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Short- and Long-Range Attraction of Cortical GABAergic Interneurons by Neuregulin-1

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Summary

Most cortical interneurons arise from the subcortical telencephalon, but the molecules that control their migration remain largely unidentified. Here, we show that different isoforms of Neuregulin-1 are expressed in the developing cortex and in the route that migrating interneurons follow toward the cortex, whereas a population of the migrating interneurons express *ErbB4*, a receptor for Neuregulin-1. The different isoforms of Neuregulin-1 act as short- and long-range attractants for migrating interneurons, and perturbing ErbB4 function in vitro decreases the number of interneurons that tangentially migrate to the cortex. In vivo, loss of Neuregulin-1/ErbB4 signaling causes an alteration in the tangential migration of cortical interneurons and a reduction in the number of GABAergic interneurons in the postnatal cortex. These observations provide evidence that Neuregulin-1 and its ErbB4 receptor directly control neuronal migration in the nervous system.

Introduction

The function of the cerebral cortex requires the coordinated action of two major neuronal types, the glutamatergic projection neurons and the GABAergic interneurons. Whereas it is well established that projection neurons of the cerebral cortex derive from the progenitor zones of the pallium, research over the past few years has provided compelling evidence that a large number of cortical GABAergic interneurons are born in the subpallial telencephalon (reviewed in Corbin et al., 2001; Marín and Rubenstein, 2001). This conclusion is based on a number of different experimental approaches, including analysis of cell dispersion using chimeras (Tan and Breen, 1993; Tan et al., 1995), neuronal migration in slice cultures (Anderson et al., 1997; De Carlos et al., 1996; Lavdas et al., 1999; Wichterle et al., 1999), genetic manipulations (Anderson et al., 1997; Casarosa et al., 1999; Sussel et al., 1999), and in vivo fate mapping of cortical GABAergic interneurons (Anderson et al., 2002; Nery et al., 2002; Wichterle et al., 2001). GABAergic interneurons born in the subpallium migrate tangentially to populate the entire cortex, including the piriform cortex, isocortex (e.g., neocortex), and hippocampus (Anderson et al., 1997; Lavdas et al., 1999; Marín et al., 2001; Pleasure et al., 2000; Sussel et al., 1999; Wichterle et al., 1999). Most tangentially migrating interneurons appear to derive from the medial ganglionic eminence (MGE), although the caudal ganglionic eminence (CGE) and, to some extent, the lateral ganglionic eminence (LGE) also give rise to cortical GABAergic interneurons (Anderson et al., 2001; Ang et al., 2003; Jiménez et al., 2002; Lavdas et al., 1999; Nery et al., 2002; Sussel et al., 1999; Wichterle et al., 1999, 2001).

Despite the detailed picture that is emerging on the origin and migratory routes followed by cortical interneurons, the mechanisms that control their migration from the subpallium to the developing cortex are still poorly characterized. Neurotrophins that activate the TrkB receptor (brain-derived neurotrophic factor [BDNF] and neurotrophin-4 [NT-4/5]), as well as hepatocyte growth factor (HGF), can acutely induce the motility of MGE-derived cells and therefore are thought to act as motogenic factors for migrating interneurons (Polleux et al., 2002; Powell et al., 2001). In addition, directional guidance of GABAergic interneurons from the MGE to the cortex appears to require the simultaneous activity of chemorepulsive and chemoattractive factors (Marín et al., 2001, 2003; Wichterle et al., 2003). Thus, MGE-derived cells actively avoid the preoptic area and the developing striatum in their way toward the cortex, whereas the developing cortex appears to produce an activity that attracts migrating interneurons. Although it is known that the repulsion of cortical interneurons by the striatal mantle is mediated by semaphorin/neuropilin interactions (Marín et al., 2001), the molecules responsible for the other activities have not been identified.

In an attempt to determine the molecular nature of the attractive activity present in the developing cortex, we have focused our analysis on one candidate, Neuregulin-1 (NRG1), a member of the neuregulin family of proteins. Neuregulins contain an epidermal growth factor (EGF)-like motif that activates membrane-associated tyrosine kinases related to the EGF receptor (also known

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as ErbB1). NRG1 directly binds to ErbB3 and ErbB4 receptors, which alone or in combination with ErbB2 mediate a large range of functions (Buonanno and Fischbach, 2001; Falls, 2003). In the central and peripheral nervous system, NRG1 signaling is of central importance for glial cell survival, proliferation, and differentiation (Adlkofer and Lai, 2000; Buonanno and Fischbach, 2001; Garratt et al., 2000). In neurons, NRG1 functions range from the control of proliferation to late differentiation events such as the regulation of ligand- and voltage-gated channels' expression (reviewed in Buonanno and Fischbach, 2001). In cortical structures such as the cerebellum and the neocortex, NRG1 signaling is important for the formation and maintenance of radial glial cells, which in turn are required to support neuronal migration (Anton et al., 1997; Rio et al., 1997; Schmid et al., 2003). Interestingly, NRG1 directly promotes the motility of Schwann cells (Mahanthappa et al., 1996; Meintanis et al., 2001), raising the possibility that it may also play a similar role for some neuronal types. Here, we present evidence that NRG1 is a chemoattractive factor for MGE-derived neurons and that NRG1/ErbB4 signaling plays a prominent role in the directional guidance of GABAergic interneurons from the subpallium to the developing cortex.

Results

Expression of *Nrg1* Isoforms in the Developing Telencephalon

Previous studies have shown that ErbB4 is expressed in a subpopulation of interneurons migrating from the MGE to the cortex (Yau et al., 2003) (Figure 1A), raising the possibility that ErbB4 and its high-affinity ligand, NRG1, might be involved in controlling cortical interneuron migration. The *Nrg1* gene is subject to differential promoter usage and alternative splicing, resulting in the expression of distinct protein isoforms (Buonanno and Fischbach, 2001; Falls, 2003). Three major classes of NRG1 proteins can be distinguished on the basis of their domain architecture. Types I and II comprise secreted isoforms that contain an extracellular immunoglobulin (Ig)-like domain (NRG1-Ig). Type III, on the other hand, corresponds to membrane bound isoforms (NRG1-CRD), which contain an extracellular cysteine-rich domain (CRD). The three types of NRG1 proteins share high sequence homology in their EGF-like domain, which is sufficient to elicit ErbB receptor dimerization, tyrosine phosphorylation, and the activation of downstream signaling pathways.

