GDNF/GFRα1 Complex Abrogates Self-Renewing Activity of Cortical Neural Precursors Inducing Their Differentiation

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SUMMARY
The balance between factors leading to proliferation and differentiation of cortical neural precursors (CNPs) determines the correct cortical development. In this work, we show that GDNF and its receptor GFRα1 are expressed in the neocortex during the period of cortical neurogenesis. We show that the GDNF/GFRα1 complex inhibits the self-renewal capacity of mouse CNP cells induced by fibroblast growth factor 2 (FGF2), promoting neuronal differentiation. While GDNF leads to decreased proliferation of cultured cortical precursor cells, ablation of GFRα1 in glutamatergic cortical precursors enhances its proliferation. We show that GDNF treatment of CNPs promoted morphological differentiation even in the presence of the self-renewal-promoting factor, FGF2. Analysis of GFRα1-deficient mice shows an increase in the number of cycling cells during cortical development and a reduction in dendrite development of cortical GFRα1-expressing neurons. Together, these results indicate that GDNF/GFRα1 signaling plays an essential role in regulating the proliferative condition and the differentiation of cortical progenitors.

INTRODUCTION
The mammalian cerebral cortex consists of diverse areas with specific functions and its cellular organization must be strictly regulated to ensure proper functionality (Bystron et al., 2008; Kowalczyk et al., 2009; Rakic, 2006). The development of this structure follows an organized generation of neurons and glial cells from local neural stem cells (NSCs). These cells are critical for the generation of the two main classes of neurons that populate the cerebral cortex, the excitatory projection neurons, which use glutamate as neurotransmitter, and the inhibitory interneurons, which use γ-aminobutyric acid (GABA) (Anderson et al., 1997, 2002). While excitatory cortical neurons are generated from NSCs from the dorsal telencephalon, inhibitory cortical interneurons are mainly generated from the germinal zones of the ventral telencephalon. Different subtypes of excitatory cortical projection neurons are born in overlapping temporal waves from local NSCs to generate defined cortical layers establishing the six-layered structure of the mature neocortex with layers VI and V generated first, followed by layers IV, III, and II (Molyneaux et al., 2007). On the other hand, interneurons generated from NSCs from the ventral telencephalon, migrate tangentially a long distance to populate the dorsal cortical structure. A correct balance between excitatory glutamatergic neurons and inhibitory (GABAergic) interneurons is necessary for the correct functioning of the cerebral cortex. Thus, a correct regulation of this process is necessary, since any inconvenience may cause possible nervous system disorders, such as cerebral malformations or psychiatric diseases. Emerging evidence indicates that intrinsic and extrinsic cues are involved in cortical precursor development. Particularly, fibroblast growth factors (FGFs) are secreted signaling molecules that play cue roles in the cerebral cortex formation (Vaccarino et al., 1999a) and patterning (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003; Hebert and Fishell, 2008). Several FGF ligands, including FGF2, 3, 7, 8, 10, 15, 17, and 18 are expressed in the rostral telencephalic midline and early cortical primordium (Cholfin and Rubenstein, 2007; Garel et al., 2003; Vaccarino et al., 1999b), and have been involved in neurogenesis (Borello et al., 2008; Raballo et al., 2000). Among the four FGF receptors (FGFRs), FGFR 1–3 are expressed in the developing CNS (Ford-Perriss et al., 2001). Despite FGFRs having been involved in the control of CNS growth including the hippocampus (Ohkubo et al., 2004) and the cerebral cortex (Kang et al., 2009; Stevens et al., 2010), the cellular and molecular mechanisms of FGFR function in the process of neurogenesis and cortical surface area expansion is still unclear (Rash et al., 2011). Most FGF ligand studies focused on the FGF2 or basic FGF ligand, which binds with high affinity to FGFR1 and certain FGFR2, 3, and 4 isoforms. FGF2 is highly expressed in the developing nervous system, especially in the ventricular zone of the cerebral cortex (Weise et al., 1993), and also functions as a mitogen for neural progenitors in culture (Qian et al., 1997). Other extrinsic cues such as BMPs, Wnts, as well as neurotrophins NT3 and BDNF, have been involved in the control of cortical progenitor development (Barnabe-Heider and Miller, 2003; Bartkowska et al., 2007). Recent evidence suggests that members of the glial cell line-derived neurotrophic factor (GDNF) family could have an important role in cortical development (Yuzwa et al., 2016).
GDNF was the first identified member of the family of neurotrophic factors, consisting of GDNF, neurturin (NTRN), persephin (PSPN), and artemin, and together make up the GDNF family of ligands (GFLs) (Saarma, 2000). These factors are best known for regulating the biology of peripheral and CNS neurons. Signaling by GFLs is mediated by alternative multicomponent receptor complex containing a glycosylphosphatidylinositol-anchored subunit named GDNF family receptor alpha (GFRα) together with either RET receptor tyrosine kinase or the neural cell adhesion molecule (NCAM). Four GPI-anchored subunit named GDNF family receptor alpha (GFRα) have been described which provide ligand-specific binding activity for each GFLs. Thus, GDNF binds preferentially to GFRα1 receptor, while NRTN signals through GFRα2, ART through GFRα3, and PSPN through GFRα4. Throughout the nervous system and, particularly in the forebrain, GFRα receptors are more widely expressed than RET, suggesting that GFRαs may signal independently of RET, presumably in collaboration with NCAM. Physiological functions of GDNF signaling in the absence of RET have been reported in different systems including GABAergic neuron development and commissural neurons of the spinal cord (Canty et al., 2009; Charoy et al., 2012; Paratcha et al., 2003, 2006; Pozas and Ibanez, 2005). Interestingly, syndecan-3 has been described as an alternative signaling partner of GDNF and NTRN in the brain without the involvement of their conventional receptors. GDNF signaling through syndecan-3 has been involved in migration of GABAergic neurons during cortical development (Bespalov et al., 2011). During the last years, we have described that GDNF through GFRα1 and NCAM receptors play a crucial role in glutamatergic hippocampal dendrite development, and that this complex is required for proper hippocampal connectivity (Irala et al., 2016).

