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GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream

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Olfactory bulb (OB) interneurons are generated from neuroblast cells derived from the anterior subventricular zone (SVZa) of the forebrain. The mechanisms guiding the rostral migration of these neuronal precursors are not well understood. Here, we show that glial cell line-derived neurotrophic factor (GDNF) is produced in the olfactory bulb but distributed along the rostral migratory stream (RMS) in a pattern concordant with the expression of its GPI-anchored receptor GFRα1. We demonstrate that GDNF is a chemoattractant factor for RMS-derived neuronal precursors, but not for SVZa neuroblast cells. In agreement with this, GDNF increased Cyclin-dependent kinase 5 (Cdk5) activity in RMS cells, a kinase critically involved in neuronal migration and guidance. GDNF-mediated cell chemoattraction was abrogated in RMS explants treated with the Cdk5 inhibitor Roscovitine as well as in RMS explants isolated from *Ncam* mutant mice. Chemical cross-linking assays showed that ^{125}I-GDNF is able to interact directly with NCAM in RMS-derived cells. Taken together, these data demonstrate that GDNF is a direct chemoattractant factor for neuroblast cells migrating along the RMS and support the participation of NCAM during this guidance process.

Keywords: GDNF; Chemoattraction; Neuronal precursors; Rostral migratory stream; NCAM

Introduction

Cell migration is a fundamental process in the development of the central nervous system (CNS), because both neuronal and non-neuronal cells are usually generated in sites that differ from those in which they eventually reside.

Cells originating from the lateral ganglionic eminence (LGE) migrate profusely to the olfactory bulb (OB) at embryonic stages to give rise to periglomerular and granule cells (Wichterle et al., 2001). During postnatal stages, this rostral migration of cells from the subventricular zone (SVZ) of the LGE to the OB proceeds continuously into adulthood (Altman, 1969; Luskin, 1993; Lois and AlvarezBuylla, 1994; De Marchis et al., 2001). Thus, cells generated in the SVZ migrate tangentially along the rostral extension of the SVZ up to its distal portion into the OB, following a well-defined route, the rostral migratory stream. Upon reaching the core of the OB, these cells migrate radially toward the granule and periglomerular layers of the OB.

Our understanding of the cellular and molecular mechanisms guiding neuronal migration from the forebrain to the OB is still limited. At the cellular level, the septum and the ventricular zone have been shown to contain repulsive activities for anterior SVZ (SVZa) neuroblast cells (Hu and Rutishauser, 1996; Zhu et al., 1999). It has been suggested that Slit protein, a secreted repellent for axons, is expressed in the septum and ventricular zone and can repel both embryonic LGE and postnatal SVZa neuronal precursors (Hu, 1999). However, it is difficult to envision how a single repellent activity can guide neuroblast migration along the entire length of the RMS. In addition, directional guidance from SVZa to the OB appears to require a diffusible attractant for SVZa cells which persists in the OB from embryonic to adult stages (Liu and Rao, 2003). It has been demonstrated that removal of the rostral OB significantly reduces the migration of SVZa cells in the RMS toward the OB (Liu and Rao, 2003). Recently, it has been also shown that Prokineticin2 is an OB-derived molecule that functions as a chemoattractant factor for SVZ-derived neuronal progenitors (Ng et al., 2005). Several extracellular molecules have been implicated in the control of neuronal migration in this migratory pathway (Mason et al., 2001; Guan and Rao, 2003). Activation of Eph receptors by ephrins as well as ErbB4 signaling can acutely induce the motility of RMS-derived cells and therefore are thought to act as motogenic factors for migrating OB neuronal precursors (Marin and Rubinstein, 2003; Anton et al., 2004). Thus, the migration of neuronal precursors into the granule and periglomerular layers of the OB would seem to be controlled by the concerted activities of repulsive factors in the septum, motogenic factors present in the SVZa and the RMS, as well as chemoattractive factors derived from the OB.