Using isoform-specific RNA probes, we found that *Nrg1-CRD*$^+$ is expressed throughout the LGE, from the subventricular zone to the developing striatal mantle (Figures 1B, 1G, and 1H). As previously reported, *Semaphorin3A* (*Sema3A*) and *Semaphorin3F* (*Sema3F*) were found to be expressed in the striatal mantle (Figures 1E–1G), where they create an inhibitory territory that migrating cortical interneurons avoid in their way toward the cortex (Marín et al., 2001). Interestingly, the analysis of adjacent sections revealed that the tangentially migrating *ErbB4*-expressing cells follow a corridor through the LGE that is *Nrg1-CRD*$^+$ and that lacks *Semaphorin3A/3F* (*Sema3A/3F*) expression (Figures 1A, 1B, and 1E–1I and Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/). In addition, the developing cortex—the target of the migrating ErbB4$^+$ interneurons—specifically expressed the diffusible form of the *Nrg1* gene, *Nrg1-Ig* (Figure 1C and Supplemental Figure S1). At the stage when migrating interneurons first enter the cortex (approximately at E13), *Nrg1-Ig* expression is stronger in lateral than medial regions of the cortex throughout the whole rostrocaudal extent of telencephalon (Supplemental Figure S1). *Nrg1-Ig* expression, however, expands to more medial regions of

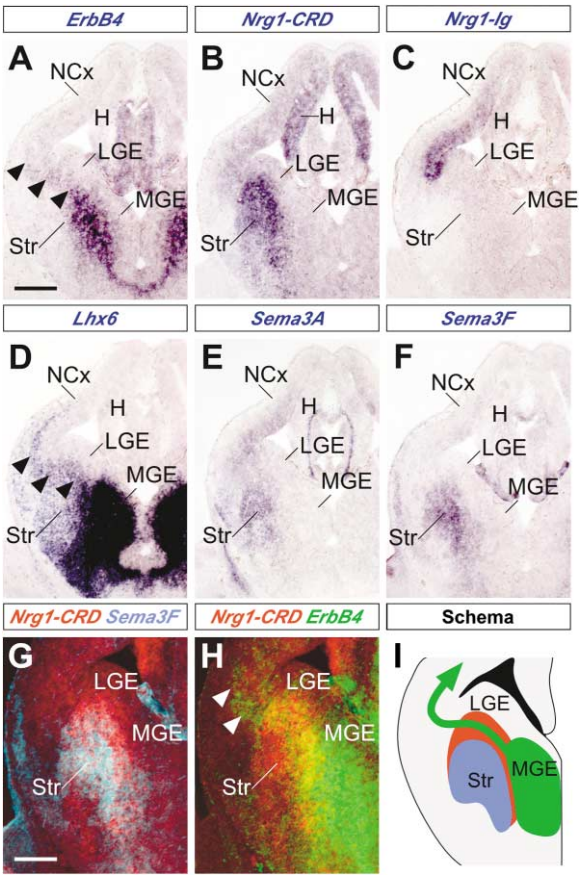

Figure 1. Complementary Expression of *Nrg1* Isoforms and Their *ErbB4* Receptor in the Developing Telencephalon during the Period of Interneuron Migration to the Cortex

Serial coronal sections through the telencephalon of E13.5 embryos showing mRNA expression for *ErbB4*, *Nrg1-CRD*, *Nrg1-Ig*, *Sema3A*, and *Sema3F*. (A and H) *ErbB4* mRNA expression in immature interneurons migrating toward the cortex (NCx; solid arrowheads). (D) *Lhx6* expression in immature interneurons migrating toward the cortex (NCx; solid arrowheads). (B, G, and H) *Nrg1-CRD* expression in the route of interneuron migration toward the cortex, in the developing striatum (Str) and in the anlagen of the hippocampus (H). (C) *Nrg1-Ig* expression in the cortex (NCx). (E) *Sema3A* expression in the striatum. (F and G) *Sema3F* expression in the striatum. *ErbB4*-expressing interneurons reach the cortex through a cellular corridor expressing *Nrg1-CRD* (G, H, and I), avoiding the striatal mantle due to Sema3A/3F-mediated chemorepulsion (for details, see Marín et al., 2001). Double in situ images (G and H) were composed from adjacent sections using Photoshop software. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bars equal 200 μm.

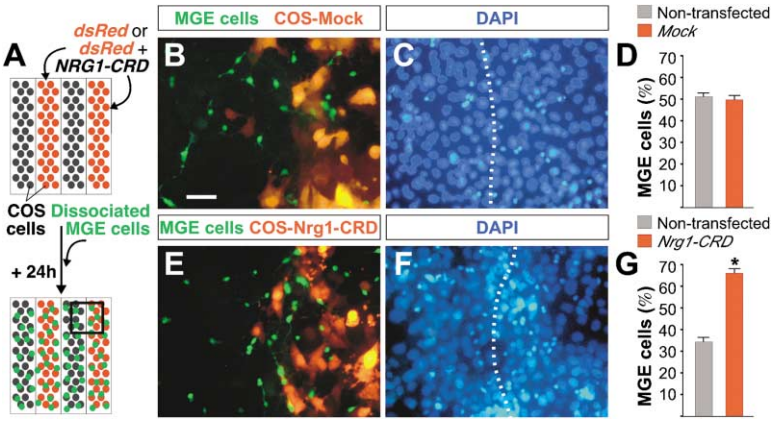

Figure 2. MGE-Derived Cells Prefer a *Nrg1-CRD*-Expressing Substrate in the Stripe Choice Assay

(A) Schematic of the experimental paradigm used in the stripe choice assay. Slides were coated with alternating stripes of nontransfected COS cells (dark gray dots) and either mock-transfected or Nrg1-CRD-transfected COS cells (red dots). Dissociated E13.5 MGE cells (green dots) were plated on top of the stripe carpets, and their distribution was studied 24 hr later. (B and E) Distribution of GFP MGE-derived cells in control (B) and experimental stripe carpets. (C and F) Homogeneous distribution of nontransfected and transfected COS cells as visualized with the fluorescent nuclear stain DAPI. The dotted lines indicate the boundary between nontransfected and transfected COS cells. (D and G) Quantification of the percentage of MGE cells on nontransfected and mock-transfected COS cells (D), or over nontransfected and Nrg1-CRD-transfected COS cells (G). $F = 21.65$; $^*p < 0.0005$ (data from three independent experiments). Scale bar equals 50 μm.

the cortex as development proceeds, in parallel with the progressive advance of migrating interneurons in medial direction (Supplemental Figure S1).