Based on the expression pattern of GDNF and its receptor, GFRα1, at early stages in cortical development, we investigated the function of this molecular system in cortical glutamatergic precursor development. In the present work, we provide evidence indicating that GDNF and its GPI-anchored receptor, GFRα1, expressed in the forebrain during the period of cortical neurogenesis, play an important role during embryonic cortical precursor development.

RESULTS

GFRα1 Localization during Early Cortical Development

To determine whether GFRα1 is expressed in neural precursors of the embryonic cortex, we analyzed expression of Gfra1 mRNA. RT-PCR of RNA isolated from rat neocortex at embryonic day 13.5 (E13.5) showed that Gfra1 is expressed at low levels at this time point, when the cortex is predominantly comprised of neural precursors, but its expression increased throughout embryogenesis and decreased in the cortex at postnatal day 0 (P0, Figure 1A). Expression of Gdnf and Ncam mRNA remained similar during the analyzed period, while the expression of other member of the GFRα receptor family, Gfra2 mRNA, was very low during the early cortical development increasing at birth (P0) (Figure 1A) and during postnatal cortical development as described previously (Burazin and Gundlach, 1999). In agreement with other works, we did not detect expression of the GDNF canonical receptor Ret mRNA during cortical development (Glazner et al., 1998; Golden et al., 1999; Lenhard and Suter-Cazzolara, 1998; Trupp et al., 1999). The expression of Fgfr1 mRNA, the most expressed FGF receptor in the CNS (Ford-Perriss et al., 2001), was clearly detected at early cortical developmental stages, and its expression was markedly decreased at later embryonic stages dropping at P0 (Figure 1A). The expression pattern of Fgfr1 mRNA is in agreement with its role as a mediator of FGF2 in early precursor proliferation (Maric et al., 2007). The realization that Gfra1 mRNA was expressed exclusively during early cortical stages prompted us to investigate a possible role of this complex in cortical development.

To define GFRα1 localization, we performed immunofluorescence analysis of E13.5 mouse cortical sections and compared the distribution of GFRα1 with SOX2, which is expressed in the proliferative ventricular zone and subventricular zone (VZ and SVZ, respectively) by NSCs (Gotz et al., 1998) and βIII-tubulin, which is expressed in the cortical plate by postmitotic neurons. We observed that GFRα1 expression was very low in cortical precursors from VZ/SVZ and high in postmitotic cells. A similar distribution was observed for NCAM (Figure 1B). GFRα1 was clearly expressed in cells positive for the postmitotic nuclear marker, Tbr1 (Figure 1C). RET immunoreactivity could not be detected in embryonic cortical sections, but neurons from cervical dorsal root ganglia, used as positive expression control, were clearly labeled (See Figure S1A). Antibody specificity was confirmed in heterologous cells transfected with Ret cDNA by immunofluorescence (Figure S1B). Moreover, we could not detect either Ret mRNA expression, or RET activity in cultured cortical precursors stimulated with GDNF and analyzed by immunoblot (Figure S1C).

Expression of GFRα1 was analyzed in dissociated cultures of rat E14.5 cortical precursors. After 5 days in vitro (DIV), most cortical precursors differentiated into postmitotic neurons in the absence of ligand, whereas cultures treated with saturating concentrations of the mitotic ligand FGF2, were composed predominantly by proliferating precursors,
positive for nestin, a specific intermediate filament of neural precursors. To determine which cells were expressing GFRα1 receptor, we performed double-label immunofluorescence analysis for GFRα1 and nestin, or βIII-tubulin. In the cultures maintained in the presence of FGF2 the majority of the cells were nestin+ (≈70%), while cultures maintained in the absence of FGF2 resulted in an enhanced neuronal differentiation and less nestin+ cells (≈20%). Expression of GFRα1 was observed in ≈40% of total cells grown in the absence of FGF2 and in ≈8% of the cells

Figure 1. Developmental Expression and Localization of GFRα1 during Early Cortical Development

(A) Analysis of developmental expression of Gdnf, Gfra1, Gfra2, Ncam, Fgfr1, and Ret mRNA by semiquantitative RT-PCR (27 cycles) in rat cortex at embryonic day (E) 13.5, E15.5, E17.5, and newborn (P0). Expression of the housekeeping gene TATA binding protein (Tbp) was analyzed as control. Ret mRNA expression was analyzed in rat E18.5 dorsal root ganglia (DRG) and in PC12 cells stimulated with nerve growth factor (NGF), a treatment that induces Ret expression.

(B) Localization by immunofluorescence of GFRα1 in coronal sections of mouse E14.5 cortex. Expression of GFRα1 (red) and Sox2 (green) are shown in the upper panel, GFRα1 (red) and βIII-tubulin (green) in the middle panel, GFRα1 (red), and NCAM (green) in the lower panel. VZ, ventricular zone; SVZ, subventricular zone; CP, cortical plate. Scale bar, 100 μm.

(C) Localization by immunofluorescence of GFRα1 (green) and the neuronal nuclear marker Tbr1 (red) in CP. Scale bar, 50 μm. High-magnification image of boxed area is shown. The arrows indicate cells expressing GFRα1 with nuclei positive for Tbr1. Scale bar, 20 μm.

(D) Analysis of cortical progenitors cultured in the absence or presence of FGF2 (40 ng/mL) for 5 days in vitro (DIV) and stained for nestin (red) or GFRα1 (green). Scale bar, 50 μm.

(E) Upper bar graph, quantification of the percentage of nestin+ cells in the presence and absence of FGF2 (40 ng/mL). Lower bar graph, quantification of the percentage of GFRα1+ cells in the presence and absence of FGF2 (40 ng/mL). Data are mean ± SEM of n = 3 independent experiments. *p < 0.05, ***p < 0.001 (Student’s t test).

