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Tangential Neuronal Migration Controls Axon Guidance: A Role for Neuregulin-1 in Thalamocortical Axon Navigation

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SUMMARY

Neuronal migration and axon guidance constitute fundamental processes in brain development that are generally studied independently. Although both share common mechanisms of cell biology and biochemistry, little is known about their coordinated integration in the formation of neural circuits. Here we show that the development of the thalamocortical projection, one of the most prominent tracts in the mammalian brain, depends on the early tangential migration of a population of neurons derived from the ventral telencephalon. This tangential migration contributes to the establishment of a permissive corridor that is essential for thalamocortical axon pathfinding. Our results also demonstrate that in this process two different products of the *Neuregulin-1* gene, CRD-NRG1 and Ig-NRG1, mediate the guidance of thalamocortical axons. These results show that neuronal tangential migration constitutes a novel mechanism to control the timely arrangement of guidance cues required for axonal tract formation in the mammalian brain.

INTRODUCTION

The neural assembly underlying the formation of functional networks in the central nervous system (CNS) is probably the most complex biological system in vertebrates. Ultimately, brain function depends on the ability of specific populations of neurons to connect with a restricted number of appropriate synaptic partners among an astonishing number of undesired targets. This pattern of connections, which is highly reproducible among different individuals of the same species, is established during development through a series of consecutive events. This program begins with the process of neural induction and the differentiation of distinct classes of neurons from progenitor cells (Jessell, 2000). Once distinct neuronal populations have been generated, immature neurons migrate from progenitor regions to more superficial positions of the neural tube, where axonal connections eventually occur (Hatten, 2002). Neurons then extend axons, which navigate through the developing brain following highly stereotyped routes to find specific targets (Tessier-Lavigne and Goodman, 1996). Finally, refinement of axonal terminals shapes the pattern of synaptic connections that will ultimately imprint the behaviors of the adult organism (Benson et al., 2000). Somewhat surprisingly, these different events are normally analyzed as independent processes, although it is evident that they must have been efficiently linked through evolution to ensure the precise formation of neural circuits.

Axons are guided along specific pathways by guidance molecules positioned in the extracellular environment (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). They navigate following a series of distinct steps in which specific guidance cues located at defined decision points determine their direction. In axon pathfinding, therefore, not only guidance factors are important; the precise distribution of guidance molecules in time and space constitutes an essential part of the process. Much progress has been made during the past ten years in the identification of molecules controlling growth cone guidance (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). In contrast, our understanding of the mechanisms controlling the precise timing and arrangement of guidance cues is much more limited. Patterning mechanisms contribute to ensure that growth cones find their way in the brain by inducing the expression of appropriate sets of guidance molecules in specific groups of neuroepithelial cells. In addition to neuroepithelial cells, postmitotic neurons also contribute to display guidance information as the brain

develops. Patterning information—and therefore the expression of guidance cues—is transferred from neural progenitors to postmitotic cells through the process of radial migration, in which neurons that are born nearby occupy adjacent locations in the mantle (Rakic, 1988). Thus, radial migration contributes to the arrangement of guidance cues for axon guidance by faithfully conveying patterning information from progenitors to postmitotic neurons.

Tangential migration represents a second general mechanism of neuronal translocation in the developing CNS (Hatten, 2002). This mode of migration is a primitive trait of the vertebrate brain, and it is thought to have evolved as a mechanism to increase the complexity of neuronal circuits because it allows neurons born from distant progenitor zones to intermingle in a final common destination (Corbin et al., 2001; Marín and Rubenstein, 2001). The ability of tangential migration to supply distinct regions of the nervous system with *immigrant* neurons raises the intriguing question of whether this mode of migration may contribute to axonal pathfinding by providing with novel guidance cues for growing axons. Tangential migration occurs extensively throughout the nervous system but is more prominent in the ventral telencephalon, through which various major axonal tracts, such as the thalamocortical connection, traverse. Thalamocortical projections constitute one of the most prominent higher-level processing connections in the mammalian brain. Thalamocortical axons (TCAs) convey sensory and motor inputs to the cerebral cortex, where integration of this information leads to perception and the organization of appropriate responses. The functional complexity of the thalamocortical projection is the consequence of an extremely elaborate process of axon guidance, orderly linking the various thalamic nuclei with specific cortical regions.

Here we provide evidence for a novel mechanism of axon guidance by demonstrating that the tangential migration of a specific neuronal population is essential for the normal guidance of thalamocortical projections. These neurons, which we have designated corridor cells, migrate tangentially within the ventral telencephalon to generate an intermediate target for TCAs, forming a permissive bridge through an otherwise nonpermissive territory for the growth of TCAs. Extension of TCAs through this permissive corridor also requires the existence of axon-growth-promoting factors generated in the developing cortex. The molecular basis for this novel guidance mechanism relies on different forms of the *Neuregulin-1* (*Nrg1*) gene and their ErbB4 receptor, which coordinately represent the first signaling system identified to mediate the role of tangentially migrating corridor cells in the guidance of thalamocortical projections.

RESULTS

TCAs Navigate through a Corridor Generated by Tangential Migration

TCAs follow a highly stereotyped pathway from their origin in the dorsal thalamus to their final target, the cerebral cortex (López-Bendito and Molnár, 2003; Garel and Rubenstein, 2004). They run rostrally toward the telencephalon, make a sharp turn dorsally to enter the mantle region of the medial ganglionic eminence (MGE), and then advance through the striatum to finally reach the developing cortex (Figures 1A and 1I). As they first enter the telencephalon around embryonic day (E) 13, TCAs navigate through a narrow corridor located superficial to the progenitor zones of the MGE and deep to the developing mantle, where the globus pallidus is starting to form (Figure 1A). The existence of this corridor through which TCAs specifically extend suggests it may be involved in their guidance. To test this possibility, we first examined the molecular identity of cells present in this domain.

Cells located within the MGE corridor do not express detectable levels of genes characteristic of this region, such as Nkx2-1 or *Lhx6* (Figures 1B and S1) (Sussel et al., 1999). In contrast, we found that corridor cells specifically express markers of lateral ganglionic eminence (LGE) derivatives. In particular, corridor cells express Islet1, *Ebf1,* and *Meis2* (Figures 1C–1F and S1), three transcription factors present in the neighboring striatum, the main LGE mantle derivative. Corridor cells also express the γ-aminobutyric acid (GABA) synthesis enzyme *Gad67*, suggesting they are GABA-containing (GABAergic) neurons (Figure S1). Double immunohistochemistry using Calretinin as a marker for TCAs demonstrated that incoming axons grow in close contact with Islet1-expressing corridor cells (Figures 1G and 1H). In sum, the MGE domain used by TCAs to first grow into the telencephalon is unexpectedly made of neurons expressing a combination of molecular markers common to LGE derivatives (Figure 1I).

To understand how the corridor forms within the MGE, we next examined the expression of the LGE markers at early stages of development. A progressive expansion of LGE markers into the MGE was found between E11.5 and E13.5 (Figures 1E and 2A–2D), raising the possibility that corridor cells may migrate tangentially from the LGE to the MGE before TCAs reach this region. To test this hypothesis, we performed homotypic and isochronic transplants of LGE progenitor zones from transgenic embryos expressing green fluorescent protein (GFP) into wild-type host slices (Figure 2E). In addition to an expected striatal radial migration (Figure 2F), transplants generated a stream of GFP-positive cells migrating tangentially into the MGE ($n = 17$ at E12.5; $n = 23$ at E13.5) (Figures 2F and 2G). These cells displayed a morphology characteristic of tangentially migrating neurons in the developing telencephalon (Figure 2H) (Marín and Rubenstein, 2001) and expressed the LGE marker Islet1 (Figure 2I), reinforcing the idea that MGE corridor cells derive from the LGE.

To confirm that the majority of corridor cells originate in the LGE, we mechanically blocked their ventral migration by inserting a semipermeable membrane between the LGE and the MGE in E11.5-E12 telencephalic slices (Figure 2J). After 48 hr in culture, the distribution of Islet1-expressing cells was normal in control slices (Figure 2K), whereas the insertion of a semipermeable membrane