NRG1-CRD Is a Permissive Factor for MGE-Derived Neurons

The pattern of expression of the different *Nrg1* isoforms and their *ErbB4* receptor is consistent with a model in which ErbB4-expressing interneurons preferentially use *Nrg1-CRD*-expressing cells as substrate en route through the LGE toward the cortex. To test this hypothesis, we performed a stripe choice assay in which E13.5 MGE-derived cells were given the possibility to migrate on top of COS cells transfected with a control plasmid or with *Nrg1-CRD* (Figure 2A). In brief, we plated alternative stripes of COS cells that were either nontransfected or transfected with *dsRed* alone (control) or *dsRed* and *Nrg1-CRD* (experimental cases). Dissociated MGE cells that were obtained from green fluorescence protein (GFP)-expressing mice (Hadjantonakis et al., 1998) were plated on top of the stripes, and their final location was assayed 24 hr later. MGE cells showed no preference for either nontransfected or mock-transfected stripes in control experiments (Figures 2B–2D) but displayed an ~2-fold preference for *Nrg1-CRD*-expressing cells compared to nontransfected stripes ($n = 3$ independent experiments, 20 fields examined per experiment; Figures 2E–2G). Thus, MGE-derived cells preferred a *Nrg1-CRD*-expressing substrate, suggesting that NRG1-CRD may play a permissive role in the guidance of interneurons toward the cortex.

NRG1-Ig Is a Chemoattractant for MGE-Derived Neurons

Guidance of interneurons requires not only the existence of a permissive corridor toward the cortex but also the existence of a diffusible attractive activity that confers directionality to the migration (Marín et al., 2003; Wichterle et al., 2003). To test whether secreted NRG1 attracts cortical interneurons, we cocultured E13.5 MGE explants with aggregates of COS cells expressing the secreted *Nrg1-Ig* isoform in a three-dimensional matrix. In coculture experiments, the majority of MGE-derived cells were directed toward the source of NRG1 ($n = 38$; Figures 3A–3D). NRG1-Ig did not affect the mitotic index in the explants (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/), suggesting that the increased migration induced by NRG1-Ig was not caused by a rise in the number of cells produced in the MGE explant. Thus, NRG1-Ig is a chemoattractive factor for immature interneurons migrating from the MGE.

To test whether interneurons migrating to the cortex respond to NRG1-Ig in a more physiologically relevant context, we placed aggregates of *Nrg1-Ig*-expressing

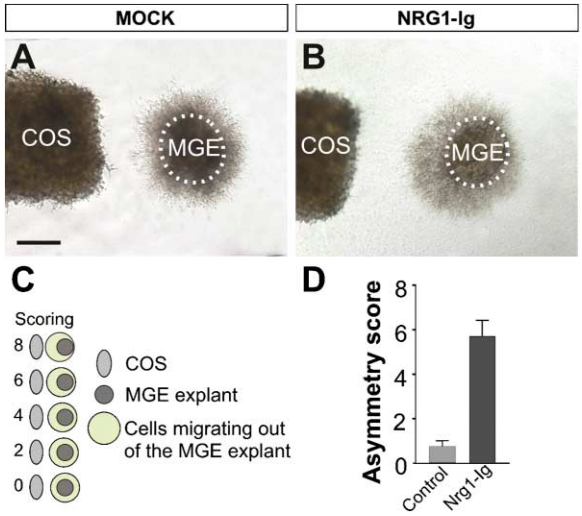

Figure 3. NRG1-Ig Is a Chemoattractant for MGE-Derived Cells

(A and B) Medial ganglionic eminence (MGE) explants from the telencephalon of E13.5 embryos were cultured in Matrigel adjacent to mock-transfected (A) or *Nrg1-Ig*-transfected (B) COS cell aggregates. (C) Scoring scheme modified from Zhu et al. (1999). (D) Quantification of the attraction of MGE cells by *Nrg1-Ig*-transfected COS cell aggregates ($n = 29$ explants). In addition to directing the migration of MGE-derived cells, NRG1-Ig enhanced the length of their leading process. Mean length of leading processes was 56.32 ± 2.18 μm (average ± SEM) in controls and 65.82 ± 3.31 μm in experimental cases. $F = 6.41$; $p < 0.014$. Scale bar equals 300 μm.

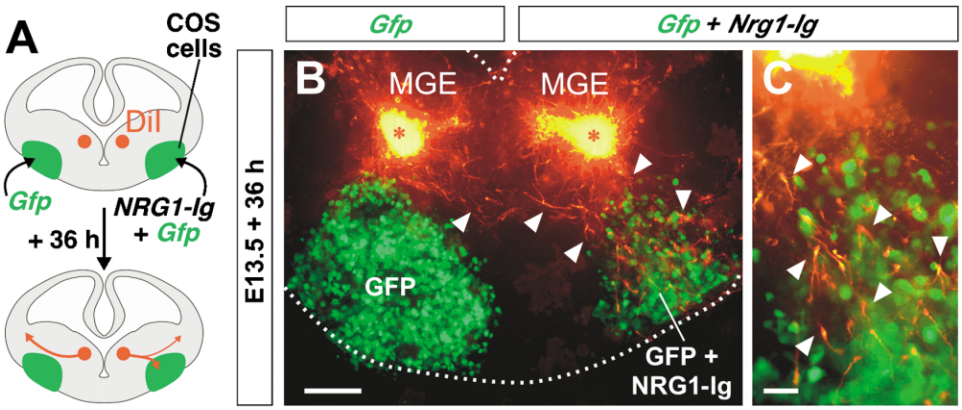

Figure 4. Ectopic Expression of NRG1-Ig Redirects the Migration of MGE-Derived Cells in Slice Cultures

(A) Schematic of the experimental paradigm used to analyze the migration of MGE-derived cells in slice cultures in the presence of control and *Nrg1-Ig*-transfected COS cells. (B) Coronal slice through the telencephalon with cell aggregates formed with control (left) and *Nrg1-Ig* (right) transfected COS cells. DiI-labeled cells (arrowheads) from both the ipsilateral and contralateral MGE (asterisk) migrate ectopically in a ventrolateral direction toward the COS cell aggregate expressing NRG1-Ig. (C) Higher magnification of the experimental side in (B). Arrowheads, DiI-labeled cells. Dotted lines, slice outline. Scale bars equal 200 μm (B) and 50 μm (C).

COS cells in the ventrolateral telencephalon in slice cultures (Anderson et al., 1997) and followed the migration of DiI-labeled E13.5 MGE cells (Figure 4A). As a control, aggregates of GFP-expressing COS cells were placed on the opposite side of the slices (Figure 4A). Cell aggregates expressing *Nrg1-Ig* attracted migrating interneurons toward the ventrolateral telencephalon (Figures 4B and 4C), diverting them from their normal route of migration toward the cortex (n = 25). Similar results were obtained when heparin beads soaked in recombinant NRG1 protein were used instead of cell aggregates (Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/). Control beads did not modify the migration of DiI-labeled MGE-derived cells toward the cortex (n = 25; Supplemental Figure S3B), whereas many MGE-derived cells abnormally migrated toward a NRG1-coated bead located in the ventrolateral telencephalon (n = 17/20; Supplemental Figure S3B). Thus, MGE-derived cells migrating toward the cortex are attracted by secreted NRG1.