(F) Co-localization of GFRα1 (green) and the neuronal marker βIII-tubulin (red) in dissociated cortical precursors maintained in the absence of FGF2. Scale bar, 50 μm.

(G) Analysis by semiquantitative RT-PCR of Gfra1, Ncam and Ret in proliferating (+FGF2, 40 ng/mL) and non-proliferating (–FGF2) cortical precursors. Analysis of Tbp mRNA was used as control. Ret mRNA expression analyzed in E18.5 rat DRG and in PC12 cells treated with NGF, which induced Ret expression, is shown as positive control. See also Figure S1.
maintained in the presence of FGF2 (Figures 1D and 1E). All of the βIII-tubulin cells of these cultures were highly positive for GFRα1 (Figure 1F). Although the majority of the nestin+ cells were negative for GFRα1, many of them show low levels of GFRα1 expression. Analysis by RT-PCR revealed that Gfra1 and Ncam mRNA expression was higher in cultures maintained in the absence of FGF2 and lower in proliferating cultures, maintained in the presence of FGF2, while Ret mRNA could not be detected in these cells independently of the presence of FGF2 (Figure 1G).

**GDFN and FGF Regulate Opposite Processes in Cortical Precursor Cells**

It is known that FGF2 is widely distributed throughout the dorsal telencephalon. High expression of FGF2 in the VZ correlates with the initial expansion of NSCs, whereas its low expression near the pial surface is associated with neuronal differentiation. Thus, the distribution of FGF2 in vivo is consistent with the dose-dependent effect of FGF2 on self-renewing of NSCs in vitro. Similar to FGF2, FGFRs are widely distributed throughout the dorsal telencephalon and exhibit progressively reduced levels of expression along the ventriculopial gradient, being lower in the pial surface (Vaccarino et al., 1999a; Weise et al., 1993). As the expression of GFRα1 was higher in differentiated cells and lower in cells positive for SOX2 (Figure 1B), we decided to study whether FGF2 could regulate the expression levels of GFRα1. To this end, cortical precursor cells isolated from E14.5 rat cortex were cultured in the presence of different concentrations of FGF2, mimicking what happens during cortical neurogenesis. After 5 DIV, the levels of Gfra1 mRNA were analyzed by real-time PCR. We observed that cultures maintained in the presence of different concentrations of FGF2, which are enough to maintain cortical precursors in proliferation (Qian et al., 1997), expressed very low levels of Gfra1 and high levels of Fgfr1 mRNA, while cultures maintained in the absence of FGF2 resulted in high levels of Gfra1 and low levels of Fgfr1 mRNA (Figure 2A). This result indicates that Gfra1 and Fgfr1 mRNA expression levels are modulated by FGF2 in an opposite manner (Figure 2A).

To analyze the effect of GDNF on the expression of Gfra1 and Fgfr1 mRNA, cortical cultures were grown in the presence of different concentrations of FGF2 plus GDNF. We observed that, in the presence of low concentrations of FGF2 and GDNF, Gfra1 mRNA expression does not decrease as much as when the cultures were grown in the presence of FGF2 alone, indicating that GDNF could modulate the expression of its own receptor, making cortical precursor cells more responsive to the neurotrophic factor (Figure 2B). However, the presence of a high concentration of FGF2 (25 ng/mL) was enough to decrease substantially the levels of Gfra1 mRNA, even in the presence of GDNF. Similar results were observed for Gfra2 mRNA. As it has been described that GDNF induces RET expression in diverse systems (Arvidsson et al., 2001), we analyzed the levels of Ret mRNA expression in cortical precursors maintained with different concentrations of FGF2 and GDNF, but we were not able to detect expression of Ret mRNA in these conditions (Figure S1D).

We next examined the effects of GDNF on precursor cells maintained under self-renewing conditions in the presence of FGF2 for proliferation and neural differentiation. Immunostaining with antibodies against Ki67, a protein that is present during all active phases of cell cycle, but is absent from resting cells (G0) (Burdon et al., 2002), indicated that, under low concentrations of FGF2 (5 ng/mL), the GDNF treatment decreases the proportion of proliferating cells compared with controls maintained in the presence of FGF2 alone (Figure 2D). The decreased proliferation observed after GDNF exposure correlated with an increase in neuronal differentiation of precursors to postmitotic neurons positive for βIII-tubulin (Figure 2D). In the presence of higher concentrations of FGF2, the effect of GDNF on cell proliferation and neuronal differentiation was abrogated (Figure 2E). This result is in agreement with the low levels of Gfra1 mRNA observed when the precursors were exposed to high concentrations of FGF2 (Figures 1G, 2A, and 2B).

As it is known that Akt is involved in neuronal precursor proliferation, and it is activated by FGF2 (Burdon et al., 2002; Jin et al., 2005), we analyzed the effect of GDNF on FGF2-induced Akt phosphorylation. As shown in Figure 2F, stimulation of cortical precursors with 5 ng/mL of FGF2 induced activation of Akt, but when the cells were treated with FGF2 in the presence of GDNF, the phosphorylation levels of Akt were reduced. The activation of MAPK, which is also known to be induced by FGF2 was not affected by GDNF, indicating that GDNF specifically inhibits FGF2-induced Akt pathway in cortical precursors (Figure 2F).

Altogether these results indicate that GDNF counteracts the mitogenic stimulation of low concentrations of FGF2 on cortical progenitors by modulating Akt activity and promoting neuronal differentiation.

