of the thalamus on pCTA trajectory. They instead indicate that TCAs within the corridor are required to open a corticothalamic path. At cellular resolution, we found that TCAs and pCTAs navigate closely, although they do not tightly fasciculate. These results are consistent with the in vitro observations that TCAs may not act as an axonal substrate for pCTAs (Bagnard et al., 2001; Torii and Levitt, 2005). They rather indicate that either TCAs induce a modification in the local subpallial environment that in turn acts on pCTAs or TCAs provide a track for cortical axons, similarly to what has been described during the formation of peripheral motor and sensory nerves (Huetl et al., 2011; Wang et al., 2011). While further experiments will be required to investigate these issues, our findings are consistent with apparently previous contradictory studies (Molnár and Blackmore, 1995; Bagnard et al., 2001; Torii and Levitt, 2005; Chen et al., 2012). Remarkably, our work reveals that the formation of reciprocal connections between the thalamus and cortex relies on a timing

of sequential events: the migration of conserved subpallial guidepost cells defines the trajectory of TCAs (López-Bendito et al., 2006; Bielle et al., 2011a, 2011b), which in turn guide reciprocal pCTAs. This later process ensures a developmental robustness in the establishment of reciprocal projections between the thalamus and cortex, even in cases of abnormal pathfinding of TCAs (Tuttle et al., 1999; Hevner et al., 2002). Our findings furthermore open the intriguing possibility that such a guidance mechanism may more generally govern the formation of reciprocal connections in the brain.

Waiting Period Is Regulated by PlexinD1/Sema3E Signaling

Studies over the past decades have revealed the central role of families of guidance cues in the spatial control of neural circuit formation. In particular, PlexinD1, an atypical member of the Plexin superfamily that directly binds to Sema3E, controls axonal pathfinding (Chauvet et al., 2007; Bellon et al., 2010) and synaptogenesis (Pecho-Vrieseling et al., 2009; Ding et al., 2012) in major neuronal networks. In this study, we show that the dynamic changes in Sema3E/PlexinD1 signaling in cortical neurons controls their waiting period (Figures 6 and 7), without apparently affecting their neuronal identity (Figure S6 and data not shown). Indeed, PlexinD1 signaling in cortical axons induces a growth inhibition effect in response to Sema3E, which is expressed by the radial glia of the striatum and by the GP. In addition, the full inactivation of *Sema3E* or the cortex-specific inactivation of *PlexinD1* disrupted the waiting period. The premature growth of cortical axons in both mutants occurs while TCAs are still crossing the subpallium (Figure S6), contributing to an abnormal trajectory of pCTAs along a CSA-like trajectory. This difference in trajectory is not due to a change in *Sema3E* activity in the GP, since a similar pathfinding defect is observed in thalamus-ablated embryos in which *PlexinD1* and *Sema3E* are normally expressed (data not shown). Finally, these early defects in pCTA pathfinding impact on the axonal trajectory at birth, since at least some CTAs still follow a CSA-like trajectory. Taken together, these results indicate that PlexinD1/Sema3E signaling regulates the spatial pathfinding of pioneer cortical axons by modulating the timing of their progression. Our study thus reveals that the temporal regulation of a guidance cue receptor controls a major checkpoint and thereby participates in the pathfinding of reciprocal projections between the thalamus and the neocortex.

Waiting Period Is Essential for Pioneer Corticothalamic Axon Pathfinding

Assembly of neural circuits requires a precise spatial organization as well as a specific timing. While the spatial control of axon guidance and network formation has been well described, much less is known about the temporal regulation. Waiting periods of growing axons have been observed in several systems (Tosney and Landmesser, 1985; Sharma et al., 1994; Wang and Scott, 2000; Bloom et al., 2007) and have been suggested to let time for the maturation of axonal targets. However, the exact functions and regulatory mechanisms of waiting periods are largely uncharacterized. Here, we report that pCTA waiting period participates in two related but distinct processes.

On the one hand, it allows TCAs to reach the lateral subpallium, a mandatory event for the correct pathfinding of pCTAs. On the other hand, it enables corticofugal neurons to complete their final maturation and specifically express transcription factors that are required for CTA and CSA pathfinding, such as *Tbr1* and *Ctip2* (Han et al., 2011; McKenna et al., 2011). Indeed, *Ctip2* and *Tbr1* are initially coexpressed in waiting cortical neurons and become progressively downregulated in pCTAs and CSAs, respectively. This transcriptional maturation occurs independently of TCAs (Figures 2J and S2), as well as in vitro in dissociated cortical neurons (data not shown), which is consistent with a cortex-intrinsic program. High levels of *Ctip2* or *Tbr1* are probably important in governing the expression of intrinsic axonal properties essential for the guidance of distinct corticofugal axons (Han et al., 2011; McKenna et al., 2011). Consistently, in *Tbr1* mutant, all corticofugal axons express high levels of *Ctip2*, adopt a layer V identity, and follow a CSA trajectory even in the presence of TCAs (Hevner et al., 2002; Bedogni et al., 2010). In this context, our analysis intriguingly reveals that pCTAs have the capacity to follow a default CSA-like trajectory in the absence of TCAs but also have a specific property to respond to the presence of TCAs. The waiting period is thus a central temporal checkpoint, as it orchestrates the timing of two major events: the intrinsic maturation of cortical neurons, including the acquisition of specific axonal properties in CTAs, and the extrinsic arrival of reciprocal TCAs. Our study reveals the functional relevance of timing in the navigation of a major reciprocal projection of the mammalian brain and provides insights on the function of waiting periods in the precise wiring of neural networks.