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The polysialylated neural cell-adhesion molecule (PSA-NCAM) has also been shown to be an important determinant for the migration of neuroblast derived from the SVZa. During postnatal stages, migrating neuroblasts originating from the SVZ are easily identifiable by the expression of PSA-NCAM and βIII Tubulin. The most distinctive abnormality of the central nervous system (CNS) of mice lacking NCAM is a dramatic reduction in the size of the adult OB (Cremer et al., 1994). This phenotype is caused by defects in the migration of neuronal precursors in the RMS (Garcia Verdugo et al., 1998). In the absence of NCAM, neuronal precursors accumulate along the RMS, producing characteristic enlargements over the entire length of the pathway, particularly in its most caudal portion between the corpus callosum and the striatum (Chazal et al., 2000). Recently, we have discovered that the neural cell adhesion molecule NCAM can function as an alternative signaling receptor for members of the GDNF family of ligands (Paratcha et al., 2003). Thus, a number of functions previously ascribed to short-range signaling by homophilic NCAM interactions, could also be mediated by the interaction of long-range signaling messengers, such as GDNF family ligands with NCAM receptors. Members of the GNDF family bind to specific glycosyl phosphatidylinositol (GPI)-anchored co-receptors (GFRα1–α4), but signal in collaboration with the RET tyrosine kinase or NCAM transmembrane receptors (Airaksinen and Saarma, 2002; Paratcha et al., 2003). Interestingly, GDNF directly promotes the motility of Schwann cells (Paratcha et al., 2003), raising the possibility that it may also play a similar role for some subpopulations of neuronal cells. The prominent localization of GFRα1 and PSA-NCAM receptors in the developing and adult SVZa-RMS pathway prompted us to investigate whether GDNF may promote directional guidance of neuroblast cells migrating toward the OB. Here, we present evidence that GDNF is a direct chemoattractant factor for RMS-derived neuroblast cells and that NCAM function is required for this activity.

Results

Expression and localization of GDNF in the SVZa-RMS pathway and during OB development

As a first step to determine whether GDNF could represent a diffusible molecule involved in guidance of neuronal cells migrating along the SVZa-RMS pathway, we examined the developmental pattern of GDNF mRNA expression in the OB and in the SVZa-RMS of P1 newborn rats. For this purpose, the pathway was dissected into SVZa, posterior RMS (RMSp) and anterior RMS (RMSa) as described in Fig. 1A. RT-PCR analysis

