Neuron, Vol. 45, 701–713, March 3, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.01.043

GDNF and GFRα1 Promote Differentiation and Tangential Migration of Cortical GABAergic Neurons

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Summary

Cortical GABAergic neurons are generated in the ventral telencephalon and migrate dorsally into the cortex following a tangential path. GDNF signaling via GFRα1 was found to promote the differentiation of ventral precursors into GABAergic cells, enhancing their neuronal morphology and motility. GDNF stimulated axonal growth in cortical GABAergic neurons and acted as a potent chemoattractant of GABAergic cells. These effects required GFRα1 but neither RET nor NCAM, the two transmembrane signaling receptors known for GDNF. Mutant mice lacking GDNF or GFRα1, but neither RET nor NCAM, showed reduced numbers of GABAergic cells in the cerebral cortex and hippocampus. We conclude that one of the normal functions of GDNF signaling via GFRα1 in the developing brain is to promote the differentiation and migration of cortical GABAergic neurons. The lack of involvement of RET or NCAM in these processes suggests the existence of additional transmembrane effectors for GDNF.

Introduction

In the cerebral cortex, progenitors proliferate in the ventricular zone (VZ) and then migrate into the cortical layers where they differentiate into specific cell types. In the dorsal part of the telencephalon (pallium), neuronal precursors that migrate radially from the underlying ventricular zone give raise to excitatory pyramidal neurons in the cerebral cortex (Rakic, 1972, 1974; Nadarajah and Parnavelas, 2002). On the other hand, cortical inhibitory interneurons are generated in the ventral telencephalon (subpallium) and migrate tangentially into the developing cortex (Lavdas et al., 1999; Pleasure et al., 2000; Anderson et al., 2001; Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003). The main region of GABAergic neurogenesis is localized to the ganglionic eminences, transient neurogenic sites in the embryonic mammalian brain, particularly the medial ganglionic eminence (MGE), with a smaller contribution from the lateral (LGE) and caudal (CGE) eminences (Tamamaki et al., 1997; Lavdas et al., 1999; Anderson et al., 2001; Wichterle et al., 2001; Nery et al., 2002; Xu et al., 2004). As they turn postmitotic, MGE-derived precursors become highly motile, leave the ventricular and subventricular zones (VZ/SVZ) of the MGE, and express GABA and calbindin (Fonseca et al., 1995; Del Rio et al., 2000; Anderson et al., 2001; Tanaka et al., 2003). In the mouse, the first wave of MGE-derived GABAergic cells migrates into the cortex at E12.5.

The migration of GABAergic cells into the cortex appears to be supported by the combinatorial action of diverse signals: (1) motogenic factors in the MGE, (2) repulsive factors in areas surrounding the MGE, and (3) permissive as well as chemoattractive factors in the developing cortex (Marin et al., 2003; Marin and Rubenstein, 2003; Wichterle et al., 2003). The molecular identities of the signals that regulate GABAergic cell differentiation and guide their migration from the MGE to the cortex are only beginning to be elucidated. The neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) have been shown to stimulate tangential migration of MGE-derived GABAergic cells into the cortex (Polleux et al., 2002). However, it is still unclear whether these neurotrophins affect the primary differentiation of MGE precursors into GABAergic cells, as they are mainly expressed during later stages of embryonic development. Hepatocyte growth factor (HGF) has also been implicated in GABAergic cell motility (Powell et al., 2001; Levitt et al., 2004). Although expression of both HGF and its receptor MET have been detected in the VZ/SVZ of the MGE (Powell et al., 2001), it is still unknown whether this factor plays a role in the differentiation of GABAergic cells in this region. Expression of the semaphorins 3A and 3F has been detected in the developing striatum, and both of these molecules are potent chemorepellents for MGE-derived GABAergic cells (Marin et al., 2001). In addition, recent studies have indicated that ventral regions neighboring the MGE are nonpermissive for GABAergic cell migration, whereas dorsal regions leading to the cortex are increasingly permissive (Marin et al., 2003; Wichterle et al., 2003). Whether these factors represent new or previously characterized molecules has not yet been clarified.

Glial cell line-derived neurotrophic factor (GDNF) was originally discovered because of its ability to promote the survival of ventral midbrain dopaminergic neurons (Lin et al., 1993). Members of the GDNF ligand family signal by binding to GPI-anchored receptors termed GFRα1 to 4, in collaboration with signaling receptor subunits such as the RET tyrosine kinase or the $p140^{NCAM}$ isoform of the neural cell adhesion molecule NCAM (Airaksinen and Saarma, 2002; Paratcha et al., 2003). Knockout mice lacking GDNF or GFRα1 display various deficits among subpopulations of sensory and enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). However, no abnormalities during brain development have so far been uncovered in those mice, which die prematurely a few hours after birth due to kidney agenesis and lack of enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996).

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In the present work, we set out to investigate possible functions of GDNF signaling during brain development in vivo by first studying the patterns of expression of GDNF and its receptors in the embryonic forebrain. We found that GDNF and GFRα1 were expressed in the MGE and the developing cortex along the pathway of migration of GABAergic cells, which prompted us to investigate a possible role for GDNF signaling in the differentiation and migration of cortical GABAergic cells.

Results

Expression of GDNF and GFRα1 in the MGE and Along the Tangential Migratory Pathway of GABAergic Cells in the Developing Cerebral Cortex

The expression patterns of GDNF and its receptors were investigated in the MGE, the site where the majority of cortical GABAergic cells are thought to be generated, and along the tangential migratory pathway of GABAergic cells in the developing cortex. GABAergic cells in the MGE and cortex were localized by immunohistochemistry for calbindin, a well-known marker for those cells (Fonseca et al., 1995; Anderson et al., 1997; Tanaka et al., 2003). At embryonic day (E) 12.5, calbindin immunoreactivity was observed in the mantle of the MGE, but not in ventricular (VZ) and subventricular (SVZ) zones of this structure where cell proliferation takes place (Figure 1A). At this stage, expression of *Gdnf* mRNA was concentrated in the proliferative (i.e., VZ and SVZ) zones of the ganglionic eminences and in the developing cortex (Figure 1B). No labeling above background could be observed in sections hybridized with a *Gdnf* sense probe (Figure S1A in the Supplemental Data available with this article online). By E15.5, GABAergic cells have already colonized the cortical plate (Fonseca et al., 1995; Del Rio et al., 2000; Tanaka et al., 2003), and at this stage, *Gdnf* mRNA was localized to the marginal zone (MZ), cortical plate (CP), and the VZ and SVZ, but not the intermediate zone (IZ), of the dorsal developing cortex (Figure 1E). These areas of *Gdnf* mRNA expression correspond to the main migratory pathways of cortical GABAergic cells originated in the MGE, as shown by calbindin immunoreactivity in an equivalent section (Figure 1D). Expression of RET was not detected in the ganglionic eminences or developing cerebral cortex at these embryonic stages by either immunohistochemistry or in situ hybridization (data not shown). Despite the abundant expression of polysialic acid (PSA)-modified NCAM in the embryonic mouse brain (Jimenez et al., 2002), no immunoreactivity for $p140^{NCAM}$, the main NCAM isoform involved in GDNF signaling (Paratcha et al., 2003), could be observed at E12.5 in the ganglionic eminences or developing cortex using an antibody directed against the intracellular domain (ICD) of this receptor (Figure S2).

In contrast, strong GFRα1 immunoreactivity could be observed in the mantle of the MGE and along the marginal zone of the developing cortex at E12.5, correlating with calbindin (Figures 1C and 1F). GFRα1 appeared to be expressed by a subpopulation of GABAergic cells in primary cultures of the MGE (data not shown), suggesting an interesting level of heterogeneity among GABAergic cells originating from this structure. Besides their overlap in the marginal zone, expression of GFRα1 was somewhat broader than that of calbindin in the developing cortex at this age, indicating additional cell types expressing this receptor. Cells coexpressing GFRα1 and calbindin were observed in the two pathways of tangential cell migration from the MGE to the cortex (Marin et al., 2001; Marin and Rubenstein, 2003) (Figure S3) and in the marginal zone of the developing cortex (Figure 1G). At E13.5, cells coexpressing GFRα1 and Lhx6, an early marker of MGE-derived cells (Grigoriou et al., 1998; Lavdas et al., 1999), could be observed in the mantle of the MGE and in the marginal zone of the developing cortex (Figures 1H–1M). No specific GFRα1 immunoreactivity could be detected in sections from *Gfra1* knockout mice (Figure S1B). RT-PCR analyses confirmed the expression of *Gdnf* and *Gfra1* mRNAs in E12.5 MGE and E15.5. cortex, with lower levels observed in E14.5 MGE and E12.5 cortex (Figure S4). On the other hand, very low levels of *Ret* mRNA could be detected in the MGE and developing cortex by RT-PCR, while *Ntn* mRNA could not be detected, and *Gfra2* mRNA could only be detected after additional PCR cycles (Figure S4).

GDNF Promotes the GABAergic Phenotype of MGE-Derived Neuronal Precursors

GDNF increased the proportion of GABAergic cells in dissociated cultures from the E12.5 MGE, an effect that was comparable to that produced by a similar dose of BDNF (Figure 2B). Moreover, GDNF stimulated the morphological differentiation of these cells, visualized by GABA immunoreactivity, increasing the number of neurites per cell (Figures 2A–2C). Cell viability and proliferation were not affected by GDNF (Figure 2D and data not shown). The GDNF homolog Neurturin (NTN) had no significant effects on these cultures (data not shown). At the biochemical level, GDNF stimulated strong and rapid (5 min) phosphorylation of MAP kinases ERK1/2, as well as moderate AKT phosphorylation after 1 hr of treatment (Figure 2E). The effects of GDNF on GABAergic cell number could be abolished by the MEK1 inhibitor PD98059 (Figure 2F), indicating that MAP kinase activity is required for the ability of GDNF to promote GABAergic differentiation of MGE cells. On the other hand, the activity of BDNF on these cells, which is known to depend on the PI3K pathway (Polleux et al., 2002), was unaffected by the MEK1 inhibitor (Figure 1F). Together, these data suggested that GDNF was able to promote the GABAergic phenotype of MGE cells without affecting their survival or proliferation.