**GDNF Decreases Proliferation of Cortical Progenitors**

To test whether culture conditions, such as adhesion can affect GDNF function, we assayed the effects of GDNF on cortical precursor grown maintained as neurospheres in the presence of FGF2 (25 ng/mL). The neurosphere assay is a useful tool for determining the proliferative capacity of a cell population because only progenitor cells with high proliferative capacity have the ability to form neurospheres in presence of FGF2. Our data indicate a significant decrease in the number and diameter of spheres formed in the medium containing FGF2 and GDNF compared with
Interestingly, in this culture condition, GDNF was able to inhibit the proliferative effects induced by high concentrations of FGF2. We evaluated the proportion of cells per sphere that were positive for Ki67+ and nestin+ by immunofluorescence. We observed that the addition of GDNF to these cultures decreased the proportion of Ki67-expressing cells, and also decreased the percentage of nestin+ progenitors expressing Ki67 (proliferating precursors) (Figure 3B). Interestingly, a robust increase in the number of differentiated βIII-tubulin+ cells was observed in neurospheres grown in the presence of GDNF (see Figure S3). Cell death was not affected as similar results were obtained in the presence of the apoptosis inhibitor, Z-VAD-FMK (see Figures S2A–S2C).

As the GDNF transmembrane signaling receptor NCAM, was detected in cortical precursors (Figure 1B), we analyzed whether NCAM could be involved in the effects of GDNF on proliferation. To investigate this, neurospheres were grown in the presence of NCAM ECD function-blocking antibody. Interestingly, the effects of GDNF on proliferation could be reverted in the presence of the anti-NCAM ECD antibody.

**Figure 2. GDNF Regulates Cortical Precursor Response to FGF2**

(A and B) Analysis of Gfra1 and Fgfr1 mRNA expression by semiquantitative RT-PCR (27 cycles) in cortical precursor cultures maintained in the absence (A) or presence of GDNF (B), plus different concentrations of FGF2. Tbp mRNA expression was analyzed as control. The bar graphs show the levels of Gfra1 mRNA measured by real-time PCR using the expression of the housekeeping gene Tbp for normalization. Shown are mean ± SEM of triplicate determinations. ***p < 0.001, *p < 0.05 (Student’s t test).

(C) Representative images of proliferating cortical progenitors grown for 5 DIV in the absence or presence of GDNF (100 ng/mL) plus different concentrations of FGF2 stained with antibodies against βIII-tubulin (green) and Ki67 (red). Scale bar, 20 μm.

(D and E) Bar graphs showing the percent of Ki67+ (left) and βIII-tubulin+ (right) cells in cultures maintained in the presence of 5 ng/mL (D) or 25 ng/mL (E) of FGF2 with or without GDNF (100 ng/mL). Data are mean ± SEM of n = 3 independent experiments *p < 0.05, **p < 0.005 (Student’s t test).

(F) Analysis of Akt and MAPK activation induced by FGF2 and GDNF on cortical progenitors. After starving overnight, cultures were stimulated for 15 min with 5 ng/mL of FGF2 or FGF2 (FGF 5 ng/mL plus GDNF 100 ng/mL). The lysates were analyzed by pAkt and pMAPK immunoblotting. The same membrane was reprobed with anti-actin antibodies. Fold of change in pAkt and pMAPK relative to total actin is indicated.
antibodies, indicating the requirement of NCAM in this process (Figure 3C).

To analyze the role of GDNF on mitotic cortical cultures, we investigated the influence of GDNF on molecules known to regulate the cell cycle. To this end, neurospheres were grown in the presence of FGF2, or FGF2 plus GDNF, and the levels of different cyclins and negative regulators of the cell cycle were analyzed by real-time PCR. Neurospheres grown in the presence of FGF2 are characterized by an upregulation of the levels of cyclins as well as by a strong decrease of the cell-cycle inhibitors (Andreu et al., 2015; Mira et al., 2010). We observed that the decrease proliferation after GDNF exposure correlated with the downregulation of Cyclin D1 and Cyclin E mRNA and the upregulation of the cyclin-dependent kinase inhibitors p21 and p27 mRNAs (Figures 3D and 3E). This result indicates that GDNF has opposite effects with respect to FGF2 on the induction of the levels of positive and negative regulators of the cell cycle. Interestingly, we also observed an upregulation of GfRA1 mRNA in cells cultured in the presence of GDNF compared with neurospheres maintained only in the presence of FGF2 (Figures 3D and 3E).

Neurospheres contain cells at different stages of differentiation, with only a fraction of cells that maintains the

Figure 3. GDNF Treatment Decreases Proliferation of Cortical Precursor Cells
(A) Neurospheres from cortical precursor cells grown for 5 DIV in the presence or absence of GDNF (100 ng/mL) plus FGF2 (25 ng/mL) stained with DAPI. Scale bar, 200 μm. The bar graphs show the quantification of the number and diameter of neurospheres that were generated. The results are expressed as mean ± SEM of three independent experiments. *p < 0.05 (Student’s t test, see also Figure S2).
(B) Images of neurospheres derived from cortical precursor cells grown as described in (A) stained with nestin (red), Ki67 (green), and DAPI. Scale bar, 50 μm. The bar graphs show the quantification of the percentage of Ki67+/Ki67−/DAPI cells as well as proliferating precursor (Ki67− nestin+/nestin−) cells per sphere grown in the presence of FGF2 (25 ng/mL) or FGF2 (25 ng/mL) plus GDNF (100 ng/mL). The results are expressed as the mean ± SEM of three independent experiments. *p < 0.05 (Student’s t test).
(C) Bar graphs showing the quantification of the percent and diameter of neurospheres that were generated from cortical precursor cells grown for 5 DIV in the presence of the indicated trophic factors and blocking anti-NCAM antibodies. The results are expressed as the average percentage reduction in the number of neurospheres grown in the presence of GDNF relative to the neurospheres grown in the presence of FGF in the absence of GDNF (indicated as dotted line). The data are expressed as the mean ± SEM three independent experiments. *p < 0.05 (Student’s t test).
(D) Analysis of CyclinD1 and Cyclin E, p21 and p27, and GFRα1 mRNA from neurospheres cultured in the presence of FGF2 or FGF2 plus GDNF by semiquantitative PCR.
(E) Bar graph showing the levels of CyclinD1 and Gfrα1 mRNA analyzed by real-time PCR. The expression of the specific mRNA was normalized to that of the housekeeping gene Tbp. Shown are averages ± SEM of triplicate determinations, **p < 0.001 (Student’s t test).
initial proliferative characteristics. This small fraction of cells is the one that maintains the capacity to generate secondary neurospheres. When primary neurospheres, which were grown in the presence or absence of GDNF, where dissociated and cultured only in the presence of FGF2, we did not find differences in the number or in the diameter of secondary neurospheres. These results indicated that GDNF treatment inhibits the proliferation of neural precursors inducing its differentiation and maintaining NSCs in a resting state (see Figures S3C and S3D).