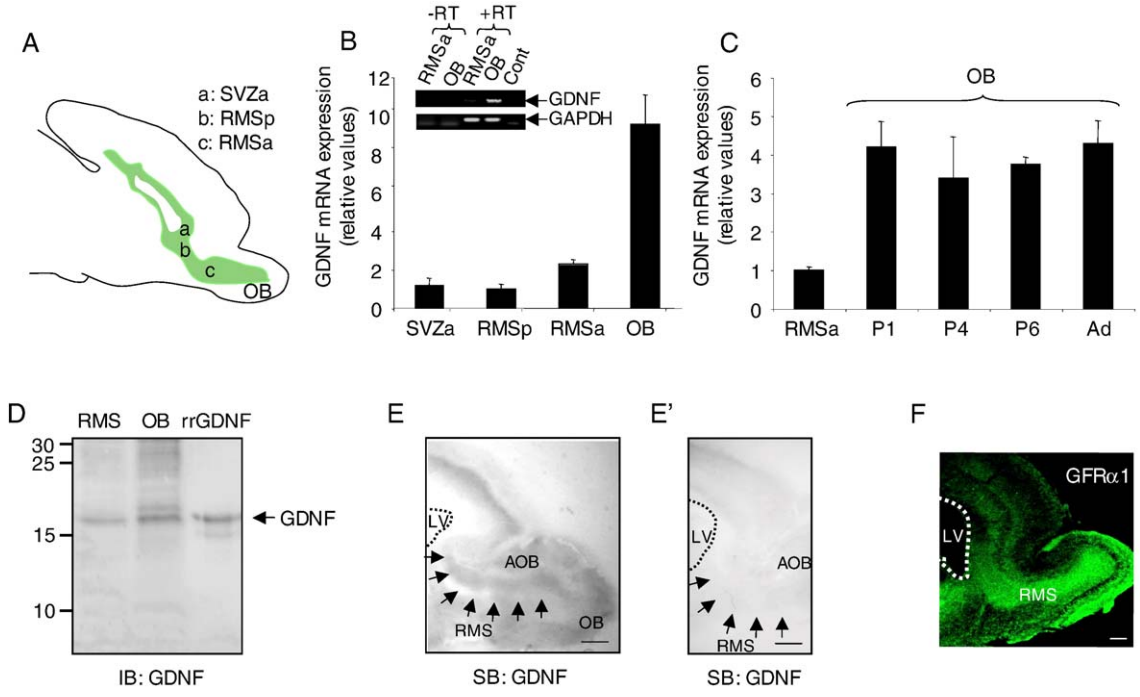

Fig. 1. Secreted GDNF protein is localized along the RMS pathway. (A) Diagram showing a sagittal section of neonatal rat forebrain indicating the anterior subventricular zone (SVZa) and different parts of the rostral migratory stream, used for different analyses in the present work. (a) Anterior part of the SVZ (SVZa); (b) posterior part of the RMS (RMSp); (c) anterior part of the RMS (RMSa). (B) Quantitative analysis of GDNF mRNA expression by real-time PCR in SVZa, RMSp, RMSa and olfactory bulb (OB) isolated from P1 newborn rats. Shown are averages ± SD of triplicate determinations. The insert shows the expression of GDNF in the RMSa and OB of P1 newborn rat examined by RT-PCR. Control samples without reverse transcriptase (−RT) are also shown. Expression of the house-keeping GAPDH was used as loading control. (C) Quantitative analysis of GDNF mRNA expression by real time PCR in the RMSa of P1 rat and OB tissue isolated during different developmental stages. Shown are averages ± SD of triplicate determinations. (D) Representative immunoblot of GDNF in total lysates prepared from both RMS and OB tissue isolated from P4 rats. Purified rat recombinant GDNF (R&D systems) was loaded in the third lane as a positive control. (E–E′) Representative photomicrographs of a GDNF slice blot obtained from sagittal sections of P4 rat forebrain probed with affinity-purified anti-GDNF antibodies (E) or anti-GDNF antibodies preadsorbed with 10^{-5} M of purified rat recombinant GDNF (E′). AOB: Accessory Olfactory Bulb. Arrows indicate GDNF staining along the RMS. (F) Immunofluorescence staining showing the expression of GFRα1 in a sagittal section of the RMS of P1 mice. Scale bar, 200 μm. LV, Lateral ventricle.

revealed that GDNF mRNA expression was barely detectable along the RMS pathway and in the SVZa, but prominently expressed in the OB of postnatal day (P) 1 newborn rats (Figs. 1B and C). During OB development, GDNF mRNA expression was maintained at high levels, relative to the RMS, from P1 to adulthood (Fig. 1C). In contrast to the low GDNF mRNA levels in the RMS, GDNF protein could be readily detected by immunoblot analysis of RMS extracts (Fig. 1D). Moreover, secreted GDNF was localized along the RMS and in the outer layers of the OB, as assessed by immunoblotting of brain slices (Fig. 1E), a technique that allows the localization of spatially patterned secretion of diffusible signals such as neurotrophic factors and chemotactic guidance molecules (Lowe, 1999). The specificity of the GDNF signal was confirmed by antigen preadsorption of GDNF antibodies (Figs. 1E and E′). Together, these data suggested that GDNF may be predominantly expressed and secreted from the OB and subsequently distributed along the rostral migratory pathway. Of note, the distribution of GDNF protein in the RMS coincided with that of its GPI-anchored receptor GFRα1 and not with its own mRNA (Fig. 1F and Paratcha et al., 2003), suggesting a role for this molecule in the localization and transport of GDNF along the RMS. Thus, the developmental and prominent GDNF expression in the OB, and the presence of soluble GDNF along the RMS are consistent with a role for this factor in chemoattraction of neuronal precursors in this pathway.

Role of GDNF in guiding neuronal precursor migration in the RMS

Next, we investigated whether GDNF family ligands could guide migration of rat P1–2 SVZa and RMS neuronal precursor cells when presented from a localized source. To this purpose, we cultured SVZa, RMSp and RMSa explants in a three-dimensional matrix together with agarose beads soaked in GDNF, Neurturin (NTN, another member of the GDNF ligand family) or BSA (control beads). In order to quantify the distribution of migrating neuroblast cells, the area surrounding each explant was divided into four quadrants (Fig. 2) and the area covered by βIII Tubulin-positive (Tuj1$^+$) cells in the quadrant proximal (P) to the agarose beads was compared to that in the distal (D) quadrant (P/D ratio in Fig. 2C). In these conditions, only beads soaked in GDNF were capable to induce a clear directional migration of RMS-derived neuroblast cells (Fig. 2). GDNF attracted neuronal cells derived from both the anterior and posterior areas of the RMS, but not from the SVZa (Figs. 2A and C). Beads soaked in BSA or NTN were completely ineffective. Similar results were obtained at later stages of development in P5–6 SVZa and RMS explants (Figs. 2B and D).

In order to define whether GDNF has a chemoattractant effect on neuronal cell bodies or axonal projections, we performed the same chemotropic assay staining the RMS explants with the