GDNF was still able to stimulate the morphological differentiation of GABAergic cells lacking RET or NCAM (Figure 3A) and to increase the proportion of GABAergic cells of either genetic background (Figure 3B), indicating that the effects of GDNF in GABAergic cell differentiation were not mediated by signaling through RET or NCAM receptors. In contrast, GABAergic cells in MGE cultures derived from mice lacking GFRα1 failed to respond to GDNF (Figures 3A and 3B). Intriguingly, the morphology of *Gfra1* mutant cells was very different from wild-type, even in the absence of GDNF, with large

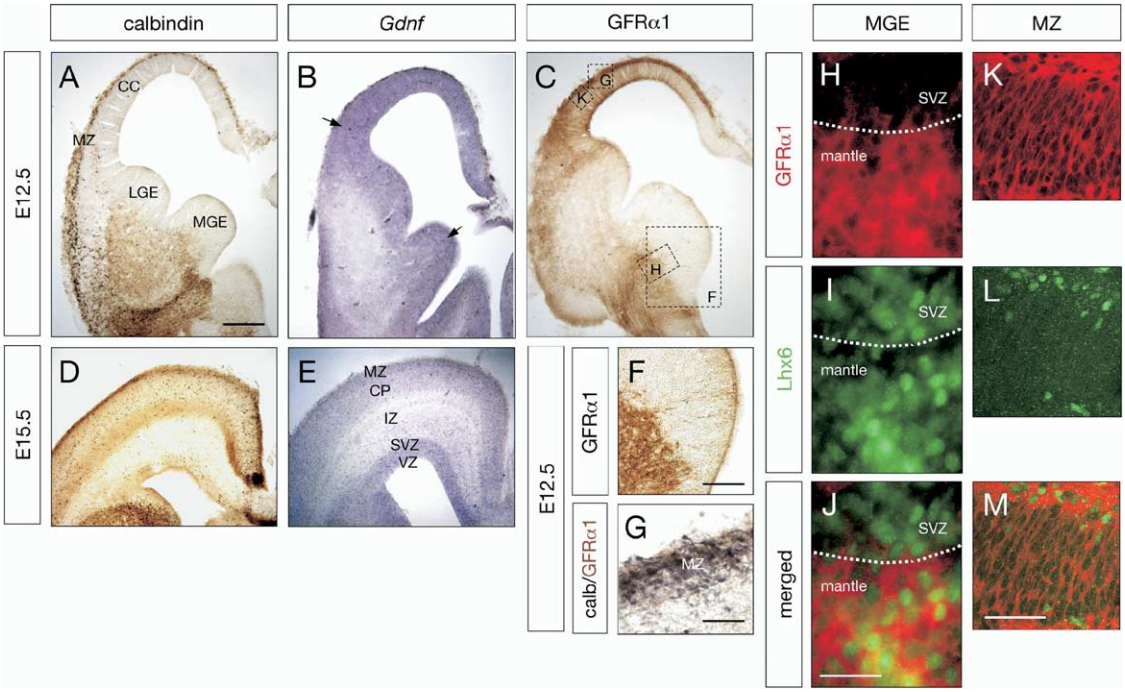

Figure 1. Expression of GDNF and GFRα1 in the MGE and Developing Cerebral Cortex

(A) Immunohistochemistry for the GABAergic cell marker calbindin at E12.5. Calbindin-expressing cells localized to the mantle of the MGE and the developing cortex. CC, cerebral cortex.

(B) In situ hybridization for *Gdnf* mRNA at E12.5. Expression of *Gdnf* mRNA appeared concentrated in the SVZ/VZ of the ganglionic eminences and in the developing cortex (arrows).

(C) Immunohistochemistry for GFRα1 at E12.5. GFRα1 immunoreactivity was observed in the mantle of the MGE and along the marginal zone and VZ/SVZ of the developing cortex.

(D) Immunohistochemistry for calbindin at E15.5.

(E) In situ hybridization for *Gdnf* mRNA at E15.5.

(F) High-magnification view of the region boxed in panel (C) showing scattered GFRα1-expressing cells in the SVZ of the E12.5 MGE as well as strong GFRα1 labeling in the MGE mantle.

(G) High-magnification view of the region boxed in panel (C) showing cells coexpressing GFRα1 (brown) and calbindin (black) in the MZ of the E12.5 cortex.

(H–M) Expression of GFRα1 (H and K) and Lhx6 (I and L) at E13.5 in the mantle and SVZ of the MGE (H–J) and cortex marginal zone (K–M) analyzed by immunofluorescence and confocal microscopy. The approximate locations of these images are denoted in panel (C). Note that Lhx6 has a nuclear localization. Merged images are shown in panels (J) and (M).

Scale bars, 250 μm (A–E); 100 μm (F); 50 μm (G); 12 μm (H–J); 25 μm (K–M).

lamellipodia-like protrusions and a complete absence of neuritic processes (Figure 3A), suggesting that signaling by endogenous GDNF contributes to the differentiation of GABAergic cells in wild-type, untreated cultures. Moreover, GDNF was unable to stimulate phosphorylation of ERK1/2 in MGE cells lacking GFRα1, while wild-type cells or cells lacking NCAM were still able to respond to this factor (Figure 3C). Thus, the effects of GDNF on GABAergic cells from the MGE were mediated by GFRα1 but not RET or NCAM.

We investigated further the effects of GDNF on precursor cells from the MGE by preparing neurosphere cultures from this structure, which selectively amplifies progenitor cells with proliferative capacity. GDNF treatment did not affect the proportion of Tuj1$^+$ neuroblasts in neurosphere-derived dissociated cultures (Figures S5A–S5C). However, GDNF treatment increased the proportion of Tuj1$^+$ cells that were also GABAergic and promoted the morphological differentiation of these cells (Figure S5A). Together, these data are consistent with a role of GDNF in the GABAergic differentiation of Tuj1$^+$ neuronal precursors in the MGE.

GDNF Promotes the Morphological Differentiation of Cortical GABAergic Neurons

At E15.5, cortical GABAergic neurons in wild-type cultures grown under control conditions showed pyramidal or stellate morphologies and possessed a long process covered with fillopodia and tipped with a growth cone (Figure 4A). GDNF treatment stimulated neuritic arborization of cortical GABAergic cells in wild-type cultures and promoted elongation of the main neuritic process (Figures 4A–4C). GABAergic neurons isolated from the cortices of mice lacking GDNF or GFRα1 displayed a stunted arborization and appeared unable to elaborate a long, main process under control conditions (Figures 4A and 4B), suggesting a role for endogenous GDNF signaling in the morphological differentiation of cortical GABAergic neurons. GDNF treatment was able to stimulate neuritic arborization in GABAer-

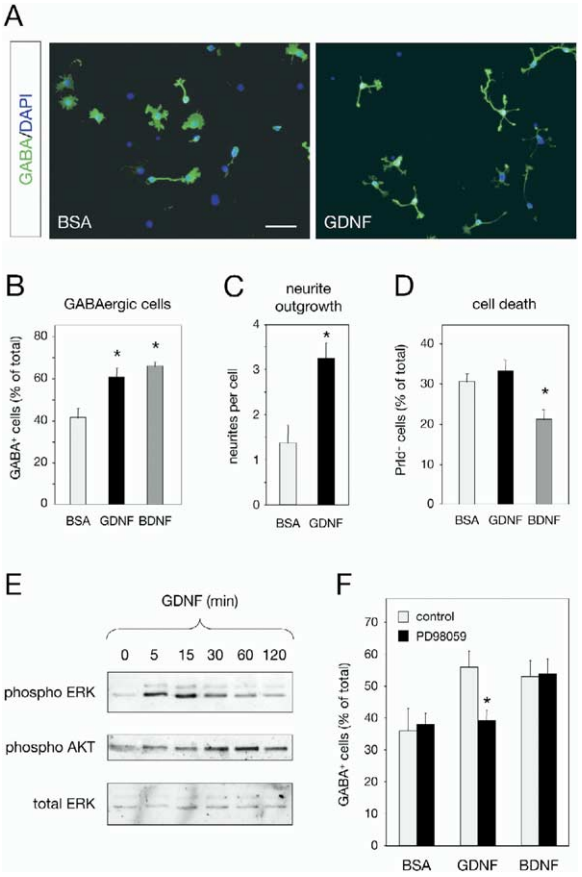

Figure 2. GDNF Promotes the GABAergic Phenotype of MGE Cells

(A) Photomicrographs of dissociated cultures of E12.5 MGE treated with BSA or GDNF visualized by GABA immunofluorescence (green) and DAPI nuclear staining (blue). Scale bar, 50 μm.

(B) GABAergic cells in MGE cultures treated with BSA, GDNF, or BDNF (50 ng/ml) for 48 hr as indicated. Results represent the percentage of GABA-positive cells relative to the total number of cells and are presented as average ± SEM of four independent experiments. *$p < 0.01$ compared to BSA.

(C) Neurite outgrowth in MGE cultures treated with BSA or GDNF. Results (average ± SEM of three independent experiments) represent the number of neurites per cell. *$p < 0.01$.

(D) Cell death in MGE cultures treated with BSA, GDNF, or BDNF. Results represent the percentage of PrId-positive cells relative to the total number of cells and are presented as average ± SEM of four independent experiments. *$p < 0.01$.

(E) Phosphorylation of ERK and AKT in MGE cultures stimulated with GDNF (50 ng/ml) for 5–120 min. Phosphorylation was detected by the indicated anti-phospho antibodies and controlled by reprobing with antibodies against total ERK.