GDNF/GFRα1 Complex Is Required to Control Cortical Precursor Proliferation in Response to FGF

To analyze the contribution of GDNF through its GPI receptor GFRα1 to the inhibition of cortical precursor proliferation, the neurosphere assay was performed using cortical progenitors derived from control or GFRα1 mutant mice. To this end, we used mice carrying a cassette composed of floxed Gfra1 cDNA followed by GFP cDNA. In these mice, Cre-mediated excision of floxed Gfra1 cDNA converts the floxed Gfra1 allele into a GFP reporter allele (Uesaka et al., 2007). We generated conditional Gfra1-mutant mice by breeding Emx1-Cre ones (Mizusawa et al., 2000; Weisstaub et al., 2006) with mice carrying floxed Gfra1 alleles. Because Emx1 is expressed in glutamatergic neuronal progenitors, Emx1-Cre;Gfra1<sup>lox/lox</sup> mice (conditional Gfra1 mutants) represent a model in which Gfra1 is deleted from glutamatergic precursors (Gorski et al., 2002; Irala et al., 2016). To analyze the contribution of GDNF/GFRα1 to cortical precursor development, we used Gfra1-mutant mice homozygous for Gfra1 deletion, Emx1-Cre;GFRα1<sup>lox/lox</sup>, and heterozygous Emx1-Cre;GFRα1<sup>lox/+:</sup> as control. There was a significant decrease in the number of neurospheres formed from E13.5 cortical precursors derived from control Emx1-Cre;GFRα1<sup>lox/1</sup> when they were grown in the presence of FGF2 plus GDNF compared with the neurospheres grown in the presence of FGF2 alone. However, cortical precursor cells derived from animals deficient in GFRα1 gave rise to an increased number of spheres compared with the ones derived from control animals, independently of the presence of GDNF (Figures 4A and 4B). In addition, the average diameter of the spheres derived from GFRα1 mutant mice was bigger than the diameter of the spheres derived from control animals. The average diameter of the spheres derived from GFRα1-deficient mice cortical progenitors grown in the presence of FGF2 was similar to the spheres grown in the presence of FGF2 plus GDNF (Figures 4A and 4B). The absence of GFRα1 expression in the neocortex of Emx1-Cre;GFRα1<sup>lox/lox</sup> mice was controlled by immunofluorescence (see Figure S4). Although GFRα1 is the main binding receptor for GDNF, this neurotrophic factor can also bind with lower affinity to GFRα2. However, the low levels of Gfra2 mRNA expression during the early cortical development (Figure 1A), together with the complete absence of effect of GDNF in the neurosphere assay from cells coming from mice lacking Gfra1, suggested that GDNF specifically inhibits FGF2-induced cortical precursor proliferation acting through GFRα1.

We also analyzed, by qPCR, the expression levels of cell-cycle regulatory genes in neurospheres derived from GFRα1 mutant mice compared with control mice. In neurospheres derived from GFRα1-deficient mice, the mRNA expression level of the cell-cycle promoter CyclinD1 increased significantly compared with the levels detected in neurospheres derived from control animals in the presence of FGF2. In contrast, the mRNA expression level of cell-cycle inhibitor p27 was decreased (Figure 4C). These results indicate that GDNF, through its receptor GFRα1, negatively controls the proliferation of neural precursor cortical progenitors.

To analyze the requirement of the GDNF/GFRα1 system in the proliferation of neuronal precursors in vivo, we performed immunofluorescence against Ki67 in cortical sections of GFRα1-deficient or control mice at E13.5. The expression of this proliferation marker was analyzed in the different layers of the cortex, the high proliferative layer, VZ/SVZ (where the apical progenitors resides); the intermediate layer, IZ (intermediate zone) populated mostly by basal progenitors); and the cortical plate where the neurons generated in this period are located (Molyneaux et al., 2007). In agreement with the in vitro results, a significant increase in Ki67-expressing cells was observed in GFRα1-deficient animal sections, both in the VZ/SVZ and IZ regions, suggesting that GFRα1 is required in vivo for a correct regulation of the proliferation of apical and basal neuronal progenitors (Figures 4D and 4E).

GDNF and Its Receptor GFRα1 Are Involved in Neuronal Differentiation of Cortical Precursors

As we mentioned earlier, GDNF counteracts the mitogenic stimulation of FGF2 on cortical progenitors promoting neuronal differentiation in vitro. To characterize the contribution of GDNF on neuronal differentiation, cortical progenitors were cultured in the presence of a low concentration of FGF2 (5 ng/mL), with or without GDNF, followed by staining with the neuronal marker βIII-tubulin and glutamatergic (VGluT, glutamate vesicular transporter) or GABAergic (VGAT, GABA vesicular transporter) markers. We observed that, under low concentrations of FGF2, GDNF induced an increase in the VGluT<sup>+</sup> neuronal population. Few VGAT<sup>+</sup> cells were detected in the cultures, and no difference was detected in their number in the presence or absence of GDNF (Figures 5A and 5B). Then, we analyzed whether GDNF treatment had any effect on neurite development of postmitotic neurons. The complexity of the βIII-tubulin cells was...
evaluated by Sholl analysis, which quantifies the number of neuritic branches intersecting concentric circles of increasing radius centering the reference point on the cell body (Sholl, 1953). Morphological parameters, such as total neurite length and branching points, were evaluated (Figures 5C–5E). We found that postmitotic neurons treated with GDNF showed a significant increase in neuritic arbor development compared with neurons non-treated with exogenous GDNF (Figures 5C–5E and S2D–S2F). The morphological effect on postmitotic neurons was also demonstrated by staining the cells with the dendrite marker MAP2 (see Figure S5). The effect of GDNF on these cells was observed in the presence of low concentrations of FGF2, but was abrogated under high concentrations of this growth factor (data not shown), indicating that GDNF promotes morphological neuronal differentiation of cortical precursors depending on the levels of GFRα1 expression modulated by FGF2.