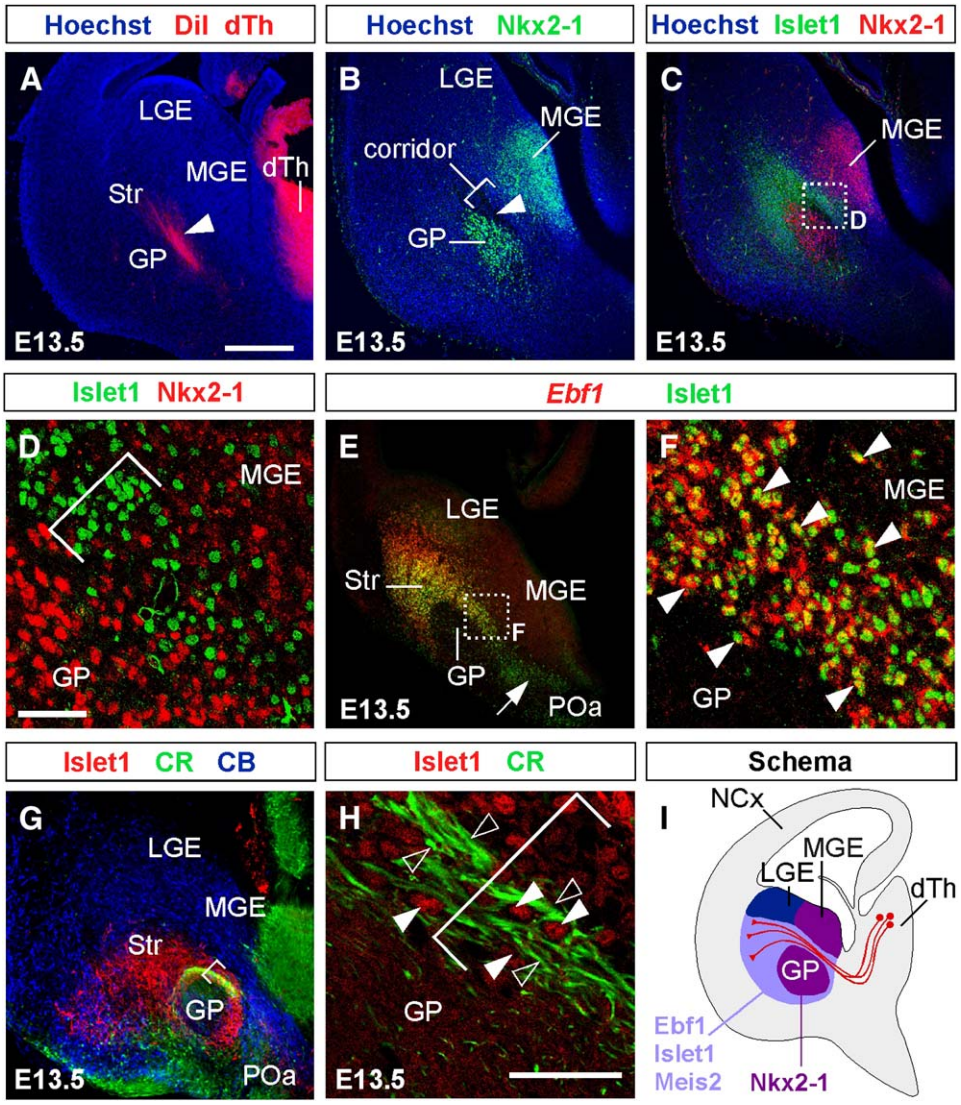

Figure 1. TCAs Enter the Telencephalon through a Restricted Corridor in the MGE

(A) E13.5 coronal mouse telencephalic section showing axonal tracing of dorsal thalamic (dTh) axons (arrowhead) by insertion of a DiI crystal. Coronal sections through the telencephalon of E13.5 embryos showing the expression pattern of Nkx2-1, *Islet1*, *Ebf1*, and Calretinin.

(B) Nkx2-1 expression is not detected in a corridor of cells (bracket) between the ventricular and subventricular zones (VZ/SVZ) of the medial ganglionic eminence (MGE) and the globus pallidus (GP), where TCAs navigate (arrowhead).

(C) Complementary expression of Islet1 and Nkx2-1 proteins.

(D) Higher magnification of the area boxed in (C).

(E) Coexpression of *Ebf1* mRNA and Islet1 protein in the striatum (Str) and in the MGE corridor but not in preoptic area (POa, solid arrow).

(F) Higher magnification of the area boxed in (E), showing coexpression of *Ebf1* and Islet1 in corridor cells (arrowheads).

(G) Calretinin-expressing TCAs navigate through the MGE corridor formed by Islet1-expressing cells. The corridor is just superficial to the route used by Calbindin-expressing interneurons to migrate toward the cortex. The triple staining image was composed from immediate adjacent sections using Adobe Photoshop software.

(H) Higher magnification of the corridor, showing that Calretinin-expressing TCAs (open arrowheads) navigate through the superficial part of the MGE corridor, in close contact with Islet1-expressing cells (solid arrowheads). The bracket indicates the width of the corridor.

(I) Schema summarizing gene expression during TCA pathfinding in the ventral telencephalon. NCx, neocortex; LGE, lateral ganglionic eminence. Scale bars = 300 μm (A, B, C, E, and G) and 50 μm (D, F, and H).

drastically reduced (n = 14) or abolished (n = 5) the presence of Islet1-positive cells in the MGE corridor (Figures 2L and 2L'). Taken together, our results show that the MGE corridor is largely generated by tangential migration from the LGE prior to the entrance of TCAs in the telencephalon.

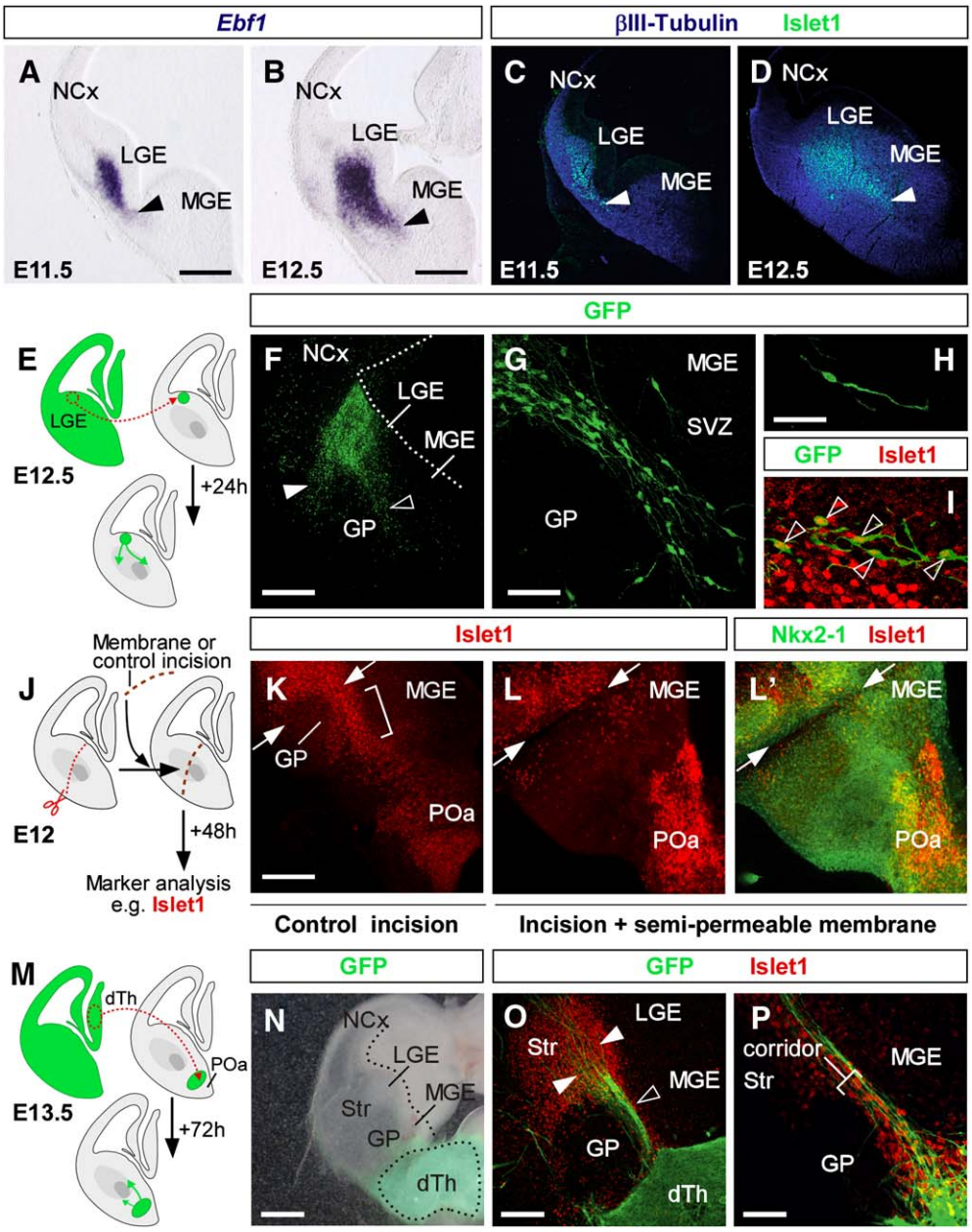

Figure 2. MGE Corridor Cells Derive from the LGE and Are Permissive for TCAs Outgrowth

(A–D) Coronal sections through the telencephalon of E11.5 (A and C) and E12.5 (B and D) embryos showing the expression pattern of *Ebf1* (A and B) and double immunohistochemistry Islet1 and βIII-tubulin (C and D).

(E) Experimental paradigm used to test the origin of corridor cells.

(F) GFP immunohistochemistry showing LGE-derived cells in the striatum (solid arrowhead), neocortex (NCx), and MGE mantle (open arrowhead).

(G) Higher magnification of LGE-derived GFP cells forming a stream superficial to the globus pallidus (GP).

(H and I) Migratory morphology of GFP cells at the MGE corridor (H). Most of them express Islet1 (I, open arrowheads).

(J) Experimental paradigm used to block cell migration between the LGE and MGE.

(K and L) Expression of Islet1 in control (K) and experimental slices (L). Note that the membrane (delineated by arrows) does not affect Islet1-positive cells in the POa. Arrows in (K) indicate the location of the control incision.

(L') Double immunohistochemistry for Islet1 and Nkx2-1 in the same slice shown in (L).

(M) Experimental paradigm used to test the growth of E13.5 GFP dorsal thalamic (dTh) in the MGE.

(N) Bright-field image of a slice with a GFP dTh explant in the POa after 72 hr in culture.

(O and P) Islet1 and GFP immunohistochemistry showing that TCAs grow preferentially through the MGE Islet1-positive corridor (open arrowhead, bracket in [P]) before fanning out in the striatum (Str; solid arrowheads). VZ/SVZ, ventricular/subventricular zone. Scale bars = 100 μm (A, C, and O), 200 μm (B, D, K, L, L', and N), 300 μm (F), 60 μm (G), 20 μm (H and I), and 70 μm (P).