ErbB4 Function Is Required for Interneuron Migration In Vitro

To determine if the attractive effect of NRG1 on MGE-derived cells was mediated by its high-affinity receptor, ErbB4, we next performed loss-of-function experiments. To this aim, we first analyzed the migration of MGE-derived interneurons expressing a dominant-negative form of *ErbB4* (*dnErbB4*) (Jones et al., 1999). In a three-dimensional matrix, cells derived from an isolated E13.5 MGE explant migrated uniformly in all directions (see, for example, Figure 3A). Thus, when an explant was subdivided into eight sectors of equal size, migrating cells within each of these sectors dispersed with an angle of roughly 45° (46 ± 4.35 [average ± SE]; n = 6; Supplemental Figure S4 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/). When MGE explants were cocultured along with a piece of embryonic cortex, the maximum angle adopted by neurons migrating within sectors 1 and 4 increased to roughly 90° (87 ± 1.3 [average ± SE]; n = 5; Figures 5A and 5B), because many neurons derived from the MGE were attracted toward the cortical explant (Marín et al., 2003; Wichterle et al., 2003). In contrast, MGE cells expressing *dnErbB4* became largely unresponsive to the cortical attractant, as revealed by the roughly 45° angle of migration adopted by the electroporated cells in the presence of a cortical explant (41 ± 4.4 [average ± SE]; n = 13; Figures 5A and 5C). It is of note that the rate of migration of GFP/dnErbB4-expressing cells was indistinguishable from that observed in cells electroporated with GFP alone (data not shown), suggesting that expression of dnErbB4 does not nonspecifically impair cell migration. Thus, ErbB4 appears to mediate, at least in part, the response of interneurons to the cortical attractive activity present in the developing cortex.

We next analyzed the pattern of migration of MGE cells expressing the dnErbB4 in slice cultures. For this set of experiments, we focally electroporated the MGE of slices obtained from E13.5 brains and analyzed the distribution of migrating cells after 36 hr in culture (Marín et al., 2001) (Figure 5D). In control experiments, GFP-expressing cells largely migrated through the Nrg1-CRD^{+}/Sema3A/F^{-} LGE corridor deep to the striatum on their way to the cortex (at least 200 cells in the cortex in 24 of 24 slices; Figures 5E and 5E′). In contrast, in slices electroporated with both *Gfp* and *dnErbB4*, most GFP-expressing cells failed to migrate to the neocortex and hippocampus (fewer than ten cells in the cortex in 16 of 19 slices) and instead accumulated in the basal telencephalon (Figures 5F and 5F′). These results suggest that decreased ErbB4 signaling interferes with the normal pattern of interneuron migration to the cortex and are in agreement with the hypothesis that NRG1 signaling through ErbB4 receptors mediates the migration of at least a population of cortical interneurons. To test this, we examined cortical interneurons in mice lacking either *ErbB4* or *Nrg1*.