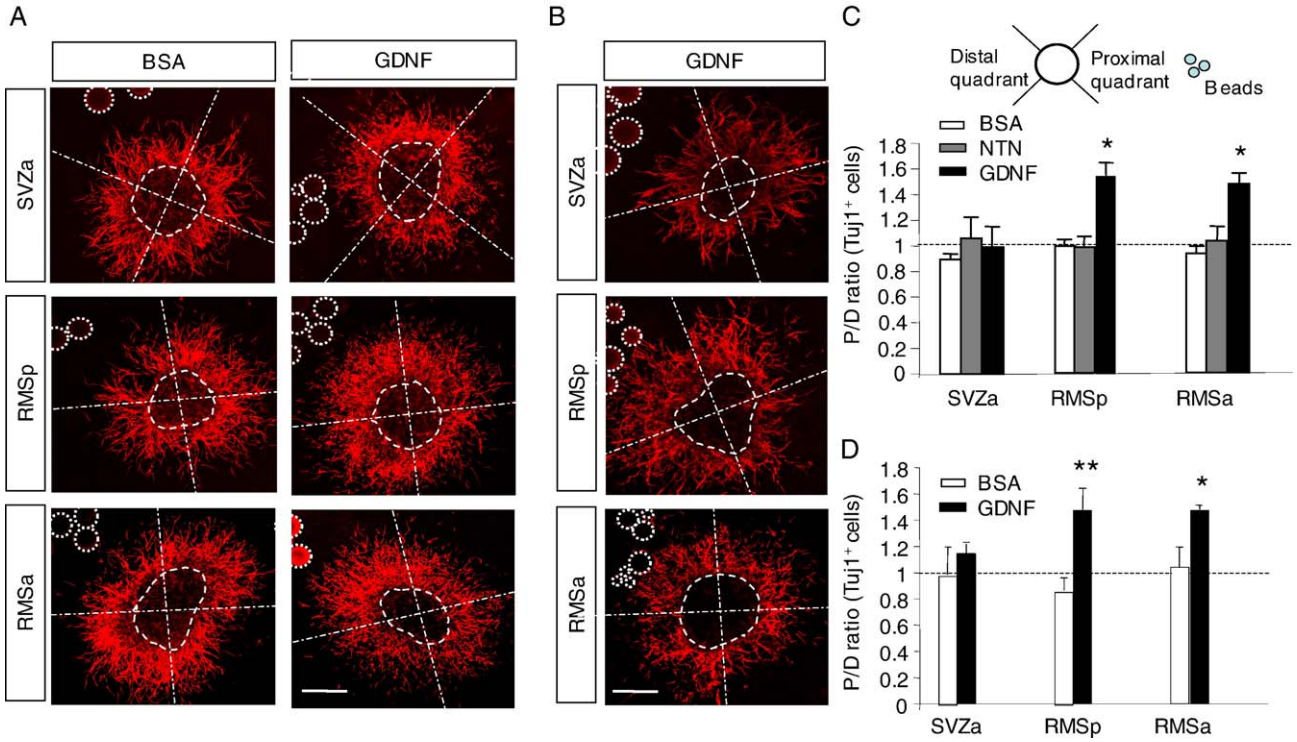

Fig. 2. A local source of GDNF induces directional guidance of RMS-derived neuronal cells. (A) SVZa, RMSp and RMSa explants (P1–2 rat) were cultured in a tridimensional matrix containing agarose beads soaked in BSA or GDNF. After 36 h, the explants were fixed and stained with anti-βIII Tubulin antibodies. Scale bar, 200 μm. (B) Representative photomicrographs of βIII-Tubulin immunofluorescence staining of SVZa, RMSp and RMSa explants (P5–6 rat) cultured in tridimensional matrices adjacent to beads soaked in GDNF. Scale bar, 200 μm. (C–D) Quantitative analysis of neuronal chemoattraction of P1–2 (C) and P5–6 (D) rat explants. Histograms showing the quantification of neuronal chemoattraction expressed as the ratio between the area covered by neuroblast cells migrating in the quadrant proximal (P) to the beads relative to the area occupied by neuroblast cells in the distal (D) quadrant (P/D ratio). Dotted line indicates the theoretical value expected for an explant growing symmetrically (P/D = 1). (C) Results are presented as average ± SEM. For each condition, a total of 25 to 34 explants were evaluated in three independent experiments. *$P < 0.01$ vs. BSA or NTN soaked beads (one-way ANOVA). (D) Results are presented as average ± SD. For each condition, a total of 10 to 23 explants were evaluated in two independent experiments. *$P < 0.05$; **$P < 0.005$ (Student's t test).