Territories Derived from the Medial Ganglionic Eminence Are Nonpermissive for TCAs

To investigate how this early LGE migration relates to TCAs pathfinding, we analyzed the ability of TCAs to grow into MGE- and LGE-derived territories using a slice coculture assay. In this assay, we dissected dorsal thalamic (dTh) explants from GFP-expressing embryonic brains and confronted them with wild-type telencephalic slices for 72–96 hr (Figure 2M). When confronted with the MGE, TCAs preferentially grew into the LGE-derived corridor, avoiding the MGE ventricular and subventricular zones (VZ and SVZ, respectively) and globus pallidus (n = 38) (Figures 2O and 2P). The apposition of dTh explants to the striatum showed a widespread axon invasion (n = 45) (Figure S2), demonstrating that this territory is highly permissive for the growth of TCAs. Thus, our in vitro assay reproduces the in vivo behavior of TCAs in the ventral telencephalon: highly fasciculated growth in the Islet1-positive corridor of the MGE, avoiding progenitors and derivatives, and widespread growth through the striatum.

The preferential growth of TCAs through the LGE-derived corridor present in the MGE could be due to two nonexclusive mechanisms: (1) MGE-derived territories (i.e., VZ/SVZ and globus pallidus) are nonpermissive for TCAs; and (2) The corridor is specifically attractive for TCAs. To test these ideas, we first inserted small explants of the MGE VZ/SVZ (n = 8) or globus pallidus (n = 9) into the striatum and examined the behavior of GFP-positive TCAs after 72 hr in culture (Figure S2). TCAs grew normally through the striatum in control slices (n = 5), while they systematically avoided the heterotypic MGE VZ/SVZ (n = 8) or globus pallidus (n = 9) transplants (Figure S2). Thus, compared to the striatum, MGE progenitors and their derived territories are relatively nonpermissive to TCAs outgrowth (Figure S2).

Corridor Cells Are Required for TCAs Guidance

We next tested whether corridor cells could facilitate the growth of TCAs in an otherwise nonpermissive environment. In the experiments performed to validate our slice assays, we found that the most caudal part of the ventral telencephalon constitutes a nonpermissive territory for TCAs outgrowth (n = 7) (Figures 3A and 3B). In contrast, when a transplant containing corridor cells was grafted into the caudal ventral telencephalon, TCAs were found to invade the telencephalon specifically through the transplant, as visualized by Islet1 immunohistochemistry (n = 8) (Figures 3A, 3C, 3D, and 3D'). Thus, corridor cells are sufficient to provide a permissive environment for TCAs to cross a nonpermissive region.

To test if corridor cells are required for the normal growth of TCAs, we performed a series of experiments in which we prevented the formation of the MGE corridor by mechanically blocking cell migration between the LGE and the MGE (Figure 3E). Telencephalic slices in which corridor formation was blocked by the insertion of a semipermeable membrane showed a drastic reduction in TCAs navigation in the MGE domain as compared to control slices (n = 8) (Figures 3F–3H). These experiments support an essential role for tangential migration of corridor cells in TCAs pathfinding.

To establish the involvement of corridor cells in the guidance of TCAs in vivo, we searched for mouse mutants in which the development of corridor cells is affected. The *Mash1* mutant constitutes an excellent candidate to test our hypothesis since loss of this transcription factor leads to a defect in the early development of the basal telencephalon that correlates with an abnormal pathfinding of TCAs (Casarosa et al., 1999; Tuttle et al., 1999). We found that the MGE corridor does not form or is severely reduced in *Mash1* mutant embryos (Figures 3I and 3J and data not shown), which may cause the initial blockage of TCAs at their entry point in the telencephalon (Figures 3K and 3L). Slice experiments showed that cell migration from the LGE is drastically impaired in *Mash1* mutant embryos, most likely causing the observed defect in corridor formation (data not shown). Thus, *Mash1* mutant slices represent a corridor-free system in which we could further test the role of these cells in TCAs pathfinding.

As expected from our previous results, wild-type TCAs largely failed to transverse the MGE in rostral slices from *Mash1* mutant embryos (n = 11) (Figures 3M and 3N). We next tested whether a graft of wild-type LGE progenitor zones—the origin of corridor cells—in *Mash1* mutant slices could rescue the formation of the corridor in the MGE and thereby restore TCAs pathfinding. Wild-type LGE transplants gave rise to ventrally migrating cells in approximately half of the experiments (n = 13 out of 25 slices) (Figures 3O and 3P). In a vast majority of these experiments, migrating cells reached the dTh explants (n = 9 out of 13). Remarkably, formation of the corridor restored the growth of wild-type TCAs into the *Mash1* mutant MGE territory (Figure 3O). Analysis of the nontransplanted side to the slice, used as a control, demonstrated that TCAs consistently fail to grow toward the cortex in the absence of the corridor (n = 25 out of 25 slices). Finally, since the MGE is also affected in *Mash1* mutants (Casarosa et al., 1999), we performed additional control experiments in which wild-type MGE was homotypically transplanted in *Mash1* mutant slices. MGE transplants restored the migration of interneurons from the MGE into the cortex (which is perturbed in *Mash1* mutants; see Casarosa et al., 1999) but did not rescue the growth of TCAs through the ventral telencephalon (n = 24 slices) (Figure S3). In sum, this series of experiments indicates that the absence of LGE-derived corridor cells in *Mash1* mutants specifically contributes to the inability of TCAs to extend through the MGE. Furthermore, they show that tangential migration of corridor cells is necessary and sufficient for the normal navigation of TCAs in the ventral telencephalon.

CRD-NRG1 Is Expressed by Corridor Cells and Contributes to TCAs Guidance

We next investigated the molecular basis for the role of corridor cells in TCAs guidance. We have recently described that different isoforms of the *Neuregulin-1* (*Nrg1*) gene act

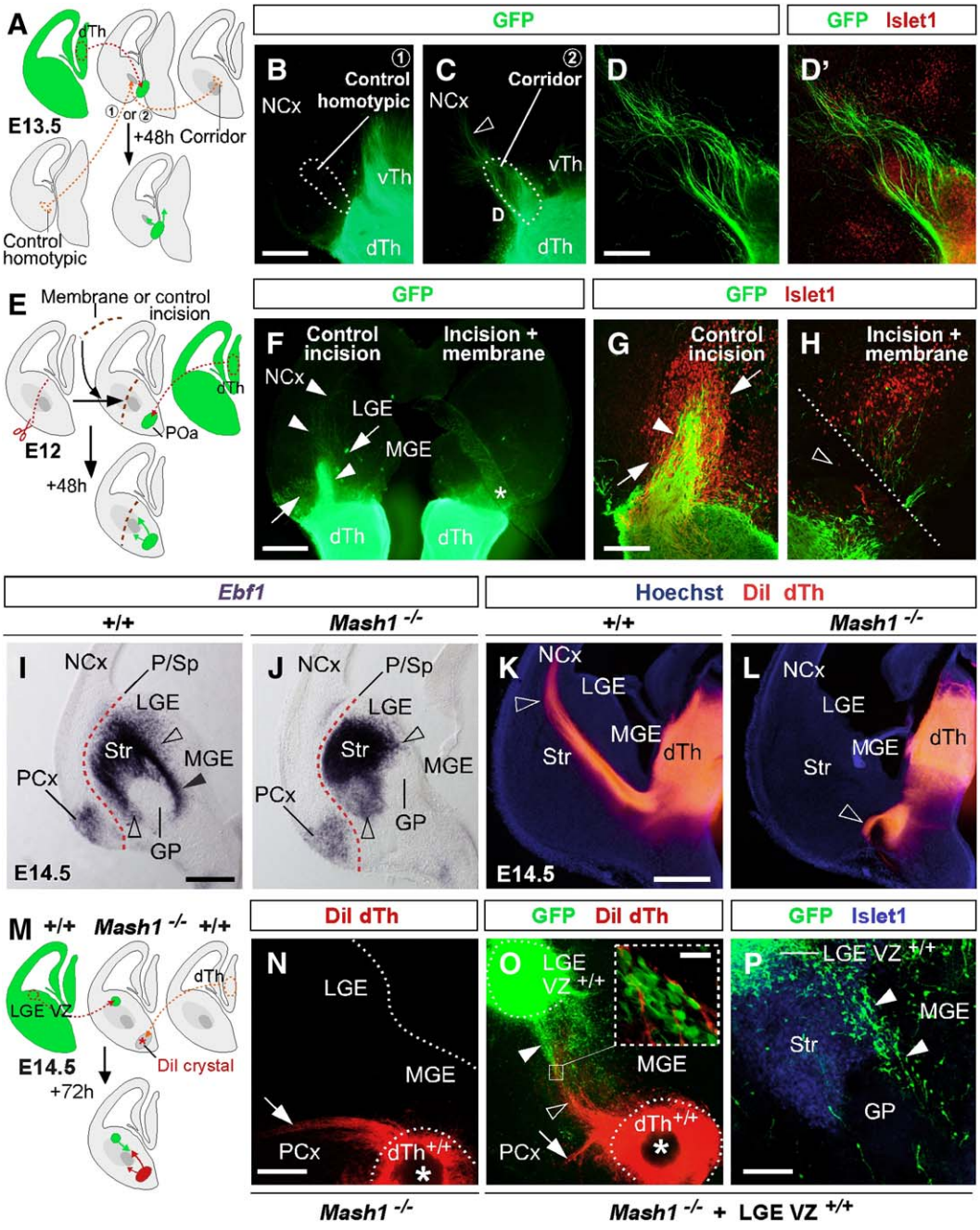

Figure 3. The Corridor Is a Permissive Territory Necessary and Sufficient for TCA Pathfinding

(A) Experimental paradigm used to test whether the medial ganglionic eminence (MGE) corridor is necessary for TCAs extension.