Differentiation during brain development involves not only the growth of neurites but also the correct establishment of synaptic connections. Because of this, we decided to evaluate whether GDNF could affect the ability of postmitotic neurons to form dendritic spines, where the majority of excitatory synapses are established. A significant increase in spine density was observed in cells maintained in the presence of GDNF and low concentration of FGF2 relative to cultures maintained only in the presence of FGF2 (Figure 5F). These results collectively are in agreement with a role of GDNF as an inhibitor of cortical precursor proliferation during early development and as a
GFRα1 Is Required for Proper Glutamatergic Neuronal Differentiation

To analyze the contribution of GDNF/GFRα1 to neuronal differentiation we cultured cortical progenitors derived from GFRα1 mutant mice Emx1-Cre:GFRα1^flox/flox, in which Cre-mediated excision of floxed GFRα1 cDNA converts the floxed GFRα1 allele into a GFP reporter (Emx1-Cre: GFRα1^GFP/GFP). We performed cultures from GFRα1-deficient mice homozygous for GFP, Emx1-Cre:GFRα1^flox/flox (Emx1-Cre:GFRα1^GFP/GFP) and heterozygous, Emx1-Cre: GFRα1^flox/+ (Emx1-Cre:GFRα1^vGFP) as control, in the
presence of GDNF and low concentrations of FGF2 (Figure 6A). No difference in the number of βIII-tubulin+ cells was observed in cultures derived from GFRα1-deficient mice compared with the control mice. Morphological parameters such as total neuritic length and branching were also evaluated. We found a significant reduction in total neurite complexity as demonstrated by Sholl analysis, and length and branchpoint number, in neurons derived from GFRα1 mutant mice compared with cells obtained from control mice when they were maintained in the presence of GDNF, indicating that GDNF/GFRα1 is required for proper neuronal development (Figures 6A–6C).

Finally, we analyzed the morphological features of GFRα1-deficient neurons in the postnatal cortex of GFRα1−/− mice. Representative images of cortical neurons from P0 GFRα1-deficient and control littermate mice. Scale bars, 500 μm (left panel) and 100 μm (right panel). Bar graph shows the quantification of total dendritic length of cortical neurons from GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) versus control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) mice. The results shown are mean ± SEM, *p < 0.05 (Student’s t test). Quantifications were performed in neurons from n = 3 GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) and control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) littermate mice.

Figure 6. The Complex GDNF/GFRα1 Is Required for Neuronal Differentiation from Cortical Progenitors
(A) Representative pictures of dissociated rat cortical progenitors obtained from GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) and control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) mice cultured in the presence of GDNF (100 ng/mL) plus FGF2 (5 ng/mL). After 5 DIV the cells were stained with antibodies against βIII-tubulin (red) and GFP. Scale bar, 30 μm.
(B) Quantification of dendrite complexity by Sholl analysis of postmitotic GFP+ neurons described in (A) maintained in the indicated conditions. Graph bar represents the cumulative neurite crossings obtained by Sholl analysis.
(C) Bar graphs show the quantification of total neuritic length and branching from cortical precursors obtained from GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) and control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) mice maintained in the indicated conditions. Data of (B) and (C) represent mean ± SEM of n = 3 independent experiments. **p < 0.001, ***p < 0.0001; ns, non-significant (ANOVA, followed by a Newman-Keuls test).
(D) GFRα1 deletion results in a reduction of cortical dendrite development in vivo. Representative images of cortical neurons from P0 GFRα1-deficient and control littermate mice. Scale bars, 500 μm (left panel) and 100 μm (right panel). Bar graph shows the quantification of total dendritic length of cortical neurons from GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) versus control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) mice. The results shown are mean ± SEM, *p < 0.05 (Student’s t test). Quantifications were performed in neurons from n = 3 GFRα1-deficient (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) and control (Emx1-Cre:GFRα1flox/flox, Emx1-Cre:GFRα1GFP/GFP) littermate mice.
mutant mice. Although GFRα1 is expressed at very low levels in the majority of postnatal cortical neurons, a subpopulation of neurons from the cingulate cortex showed a clear GFP expression in transgenic Emx1:Cre-GFRα1^lox/lox (Emx1:Cre-GFRα1^{GFP/GFP}) or Emx1:Cre:GFRα1^+/- (Emx1: Cre-GFRα1^{GFP/Δ}), indicating that GFRα1 is expressed in these neurons. The complexity of the dendritic arbors was examined using an anti-GFP antibody, which allows visualization of individual cells and follows dendritic arbors on P0 cortical sections. We analyzed whether loss of GFRα1 expression affects dendritic morphological features in GFP-expressing neurons. In control mice, a typical pyramidal morphology of these neurons was observed with a single apical dendrite extending toward the pia and secondary branches. In GFRα1 mutant mice Emx1:Cre-GFRα1^lox/lox, the dendrite morphology was abnormal, a significant reduction in the total length of apical dendritic arbors was observed, suggesting that GDNF/GFRα1 signaling may be required in vivo for normal dendritic elaboration (Figure 6D). Taken together, these results indicate that the GDNF/GFRα1 system is involved in the neuronal differentiation of glutamatergic neurons from cortical precursors.

**DISCUSSION**

During embryogenesis, neural precursor cells (NPCs) in the developing cortex are exposed to different intrinsic and extrinsic cues, the expression of which is under strict spatial and temporal control. External cues include growth factors such as FGF2, epidermal growth factor, Wnts, and neurotrophins, neurotransmitters such as GABA, glutamate, acetylcholine, and PACAP (Antonopoulos et al., 1997; Bartkowska et al., 2007; Haydar et al., 2000; Li et al., 2001; LoTurco et al., 1995; Ma et al., 2000), and contact-mediated signals, such as Notch (Chambers et al., 2001; Shen et al., 2002).