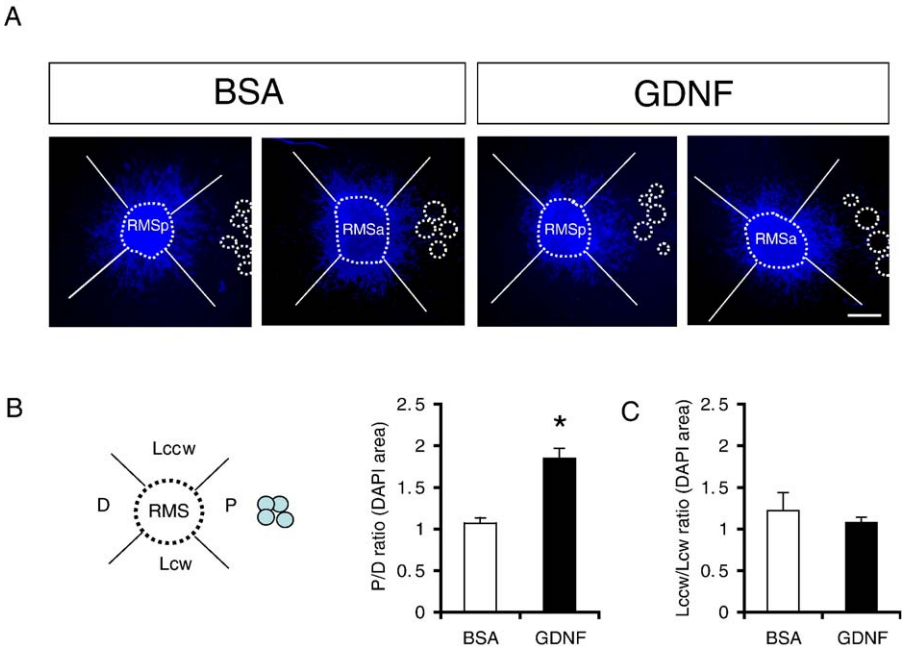

Fig. 3. Chemoattractant effects of GDNF on RMS cell bodies. (A) RMSp and RMSa explants (P1–2 rat) were cultured in a tridimensional matrix containing agarose beads soaked in BSA or GDNF. After 36 h, the explants were fixed and stained with the nuclear marker DAPI. Scale bar, 200 μm. (B) Quantitative analysis of cell chemoattraction of P1–2 rat explants. Histograms showing the quantification of cell chemoattraction expressed as the ratio between the area covered by DAPI-stained cells migrating in the quadrant proximal (P) to the beads relative to the area occupied by DAPI-stained cells in the distal (D) quadrant (P/D ratio). Results are presented as average ± SEM. For each condition, a total of 16 and 30 RMS explants were evaluated in three independent experiments. *$P < 0.005$ vs. BSA soaked beads (Student's t test). (C) Quantitative analysis of cell distribution expressed as the ratio between the area covered by DAPI-stained cells migrating in the lateral contraclockwise (Lccw) quadrant to the beads relative to the area occupied by DAPI-stained cells in the lateral clockwise (Lcw) quadrant (Lccw/Lcw ratio). For this analysis, a total of 16 and 30 RMS explants were evaluated in three independent experiments.

nuclear marker DAPI (Fig. 3A). This analysis showed that beads soaked in GDNF were capable to induce a clear attraction of RMS-derived neuronal cell bodies (Fig. 3B). At the same time, the similar distribution of neuronal cell bodies between both lateral quadrants (Lccw/Lcw ratio) provides additional support to this chemoattractant effect (Fig. 3C). Furthermore, we investigated whether, in addition to directing the migration of RMS cells, GDNF could also modify the length of their leading processes. To test this, we cultured RMS neuronal cells in the absence or in the presence of GDNF (100 ng/ml) for 36 h. After fixation and staining with βIII-Tubulin, the length of the leading process was measured. Mean length of the leading process was 59.4 ± 6 μm (average ± SD) in controls and 63.5 ± 14.3 μm in GDNF-treated cells, indicating that GDNF had no additional effects on the length of the leading process.

Additional BrdU labeling experiments indicated that GDNF did not affect the mitotic index of BrdU-positive neuroblast cells ($Tuj1^+/BrdU^+$ cells) derived from SVZa or RMS (data not shown), thus ruling out possible cell proliferation effects in the chemoattractant activities of localized GDNF. Double-immunocytochemistry using antibodies against βIII Tubulin and GABA confirmed the GABAergic nature of the neuronal precursors that migrated from SVZa and RMS explants (data not shown).

GDNF increases the activity of Cyclin-dependent kinase 5 (Cdk5) in RMS cells

Cdk5 is an atypical member of the serine/threonine cyclin-dependent kinase family that is highly expressed in post-mitotic neurons in the nervous system, and that has been implicated in neuronal cell migration and axon guidance (Dhavan and Tsai, 2001). In our previous work, we identified a role for Cdk5 in the effects of GDNF signaling on axon guidance in sympathetic and sensory neurons (Ledda et al., 2002). We therefore investigated whether GDNF was able to activate Cdk5 in cells derived from the RMS. In the first set of experiments, we used dissociated cultures of cells derived from the whole RMS stimulated with GDNF (100 ng/ml) for different periods of time and assessed Cdk5 activity in an in vitro kinase assay with histone H1 as exogenous substrate (Fig. 4A). GDNF treatment promoted an increase in Cdk5 kinase activity (1.5-fold) after 15 min (Fig. 4A), which could be blocked by pretreatment with the specific Cdk5 inhibitor Roscovitine (Fig. 4B). In a second set of experiments, cells were dissociated from SVZa, RMSp and RMSa areas. Interestingly, GDNF was able to stimulate Cdk5 activity only in cells derived from the RMS (both anterior and posterior), but not from the SVZa (Fig. 4C). This observation was in agreement with the differential chemotropic activities of GDNF on those cells.