(F) GABAergic cells in MGE cultures treated with the MEK1 inhibitor PD98059, BSA, GDNF, or BDNF (50 ng/ml) for 48 hr as indicated. The effects of GDNF, but not of BDNF, on GABA cell number were abolished by the inhibitor. Results represent the percentage of GABA-positive cells relative to the total number of cells and are presented as average ± SEM of three independent experiments. *$p < 0.01$ compared to BSA.

gic neurons isolated from *Gdnf* mutants but not from *Gfra1* mutants (Figures 4A and 4B), indicating the requirement of this receptor for the effects of GDNF in these cells. In contrast, cells isolated from mice lacking RET or NCAM were still able to respond to GDNF in a manner indistinguishable from wild-type cells (Figures 4A and 4B). NTN had no effect on the morphology of cortical GABAergic neurons (data not shown). BDNF, on the other hand, was able to stimulate neuritic arborization in GABAergic neurons from all genotypes, including those lacking GFRα1 (Figure 4B). However, the neuritic growth induced by BDNF was different from that induced by GDNF in that it mainly consisted of short, dendritic-like processes emanating from the cell body, without significant effects in axonal elongation (Figure 4A).

Role of GDNF in MGE Cell Motility and Tangential Migration

The effects of GDNF on MGE cell motility were assessed as the ability of cells to exit explants of E12.5 MGE cultured in matrigel. GDNF had no significant effects when added to the medium of cultures obtained from wild-type MGE (Figures 5A and 5B). In contrast to wild-type cultures, fewer cells could be seen leaving MGE explants isolated from mice lacking GDNF when grown under control conditions (Figures 5A and 5B). Importantly, addition of GDNF restored the ability of the cells to leave GDNF-deficient explants and colonize the surrounding substrate (Figures 5A and 5B). Together, these data suggested that endogenous, MGE-derived GDNF can stimulate the intrinsic motility of MGE cells.

To investigate whether GDNF could act as a chemoattractant factor for MGE cells, we cultured E12.5 MGE explants together with aggregates of COS cells expressing GDNF in a collagen matrix. While no chemoattractant activity could be observed toward control transfected COS cells, COS cell aggregates expressing GDNF consistently produced a larger area or cells exiting the proximal side of MGE explants (Figure 5B), suggesting that GDNF can act as a chemoattractant factor for MGE cells. We investigated this possibility further by coculturing MGE explants together with cortical explants obtained from either wild-type or *Gdnf* $^{-/-}$ mice. Explants from wild-type cortex displayed significant chemoattraction toward MGE cells (Figure 5C). This activity was reduced, but not completely abolished, in explants isolated from mice lacking GDNF (Figure 5C), indicating that cortex-derived GDNF is a component of the chemoattractant activity of this structure toward MGE cells.

Chemoattractant Activity of GDNF Toward Cortical GABAergic Cells

Next, we investigated whether an ectopic source of GDNF was able to redirect the migration of cortical GABAergic cells in organotypic slice cultures. Agarose beads soaked in BSA, GDNF, or BDNF were placed below the cortical plate along the migratory pathway of GABAergic cells in coronal slices of E13.5 brains (see diagram in Figure 6A). Beads soaked in BSA were largely ignored by calbindin-expressing cells in the developing cortex (Figure 6A). In contrast, cortical GABAergic cells were strongly attracted by beads soaked in GDNF, forming a compact halo around, and sometimes invading, the beads (Figure 6A). BDNF also displayed a strong chemoattractant effect (Figure 6A). Beads soaked in NTN had no effect (data not shown). We then quantified the chemoattractant effects of GDNF and BDNF in cortical slices from

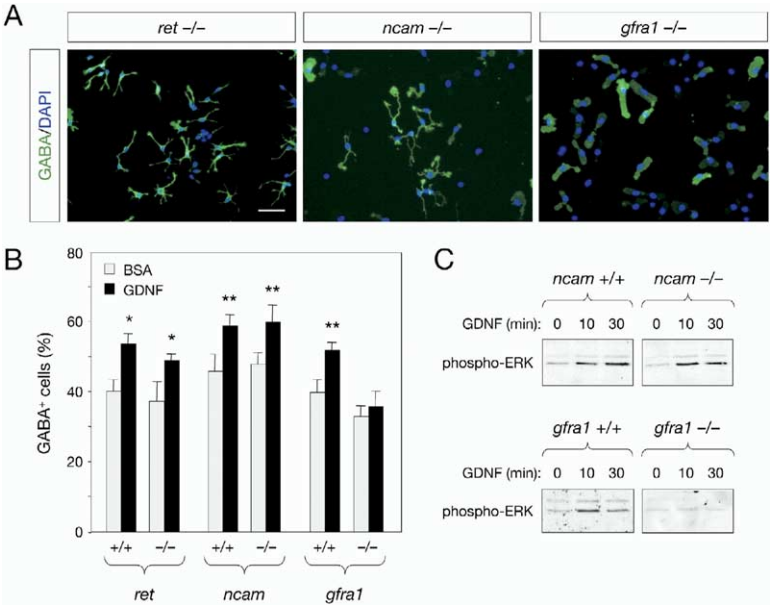

Figure 3. The effects of GDNF on MGE Cells Are Mediated by GFRα1 but Not RET or NCAM

(A) Photomicrographs of dissociated cultures of E12.5 MGE from the indicated mutant mice treated with GDNF and visualized by GABA immunofluorescence (green) and DAPI nuclear staining (blue). Scale bar, 50 μm.
(B) GABAergic cells in MGE cultures from the indicated genotypes treated with BSA (gray bars) or GDNF (solid bars). Results represent the percentage of GABA-positive cells relative to the total number of cells and are presented as average ± SEM of three animals per genotype. *p < 0.05; **p < 0.01 compared to BSA.
(C) Phosphorylation of ERK in MGE cultures of E12.5 *Ncam* or *Gfra1* mutant mice stimulated with GDNF (50 ng/ml) for 10–30 min. Phosphorylation was detected with anti-phospho antibodies and controlled by re-probing with antibodies against total ERK .

mice lacking RET, NCAM, or GFRα1, as well as their corresponding wild-type littermates. GDNF induced comparable chemoattraction of GABAergic cells in slices from all wild-type backgrounds, as well as in slices lacking RET or NCAM (Figures 6A and 6B), indicating that these two receptors were not involved in the chemoattractant effects of GDNF toward cortical GABAergic cells. In contrast, beads soaked in GDNF had no effect in slices isolated from mice lacking GFRα1 (Figures 6A and 6B), indicating that this receptor was required for the chemoattractant activity of GDNF. Calbindin-expressing cells from all genotypes, including those lacking GFRα1, were able to respond to BDNF in this chemoattraction assay (Figure 6B). Thus, similar to the activities observed in the MGE, the effects of GDNF on cortical GABAergic cell morphology and migration were mediated by GFRα1 but not RET or NCAM.

Abnormal Tangential Migration and Reduced Number of Cortical GABAergic Neurons in Mutant Mice Lacking GDNF or GFRα1

In the wild-type E13.5 MGE, calbindin-expressing cells are normally confined to the mantle zone; a sharp boundary separates them from the proliferative SVZ/VZ regions of the MGE where they are very rarely seen (Figure 7A). In contrast, ectopic extrusions of calbindin-

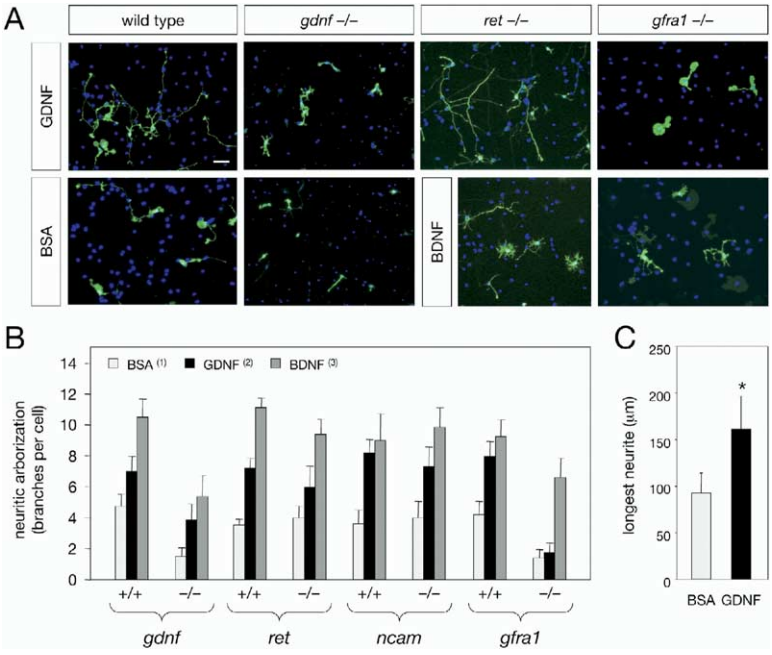

Figure 4. GDNF Promotes the Morphological Differentiation of Cortical GABAergic Cells

(A) Photomicrographs of dissociated cultures of E15.5 cortex from the indicated mutant mice treated with BSA, GDNF, or BDNF visualized by GABA immunofluorescence (green) and DAPI (blue). Scale bar, 50 μm.
(B) Neuritic arborization in cortical cultures from the indicated genotypes treated with BSA, GDNF, or BDNF as indicated. Results represent the total number of neuritic branches per cell and are presented as average ± SEM of at least three animals per genotype. (1) No significant differences between +/+ and –/– in any genotype except *Gdnf* and *Gfra1* ($p < 0.01$). (2) Significantly different from BSA in all genotypes ($p < 0.01$) except *Gfra1*$^{-/-}$. (3) Significantly different from BSA in all genotypes ($p < 0.01$).
(C) Length of the longest neurite (average ± SEM of four wells) in cultures of E15.5 cortex treated with BSA or GDNF. *p < 0.01.