In the present work, we described the expression of Gdnf mRNA during early cortical developmental stages and provided evidence indicating that GDNF counteracts FGF2 self-renewal activity on NSCs, promoting the neural differentiation. Our data show that the GDNF receptor, Gfra1, mRNA is expressed during cortical development, increasing between E12 and E18, while the levels of the main FGF2 receptor in the CNS, Fgfr1, decrease during this period, indicating a negative correlation between the expression of the two receptors. While Fgfr1 is highly expressed in proliferating cultures of NPCs maintained in the presence of FGF2, Gfra1 is mainly expressed in non-proliferating cultures maintained in the absence of FGF2. Using both adherent and neurosphere cell cultures, we found that GDNF acts by restricting NPC division in the presence of a mitogenic stimulus. Our work provides evidence showing that GDNF reduces Akt activation and inhibits the expression of Cyclin D1 and E, which are induced by FGF2, and promotes the expression levels of cell-cycle inhibitors, such as p27 and p21. Selective ablation of GFRα1 in glutamatergic cortical precursors results in an increase in the number of cortical precursors positive for the proliferation marker Ki67 in vivo. In agreement with this, we obtained more and bigger neurospheres from GFRα1-deficient mice than the ones obtained from control animals. The decrease in proliferation induced by GDNF/GFRα1 is due in part to an increase in neuronal differentiation of the NPCs and to maintaining stem cells in a resting state. Addition of GDNF enhances the number of postmitotic neurons on cultures of cortical precursor cells. However, although our experiments indicate that GDNF can influence neuronal differentiation, it is likely that other factors can contribute to the process as well, since animals with target disruptions of GFRα1 in glutamatergic precursors do not have a clear deficit in neuronal number in the postnatal cortex or hippocampus (Irala et al., 2016). Although it is possible that other factors can compensate for the loss of GFRα1, it could also be possible that the deficit in GFRα1 could delay neuronal differentiation. In addition to promoting the withdrawal of precursor cells from cell cycle and inducing an increase in postmitotic neurons, GDNF also regulates dendrite development of these neurons. In agreement with a previous report (Irala et al., 2016) in which we described that GDNF through GFRα1 promotes dendrite arborization and neurite complexity in hippocampal pyramidal cells, in the present work we show that the addition of GDNF to cultures of NPCs induced an increase in dendrite length and complexity, as well as dendritic spine-like protrusion in the presence of FGF2. These results revealed an important role of GDNF and GFRα1 in hippocampal and cortical connectivity. Recently it has been described that glutamatergic cortical neurons from patients with Alzheimer's disease (AD) lacked a response to GDNF. In that work, the authors showed evidence indicating that cultured cortical neurons and postmortem brain tissue from patients with AD have lower levels of GFRα1 receptors compared with neurons derived from normal brains (Konishi et al., 2014). Based on these data it will be important to continue investigating the contribution of GDNF/GFRα1 complex in neurodevelopmental disease characterized by cognitive impairments.

**GDNF in Cortical Development**

Previous reports have described a role for GDNF in GABAergic neuron migration and differentiation during early cortical development. These reports indicate that GDNF promotes differentiation and tangential migration of cortical GABAergic neuroblast from the ganglionic
eminence toward the cortex (Poza and Ibanez, 2005; Bespalov et al., 2011). In the present work, we described a role of GDNF as a differentiation factor of glutamatergic cortical precursors acting through its receptor GFRα1. In agreement with our results, a recent work using a transcriptome profiling comparing ligands produced by NPCs and neurons, identified GDNF and another member of the GDFN family, NRTN, as proneurogenic factors. The authors claim that these effects are mediated by the tyrosine kinase receptor, RET; however, we could not detect the expression of this receptor by RT-PCR (Figure 1), immunostaining or immunoprecipitation (Figure S1). To define the mechanism involved in GDNF effect on cortical development, we consider that it will be necessary to perform the in vivo experiments in RET-deficient mice.

In the present work, we described that GDNF, through GFRα1, promotes dendritic growth and a complexity of glutamatergic neurons derived from cortical precursors. Although GFRα1 is highly expressed in hippocampal neurons, the expression of GFRα1 in the postnatal cortex is restricted to a small population of cortical neurons. Analysis of newborn conditional GFRα1-deficient mice shows a reduction in dendritic length in a subpopulation of cingulate cortical neurons. This result is in agreement with our previous results indicating that the GDNF/GFRα1 complex plays a crucial role in the development of dendritic arbors and establishment of excitatory synaptic contacts. In hippocampal neurons these effects are mediated by the neural cell adhesion molecules NCAM-180 (Irala et al., 2016).

**GDNF as a Proliferative or Differentiation Factor**

Interestingly, GDNF has been described as a promotor of proliferation in different systems such as enteric progenitors and spermatogonial stem cells (SSCs) (Meng et al., 2000; Sasselli et al., 2012; Yan et al., 2004). In these systems, the GDNF proliferative effects depend on tyrosine kinase receptor, RET, signaling. Addition of GDNF to enteric neuron precursors, which express RET, increases the number of neurons and enteric glia. Furthermore, it has also been described that GDNF signaling is indispensable for SSC self-renewal by binding to the GFRα1/RET receptor. In this system, GDNF and FGF2 induce SSC self-renewal by different molecular mechanisms (Takashima et al., 2015).