EXPERIMENTAL PROCEDURES

Mouse Lines

The different mouse lines used in this study are described in detail in the Supplemental Experimental Procedures. The day of vaginal plug was considered as E0.5. Animals were kept under French and EU regulation.

In Situ Hybridization, Immunohistochemistry, and Axonal Tracing

For in situ hybridization, mouse brains were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. We hybridized 100 μ m free-floating vibratome sections as described before (López-Bendito et al., 2006) with the following digoxigenin-labeled probes: *Sema3E* and *PlexinD1* (Chauvet et al., 2007) and *Ebf1* (Garel et al., 1997). For axonal tracing, embryonic brains were fixed at least overnight at 4°C and cultured slices for 30 min in 4% PFA at room temperature (RT). Small crystals of Dil (1,1'-dioctadecyl 3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes) or DiA (4,4-dihexadecyl aminostyryl N-methyl-pyridinium iodide; Molecular Probes) were inserted into the thalamus, the cerebral cortex, the internal capsule, and the GP or the cerebral peduncle after hemidissection of the brains or into cultured slices and let diffuse at 37°C (from 2 days to up to a month). Status of dye diffusion was assessed by whole-brain or whole-explant examination under a fluorescent binocular set up (Leica MZ16 F). Subsequently, brains were cut into 100- μ m-thick vibratome sections and tracing specificity was systematically checked after diffusion on serial sections adjacent to the site of crystal insertion. Some sections were processed for immunohistochemistry as described below.

For immunohistochemistry, cultured slices/explants and embryos were fixed in 4% PFA at 4°C for 30 min and for 4–12 hr, respectively. Immunohistochemistry was performed on culture slices or 80–100 μ m free-floating vibratome sections. The slices were incubated 1 hr at RT in a blocking solution adapted to the primary antibody used (see Supplemental Experimental Proce-

dures) and incubated in the same blocking solution with primary antibodies overnight at 4°C. Sections were rinsed several times in PBS and incubated overnight at 4°C with the secondary antibodies. Details on the primary and secondary antibodies used in this study are described in the Supplemental Experimental Procedures. For immunohistochemistry on slices containing Dil staining, primary antibodies were used in a blocking solution adapted from Matsubayashi et al. (2008) and contained 3% normal goat serum, 2% DMSO, and digitonin 1mg/ml (Calbiochem). Hoechst (Sigma) was used for fluorescent nuclear counterstaining.

Slice Culture Experiments

Organotypic slice cultures of the embryonic mouse were prepared as previously described (López-Bendito et al., 2006). Host slices were cut along a 45° section plane (between sagittal and coronal), which encompass the trajectory of TCAs, or along a coronal plane and only intermediate telencephalic slices were selected because they consistently lacked TCAs. Brain slices were cultured on polycarbonate culture membranes (8 μ m pore size; Whatman) or on PET cell inserts (1 μ m pore size; Beckton-Dickinson) in organ tissue dishes containing 1.8 ml of medium BME/HBSS (Invitrogen) supplemented with 20 mM glucose, 1 mM glutamine, 5% horse serum, and 1 mM penicillin/streptavidin (Invitrogen). All explants were cut into cubes of approximately 250 μ m hedge length. Cortical explants from *Gfp*-expressing or *tdTomato*-expressing mice (Hadjantonakis et al., 1998; Muzumdar et al., 2007) were grafted at 0 days in vitro (DIV) and thalamus explants from wild-type or *tdTomato*-expressing mice were grafted at 1 DIV and slices were cultured for 1, 2, or 4 DIV, depending on the experiment.

Collapse Assays

Dissociated cultures from E13.5 or E15.5 neocortex and lateral cortex were performed as described in Chauvet et al. (2007). After 24–36 hr of culture, neurons were incubated with control or 10 nM AP-Sema3E supernatant for 30 min at 37°C, fixed, immunostained with mouse anti-tubulin antibody (1/2,000, Sigma-Aldrich), and labeled with Texas Red-X Phalloidin (1/40, Invitrogen) to analyze growth cone morphologies. Production of mouse AP-Sema3E was performed as described in Chauvet et al. (2007) and collapsed growth cones were scored as in Castellani et al. (2000).

Image Acquisition and Analysis

Images were acquired with fluorescence binocular microscope (Leica MZ16 F), fluorescence microscope (Leica DM5000 B), and confocal microscopes (Leica TCS SP2AOPS and TCS SP5). Image analyses were performed with ImageJ, Bitplane Imaris, and Adobe Photoshop software. The chi-square test was used to determine statistical significance of results expressed as percentages of slices or axons.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2012.11.031>.

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