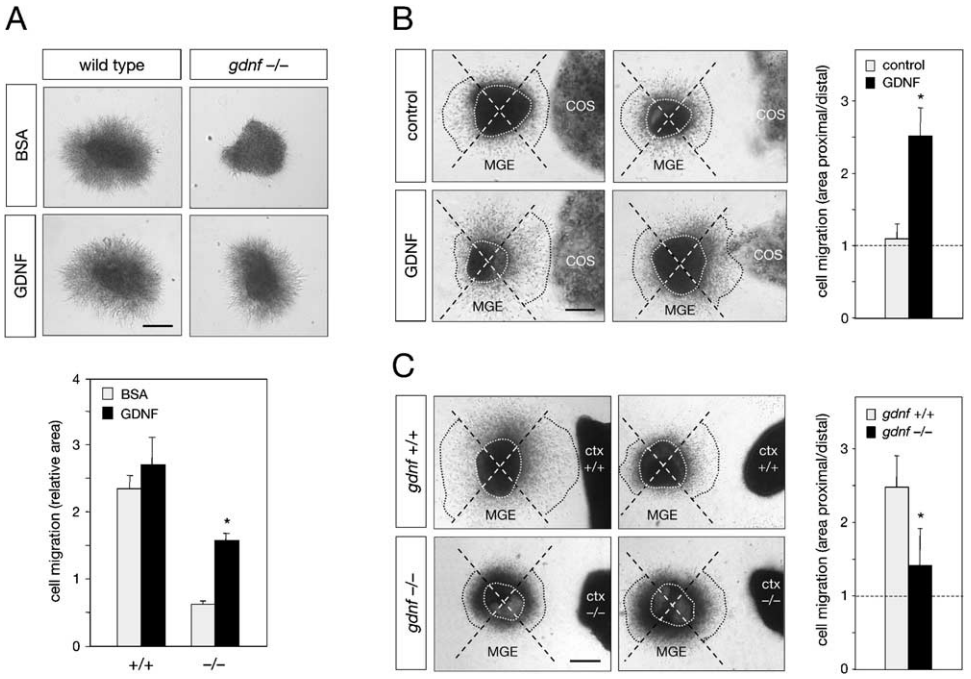

Figure 5. Role of GDNF in MGE Cell Motility and Tangential Migration

(A) Representative phase contrast photomicrographs of E12.5 MGE explants from wild-type or *Gdnf* mutant mice cultured in matrigel and treated with BSA or GDNF for 48 hr. Scale bar, 200 μm. The histogram below shows the quantification of cell migration in MGE explants from wild-type (+/+) or *Gdnf* mutant (–/–) mice presented as average ± SEM of a minimum of ten explants from four animals from each genotype. See Experimental Procedures for details. *$p < 0.01$.

(B) E12.5 MGE explants from wild-type mice cultured in collagen together with aggregates of COS cells expressing GDNF or control COS cells. Scale bar, 200 μm. The histogram shows the quantification of cell chemoattraction expressed as the ratio between the area occupied by MGE cells in the quadrant facing the COS cells (proximal) relative to the area occupied by cells in the opposite quadrant (distal). The maximal extent of cell migration used to calculate the corresponding areas is indicated by black dotted lines. Results are presented as average ± SEM. For each condition, a total of 30 explants were evaluated in three independent experiments. *$p < 0.01$.

(C) Wild-type E12.5 MGE explants cultured in collagen together with cortical explants obtained from either wild-type (+/+) or *Gdnf*$^{-/-}$ mutant mice. Scale bar, 200 μm. The histogram shows the quantification (average ± SEM) of cell chemoattraction calculated as in (B). A total of 20 to 25 explants were evaluated from four different animals of each genotype. *$p < 0.01$.

expressing cells were observed deep into the proliferative layers of the MGE in mice lacking GFRα1 (Figure 7A). Calbindin-expressing cells in this region of the mutant MGE appeared misrouted, with their leading process directed toward the cerebral ventricle. Tangentially migrating cells converging into the ventral region of the developing cortex were examined by calbindin and Lhx6 immunohistochemistry in wild-type and *Gfra1* mutant brains (Figure 7B). A reduction (25% to 35%) in the number of cells migrating through the initial segments of the marginal zone and cortical plate was observed in the mutants (Figure 7B), supporting a role for this receptor in the tangential migration of MGE cells to the cortex.

At E15.5, the complement of calbindin-expressing cells in the developing cortex of mice lacking GFRα1 was reduced to approximately two-thirds of that observed in wild-type littermates (Figure 7C). No abnormalities could be observed by Nissl staining in the mutant cortex (data not shown). The reduction in calbindin-expressing cells was most pronounced in the marginal zone and cortical plate (MZ/CP) (Figure 7C), where the majority of migrating GABAergic cells are normally located at E15.5. The absolute number of calbindin-expressing cells was actually increased in the intermediate zone (IZ) of the mutant cortex (Figure 7C), suggesting an alteration in the migratory pathway of cortical GABAergic cells in the absence of GFRα1. Interestingly, the IZ was the region that expressed lowest levels of *Gdnf* mRNA in the developing cortex (Figure 1B).

The premature death of mice lacking GDNF, RET, or GFRα1, around 12 hr after birth, limited the analysis of later stages to newborn animals. In agreement with our observations made during embryonic stages, the cortex and hippocampus of newborn mice lacking GFRα1 showed significantly reduced numbers of calbindin-expressing cells (Figures 8A and 8B). A comparable reduction in the number of cells expressing *Gad* mRNA, encoding the GABA-synthesizing enzyme, was also observed in the cortex of *Gfra1* mutants (Figure 8C), suggesting an absence of cortical GABAergic cells rather than a specific downregulation of the calbindin marker. In agreement with this, reduced numbers of cells expressing *Lhx6* mRNA were also seen in somatosensory cortex of *Gfra1* mutants (Figure 8D). Unlike calbindin and *Gad* mRNA, however, the majority of the cells labeled by this marker appeared to be non-GABAergic at this stage (compare the scales of Figures 8C and 8D).

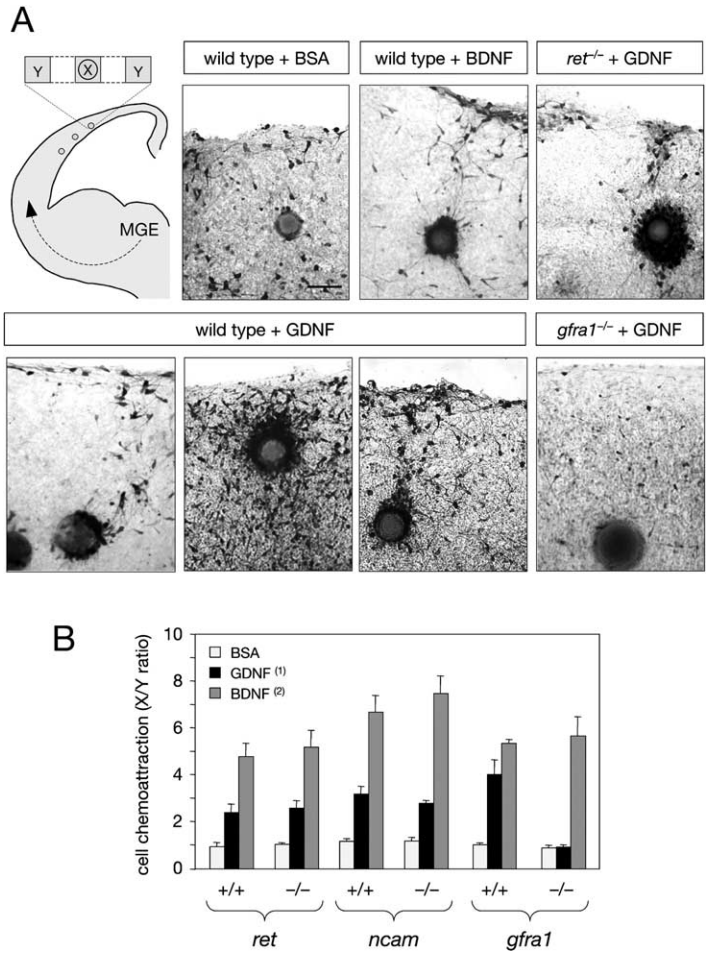

Figure 6. Chemoattractant Activity of GDNF on Cortical GABAergic Cells

(A) Calbindin immunohistochemistry in sections of organotypic slice cultures of E13.5 cerebral cortex treated with agarose beads soaked in BSA or BDNF (wild-type) or GDNF (wild-type, *Ret*$^{-/-}$ or *Gfra1*$^{-/-}$ as indicated). Three examples of the effects of GDNF-coated beads placed at different dorso-ventral levels are shown. Slices from *Gfra1* mutant mice displayed an overall lower densitiy of calbindin-positive cells (in agreement with the data shown below in Figure 7B). The diagram illustrates the set up of the experiment with the approximate position of the beads and a scheme of the areas used for quantification of cell chemoattraction. See Experimental Procedures for details. Scale bar, 50 μm.

(B) Quantification of cell chemoattraction by BSA, BDNF, and GDNF in the indicated genotypes. Cell chemoattraction was expressed as the ratio between the number of calbindin-expressing cells in square X (around the bead) and the average number in squares Y (away from the beads). See Experimental Procedures for details. Results are presented as average ± SEM of at least four animals per genotype. (1) Significantly different from BSA in all genotypes ($p < 0.01$) except *Gfra1*$^{-/-}$. (2) Significantly different from BSA in all genotypes ($p < 0.01$).

In contrast to its importance for GABAergic cells, GFRα1 was not required for the development of calretinin-expressing Cajal-Retzius cells, another neuronal subpopulation of the cortical marginal zone (Figure 8A). A reduction in the number of calbindin-expressing cells was also observed in the cortex and hippocampus of mice lacking GDNF (Figure 8B). Interestingly, and in agreement with our previous results, no deficits in cortical GABAergic cells could be detected in animals that lacked RET or NCAM (Figure 8B).