Our present results indicate that GDNF inhibits the proliferation of cortical precursors. The mechanism by which GDNF suppress NPCs proliferation it is not clear yet. Our findings indicate that GDNF antagonizes the proliferative effect of FGF by inhibiting intracellular signaling, such as the Akt pathway, which leads to increased levels of the cell-cycle inhibitor p27 and reduction in the levels of the cyclin D1 expression, as well as downregulation of FGFR1 expression. We provide evidence indicating that this effect could be mediated by GDNF signaling acting through GFRα1 and NCAM receptors. Recently, NCAM and NCAM-derived peptides have been reported to interact with FGFRs (Christensen et al., 2006; Francavilla et al., 2009; Kiselyov et al., 2003), modulating FGFR-mediated cellular functions (Hansen et al., 2008). Interestingly, it has been demonstrated that NCAM expression reduces FGF-stimulated cell proliferation in fibroblasts (Francavilla et al., 2007). Thus, one possibility is that GDNF acting through its receptors GFRα1 and NCAM could interfere FGFR signaling in NPCs.

The proliferation of cortical precursor cells appears to be regulated by FGF during early cortical development in the ventricular zone, when FGF2 prevents NPC differentiation. Subsequently, GDNF acts on precursor cells inducing cell-cycle withdrawal and neuronal differentiation. Later in the development GDNF acts on postmitotic neurons promoting dendrite complexity. The dual effect of GDNF depends on the levels of GFRα1 expression. Our findings show that the expression of GFRα1 is regulated by FGF2 treatment in cortical precursors, while the expression of FGFR1 is modulated by GDNF. Thus, during cortical development, the levels of FGF2 decrease downregulating the expression of its receptor FGFR1 and upregulating the levels of GFRα1 in postmitotic neurons. The absence of RET in cortical postmitotic neurons suggests that the effect on neurite complexity could be mediated by NCAM signaling, as we have described previously in CA1 and CA3 pyramidal neurons (Irala et al., 2016). The evidence obtained during the last years about the role of GDNF on hippocampal and cortical neurons gives new opportunities to study the role of GDNF in neurodevelopmental diseases characterized by cognitive deficits.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**

*Gfra1floxflox* mice were generously provided by Dr J. Milbrandt (Washington University School of Medicine, St Louis, MO, USA) (Irala et al., 2016). *Gfra1floxflox* mice were mated with *Emx1-Cre* mice generously provided by Dr N. Weisstaub (School of Medicine, University of Buenos Aires, Argentina) (Iwasato et al., 2000; Weisstaub et al., 2006). All transgenic strains were genotyped by PCR. PCR primer sequences are available upon request. The use of animals was approved by the Animal Care and Use Committee of the School of Medicine, University of Buenos Aires, ethical permit number 67341/2013.

**PCR and Real-Time PCR**

PCR and real-time PCR were performed using standard methodologies. See Supplemental Experimental Procedures for details.
DNA Constructs, Cell Transfection, and Recombinant Proteins
Details of DNA constructs, cell transfection, and recombinant proteins used in the work can be found in Supplemental Experimental Procedures.

Primary Cortical Progenitor Cultures and Neurosphere Assay
Rat and mouse cortical progenitor cells were isolated from E14.5 Wistar rats and E13.5 C57/B16 mice, respectively (School of Medicine, University of Buenos Aires, Argentina) to perform primary cultures. Details about the cultures can be found in Supplemental Experimental Procedures.

Immunostaining and Confocal Microscopy
For immunofluorescence, cells were washed, fixed with 4% paraformaldehyde (PFA) in PBS, permeabilized with 0.3% Triton X-100, blocked with 10% donkey normal serum (Jackson ImmunoResearch) in PBS, and incubated overnight at 4°C with the indicated antibodies (see Supplemental Experimental Procedures).

For tissue immunofluorescence assays, rat and mouse brains were isolated from animals fixed with 4% PFA for 4 hr, maintained in sucrose 30% in PBS overnight, and then embedded in O.C.T. (Tissue-Tek) and sectioned at 25 μm. Cryostat sections were permeabilized with 0.1% Triton X-100, blocked with 10% donkey normal serum, and incubated overnight at 4°C with primary antibodies. The primary antibodies used for these stainings are described in Supplemental Experimental Procedures. The nuclear marker DAPI (1/10000, Sigma) was used to stain cells in culture and tissue sections. The secondary antibodies were from Jackson ImmunoResearch.

After immunostaining, microscopy was performed using an Olympus IX-81 inverted microscope or an Olympus FV-1000 confocal microscope, using identical settings between control and experimental images.

Total Cell Lysates and Western Blot
Cell cultures were lysed at 4°C in TNE buffer (25 mM Tris-ClH [pH 7.4]; 1 mM EDTA; 137 mM NaCl) containing 0.5% Triton X-100, plus protease and phosphatase inhibitors. Proteins lysates were clarified by centrifugation and analyzed by western blotting as described previously (Paratha et al., 2003). The antibodies used for western blotting are described in Supplemental Experimental Procedures. The blots were scanned in a Storm 845 PhosphorImager (GE Healthcare Life Sciences), and quantifications were done with ImageQuant software (GE Healthcare Life Sciences). Numbers below the lanes indicate fold of induction relative to control normalized to total levels of target protein.

Image Analysis and Quantification
Neuritic growth assays for neurons derived from cortical precursors were performed in primary E14.5 rat or E13.5 mouse dissociated cultures. Images of dissociated neurons to perform the morphological analyses were obtained using an Olympus IX-81 microscope, in a 20× objective. Neuritic complexity measurements were performed, as described in Supplemental Experimental Procedures, on neuronal cells that showed a pyramidal type morphology, containing a principal dendrite and several fine dendrites.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism. Details are provided in each figure legend. For quantification of in vitro or in vivo assays, 10–30 neurons were chosen randomly per condition in each experiment from at least three independent experiments. The results are shown as mean ± SEM as indicated in the figure legends, and significance was accepted at p < 0.05. Statistical significance was calculated using two-tailed Student’s t test or ANOVA followed by post hoc multiple comparison test as indicated.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.01.019.

AUTHOR CONTRIBUTIONS
A.B., G.P., and F.L. designed the experiments. A.B. and P.A.F. performed the experiments. A.B., P.A.F., G.P., and F.L. analyzed and discussed the experiment results. F.L. wrote the manuscript.

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