Requirement of Cdk5 activity for the chemoattractant effect of GDNF on RMS neuronal cells

We then investigated whether Cdk5 could be an important downstream mediator of the chemoattractive effects of GDNF in cells derived from the RMS. To examine this, we cultured RMS explants from P2 rats in three-dimensional gel matrices together with beads soaked in GDNF in the absence (control) or in the presence of the Cdk5 inhibitor Roscovitine at 20 μM, a

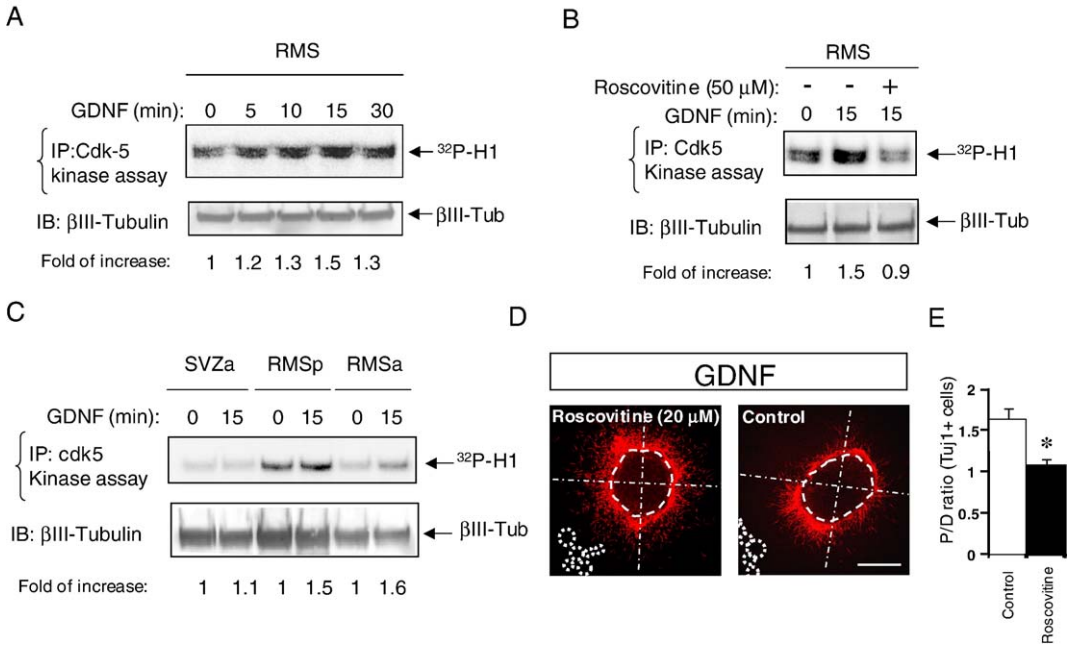

Fig. 4. Activation of Cyclin-dependent kinase 5 (Cdk5) by GDNF in RMS-derived cells. (A) Cdk5 kinase assay in RMS-derived cells cultured 48 h and treated with GDNF (100 ng/ml) for the indicated times. Immunoprecipitation of Cdk5 was followed by in vitro kinase assay using histone H1 as substrate and autoradiography. Reprobing control performed with antibodies against βIII-Tubulin is shown below. (B) Inhibition of Cdk5 activity in RMS-derived cells stimulated with GDNF (100 ng/ml) by the Cdk5 inhibitor Roscovitine (50 μM). Reprobing control was done with antibodies against βIII-Tubulin. (C) Cdk5 kinase activity in dissociated SVZa, RMSp and RMSa-derived cells. Cultures were treated with GDNF (100 ng/ml) for 15 min. Immunoprecipitation of Cdk5 was followed by in vitro kinase assay using histone H1 as substrate and autoradiography. Numbers below the lanes indicate fold induction relative to control normalized to the levels of the reprobing control. The experiments were repeated 2–3 times with similar results. (D) Representative photomicrographs showing the inhibition of the chemoattractive effect of GDNF on RMSp neuronal cells by Roscovitine. RMS explants were cultured in a tridimensional gel containing agarose beads soaked in GDNF in the absence (control) or presence of Roscovitine (20 μM). After 36 h, the explants were fixed and stained with anti-βIII Tubulin antibodies. Scale bar, 200 μm. (E) Results are presented as average ± SD. For each condition, a total of 18 control and 15 Roscovitine-treated explants were evaluated in two independent experiments. *$P < 0.005$ (Student's t test).

concentration that does not affect the overall neuronal migration (data not shown). In the control condition, Tuj1-stained neuronal cells were attracted toward the beads containing GDNF (Figs. 4D and E). However, in the presence of Roscovitine (20 μM), the attraction of neuronal cells toward GDNF soaked beads was inhibited (Figs. 4D and E). Taken together, these results demonstrate that GDNF can activate Cdk5 in RMS cells and that this activity is required for the chemoattractant effect of GDNF on these cells.