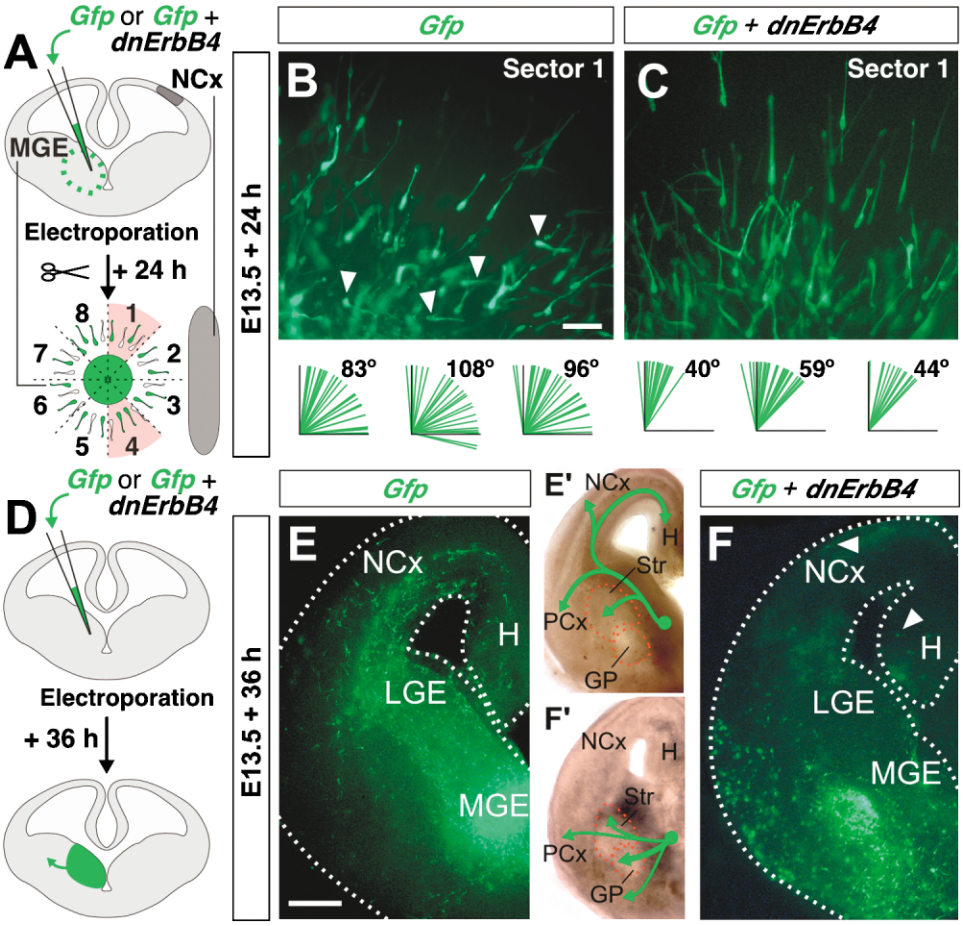

Figure 5. Loss of ErbB4 Function Perturbs Interneuron Migration to the Cortex

(A) Schematic of the experimental paradigm used to analyze the migration of MGE-derived cells expressing *Gfp* or *Gfp* + *dnErbB4*. Expression vectors were pressure injected focally into the MGE of coronal slice cultures, the slices were electroporated, and the electroporated MGE explants were then cultured in Matrigel adjacent to a piece of embryonic cortex. Explants were subdivided into eight sectors, and the migration angle adopted by MGE cells was quantified in sectors 1 and 4. (B and C) Migration of MGE-derived cells electroporated with *Gfp* (B) or *Gfp* and *dnErbB4* (C). Arrowheads point to cells directed toward the cortical explant (to the right in both [B] and [C]). Vector diagrams represent the orientation of migrating cells in three different cases of control (B) or experimental (C) explants, and the numbers illustrate the maximum angle range adopted by migrating cells in each case. Note that many MGE-derived cells in control experiments (B) migrated within the angle range expected for cells not attracted by the cortex (45°; Supplemental Figure S4 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/); a substantial number of MGE-derived cells do not migrate to the cortex in vivo but instead remain within the basal telencephalon (reviewed in Marín and Rubenstein, 2003). This suggests that a number of MGE-derived cells are normally unresponsive to the cortical attractant(s). (D) Schematic of the experimental paradigm used to analyze the migration of MGE-derived cells expressing *Gfp* or *Gfp* and *dnErbB4* in slice cultures. Dotted lines, slice outline. (E and F) Migration of cells electroporated with a *Gfp* expression vector alone (E) or with *Gfp* and *dnErbB4* expression vectors (F). Arrowheads point to a few cells that have reached the cortex in the experimental case. (E′ and F′) Schematic representation of migratory routes in the slices shown in (E) and (F), respectively. Scale bars equal 50 μm (B and C) and 200 μm (E and F).

Reduced Numbers of Cortical GABAergic Interneurons in *ErbB4* and *Nrg1* Mutants

Targeted inactivation of *ErbB4* results in midembryonic lethality due to failed development of myocardial trabeculae (Gassmann et al., 1995), precluding the analysis of interneuron migration in these animals. To circumvent this problem, we examined *ErbB4* mouse mutants in which the heart defects have been rescued by the expression of human *ErbB4* (*HER4*) under a cardiac-specific myosin promoter (Tidcombe et al., 2003). Consistent with our in vitro experiments, ErbB4 mutant interneurons largely failed to enter the NRG1-CRD^{+} corridor in the LGE (see Figure 1G) as they migrate toward the cortex (Figures 6A and 6B). As expected from these initial migratory defects, the embryonic cortex of *ErbB4*$^{-/-}$ *HER4*^{heart} mutant embryos contained significantly fewer migrating interneurons than controls. This was observed by immunohistochemistry against Calbindin (control, 53.67 ± 5.13; mutant, 39.06 ± 2.65 [cells, average ± SD]; $p < 0.026$; $n = 3$; Figures 6C and 6D), a well-established marker for migrating embryonic GABAergic interneurons (Anderson et al., 1997), or by in situ hybridization with other markers of cortical interneurons, such as *Lhx6* (data not shown).

In agreement with our previous experiments suggesting that NRG1 largely mediates the attraction of

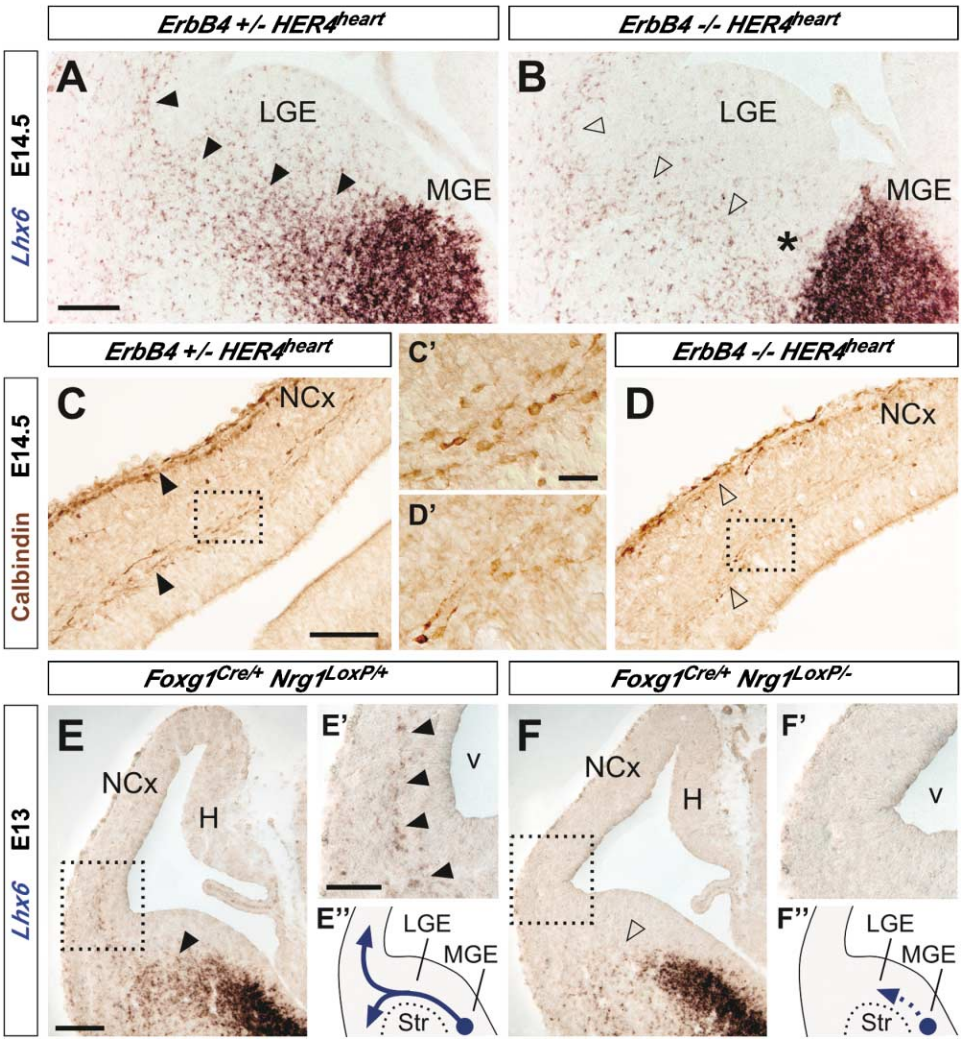

Figure 6. Loss of ErbB4/NRG1 Signaling Decreases the Number of GABAergic Interneurons in the Embryonic Cortex

(A and B) Coronal sections through the telencephalon of control (A) and *ErbB4* mutant (B) E14.5 embryos, showing *Lhx6* mRNA expression. Arrowheads point to migrating cells entering the LGE NRG1^{+} corridor in controls, which are disseminated in mutant embryos (empty arrowheads). Very few *Lhx6* neurons enter the LGE corridor at this early stage of the migration in ErbB4 mutants (asterisk). (C and D) Coronal sections through the cortex of control (C) and *ErbB4* mutant (D) E14.5 embryos, showing Calbindin immunohistochemistry. (C′ and D′) Details of the boxed areas in (C) and (D), respectively. Arrowheads point to cells migrating through the marginal zone or the subventricular zone in controls, most of which are missing in mutant embryos (empty arrowheads). (E and F) Coronal sections through the cortex of control and *Nrg1* mutant E13 embryos, showing *Lhx6* RNA expression. (E′ and F′) Details of the boxed areas in (E) and (F), respectively. Arrowheads point to migrating cells entering the cortex in controls, which are absent in mutant embryos. (E″ and F″) Schemas depict the migratory route followed by *Lhx6* cells in control (E) and *Nrg1* mutants (F). H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum. Scale bars equal 50 μm (C′ and D′) and 100 μm (A, B, E, F, E′, and F′).