(B and C) GFP immunohistochemistry showing the behavior of TCAs in control (B) and experimental slices (C).

(D and D') Higher magnification of TCAs in (C) showing the presence of Islet1-positive corridor cells.

(E) Experimental paradigm used to test the requirement of LGE to MGE migration for TCA guidance.

(F) GFP immunohistochemistry showing that a control incision (left hemisphere) does not affect TCAs growth toward the neocortex (NCx, open arrowheads), whereas insertion of a membrane (asterisk, right hemisphere) impairs the growth of TCAs.

(G and H) A higher magnification of control (G) and a membrane inserted (dashed line in H) slices showing GFP and Islet1 immunohistochemistry. TCAs outgrowth correlates with the presence of Islet1-expressing cells (open arrowhead in [G]).

(I and J) *Ebf1* mRNA expression at E14.5 shows that MGE corridor formation (solid arrowhead in [I]) is impaired in *Mash1* mutant embryos (J). Open arrowheads mark the LGE/MGE boundary, and a red dashed line delineates the pallium/subpallium boundary (P/Sp).

(K and L) DiI labeling of TCAs (open arrowheads) in coronal sections through E14.5 brains in control (K) and *Mash1* mutant embryos (L).

(M) Experimental paradigm used to test the ability of LGE-derived MGE corridor cells to restore TCAs growth in the *Mash1* mutant telencephalon.

as short- and long-range attractants for migrating cortical interneurons (Flames et al., 2004). Interestingly, we observed that the LGE-derived corridor found within the MGE expresses high levels of membrane bound isoforms of *Nrg1* (*CRD-Nrg1* or type III NRG1) (Figures 4A–4C), raising the possibility that CRD-NRG1 may participate in controlling TCAs navigation. To test this hypothesis, we adapted an assay in which the complete TCA pathway is preserved in a single slice culture (Agmon and Connors, 1991) and placed aggregates of *CRD-Nrg1*-expressing COS cells in the ventral telencephalon prior to the entrance of TCAs in this territory (Figure 4E). TCAs that encountered COS cell aggregates expressing *CRD-Nrg1* were diverted from their normal pathway (n = 21) (Figures 4I–JK), whereas control cell aggregates did not influence the guidance of TCAs (n = 28) (Figures 4F–4H). Thus, TCAs preferentially grow in contact with CRD-NRG1-expressing cells in slice cultures.

To assess the function of CRD-NRG1 in the guidance of TCAs in vivo, we examined mutant embryos in which CRD-containing isoforms of NRG1 are disrupted through gene targeting, but diffusible NRG1 proteins (type I and type II NRG1, also known as Ig-NRG1 isoforms) are still produced (Wolpowitz et al., 2000). We analyzed TCAs development in these mutant mice at E14.5 by placing crystals of DiI in the developing dTh. These experiments revealed a disorganized arrangement of TCAs as they progress through the MGE in *CRD-Nrg1* mutants (n = 3) (Figures 4P, 4Q, and 4S) as compared to controls (n = 3) (Figures 4L, 4M, and 4O). Labeling of TCAs using L1 immunohistochemistry (Fukuda et al., 1997) confirmed this observation (n = 3) (Figure S4) and showed that fewer TCAs reached the neocortex in *CRD-Nrg1* mutants than controls (n = 3) (Figures 4N, 4O, 4R, and 4S). Thus, CRD-NRG1 expression in corridor cells contributes to the navigation of TCAs within the ventral telencephalon in vivo.

Different Isoforms of NRG1 Cooperate to Control TCAs Pathfinding

The previous results prompted us to search for additional molecules that could contribute to the growth of TCAs even in the absence of the permissive substrate that CRD-NRG1 represents. Since *Ig-Nrg1* is expressed in the cortex at the time TCAs first enter the telencephalon (Figure 5A), we wondered whether the diffusible forms of NRG1 could also contribute to their guidance. To test this hypothesis, we cocultured E13.5 dTh explants with COS cells aggregates expressing *Ig-Nrg1* in a three-dimensional matrix. In these experiments, axons did not specifically extend toward the source of NRG1, but instead Ig-NRG1 dramatically promoted their outgrowth (n = 21) (Figures 5B–5D and S5). This effect was observed independently of the type of three-dimensional matrix used and was reproduced by the purified EGF-like domain of human Ig-NRG1 (Figure S5).

We next examined the consequences of loss of Ig-NRG1 in the outgrowth of TCAs within the telencephalon. Although *Ig-Nrg1* expression is found throughout the cerebral cortex around E15.5 (Flames et al., 2004), the domain of *Ig-Nrg1* expression is largely confined to ventral and lateral divisions of the pallium when the earliest TCAs enter the telencephalon, around E13-E13.5 (Figure 5A). Based on this observation, we predicted that ablation of the VZ at the pallial/subpallial boundary—the "angle" region—would result in a reduction of secreted NRG1, thereby influencing TCAs in our slice culture system (Figure 5E). While in control experiments TCAs reached the cortex within 72 hr in culture (n = 8) (Figures 5F and 5H), complete angle ablations drastically affected axonal navigation, preventing TCAs to reach the cortex (n = 19 out of 21) (Figures 5G and 5H). These experiments suggest that the angle region contributes to the growth of TCAs through long-range factors since it influences axonal outgrowth long before they reach the pallium.

To directly test if the TCA outgrowth blockage produced by the angle ablation was partly due to a reduction of Ig-NRG1 levels, we supplied angle-ablated slice cultures with exogenous *Ig-Nrg1*-expressing COS cells, placed in the lateral cortex or at the pallial/subpallial boundary (Figure 5I). While ablation experiments with control cells drastically affected TCA navigation (n = 16 out of 19) (Figures 5J and 5J' and data not shown), addition of *Ig-Nrg1*-expressing COS cells to the slice cultures rescued the growth of TCAs toward the cortex (n = 16 out of 27) (Figures 5K–L'). In addition, these experiments demonstrated that in a physiologically relevant context Ig-NRG1 controls the oriented growth of TCAs since laterally placed aggregates were able to partially derail growing axons from their normal trajectory to the neocortex (Figures 5K and 5K').

To reveal the contribution of Ig-NRG1 to the guidance of TCAs in vivo, we next analyzed embryos in which all forms of NRG1 were disrupted through gene targeting specifically restricted to the telencephalon. L1 staining and DiI tracing at E14 revealed that in the absence of telencephalic NRG1 TCAs entered the telencephalon as in controls but defasciculated through the MGE corridor and largely failed to progress toward the cortex (n = 3) (Figures 6 and S4). This defect was found to be partially persistent in neonatal embryos (n = 3) (Figure S6) and was not due to an absence of corridor formation, as shown by Islet1

(N) DiI-labeled TCAs do not grow toward the neocortex in *Mash1* mutant slices, although they can ectopically invade the piriform cortex (PCx; arrow).
(O) GFP expression showing that a graft of GFP-LGE VZ/SVZ (dotted circle) into the LGE of *Mash1* mutant slices generates cells that migrate tangentially into the MGE (solid arrowheads), forming a corridor used by DiI-labeled TCAs (open arrowhead) to extend toward the NCx.
(P) GFP and Islet1 immunohistochemistry showing that wild-type GFP-expressing neurons migrate from the LGE into the MGE of *Mash1* mutant slices. GP, globus pallidus; Str, striatum; vTh, ventral thalamus; VZ/SVZ, ventricular/subventricular zone. Scale bars = 200 μm (B, C, and F), 100 μm (D, D', G, and H), 300 μm (I–L), 150 μm (N and O), and 100 μm (P).