Role of NCAM in the chemoattractant effects of GDNF on RMS cells

Recently, we have identified NCAM as an alternative signaling receptor for members of the GDNF ligand family (Paratcha et al., 2003). Because RET expression is absent in the RMS, we tested whether the attractive effect of GDNF on RMS cells was mediated by NCAM receptors. To this end, we performed chemotropic assays as above using anterior and posterior RMS explants taken from wild type and *Ncam*-deficient mice. In these experiments, NCAM-deficient cells were totally unresponsive to the chemoattractant effects of GDNF, while wild type cells responded normally (Figs. 5A and B). It has been demonstrated that migration from non-stimulated NCAM −/− explants is lower than from non-stimulated NCAM +/+ explants, raising the possibility that the absence of response to GDNF in the NCAM −/− explants might be non-specific, because these cells cannot respond to signals that normally regulated their migration. To explore this, we assessed whether guidance of NCAM −/− cells could be modulated by Slit, a prototypic repulsive factor for those cells (Wu et al., 1999). To test this, we co-cultured RMS explants from P2 rats together with aggregates of COS cells expressing human Slit-2N in tridimensional gel matrices for 36–48 h. These experiments showed that the migration of NCAM −/− RMS cells could still be modulated by the repulsive signal Slit-2N (Fig. 5C), supporting the role of NCAM as a specific mediator of the chemoattractive activity of GDNF on these cells. In agreement with this, chemical cross-linking assays using ^{125}I-GDNF confirmed that this neurotrophic factor was able to interact directly and specifically with PSA-NCAM molecules in dissociated primary cultures of RMS neuroblast cells (Fig. 5D). Immunoprecipitation with antibodies against RET did not bring down any affinity-labeled complex from these cells (Fig. 5D). Thus, these experiments demonstrate that NCAM function is required for the chemoattractive effects of GDNF on neuroblast cells from the RMS.

Discussion

In the present study, we have demonstrated that GDNF is a direct chemoattractant molecule for neuronal cells in the RMS, but not for SVZa neuroblasts. This chemoattractant effect was abolished in RMS explants isolated from *Ncam*-deficient mice, supporting the involvement of NCAM receptors during this guidance process.