Discussion

The results from the present study demonstrate the involvement of GDNF in several steps of the development of cortical GABAergic cells, including their maturation and morphological differentiation, their initial motility in the subpallium, and their subsequent migration toward and through the cortex. Although a variety of roles were initially anticipated for GDNF in mammalian brain development, the analyses of mutant mice carried out previously had not uncovered any defect caused by lack of GDNF signaling in the developing brain. The requirement of GDNF signaling via GFRα1 for cortical GABAergic cell development revealed in this study represents the first function to be demonstrated for this neurotrophic factor during brain development in vivo. The fact that neither of the known GDNF signaling receptors—RET and NCAM—appeared to be involved in the effects reported here suggests the existence of novel signaling pathways, including unidentified transmembrane effectors, mediating the activities of this factor. While this paper was under review, Enomoto et al. (2004) have argued that GFRα1 expression in cells lacking RET is dispensable for CNS development, and indicated that validation of the functional importance of RET-independent signaling will require the identification of deficits that are unequivocally present in *gfra1* but not in *ret* mutant mice. Our present paper now provides that evidence.

GDNF Signaling in the Development of Cortical GABAergic Interneurons

The activities of GDNF in the development of cortical GABAergic cells displayed a relatively broad time window, from the early stages of cell differentiation in the MGE, to subsequent tangential migration and later maturation of GABAergic neurons in the cortex and hippocampus. This is in accordance with the early expression of GDNF and GFRα1 in the MGE, along the tangential pathways of GABAergic cell migration in the developing cortical plate, and later in the cortex,

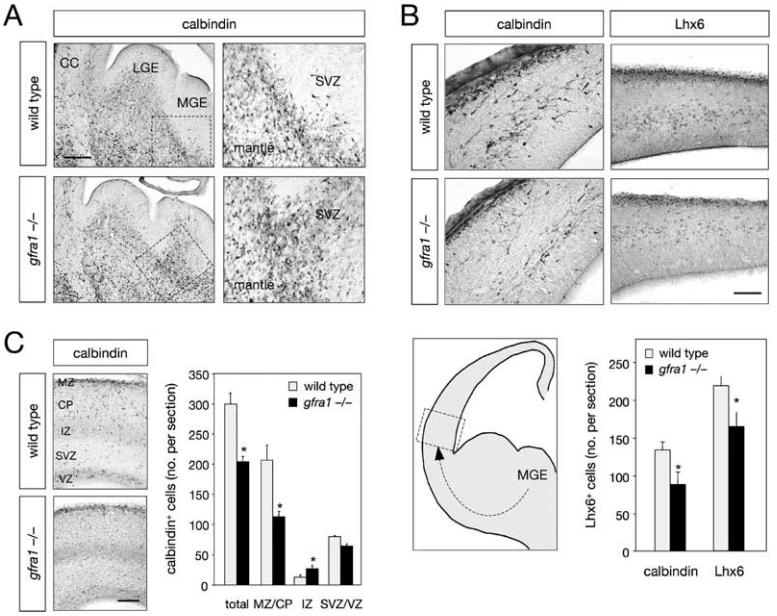

Figure 7. Abnormal Migration and Reduced Number of Tangentially Migrating GABAergic Neurons in Mutant Embryos Lacking GFRα1

(A) Calbindin immunohistochemistry in coronal sections through the E13.5 ganglionic eminences of wild-type and *Gfra1* mutant mice. Panels to the right show high-magnification views of the boxed areas. Scale bar, 200 μm.

(B) Calbindin and Lhx6 immunohistochemistry in coronal sections of the E13.5 cortex of wild-type and *Gfra1* mutant mice. Scale bar, 50 μm. The diagram illustrates the region shown in the micrographs and used for quantification. The histogram shows the quantification of calbindin- and Lhx6-expressing cells in the boxed area. *$p < 0.01$.

(C) Calbindin immunohistochemistry in coronal sections through the E15.5 cortex of wild-type and *Gfra1* mutant mice. Note the paucity of calbindin-expressing cells in the mutant cortex. Scale bar, 100 μm. The histogram shows the quantification (average ± SEM) of calbindin-expressing cells in different cortical layers of wild-type and *Gfra1* mutant mice. MZ/CP, marginal zone/cortical plate; IZ, intermediate zone; SVZ/VZ, subventricular zone/ventricular zone.

during the maturation and radial dispersion of cortical GABAergic interneurons. Several of our observations suggest a role for endogenous GDNF signaling in GABAergic cell differentiation and tangential migration from the MGE: (1) MGE cells lacking GFRα1 displayed a striking immature morphology in vitro, essentially lacking neuritic processes, (2) MGE cells derived from explants lacking GDNF had reduced motility, (3) cortical explants derived from mice lacking GDNF had reduced chemoattractant activity toward MGE cells, and (4) MGE-derived calbindin- and Lhx6-expressing cells from mice lacking GFRα1 presented an aberrant pattern of tangential migration in vivo. Together with the ability of exogenous GDNF to increase GABAergic cell number and neuritogenesis in cultured MGE cells, these results indicate that endogenous GDNF signaling through GFRα1 may contribute to several fundamental aspects of the development of these cells in the MGE, including (1) the acquisition of a GABAergic phenotype by MGE neuronal precursors, (2) the ability of GABAergic cells to elaborate neuritic processes, and (3) the overall motility of these cells in situ.

The ability of GDNF to increase the number of GABAergic cells in MGE cultures without affecting cell proliferation or survival suggests that GDNF is a differentiation factor for MGE precursors, stimulating their acquisition of a GABAergic phenotype at the expense of other fates. This notion is supported by similar effects of GDNF on secondary MGE precursors selectively amplified through neurosphere cultures. The requirement of GFRα1 for the development of neuritic processes by MGE-derived GABAergic cells, together with the ability of GDNF to stimulate neurite outgrowth in these cells, suggests a role for endogenous GDNF signaling in the morphological differentiation of GABAergic cells in the MGE. It is well known that migrating neuronal cells utilize a leading process not only to guide cell movement but also to help propell the cell body along the migratory path (Polleux et al., 2002; Ang et al., 2003). Thus, the ability of GABAergic MGE cells to elaborate neuritic processes is likely to be instrumental for their migration. Several of our observations suggest that MGE-derived GDNF may contribute to the overall motility of GABAergic cells within this structure. Directionality in MGE cell migration could arise as a result of the combined action of MGE-derived motogenic factors, such as GDNF, and repulsive activities located in areas surrounding the MGE (Marin et al., 2001, 2003; Wichterle et al., 2003). While this paper was under review, Flames et al. (2004) identified Neuregulin-1 as a chemoattractant factor for MGE-derived GABAergic interneurons.

In the cortex, expression of GDNF and GFRα1 coincided with the migratory pathways of GABAergic cells (Fonseca et al., 1995; Del Rio et al., 2000; Denaxa et al., 2001; Tanaka et al., 2003). Our results indicate that endogenous GDNF signaling in the cortex contributes to the migration as well as the morphological differentiation of cortical GABAergic cells. The mislocalization of calbindin cells observed in the developing cortex deficient in GFRα1 suggests that GDNF signaling contributes to maintaining the correct migratory pathway of those cells. While both GDNF and BDNF were able to increase the arborization of cortical GABAergic cells in culture, the morphologies of neuritic processes induced by each factor were quite different. Treatment with GDNF favored the elongation and complexity of a single main process, while BDNF increased the elaboration of short, highly branched neurites from the cell body. These results are in line with earlier work indicating the ability of BDNF to stimulate the growth and branching of dendritic processes in cortical and hippocampal neurons (McAllister et al., 1995, 1997; Pappas and Parnavelas, 1997; Horch et al., 1999; Danzer et al.,

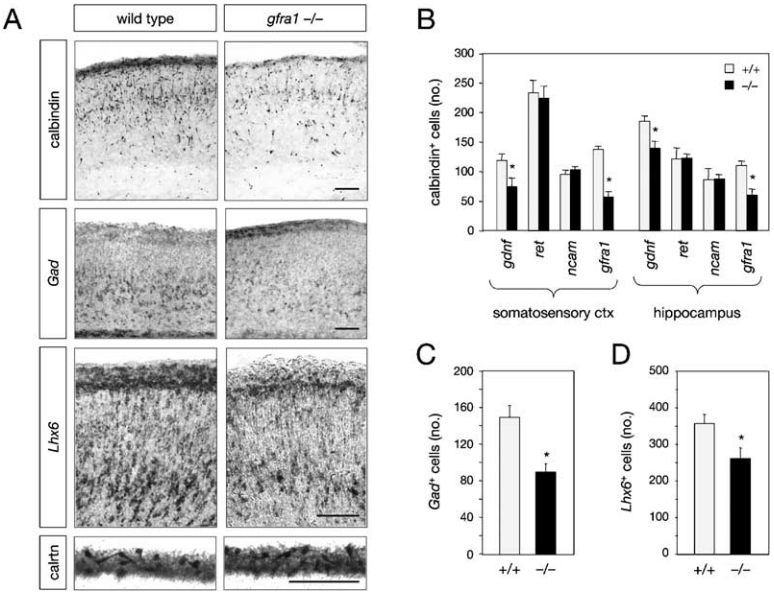

Figure 8. Reduced Number of Cortical GABAergic Neurons in Newborn Mice Lacking GDNF or GFRα1 but Not RET or NCAM

(A) Calbindin, *Gad* mRNA, *Lhx6* mRNA, and calretinin expression in coronal sections through the somatosensory cortex of newborn wild-type and *Gfra1* mutant mice. The bottom panel shows calretinin-expressing Cajal-Retzius cells in the marginal zone. Scale bar, 100 μm.