ErbB4^{+} cortical interneurons, tangentially migrating cells also failed to enter the LGE corridor and reach the cortex in *Nrg1* conditional mutant embryos at the onset of migration (n = 3; Figures 6E and 6F). Moreover, similar to *ErbB4*$^{-/-}$ *HER4*^{heart} mutants, the number of migrating interneurons in the cortex of *Nrg1* conditional mutants was also reduced at E14.5 (data not shown). Thus, the embryonic phenotypes of the two mutant lines fully support the in vitro evidence that ErbB4/Nrg1 signaling participates in controlling the tangential migration of interneurons to the cortex.

Although *Nrg1* conditional mutants die perinatally (A.N.G. and C.B., unpublished data), *ErbB4*$^{-/-}$ *HER4*^{heart} mice develop to adulthood, allowing the examination of the cerebral cortex in postnatal mice. Gross anatomical observation of Nissl-stained coronal sections through the telencephalon of rescued *ErbB4* mutant mice did not reveal gross morphological defects in 3-week-old mice (Figures 7A and 7B). Nevertheless, immunohistochemical analysis using anti-GABA antibodies demonstrated a significant decrease in the number of GABA+ cells in the postnatal cortex of rescued *ErbB4* mutant mice compared to controls (n = 3; Figures 7C and 7D). Quantification of these defects at different rostrocaudal levels of the cerebral cortex showed that the number of GABA+ interneurons was normal at rostral cortical levels, but there was a significant reduction at midcortical and caudal cortical levels (Figure 7E). The decrease

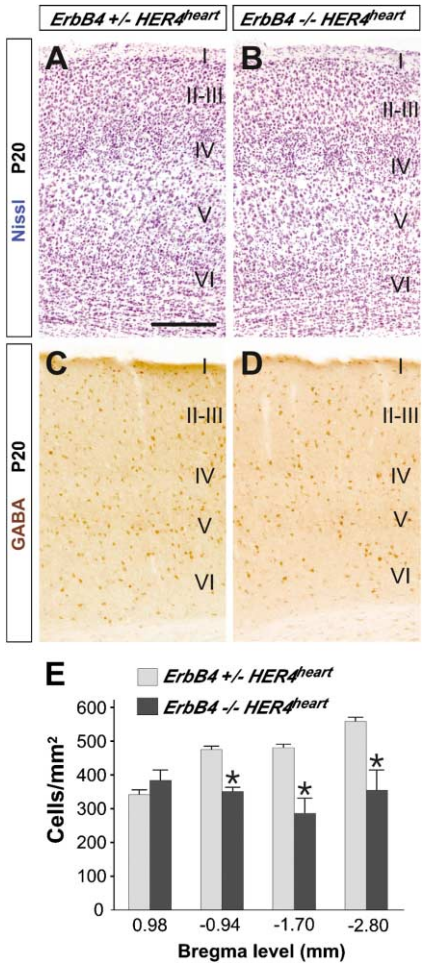

Figure 7. Loss of ErbB4 Signaling Decreases the Number of GABAergic Interneurons in the Postnatal Cortex

Coronal sections through the parietal cortex of P20 control (A and C) and ErbB4 mutant (B and D) mice. Roman numerals designate cortical layers. (A and B) Nissl staining. (C and D) GABA immunohistochemistry. (E) Quantification of the number of $GABA^+$ interneurons at different rostrocaudal levels of the cortex in control (light gray) and *ErbB4* mutant (dark gray) mice. $F = 1.88$, $p > 0.20$ (Bregma 0.98); $F = 68.29$, $^*p < 0.0005$ (Bregma −0.94); $F = 10.03$, $^*p < 0.02$ (Bregma −1.70); $F = 10.15$, $^*p < 0.02$ (Bregma −2.80). Scale bar equals 200 μm.

in the number of GABAergic neurons appeared to be uniform across all cortical layers. In addition, the number of GABAergic cells was also significantly reduced in the hippocampus of rescued *ErbB4* mutant mice compared to controls (Supplemental Figure S5 at http://www.neuron.org/cgi/content/full/44/2/251/DC1/). Thus, ErbB4/NRG1 signaling appears to be required for normal development of a subpopulation of cortical GABAergic interneurons in vivo.

Discussion

Our results demonstrate that NRG1 is a chemoattractant for a population of cortical interneurons and that this effect is mediated, at least in part, through ErbB4 receptors. First, a subpopulation of MGE-derived interneurons migrating toward the cortex expresses *ErbB4*. In the LGE, these cells move through a corridor that expresses a membrane bound form of NRG1, which may act as a permissive cue for their migration. Moreover, the developing cortex expresses a diffusible isoform of NRG1, which in vitro acts as a potent attractant for MGE-derived cells. Furthermore, loss of ErbB4 or NRG1 function perturbs interneuron migration to the cortex and alters the number of GABAergic interneurons in the postnatal cortex. Consequently, we suggest that NRG1/ErbB4 interactions mediate both short- and long-range attraction for tangentially migrating interneurons at different stages of their journey and that this signaling system is required for the normal development of a subpopulation of cortical interneurons.

ErbB Receptors and Neuronal Migration in the Nervous System

NRG1 directly binds to ErbB3 and ErbB4 receptors, which alone or in combination with ErbB2 mediate the large range of functions attributed to this factor during the development of the nervous system (Buonanno and Fischbach, 2001; Falls, 2003). In the brain, NRG1 is required for the normal development of radial glial cells, and this function appears to depend on either ErbB2 or ErbB4 receptors (Anton et al., 1997; Schmid et al., 2003). In the cerebral cortex, for example, disrupting ErbB2 function leads to abnormal radial glia formation, indirectly perturbing the radial migration of projection neurons (Anton et al., 1997; Schmid et al., 2003). In the cerebellum, NRG1 induces astrocytes to adopt a radial glia phenotype in vitro, which is in turn required to support neuronal migration (Rio et al., 1997). Thus, ErbB2 and ErbB4 appear to have only an indirect role in the process of radial migration in the developing central nervous system, contributing to the development of the supporting elements necessary for the radial migration of neocortical projection neurons and cerebellar granule cells.