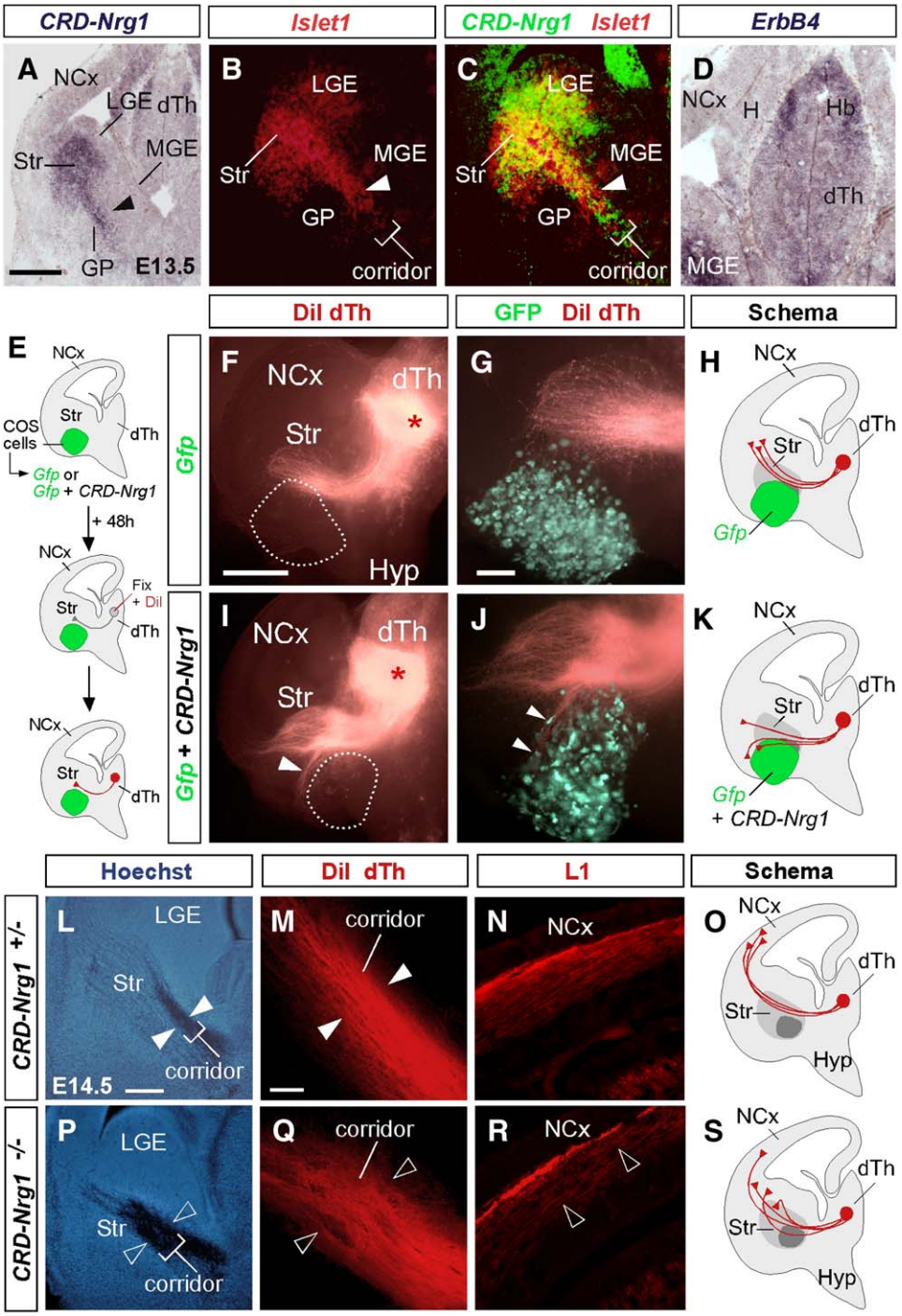

Figure 4. *CRD-Nrg1* Is Expressed in MGE Corridor Cells and Contributes to TCA Pathfinding

Serial coronal sections through the telencephalon of E13.5 embryos.

(A) *CRD-Nrg1* expression in the striatum (Str) and in cells forming the medial ganglionic eminence (MGE) corridor (arrowhead).

(B) *Islet1* expression in the Str and in cells forming the MGE corridor (arrowhead, bracket).

(C) *Islet 1* and *CRD-Nrg1* in MGE corridor cells (arrowhead, bracket). Double in situ image was composed from immediate adjacent sections using Adobe Photoshop software.

(D) *ErbB4* expression in the dorsal thalamus (dTh).

(E) Experimental paradigm used to analyze the response of TCAs to *CRD-Nrg1*-transfected COS cell aggregates in slice cultures.

(F and I) DiI-labeled TCAs traveled normally through the telencephalon toward the neocortex (NCx) in controls (F) but derailed from their normal path (arrowhead in [I]) when they contact a COS cell aggregate expressing CRD-NRG1. Asterisks mark DiI placements in the dorsal thalamus (dTh).

expression in the *Nrg1* mutant MGE (Figure S7). Altogether, these experiments suggest that CRD-NRG1 and Ig-NRG1 proteins cooperate in vivo to guide TCAs through the MGE corridor on their way to the neocortex.

ErbB4, a NRG1 Receptor, Is Required for TCAs Navigation

NRG1 directly binds to ErbB3 and ErbB4 receptors, which alone or in combination with ErbB2 mediate a large range of functions (Falls, 2003). ErbB4 receptors are expressed by thalamic neurons at the time they start extending their axons toward the telencephalon (Figure 4D and data not shown), suggesting that ErbB4 signaling may underlie the function of NRG1 in TCAs guidance. To test this hypothesis, we analyzed the thalamocortical projection by DiI tracing at E14 in a strain of *ErbB4* mutant embryos (Tidcombe et al., 2003). As in the case of *Nrg1* mutant embryos, TCAs largely failed to progress normally through the MGE in *ErbB4* mutants, extending in all directions within ventral telencephalic region ($n = 3$) (Figures 7A–7F).

Further analysis of *ErbB4* mutants revealed that the migration of LGE-derived corridor cells to the MGE does not depend on ErbB4 function (Figure S7), suggesting that the observed defects were cause by a cell-autonomous mechanism. To directly test this, we performed two sets of experiments: (1) We expressed a dominant-negative form of *ErbB4* (*dnErbB4*) in the dTh by focal electroporation in embryonic slices (Figure 7G); and (2) We recombined wild-type telencephalic slices with dTh explants from *ErbB4* mutant embryos (Figure 7J). In control electroporation experiments ($n = 26$) (Figure 7H) or recombination experiments ($n = 10$ out of 10 slices) (Figures 7K), axons formed a tight organized bundle in the ventral telencephalon before reaching the cortex. In contrast, in slices electroporated with *dnErbB4*, axons failed to organize in a compact bundle through the MGE, navigating randomly in all directions within the ventral telencephalon ($n = 27$) (Figure 7I). Similarly, axons derived from *ErbB4* mutant explants largely fail to reach the neocortex (n = 22 out of 23 slices) (Figures 7L). Thus, loss of ErbB4 function in the dTh resulted in a similar phenotype to the *ErbB4* mutant, in which TCAs enter the telencephalon but fail to progress efficiently toward the cortex (Figures 7C and 7F). Altogether, these results strongly suggest that ErbB4 signaling in TCAs is required for their proper navigation in the ventral telencephalon and are in agreement with the hypothesis that ErbB4 mediates the function of NRG1 in this process.

DISCUSSION

During development of the nervous system, axons are guided by specific cues presented at defined decision points along their pathway. The precise distribution of the various guidance cues frequently depends on early patterning mechanisms, which control their timely expression in the neuroepithelium. We have shown that tangential migration of intermediate targets constitute a novel mechanism to effectively position guidance cues—both in time and space—for growing axons. Specifically, the normal development of the thalamocortical projection, one of the most prominent tracts in the forebrain, depends on the early tangential migration of GABAergic neurons from the LGE to the MGE. This tangential migration is essential to form a permissive corridor required for TCAs to navigate through the telencephalon (Figure 8A). Our results also demonstrate that ErbB4 and two different products of the *Nrg1* gene, CRD-NRG1 and Ig-NRG1, control the guidance of TCAs in the telencephalon.

Corridor Cells Constitute an Essential Territory for the Guidance of TCAs

TCAs convey sensory and motor inputs to the cerebral cortex, where integration of this information leads to the organization of appropriate responses to internal and external stimuli. To reach the cortex, TCAs follow a very complex path that includes multiple guidance decision points (Braisted et al., 1999; Auladell et al., 2000). In this study, we have characterized the territory used by TCAs to initially extend through the telencephalon. Somewhat surprisingly, this MGE domain is formed by LGE-derived GABAergic neurons that migrate tangentially to their final position before TCAs reach the telencephalon. These neurons, which we have named corridor cells, appear to be essential for the proper pathfinding of TCAs at early stages of development. This is well illustrated by the analysis of *Mash1* mutants, in which corridor cells fail to invade the MGE and TCAs can hardly progress through the ventral telencephalon. The requirement of corridor cells in the normal guidance of TCAs is further supported by experiments in which rescue of the corridor domain in *Mash1* mutant slices restores thalamocortical projections. Thus, corridor cells form a bridge between two permissive territories for TCAs, the prethalamic region and the striatum, which are initially separated by nonpermissive MGE-derived cells (Figure 8A). This conclusion is supported by the analysis of *Nkx2-1* mutant embryos, in which

(G and J) Higher magnifications of the images shown in (F) and (I), respectively.
(H and K) Schematic representation of the pathway taken by TCAs in response to control and *CRD-Nrg1* transfected COS cell aggregates.
(L and P) Nuclear counterstain of *CRD-Nrg1* heterozygous (L) and *CRD-Nrg1* mutant (P) E14.5 coronal sections shows that TCAs abnormally defasciculate in the MGE corridor of mutants (open arrowheads and brackets in [P] and [Q]) compared to controls (arrowheads and brackets in [L] and [M]).
(M and Q) High magnifications of DiI-labeled axons in E14.5 *CRD-Nrg1* heterozygous (M) and *CRD-Nrg1* mutant (Q) showing that the MGE corridor is wider and more disorganized in *CRD-Nrg1* mutants (arrowheads) than in control brains.
(N and R) High magnification of L1-labeled axons observed in the NCx of control (N) and *CRD-Nrg1* mutants (R) at E14.5.
(O and S) Schematic representation of the pathway taken by TCAs in control and *CRD-Nrg1* mutants. H, hippocampus; Hb, habenula; Hyp, hypothalamus; LGE, lateral ganglionic eminence; GP, globus pallidus; PCx, piriform cortex. Scale bars = 200 μm (A–D, H, L, J, and P), 1 μm (F and I), 300 μm (G and J), and 100 μm (M, N, Q, and R).