(B) Quantification (average number per section ± SEM) of calbindin-expressing cells in hippocampus and somatosensory cortex of newborn mice of the indicated genotypes. *p < 0.01.

(C) Quantification of cells expressing *Gad* mRNA in somatosensory cortex of newborn wild-type (+/+) and *Gfra1* mutant (–/–) mice. *p < 0.01.

(D) Quantification of cells expressing *Lhx6* mRNA in somatosensory cortex of newborn wild-type (+/+) and *Gfra1* mutant (–/–) mice. *p < 0.01.

2002; Gorski et al., 2003) and with our own previous observations in hippocampal cells, where GDNF readily stimulates axonal elongation (Paratcha et al., 2003). Thus, the developing cortex would appear to express factors having complementary effects on the morphological differentiation of GABAergic neurons, some that primarily stimulate dendritic development, such as BDNF and others that mainly affect the growth and complexity of the axon, such as GDNF. In addition, the fact that the effects of GDNF—but not those of BDNF—on the differentiation of GABAergic interneurons were partially mediated by MAP kinase signaling, suggests an unexpected level of specificity in the intracellular mechanisms underlying the effects of these two factors.

The reduction in the number of calbindin-expressing cells observed in the cortex and hippocampus of mutant mice deficient in GDNF or GFRα1 could be accounted for by deficits in both the differentiation and migration of GABAergic cells. The fact that lower numbers of cells expressing *Gad* and *Lhx6* mRNA were also observed in the cortex of mice lacking GFRα1 suggests an overall decrease in the number of inhibitory interneurons and not simply a mere reduction in the expression of calbindin in those cells. Although the physiological consequences of a reduction in the number of inhibitory interneurons on adult brain function could not be assessed in this study, our results warrant future investigations on the possible roles of GDNF in the assembly and function of inhibitory circuits using tissue-specific mutants.

Promiscuous GDNF Signaling Mechanisms: Exception or Rule?

Among the best-studied activities of GDNF in the brain is its ability to promote the survival of ventral midbrain dopaminergic neurons in animal models of Parkinson's disease (Olson, 1997; Gash et al., 1998; Brundin, 2002; Kordower, 2003). Despite a voluminous literature on the effects of GDNF on dopaminergic neurons, mice lacking GDNF (or Ret, or GFRα1) display no abnormalities in dopaminergic neuron number or in the pattern or density of striatal dopaminergic innervation at birth. Although adult mice that are heterozygous for a null mutation in the *Gdnf* gene were found to display mild cognitive impairments (Gerlai et al., 2001) and enhanced responses to drugs of abuse (Messer et al., 2000), no developmental defects have been uncovered so far in the brains of mice lacking either GDNF, GFRα1, or RET (Airaksinen and Saarma, 2002). While the physiological function of RET signaling during brain development in vivo remains to be elucidated, our present results indicate that at least one of the normal functions of GDNF signaling via GFRα1 in the developing brain is to support the differentiation and migration of cortical GABAergic cells, an unexpected finding given what was previously known about the functions of this factor. The fact that neither RET nor NCAM were found to be required for the effects of GDNF on MGE and cortical GABAergic cells was also unanticipated and suggests the existence of alternative transmembrane signaling mediators for this factor.

GFRα1 is the only receptor known to be absolutely required for the effects of GDNF in all systems studied so far, and it is the only membrane-associated molecule known that is capable of binding GDNF with high affinity. Thus, GFRα1 is likely to function as the indispensable GDNF-sensing component of several different receptor complexes. In the majority of cases studied so far, transmembrane partner molecules have been shown to associate with GPI-anchored receptors for signal transduction. Intriguingly, a recent study has shown that the HGF receptor MET could be activated by GDNF in cells expressing GFRα1 but not RET (Popsueva et al., 2003). However, MET did not interact with either GDNF or GFRα1, and it appeared to be transactivated by an indirect mechanism (Popsueva et al., 2003). Although it is unlikely that MET forms part of a GDNF

receptor complex, the possible contribution of this receptor to the effects of GDNF in MGE and cortical GABAergic cells deserves further investigation.

GDNF has been shown to regulate the migration of enteric neuron precursors in the developing gut using a receptor complex based on RET an GFRα1 (Natarajan et al., 2002). Recent studies have indicated that GDNF signaling may contribute to the migration of neuronal precursors in the rostral migratory stream using GFRα1 and NCAM, but not RET (Paratcha et al., 2003). In the present study, GDNF was shown to regulate the tangential migration of GABAergic cells in the MGE and cortex via GFRα1 but neither RET nor NCAM. It would therefore appear that different receptor complexes are used by GDNF to regulate cell migration in different biological settings. This promiscuity of GDNF signaling mechanisms is, however, not unusual, as several similar examples abound now in the literature (Trusolino et al., 2001; Castellani et al., 2002; Barker, 2004). Thus, as we learn more about mechanisms of receptor-mediated growth factor signaling, uncanny combinations of receptor molecules are likely to become the rule rather than the exception.

Experimental Procedures

Recombinant Proteins and Mice

GDNF was purchased from R&D, and NTN was purchased from PeProtech. C57 mice were obtained from Scanbur (Denmark). *Ret* knockout mice were kindly provided by Vassilis Pachnis (Schuchardt et al., 1994), *Gdnf* knockout mice by Heiner Westphal (Pichel et al., 1996), *Gfra1* knockout mice by Arnon Rosenthal (Cacalano et al., 1998). *Ncam* knockout mice were obtained from the Jackson Laboratory (Cremer et al., 1994).

RT-PCR

Total RNA was isolated from the MGE (E12.5 and E14.5) and cerebral cortex (E12.5 and E15.5) using SNAP columns (Invitrogen). Single-stranded cDNA was synthesized using Multiscribe Reverse Transcriptase and oligo-dT primers (Perkin-Elmer). Sequences of PCR primers used in this study are available upon request.

Primary Culture

Dissociated Cultures

Mouse MGE and cerebral cortex from E12.5 and E15.5 mouse embryos, respectively, were trypsinized and cultured in Neurobasal medium supplemented with B27 (GIBCO) in the presence or absence of GDNF, NTN, BDNF, or BSA at 50 ng/ml. Tissue culture plates were coated with poly-D-Lysine (Sigma) 2 hr before plating the cells. Twenty-five thousand cells were plated per well on a 48-well plate and were cultured for 48 hr at 37°C in 10% CO_2. For viability studies, living cultures were stained with 20 μg/ml Propidium Iodide (Sigma) to specifically label dead cells. These were quantified as percentage of the total number of cells counted by phase contrast illumination. For proliferative studies, a 5 hr pulse of Bromo-deoxy-Uridine (BrdU) (10 μM) was done 24 hr after platting. MAPK activity was blocked by treatment with 50 μM PD98059 (Tocris) throughout the culture period. This treatment did not affect the viability of the cells. The number of GABA/BrdU double-positive cells was counted 24 hr later and expressed as a percentage of the total number of GABA-positive cells. Dissociated cultures were fixed in 4% paraformaldehyde (PFA), except those used for GABA immunostaining, which were fixed in 4% PFA, 0.5% glutaraldehyde. Cells were permeabilized with 0.1% Triton X-100 and stained as described below. Cultures were routinely counterstained with DAPI (Molecular Probes). Neuritic arborization was evaluated by counting the total number of branches (primary and secondary) per cell after GABA immunostaining. Axonal length was evaluated by measuring the longest neurite in each cell.

Neurospheres

Neurospheres cultures from E12.5 MGE precursors were obtained in the presence of 10 ng/ml bFGF and 20 ng/ml EGF as previously described (Parmar et al., 2002). Differentiation studies were performed with cells dissociated from passage 2 neurospheres cultured for 4–7 days as above in the presence or absence of GDNF (50 ng/ml).

Explants

Explants of the E12.5 MGE were plated in Matrigel (Beckton Dickinson) or rat tail collagen as previously described (Pozas et al., 2001). GDNF was used at 50 ng/ml. Cell migration was evaluated under phase contrast by quantifying the area covered by the cells that had exited the explant relative to the area covered by the explant itself (Alcantara et al., 2000). Chemoattraction assays were performed by culturing MGE explants together with aggregates of COS cells transiently expressing GDNF or mock-transfected control cells as described (Pozas et al., 2001). Other experiments included cortical explants derived from wild-type or mutant mice as indicated. After polymerization, the gels were overlaid with Neurobasal medium supplemented with B27 and cultured for 48 hr, fixed in 4% paraformaldehyde (or together with 0.5% glutaraldehyde for GABA immunostaining). For quantification, MGE explants were subdivided into proximal (P), distal (D), north (N), and south (S) quadrants, and the areas occupied by migrating cells in each quadrant were determined by image analysis as described previously (Pozas et al., 2001). The P/D ratio was calculated and used as a measure of chemoattraction in each case. No significant differences were observed in N/S, D/S, or D/N comparisons (data not shown).

Organotypic Slices

Coronal sections (300 μm) including the MGE, primordial neocortex and hippocampal anlagen were obtained from E13.5 mouse brains using a tissue chopper (McWilman). Slices were cultured in an air-interface culture (Stoppini et al., 1991) in Neurobasal medium supplemented with B27. Agarose beads (Sigma) were preincubated for 2 hr with either BSA, GDNF, BDNF, or NTN as previously described (Ledda et al., 2002) and then washed in PBS. Soaked beads were placed in different positions along the cortical plate and/or intermediate zone on living sections of the developing cortex. At E13.5, there is a higher density of calbindin cells in the ventral portion of the cortex—i.e., closer to the MGE—than in the dorsal region. For the quantifications, we always compared beads located at comparable dorso-ventral positions, usually rather more toward the dorsal side to facilitate cell counting. After 48 hr, the cultures were fixed in 4% paraformaldehyde for 4 hr and then processed for calbindin immunohistochemistry. For quantitative analysis of cell chemoattraction, calbindin-expressing cells were counted in a square area immediately surrounding the bead and in two equivalent squares separated approximately one bead diameter from the bead along a line parallel to the pia surface of the cortex (see diagram in Figure 7A). Cell chemoattraction was expressed as the ratio between the number of calbindin-expressing cells in the square around the bead (X) and the average of the counts in the two distant squares (Y). An X/Y ratio greater than unity was taken to imply cell chemoattraction. At least two to five beads per section in a minimum of three different sections were evaluated for each condition and experiment.