In contrast to the non-cell-autonomous effects of ErbB2 and ErbB4 signaling on radial migration, our experiments demonstrate that ErbB4 cell-autonomously directs the tangential migration of MGE-derived interneurons toward the $NRG1^+$ neocortex and thus directly implicate this signaling system in the control of neuronal migration in the developing brain. In slice cultures, migrating interneurons in which ErbB4 function has been disrupted failed to reach their target, demonstrating that the expression of this receptor confers on migrating interneurons the ability to respond to chemoattractive cues secreted by the cortex. Previous studies have also shown that a closely related receptor, the epidermal growth factor receptor (EGFR or ErbB1), can cell-autonomously induce chemotaxis of migrating neurons (Caric et al., 2001), probably in response to ligands such as heparin binding epidermal growth factor (HB-EGF) or transforming growth factor α (TGFα). ErbB1 signaling, however, appears to exclusively influence radially migrating cortical neurons but not tangentially migrating interneurons. Since ErbB4 is also expressed in tangentially migrating neurons in other regions of the developing brain, including the pontine region or the cerebellar anlagen (N.F. and O.M., unpublished data),

it appears that ErbB1 and ErbB4 receptors may have specialized in regulating different types of migration in the developing brain. Further analysis on the role of these members of the ErbB family of tyrosine kinase receptors is required to resolve this question.

Molecular Guidance of Cortical GABAergic Interneurons

The migration of immature GABAergic interneurons from the subpallium to the developing cortex involves both chemorepulsive and chemoattractive factors. Chemorepulsive cues appear to be required to prevent the migration of cortical interneurons to telencephalic structures other than the cortex. This is illustrated, for example, by the role of semaphorin/neuropilin interactions in restricting the access of migrating cortical interneurons to the developing striatum (Marín et al., 2001). A similar mechanism may restrict the ventral migration of cortical interneurons into the preoptic area (Marín et al., 2003; Wichterle et al., 2003), although the molecule(s) responsible for this process has not been determined.

Several molecules have been described that promote the migration of cortical GABAergic interneurons, such as NT4/5 and HGF (Polleux et al., 2002; Powell et al., 2001). To our knowledge, however, NRG1-Ig is the first factor that has been described to have a chemoattractive effect on cortical interneurons. Interestingly, nonsecreted forms of NRG1 also influence the migration of cortical interneurons by creating a permissive corridor used by GABAergic interneurons in their way toward the cortex. Since all isoforms of the *Nrg1* gene contain an EGF-like domain that appears to mediate all its biological functions, it is remarkable that the different membrane and secreted isoforms of *Nrg1* are expressed in such a coordinated pattern to control the guidance of ErbB4$^+$ interneurons. As cells leave the MGE, repellent signals largely govern the guidance of migrating interneurons by delineating the territories that are permissive to their migration. This is the case for Sema3A/3F expression in the striatal mantle or the uncharacterized activity present in the preoptic area. However, Nrg1-CRD expression appears to be crucial to channel ErbB4$^+$ interneurons through the LGE subventricular zone toward the pallial-subpallial boundary. This is clearly illustrated in our experiments by the permissive role of NRG1-CRD in vitro and the inability of migrating cells to enter the LGE corridor in *ErbB4* and *Nrg1* mutant embryos. In addition to the channeling role of NRG1-CRD, NRG1 function also relies on secreted isoforms (NRG1-Ig), which establish the required chemoattractive gradient that directs GABAergic interneurons toward the cortex.

It is worth noting that ErbB4 is only expressed in a subpopulation of interneurons that migrates toward the cortex, suggesting that different types of cortical interneurons may be guided by distinct factors. In addition, it is possible that the same population of interneurons respond to several migration-promoting factors at the same time, like HGF and NRG1, which will contribute to ensure proper guidance of interneurons toward the cortex. Of note, the urokinase plasminogen activator (uPA) and its receptor (uPAR) appear to be involved at some level in the role of HGF in promoting MGE migration (Powell et al., 2001), whereas both uPA and uPAR appear to mediate the cell invasive behavior that is induced by NRG1 in cancer cells (Mazumdar et al., 2001). Thus, it would be interesting to determine how these different signaling systems could interact at the molecular level to generate an integrated cellular response.

NRG1, GABAergic Interneurons, and the Etiology of Schizophrenia

The role of NRG1/ErbB4 signaling in the development of cortical GABAergic interneurons is of further interest, as NRG1 has been implicated in susceptibility to schizophrenia (Corvin et al., 2004; Harrison and Owen, 2003; Li et al., 2004; Stefansson et al., 2002, 2003; Tang et al., 2004; Williams et al., 2003; Yang et al., 2003) (reviewed in Corfas et al., 2004). Given the evidence that schizophrenia may result from abnormal neurodevelopment (reviewed in Lewis and Levitt, 2002) and that defective GABA neurotransmission may underlie its pathophysiology (Benes and Berretta, 2001; Keverne, 1999; Lewis, 2000), a defect in the development of cortical GABAergic interneurons could cause this disorder. Thus, the evidence presented herein that implicates both NRG1 and ErbB4 in cortical interneuron development provides a coherent biological context whereby NRG1 may confer susceptibility to some forms of schizophrenia. Since it is likely that NRG1 contributes to the pathogenesis of the disorder in only a subset of the patients, it will be important to test the existence of a correlation between an abnormal NRG1 genotype and migration defects in human cases. Moreover, since ErbB4 expression is maintained in a subpopulation of cortical interneurons in the postnatal cortex (Yau et al., 2003), it remains to be tested whether NRG1 may also influence later stages in the development of GABAergic interneurons, including the elaboration of axon arbors or synapse formation, which may also contribute to the pathophysiology of this disorder (Woo et al., 1998). In view of the important role that the expression of different isoforms of *Nrg1* has in the development of cortical interneurons, it is tempting to speculate that an alteration in the expression pattern of the different isoforms of the *Nrg1* gene in humans may lead to abnormal cortical GABAergic interneurons function due to defects in this neuronal population at different stages of development.