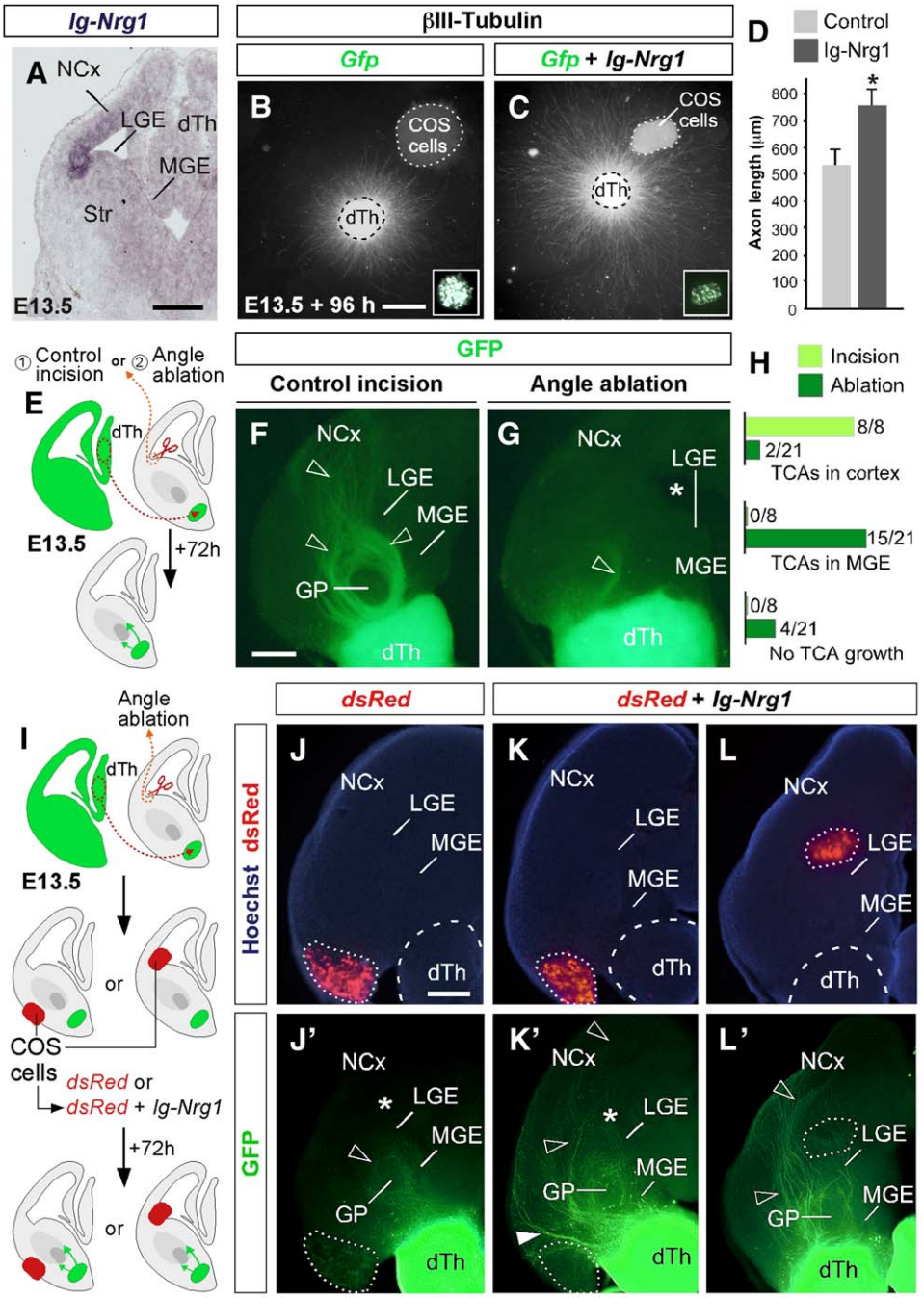

Figure 5. Ig-NRG1 Controls the Oriented Outgrowth of TCAs

(A) *Ig-Nrg1* mRNA expression in the developing cortex at E13.5.

(B and C) βIII-Tubulin immunohistochemistry showing dorsal thalamic (dTh) explants from E13.5 embryos after 96 hr in culture adjacent to mock transfected (B) or *Ig-Nrg1* transfected (C) COS cell aggregates (dotted lines). Insets show GFP expression in transfected COS cells.

(D) Quantification of axonal length in the experiments shown in (B) and (C). Additional quantifications are displayed in Figure S4.

(E) Experimental paradigm used to test the effect of ventricular zone ablations in the angle region on the growth of GFP-positive dTh axons in E13.5 telencephalic slices.

(F and G) GFP expression showing that TCAs (open arrowheads) extend through the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and neocortex (NCx) in control slices but fail to do so in angle ablation slices (asterisk in [G]).

(H) Qualification of the experiments shown in (F) and (G).

(I) Experimental paradigm used to test the effect of control or *Ig-Nrg1* transfected COS cell aggregates on the growth of GFP-positive dTh axons in E13.5 angle-ablated telencephalic slices.

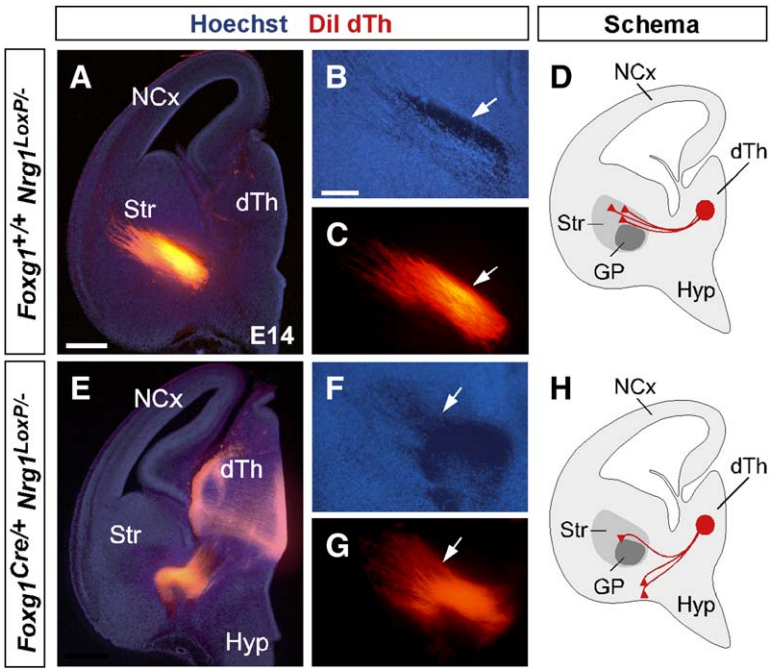

Figure 6. Abnormal Development of TCAs in the Absence of All *Nrg1* Isoforms

(A and E) Coronal sections through E14 control (A) and *Nrg1* mutant (E) embryos showing nuclear staining and DiI labeling after dorsal thalamic (dTh) injections.

(B, C, F, and G) Higher magnifications of the internal capsule region (arrows) in control (B and C) and *Nrg1* mutant (F and G) embryos.

(D and H) Schematic representation of the pathway taken by TCAs in control and *Nrg1* mutant brains. GP, globus pallidus; Hyp, hypothalamus; Str, striatum. Scale bar = 300 μm (A and E) and 200 μm (B, C, F, and G).

MGE-derived territories fail to form at the expense of an expanded LGE domain (Sussel et al., 1999) and thalamocortical connections still form normally (Marín et al., 2002).

The Angle Region Is Essential for TCA Growth

In the course of our experiments, we have found that the most ventral region of the pallium—the angle region—is essential for the growth of TCAs through the telencephalon. Specifically, removal of this small region in telencephalic slices prevents the extension of TCAs through the telencephalon. This behavior does not seem to depend on structural changes in the pathway followed by TCAs since ablation of the angle region was performed after corridor cells occupied their normal position in the MGE and the rest of the subpallium was unaffected by the manipulation. Instead, our experiments suggest that the angle region contains an activity that is necessary for the extension of TCAs.

Previous studies have proposed that the structural integrity of the pallial/subpallial boundary is required for the guidance of TCAs and corticofugal axons (Jones et al., 2002). Unexpectedly, we have found that the angle region also influences TCAs growth at a long distance, suggesting that diffusible factor(s) released by this region regulates the guidance of TCAs.

NRG1 Guides Thalamocortical Projections through the Ventral Telencephalon

Although thalamocortical projections are probably among the most studied connections in the developing brain (Wise and Jones, 1978; Ghosh et al., 1990; Molnár et al., 1998a; Garel et al., 2002; López-Bendito et al., 2002; Marín et al., 2002), little was known about the molecular nature of the signals controlling their guidance. The first guidance decision point, which enables TCAs to enter the telencephalon, appears to rely on the repulsive activity of Slit1 and Slit2 present in the hypothalamus (Bagri et al., 2002). In addition, several studies have identified cell populations in the ventral thalamus and telencephalon that grow axonal projections in opposite direction to TCAs and may facilitate TCAs guidance (Mitrofanis and Baker, 1993; Métin and Godement, 1996; Braisted et al., 1999; Molnár and Cordery, 1999), although the exact role and molecular basis of this axonal interaction remains to be determined.

Once in the telencephalon, very few guidance cues have shown a prominent effect on TCAs. Slits have been involved in the repulsion of TCAs away from the ventral midline (Bagri et al., 2002), Netrin-1 in the restriction of the internal capsule width (Braisted et al., 2000), and Sema6A and Eph/ephrins in the guidance of some TCAs (Leighton et al., 2001; Dufour et al., 2003). In addition, Netrin-G1 ligand

(J–L) Nuclear staining and dsRed expression in angle-ablated telencephalic slices with control (J) or *Ig-Nrg1* transfected (K and L) COS cell aggregates. The dotted lines delineate COS cell aggregates, whereas dashed lines delineate dTh explants.

(J'–L') GFP expression in the same slices (J–L), showing that TCAs (open arrowheads) fail to extend toward the cortex in control slices (J') but do so in angle-ablated slices containing *Ig-Nrg1* transfected COS cell aggregates (K' and L'). Scale bar = 500 μm (A–C) and 200 μm (F, G, J, J', K, K', L, and L').