Histology, Immunocytochemistry, and In Situ Hybridization

Embryos and newborn mice were perfused with 4% paraformaldehyde. Alternatively, embryonic brains were fixed directly in 4% paraformaldehyde overnight. The brains were then cryoprotected in 30% sucrose at 4°C overnight. Serial coronal sections (12 or 50 μm) were obtained in a cryostat. Primary antibodies were used in the following dilutions: PSA-NCAM (1:1000, Chemicon), $NCAM^{ICD}$ (1:500, Pharmigen), GABA (1:2000, Sigma), Calbindin K28 (1:3000, Swant or Chemicon), Calretinin (1:2000, Swant), BrdU (1:50 DAKO), GFRα1 (1:400, a gift of Michele Sanicola), RET (1:500, Santa Cruz), and Lhx6 (1:500, a gift of Vassilis Pachnis). Sections were treated for 30 min in 1% H_2O_2, 10% MetOH and then washed three times with PBS and permeabilized with 0.3% Triton X-100 in PBS. They were then incubated overnight with primary antibodies, washed with PBS-Triton X-100, incubated for 2 hr with biotin-conjugated

secondary antibodies (Jackson), washed again, and incubated for 2 hr with the ABC complex (DAKO). Sections were then developed with DAB (Sigma) or Supergrey or Novared (DAKO). For double-staining experiments, sections were extensively washed after DAB treatment and then were incubated with the new primary antibody, secondary antibody, and ABC complex as above, except that this time they were developed with Supergrey. Details of this protocol have appeared elsewhere (Pozas et al., 1997). Sections were mounted, air dried, dehydrated, overlaid with coverslips in Pertex, and observed in an Axioskop microscope (Zeiss). For immunofluorescence, primary antibodies were visualized using secondary antibodies conjugated with Rhodamine (red), Cy3 (red), or Cy2 (green), or with nonconjugated secondary antibodies followed by streptavidin conjugated to fluorochromes (Jackson). Immunofluorescence stainings were visualized in an Axiovert200M microscope (Zeiss). Confocal microscopy was performed in a Leica TCS-SP2 laser scanning microscope (Leica). Photographs were taken with an OrcaER digital camera (Hamamatsu) using OpenLab software (Improvision). For quantitative studies in brains of mutant mice, calbindin-positive cells were counted on coronal sections from at least four animals of each genotype. At E13.5, the total number of calbindin- and Lhx6-positive cells were counted in the cerebral cortex on three consecutive sections in plates 3-4 of *Chemoarchitectonic Atlas of the Development Mouse Brain* by Jacobovitz and Abbot, 1998. At E15.5, the total number of calbindin-positive cells in the dorsal cerebral cortex were counted on three consecutive sections in plates 4-5 (Jacobowitz and Abbott, 1998). At P0, calbindin- and Lhx6-positive cells were counted in sections of somatosensory cortex and hippocampus corresponding to plates 5-6 and 7-8, respectively (Jacobowitz and Abbott, 1998). All statistical analyses involved two-way comparisons and were performed with the Student's t test using the Statgraphic software package.

For in situ hybridization, probes corresponding to the extracellular domain of mouse RET, full-length mouse GDNF, and mouse GFRα1 were amplified by RT-PCR and subcloned by TOPO-TA cloning (Invitrogen). For detection of *Gad* mRNA, a mixture of antisense probes from both *Gad65* and *Gad67* genes was used (Erlander et al., 1991). Lhx6 riboprobe was kindly provided by Vassilis Pachnis. Nonradioactive in situ hybridization was performed as previously described (Pascual et al., 2004). Sections were hybridized overnight at 60°C with 100 ng/ml of the specific antisense riboprobes labeled with digoxigenin-dUTP (Roche Diagnostics) and then were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000 dilution; Roche Diagnostics) and developed with the BCPI/NTB substrate. Control hybridizations with sense riboprobes did not give any signal (Figure S1 and data not shown).

Western Blotting

Primary cultures were lysed for 30 min in TNE buffer supplemented with 0.1% Triton X-100 and proteases and phosphatases inhibitors as described previously (Paratcha et al., 2001). Protein in total cell lysates was quantified with the BCA kit (Pierce); 30 μg of total protein were run in SDS-PAGE gels and transferred to PVDF membranes (BioRad). Primary antibodies anti-ERK, anti P-ERK (Thr-202/Tyr-204), anti-AKT, and anti P-AKT (Ser 473) were from New England Biolabs. Blots were processed by Enhanced Chemifluorescence using standard protocols (Amersham). All blots were scanned in a Storm840 fluorimager (Molecular Dynamics), and quantification was done with ImageQuant software (Molecular Dynamics).

Supplemental Data

The Supplemental Data accompanying this article can be found online at http://www.neuron.org/cgi/content/full/45/5/701/DC1/.

Acknowledgments

We would like to thank Vassilis Pachnis for knockout mice and Lhx6 reagents, Arnon Rosenthal and Heiner Westphal for knockout mice, Michele Sanicola for GFRα1 antibodies, and Xiaoli Li and Carolina Svensson for secretarial help. This work was funded by grants from the Swedish Foundation for Strategic Research, the Swedish Research Council (33X-10908-10A), the Joint Program on Stem Cell Research from the Swedish Research Council and JDRF, and the European Commission (QLG3-CT-2002-01000). E.P. is the recipient of a postdoctoral fellowship from the Ministerio de Educación y Ciencia (Spain).

Received: August 16, 2004
Revised: November 5, 2004
Accepted: January 27, 2005
Published: March 2, 2005

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Supplemental Data

GDNF and GFRα1 Promote Differentiation and Tangential Migration of Cortical GABAergic Neurons

Esther Pozas and Carlos F. Ibáñez

Figure S1. Expression of *Gdnf* mRNA and GFRα1 Protein in the Developing MGE and Cortex

(A) In situ hybridization for *Gdnf* mRNA at E12.5. The upper panel shows a section hybridized with an antisense *Gdnf* riboprobe. The lower section was hybridized with a the complementary sense probe. Specific labeling (arrows) was only seen with the antisense probe.

(B) Immunohistochemistry for GFRα1 at E12.5 in sections from wild type and *Gfra1* knockout mice. Specific labeling can only be observed in wild-type tissue expressing GFRα1.

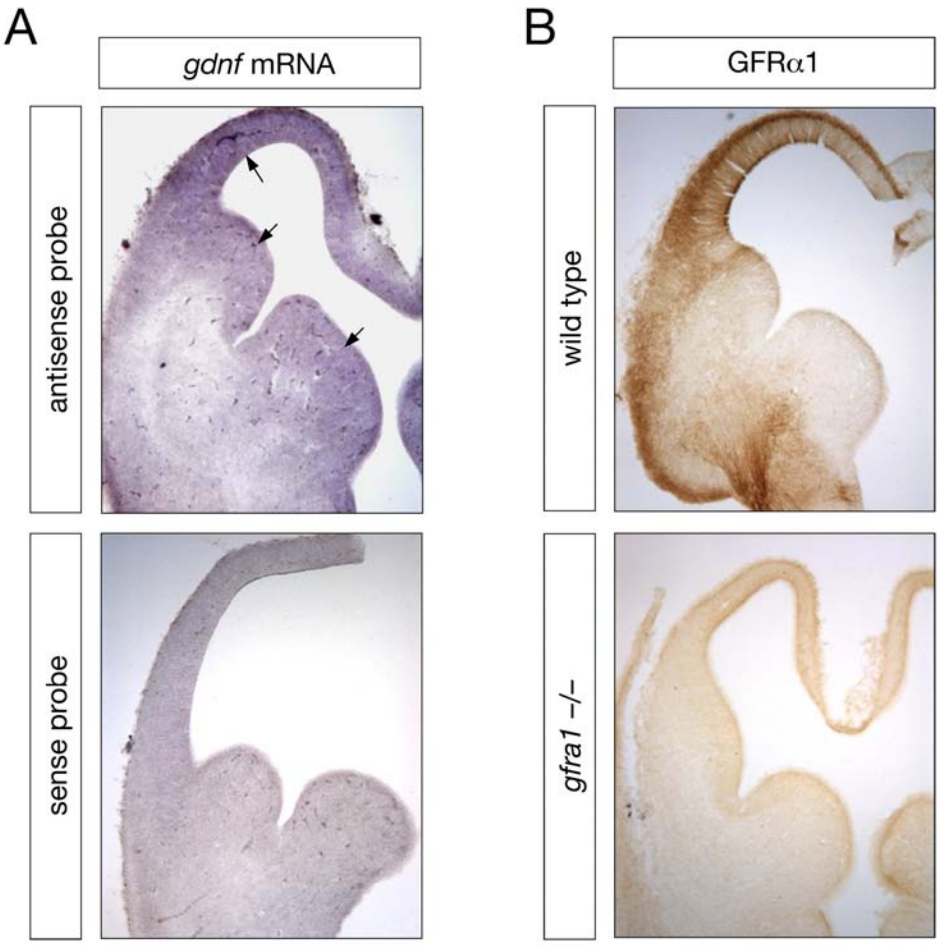


Figure S2. Expression of p140^{NCAM} at E12.5 and E15.5

(A) Immunohistochemistry for the intracellular domain of NCAM at E12.5.
(B) Immunohistochemistry for the intracellular domain of NCAM at E14.5. Strong NCAM^{ICD} immunoreactivity was observed in developing axons, but not in cortical cells at this age.