Experimental Procedures

Animals

Wild-type and GFP-expressing transgenic mice (Hadjantonakis et al., 1998), maintained in a CD1 background, were used for expression analysis and cell 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 C57b/6 and 129/SvJ background. *HER4^{heart}* transgenic mice were mated to ErbB4 heterozygous mice to eventually generate *ErbB4$^{+/-}$ HER4^{heart}* and *ErbB4$^{-/-}$ HER4^{heart}* littermate 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, due to the expression of the *HER4* transgene (Tidcombe et al., 2003). The generation of *ErbB4* mutant mice (Gassmann et al., 1995) and *HER4^{heart}* transgenic mice (Tidcombe et al., 2003) has been previously described in detail. Null and floxed alleles for the *Nrg1* gene have also been described elsewhere (Meyer and Birchmeier, 1995). *Foxg1$^{Cre/+}$* (Hebert and McConnell, 2000) mice were used to obtain telencephalic *Nrg1* mu-

tant embryos. It should be noted that, in *Foxg1^{Cre}* mice, *Cre* was knocked in the *Foxg1* locus, and therefore mice expressing Cre are also heterozygous for the *Foxg1* gene. To distinguish the effect of the *Nrg1* deletion from any possible phenotype present in the absence of one copy of the *Foxg1* gene, we systematically used *Foxg1$^{Cre/+}$* as controls in our experiments. Animals were kept at the Instituto de Neurociencias under Spanish and EU regulation. The Ethics Review Committee of the University Miguel Hernández approved all animal protocols that were used in the present study.

In Situ Hybridization and Immunohistochemistry

Twenty micrometer frozen sections were hybridized with digoxigenin-labeled probes as described before (Schaeren-Wiemers and Gerfin-Moser, 1993). The following cDNA probes were used in this study: *ErbB4, Nrg1-CRD, Nrg1-Ig*, and *Lhx6* (kindly provided by V. Pachnis); *Sema3A* and *Sema3F* (kindly provided by M. Tessier-Lavigne); and *Gad67* (kindly provided by B. Condie). *Nrg1-Ig* spans sequence number 345–845 in accession number NM_031588, one of the rat Nrg1-Ig domain-containing isoforms, whereas Nrg1-CRD spans sequence number 555–1321 in accession number AF194438, a rat Nrg1 SMDF isoform. Double in situ images shown in Figure 1 were composed from adjacent sections using Photoshop software.

For immunohistochemistry in postnatal brains, mice were anesthetized and perfused with 4% PFA. Brains were removed, cryoprotected in 30% sucrose in PBS, and cut frozen in the transverse plane on a sliding microtome at 40 μm. Free-floating sections were then incubated in 1% BSA and 0.1% TX in PBS for 30 min at room temperature and were subsequently incubated with rabbit anti-GABA antibodies (Sigma) for 36 hr at 4°C in 0.5% BSA and 0.1% TX in PBS. Sections were then incubated in biotinylated secondary antibodies (Vector), diluted 1:200, and processed by the ABC histochemical method (Vector). In each experiment, sections from homozygous mutants and their wild-type or heterozygous littermates were processed together.

For immunohistochemistry in MGE explants, Matrigel pads were fixed overnight in 4% PFA, incubated in 1% BSA and 1% TX in PBS for 30 min at room temperature, and subsequently incubated with primary antibodies (GFP, diluted 1:1000, Molecular Probes; Phospho-Histone H3, diluted 1:200, Upstate) for 36 hr at 4°C in 0.5% BSA and 1% TX in PBS. After washing, Alexa 488 or 595 secondary antibodies (Molecular Probes) diluted 1:200 were used.

COS Cells

COS7 cell aggregates expressing *Gfp*, *Nrg1-Ig*, or both were prepared by diluting transfected cells with Matrigel in a 1:1 proportion. After jellification, COS cell aggregates were cut with a scalpel in small cubes with dimensions of approximately 400 μm. In control experiments, a control vector was transfected together with *Gfp* into COS7 cells. The sequences of the cDNAs used for expression of type I NRG1 (Nrg1-Ig) and type III NRG1 (NRG1-CRD) correspond to Nrg1-typeIβ1a (accession number AY648976) and Nrg1-typeIIIβ1a (accession number AY648975), respectively.

Bead Experiments

Beads were loaded with recombinant NRG1 protein as described before (Martínez et al., 1999). The recombinant NRG1 protein was constructed by expressing the EGF-like domain of NRG-1 as a C-terminal GST fusion protein.

Stripe Choice Assay

COS cells were plated in a two-well chamber slide (Lab-Tek Chamber Slide System Permanox Slide 177429), transfected at approximately 70% confluence, and incubated overnight. To produce the stripes, transfected cells were removed with a pipette tip (one line every 2 mm), and nontransfected cells were plated on top (350.000 cells per well). Slides were subsequently incubated for 30 min to allow cells attachment to the empty stripes. The excess of cells was then washed out with three rinses of PBS, new media was added, and after 12 hr, GFP-expressing dissociated cells from the MGE were plated on top (10^5 cells per well). Analysis was performed after 24 hr.

In Vitro Focal Electroporation

Coronal slice cultures were obtained as described previously (Anderson et al., 1997). *Gfp* was cloned into pCAGGS, a chicken β-actin promoter expression vector. The expression vector was pressure injected focally into the MGE of coronal slice cultures by a Pneumatic PicoPump (Narishige) through a glass micropipette. Slices were electroporated within a setup of two horizontally oriented platinum electrodes (Protech International Inc.) powered by a T820 Electro Square Porator (BXT).

Quantification of Migration Angle

Telencephalic slices from 13.5 embryos were electroporated into the subventricular zone of the MGE with either *Gfp* expression vector (control) or a mix of *Gfp* and *dnErbB4* expression vectors. Immediately after electroporation, MGE explants were dissected out and cultured adjacent to cortical explants in Matrigel for 24 hr. Each MGE explant was subdivided into eight equal sectors. The orientation of migrating cells was estimated by drawing a vector with its origin in the nucleus and the direction of the leading process. To study the range of angles in which cells migrated out of the explant sector, the vectors of each experiment were pooled together in its origin, and the angle between the two most differently oriented cells was measured.

Quantification of GABAergic Interneurons in the Cortex

For the quantification of Calbindin+ interneurons in the embryonic cortex of *ErbB4$^{-/-}$ HER4^{heart}* mutants and controls, a 200 μm profile of the dorsal cortex at intermediate rostrocaudal levels was selected for each hemisphere, and migrating interneurons were counted throughout the thickness of the developing pallium, for three different brains from each genotype. For the quantification of GABAergic interneurons in the postnatal cortex of *ErbB4$^{-/-}$ HER4^{heart}* mutants and controls, a 300 μm profile of the lateral pallial wall, from the lateral ventricle to the pial surface, was counted in four different rostrocaudal levels (Bregma levels 0.98, −0.94, −1.70, and −2.80), for three different brains from each genotype.

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