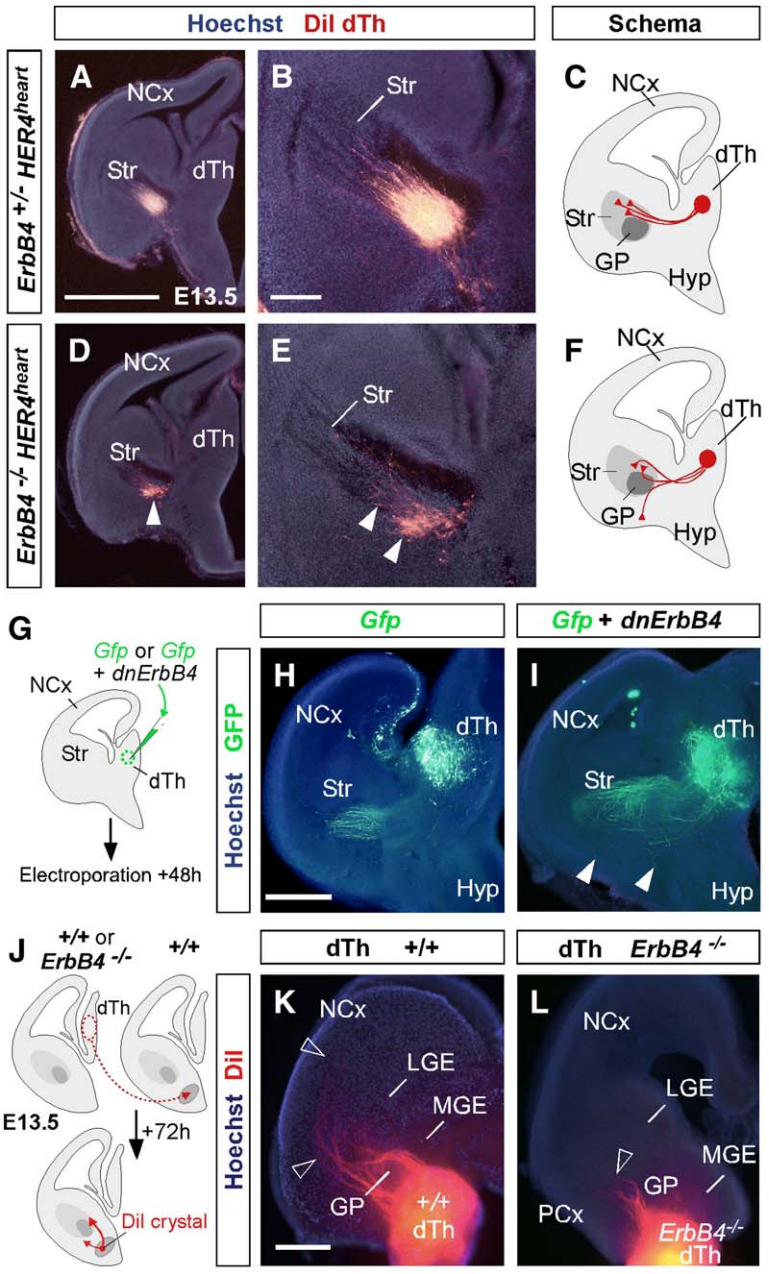

Figure 7. Loss of ErbB4 Function Perturbs TCA Guidance

(A and D) Coronal sections through E13.5 *ErbB4* heterozygous (A) and *ErbB4* mutant (D) brains showing nuclear staining and DiI labeling (arrowheads in D) after dorsal thalamic (dTh) injections.

(B and E) Higher magnifications of the images shown in (A) and (E), respectively.

(C and F) Schematic representation of the pathway taken by TCAs in a control situation (C) or in the absence of *ErbB4* function (F).

(G) Experimental paradigm used to analyze the effect of a dominant-negative form of ErbB4 (*dnErbB4*) in the guidance of dTh axons.

(H and I) GFP immunohistochemistry showing TCAs as they extend through the striatum (Str) toward the neocortex (NCx) in control (H) and *Gfp* + *dnErbB4* electroporated slices (I).

(J) Experimental paradigm used to test the growth of E13.5 wild-type or *ErbB4*$^{-/-}$ dTh explants in wild-type telencephalic slices.

(K and L) DiI labeling and nuclear staining showing wild-type (K) and *ErbB4*$^{-/-}$ (L) TCAs as they extend through wild-type telencephalic slices. GP, globus pallidus; Hyp, hypothalamus; PCx, piriform cortex. Scale bars = 1 mm (A, D, H, I, K, and L) and 200 μm (B and E).

and hepatocyte growth factor promote thalamic axons outgrowth in vitro (Lin et al., 2003; Powell et al., 2003).

Our results suggest that different isoforms of NRG1 are important signals for the guidance of TCAs and that ErbB4 is part of the receptor complex required to transduce the effect of NRG1 (Figure 8A). First, CRD-NRG1 expression in corridor cells contributes to the pathfinding of TCAs as they initially enter the developing telencephalon. The lack of a complete block in the growth of TCAs through the MGE corridor in *CRD-Nrg1* and conditional *Nrg1* mutant embryos suggests that additional factors accounting for the permissive activity of corridor cells remain to be identified. Alternatively, the nonpermissive nature of the territories surrounding the MGE corridor may force TCAs to use corridor cells as a substrate even in the absence of CRD-NRG1, especially in view of the fact that TCAs outgrowth is strongly promoted in the telencephalon.

Several lines of evidence suggest that expression of *Ig-Nrg1* in the pallium accounts at least in part for the outgrowth-promoting activity found in this region. First, this diffusible form of the *Nrg1* gene is timely expressed by progenitors cells in the most ventral region of the pallium prior to the entrance of TCAs in the telencephalon. Second, Ig-NRG1, largely via its EGF-like domain, is a prominent axonal outgrowth-promoting factor for dTh axons in vitro. Third, in the context of the slice assays, Ig-NRG1

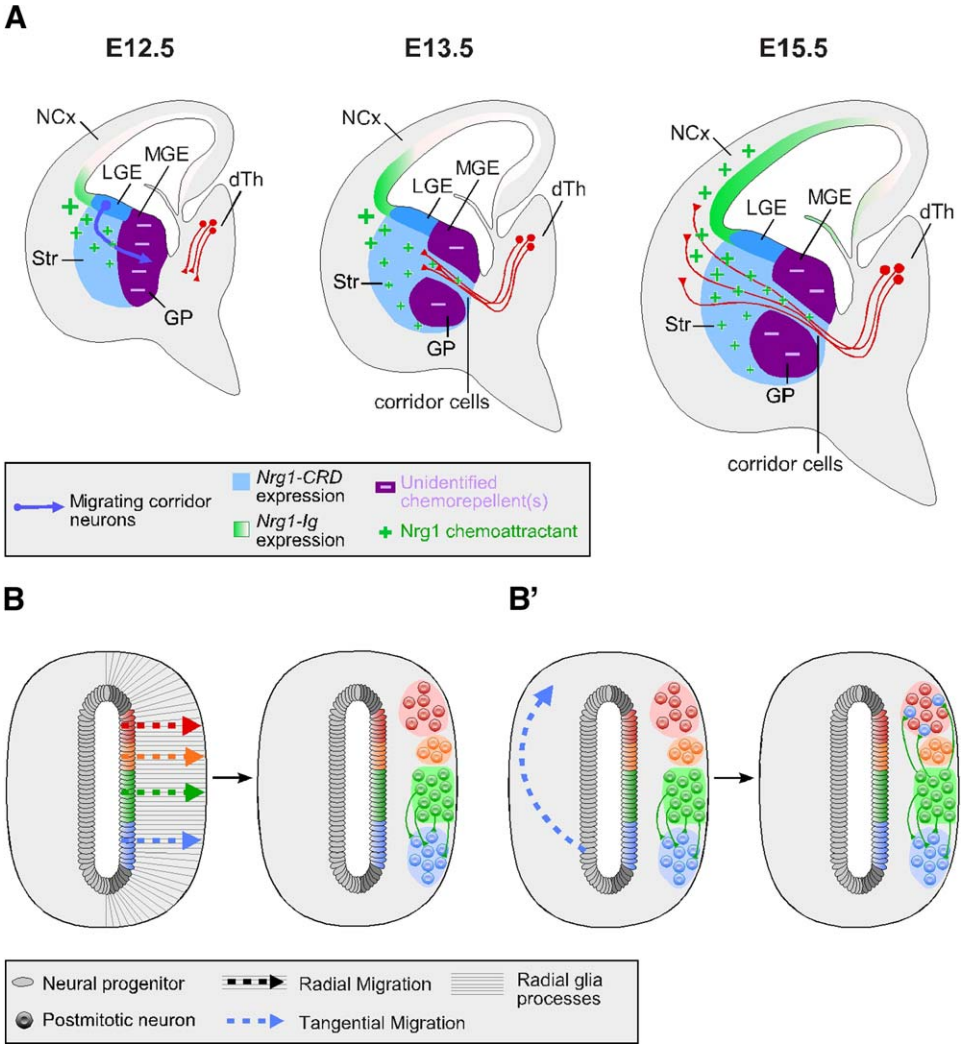

Figure 8. Tangential Migration and Axon Guidance in the Central Nervous System

(A) A model of TCAs guidance by tangential migration of corridor cells and NRG1 expression. GABAergic neurons migrate tangentially from the lateral ganglionic eminence (LGE) to form a corridor in the medial ganglionic eminence (MGE) around E12.5 (blue line) prior to the entrance of TCAs in the telencephalon. At this early stage, the MGE territory is not permissive for TCAs (dark purple area). LGE-derived neurons colonize the MGE mantle around E13.5, forming a permissive corridor for TCAs in this region. CRD-NRG1 expression by corridor cells contributes to the guidance of TCAs through this region, which also requires secreted Ig-NRG1 from the pallium (green gradient). Tangential migration and axon guidance in the developing neural tube.