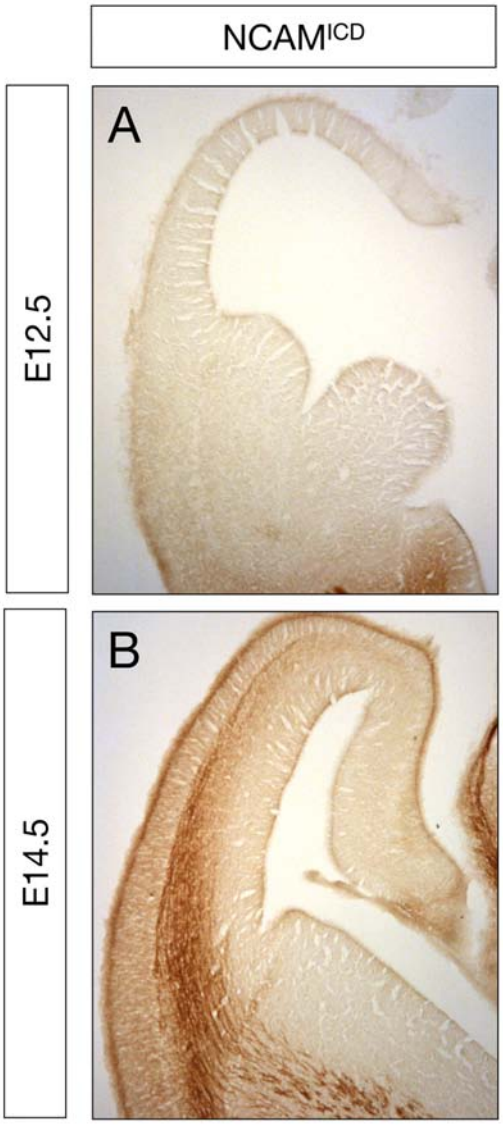


Figure S3. Tangentially Migrating Cells Coexpress Calbindin and GFRα1

(A) MGE-derived cells are believed to invade the cortex through two separate pathways or corridors flanking the future striatum (asterisk). The interior pathway runs at the border between the LGE and the future striatum (1 in A) and develops predominantly at E13.5, while the exterior pathway takes a more ventrolateral route (2 in B) and represents the main path of tangential migration at E12.5 (Marin et al., 2001; Marin and Rubenstein, 2003). The image shows GFRα1 immunostaining at E12.5. Scale bar, 250 μm.

(B–E) Cells coexpressing GFRα1 and calbindin were observed in both pathways, although more abundantly in the external corridor at E12.5. Scale bars, 60 μm (B and C); 15 μm (D and E).

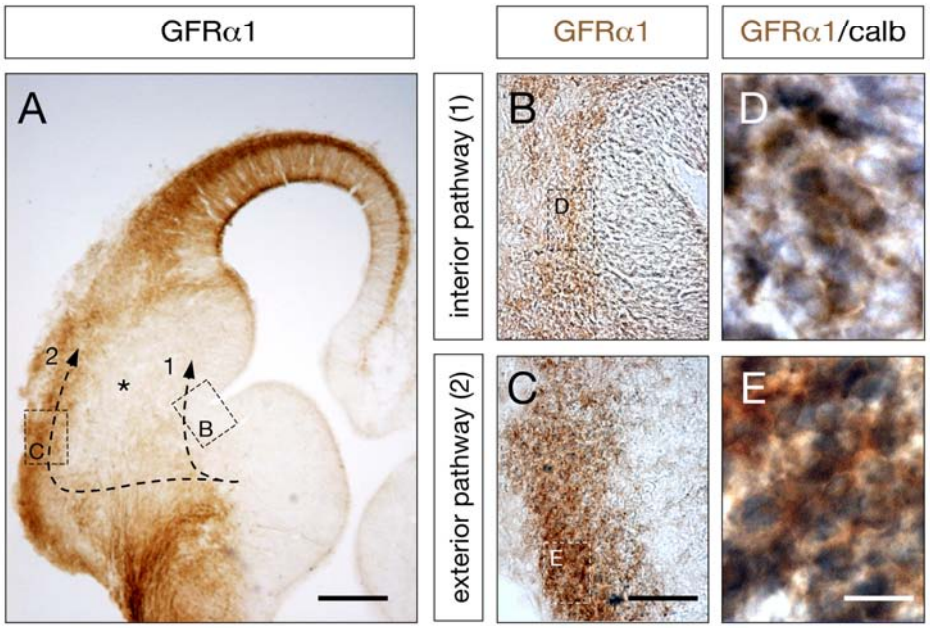


Figure S4. RT-PCR Analysis of mRNA Expression in MGE and Cortex

(A) Expression of *Gdnf*, *Ntn*, *Gfra1*, *Gfra2*, and *Ret* mRNAs in E12.5 and E14.5 MGE and E12.5 and E15.5 cortex after 20 PCR cycles. Expression of *glyceraldehyde-phosphate-*dehydrogenase (*Gapdh*) was used as control for equal amounts of total RNA between the samples. RT, reverse transcriptase.

(B) Expression of *Ntn* mRNA in the motorneuron cell line MN1 but not in E12.5 MGE even after 35 PCR cycles.

(C) Expression of *Gfra2* mRNA could first be detected in developing MGE and cortex after 35 PCR cycles, indicating very low levels of this mRNA at early embryonic stages.

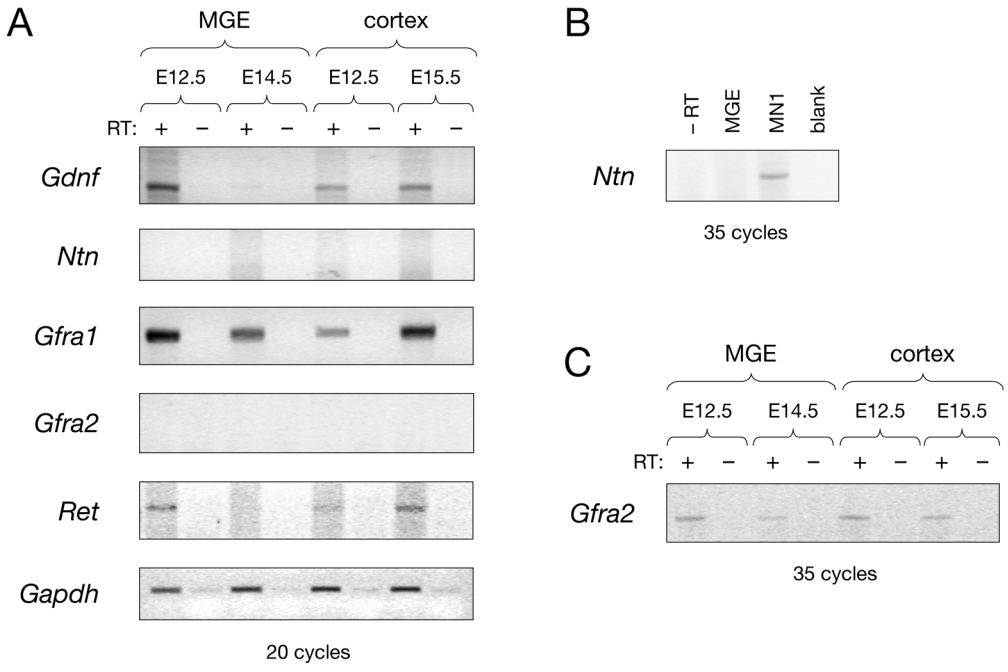
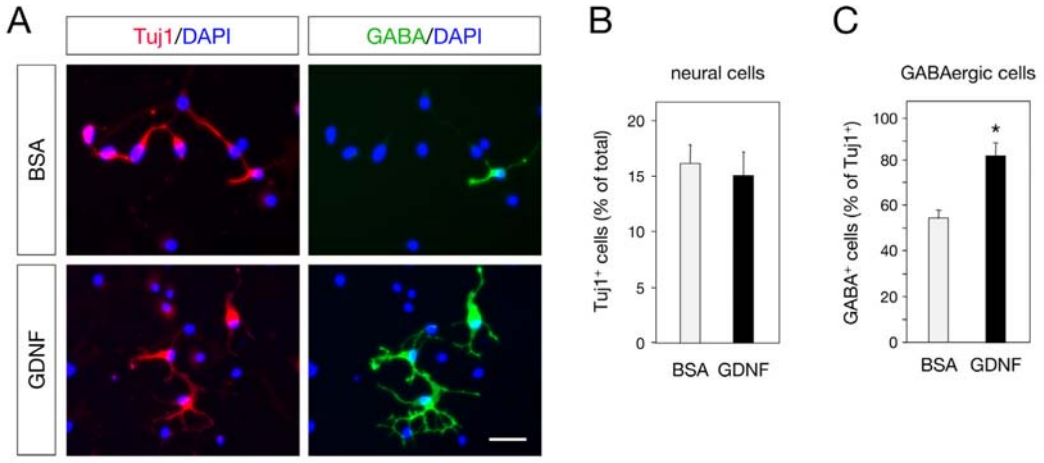


Figure S5. GDNF Increases the Number of GABAergic Cells in Cultures of MGE-Derived Neuronal Precursors

(A) Photomicrographs of dissociated cultures from passage 2 MGE-derived neurospheres treated for 4d with BSA or GDNF and visualized by immunostaining for the neural marker Tuj1 (red), GABA (green), or DAPI (blue). Scale bar, 30 μm.

(B) Neuronal cells in MGE-derived neurosphere cultures treated with BSA (gray bars) or GDNF (solid bars). Results represent the percentage of Tuj1-positive cells relative to the total number of cells and are presented as average ± SEM of three independent experiments.

(C) GABAergic cells in MGE-derived neurosphere cultures treated with BSA or GDNF. Results represent the percentage of GABA-positive cells relative to the number of Tuj1-positive cells and are presented as average ± SEM of three independent experiments. *p < 0.01 compared to BSA.



Supplemental References

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