(B) Radial glia provides structural support for radial migration, a process that results in the generation of different nuclei topographically organized in relation to their place of origin.

(B') Tangential migration is independent of radial glia processes and therefore does not respect topographical references. As a result, tangential migration produces an increase in the cellular complexity of neural circuits by providing cell types distinct from those locally generated and represents a novel mechanism for presenting cues to navigating axons.

can replace the function of the angle region in the guidance of TCAs, acting as a long-range attractant. Fourth, TCAs fail to extend normally through the telencephalon in mice with a loss of function mutation in the *Nrg1* gene. These results strongly suggest that both isoforms of NRG1 cooperate in the guidance of TCAs through the ventral telencephalon. Thus, in addition to the recent role of these two isoforms as attractive cues for interneurons migrating toward the developing cortex (Flames et al., 2004), our study demonstrates a novel involvement of NRG1 in axonal guidance. Furthermore, it shows that the same set of cues coordinates the guidance of two major inputs to cortical development and function, interneurons and TCAs. It is worth noting, however, that cortical interneurons and thalamic axons navigate in parallel, nonoverlapping routes within the ventral telencephalon (Figure S3). In addition, since both interneurons and TCAs persist in neonatal *Nrg1* and *ErbB4* mutant embryos (Flames et al.,

2004) (Figure S6 and data not shown), it is clear that this signaling system cooperates with other unidentified molecules to control their guidance.

Tangential Migration-Mediated Axon Guidance

Axon guidance depends on the precise arrangement of guidance molecules in the extracellular environment (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). During early stages of development, neuroepithelial cells are patterned to secrete most guidance cues and thereby influence the establishment of early axonal tracts. This is, for example, the case of the floor plate, which controls the guidance of spinal commissural axons through the production of Netrin-1 and Sonic hedgehog (Serafini et al., 1996; Charron et al., 2003). As development proceeds, however, additional guidance cues need to be deployed to ensure the guidance of axonal tracts that navigate away from progenitor regions. In a general sense, it has been assumed that radial migration has served as the general mechanism involved in transferring patterning information from the neuroepithelium to the mantle and is therefore responsible for positioning guidance cues at appropriate times and locations in the brain (Figure 8B). Here we have shown that neuronal tangential migration is a novel mechanism controlling the timely arrangement of guidance cues during development of the mammalian brain (Figure 8B').

During evolution, tangential migration may have evolved to increase the complexity of neuronal circuits (Marín and Rubenstein, 2001). In addition to this major evolutionary advantage, tangential migration may have contributed to the development or reorganization of axonal projections in the brain by providing additional intermediate targets and guidance cues for growing axons. Through this process, tangentially migrating neurons may have permitted developing axons to bypass nonpermissive territories, thereby contributing to the emergence of new connections. Our experiments on the development of thalamocortical projections illustrate this point. In the absence of corridor cells in the MGE domain, embryonic TCAs fail to enter the telencephalon or grow into ventral telencephalic regions, suggesting that corridor formation is a major requirement for the development of thalamic projections. Thus, our experimental evidence supports the hypothesis that tangential migration of corridor cells is likely to constitute a fundamental evolutionary step in the development of the forebrain.

Our results illustrate the importance that tangential migration has on the development of thalamocortical projections but also suggest that this may be a general mechanism controlling axonal pathfinding in the developing brain. In agreement with this idea, the development of several major tracts in the forebrain appears to be preceded by the tangential migration of an intermediate population. One clear example is the formation of the lateral olfactory tract (LOT), which transmits smell information from the olfactory bulb to the piriform cortex. Formation of the LOT correlates with the development of a subset of early-generated neurons designated as LOT cells, which reach their final destination through tangential migration (Tomioka et al., 2000) and have been suggested to guide LOT axons (Sato et al., 1998). Furthermore, a widespread network of early-born cells in the human forebrain forms tangential links between intermediate zones of the thalamus, ganglionic eminence, hypothalamus, and cortical preplate. This cellular network precedes the establishment of axonal connectivity in the forebrain and may provide guidance cues necessary for the navigation of growing axons (Bystron et al., 2005). Although this is difficult to test experimentally, this network of tangentially migrating neurons may include populations of neurons similar to the corridor cells described in our study, reinforcing the view that this process constitutes a general mechanism controlling the guidance of major axonal tracts in the forebrain of mammals, including humans.

EXPERIMENTAL PROCEDURES

Mouse Lines

Wild-type and GFP-expressing transgenic mice (Hadjantonakis et al., 1998), maintained in a CD1 or Swiss OF1 background, were used for expression analysis and tissue culture experiments. *HER4^{heart}* transgenic mice, which express a human *ErbB4* (HER4) cDNA under the control of the cardiac-specific α-HMC (myosin heavy chain) promoter, were maintained in a mixed C57Bl/6 × 129/SvJ background. *HER4^{heart}* transgenic mice were mated to *ErbB4* heterozygous mice (Gassmann and Lemke, 1997) to generate *ErbB4$^{+/-}$ HER4^{heart}* and *ErbB4$^{-/-}$ HER4^{heart}* mice, which were used in our experiments as control and *ErbB4* mutants, respectively. *ErbB4$^{-/-}$ HER4^{heart}* mice are null for the *ErbB4* gene except in the heart (Tidcombe et al., 2003). *CRD-Nrg1* heterozygous and homozygous mutant embryos were generated by crosses of heterozygous parents maintained on a mixed C57Bl/6 × 129/SvJ background. Null and floxed alleles for the *Nrg1* gene have been described elsewhere (Meyer and Birchmeier, 1995). *Foxg1$^{Cre/+}$* mice (Hebert and McConnell, 2000), a knock-in of the Cre recombinase, were used to obtain telencephalic *Nrg1* mutant embryos. *Mash1* heterozygous mice (Guillemot et al., 1993) were maintained in a mixed C57Bl6/DBA2 genetic background and crossed to produce homozygous embryos. Animals were kept under Spanish, French, and EU regulation.

In Situ Hybridization, Immunohistochemistry, and Axonal Tracing

For in situ hybridization, brains were fixed overnight in 4% paraformaldehyde in PBS (PFA). 20 μm frozen sections or 80 μm free-floating vibratome sections were hybridized with digoxigenin-labeled probes as described before (Garel et al., 2003; Flames et al., 2004). For combined fluorescent in situ hybridization and immunohistochemistry, fast Red (Roche) was used as an alkaline phosphatase fluorescent substrate.

For immunohistochemistry, cultured slices/explants and embryos were fixed in 4% PFA at 4°C for 30 min and from 6–12 hr, respectively. Immunohistochemistry was performed on: (1) culture slices; (2) dorsal thalamic explants, matrigel, or collagen pads; (3) 80 μm–100 μm free-floating embryo vibratome sections; or (4) 12–20 μm cryostat sections. The following antibodies were used: mouse anti-β3-Tubulin 1/1000 (Promega); rabbit anti-Calretinin 1/5000 (Swant); rabbit anti-GFP 1/1000 (Molecular Probes); mouse anti-Islet1 39.4D5 1/100 (Developmental Studies Hybridoma Bank); rabbit anti-Islet1/2 K5 1/5000 (a kind gift from T. Jessell); rabbit anti-Nkx2-1 1/2000 (Biopat); and rat anti-L1 1/200 (Chemicon).

For axonal tracing, embryonic brains were fixed by perfusion and overnight fixation in 4% PFA. Small DiI crystals (1,1′-dioctadecyl 3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate; Molecular Probes)

were inserted into the rostral part of the dTh thalamus after hemidissection of the brains. Brains were cut on a vibratome into 80–100 μm sections and mounted in Aquamount. Hoechst or Sytox Green (Molecular probes) was used for fluorescent nuclear counterstaining.

Slice Culture Experiments

Organotypic slice cultures of different levels of the embryonic mouse telencephalon were prepared as previously described (Anderson et al., 1997; Seibt et al., 2003). In *Mash1* mutant experiments, only rostral telencephalic slices were selected because they consistently lacked a MGE corridor. Brain slices were cultured on polycarbonate culture membranes (8 μm pore size; Corning Costar) or PET cell inserts (1 μm pore size; Beckton-Dickinson) in organ tissue dishes containing 1 ml of medium (Neurobasal/B-27 [Life Technologies] or BME/HBSS [Life Technologies] supplemented with glutamine, 5% horse serum, and pen/strep). In these assays, TCAs begin to grow after 36 hr; slices were cultured for 72–96 hr. Aggregates of COS7 transfected cells were prepared by diluting transfected cells with matrigel (Flames et al., 2004). Focal electroporation was performed as described before (Flames et al., 2004).

Quantification of Axonal Length

Dorsal thalamic explants were dissected from E13.5 wild-type mice and cultured in collagen, laminin, or matrigel for up to 96 hr. Explants were (1) confronted with COS cells aggregates transfected with *Gfp* or cotransfected with *Ig-Nrg1* and *Gfp*; or (2) cultured with medium supplemented with purified EGF-like domain from Heregulin β1 (0.1 μM, Peprotech). After fixation, dTh explants were subdivided into four sectors, and the length of the 15 longest axons was measured in every explant using Sigma Scan Pro software.

Supplemental Data

Supplemental Data include seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/1/127/DC1/.

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