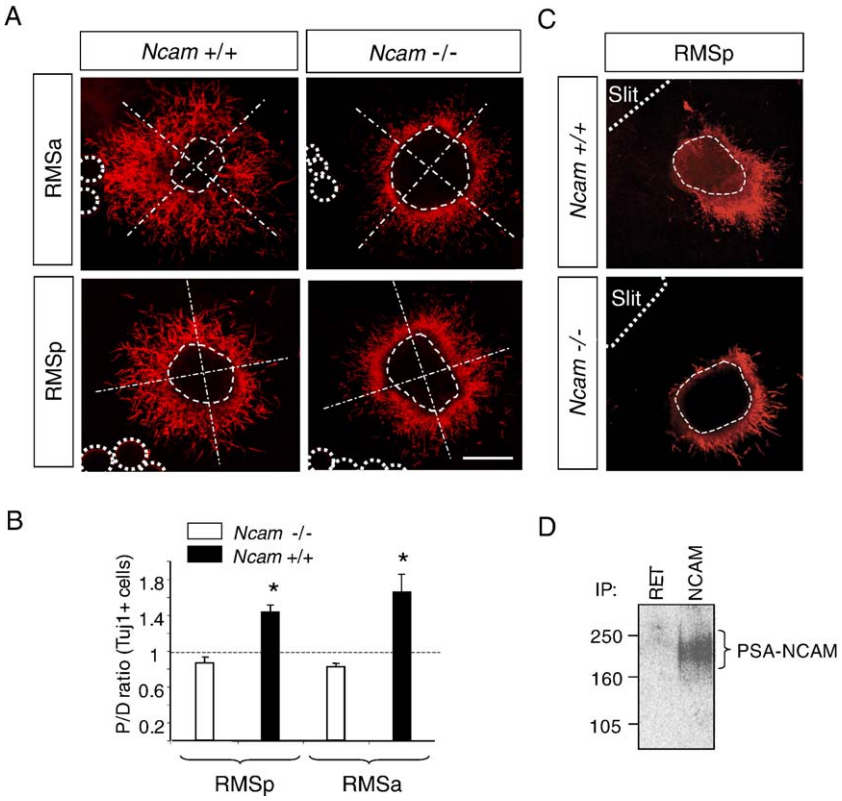

Fig. 5. GDNF-dependent chemoattraction is abrogated in RMS explants derived from *Ncam*-null mice. (A) Representative photomicrographs of βIII-Tubulin immunofluorescence staining of both anterior and posterior RMS explants from newborn (P1–2) wild type or *Ncam* mutant mice cultured in a tridimensional matrix adjacent to GDNF soaked beads. Scale bar, 200 μm. (B) Quantification of neuroblast chemoattraction expressed as P/D ratio and evaluated as indicated in Fig. 2C. Results are presented as average ± SEM. For each condition, a total of 4 to 7 animals from each genotype were evaluated. $*P < 0.005$ (Student's *t* test). (C) Guidance modulation of NCAM +/+ and NCAM −/− neuronal cells by Slit-2N proteins. RMS explants were cultured in a tridimensional matrix in the proximity of Slit-2N expressing COS cells. After 36–48 h, the explants were fixed and stained with anti-βIII Tubulin antibodies. Dotted lines indicate the location of the cell aggregates relative to the explants. (D) Affinity labeling of RMS-derived cells with ^{125}I-GDNF, followed by chemical cross-linking and immunoprecipitation (IP) with the RET or NCAM^{ICD} antibodies. Complex containing PSA-NCAM together with GDNF is indicated.

Molecular guidance cues for neuronal cells tangentially migrating in the RMS

Tangential migration in the SVZa-RMS pathway involves the coordinate action of motogenic factors as well as chemorepulsive and chemoattractive cues. As cells leave the LGE/SVZa, repellent signals largely govern the guidance of migrating neuroblasts by delineating the corridors that are permissive to their migration. Thus, chemorepulsive factors expressed in the septum and VZ appear to be required to push both the postnatal SVZa neurons (Hu, 1999) and embryonic LGE neurons (Zhu et al., 1999) into the RMS pathway. However, it is unlikely that repulsive factors (i.e., Slits) can on their own direct neuronal migration along the entire RMS (Hu, 1999; Wu et al., 1999; Chen et al., 2001). In addition to repulsive signals, chemoattractive gradients are also required to direct immature GABAergic neurons toward the OB. Recent studies have presented evidence supporting an essential role of the OB in directing neuronal migration in the RMS from the SVZa (Liu and Rao, 2003). In particular, it has been demonstrated that the OB secretes a chemotropic activity for neuronal cells, which persist from embryonic to adult stages. Our findings demonstrating that GDNF could represent an OB-derived diffusible chemoattractant factor directing migration in the RMS are in agreement with this previous study (Liu and Rao, 2003). However, it is at present unclear whether the OB can attract SVZa cells, since contrasting results have been reported for this area of the forebrain (Hu and Rutishauser, 1996; Liu and Rao, 2003). In another study, Kirschenbaum et al. (1999) have shown that RMS cells continue to migrate anteriorly in the absence of the OB, suggesting that the OB is not essential for this process. However, in this study, the authors do not demonstrate whether the rate of migration was affected in the absence of OB. Furthermore, these authors could not rule out the existence of endogenous gradients of OB-derived signals in the RMS, which could still be directing the rostral migration of precursors after OB removal. Finally, the reported increase in the size of the RMS detected after OB removal – which phenocopies the *Ncam* mutation – most likely reflects an impairment in RMS cell migration, which could be in agreement with an attractant role of OB-derived signals.

During recent years, it has been demonstrated that the effects of guidance cues can be modulated by interactions with other extracellular and/or intracellular signals, indicating that the actions of guidance factors could be context-dependent (Mason et al., 2001). Thus, the lack of GDNF chemoattraction that we observed on SVZa explants could be due to differences in the signaling pathways activated in SVZa proliferating neuronal cells, and in post-mitotic neuroblast migrating along the RMS. Although both

types of cells express GFRα1 and NCAM receptors (Paratcha et al., 2003), it has been reported that early-born vs. late-born neuronal cells may have different responses to p35/Cdk5 signaling (Gilmore et al., 1998; Gilmore and Herrup, 2001). This is in agreement with our results showing that GDNF was able to activate Cdk5 in RMS, but not in SVZa-derived cells. Although Cdk5 is rather ubiquitously expressed, its activity is strictly regulated by binding to its neuron-specific activator p35, which is absent in proliferating neuronal progenitors but restricted to post-mitotic neuronal cells (Tsai et al., 1994; Zheng et al., 1998). Thus, the Cdk5/p35 pathway is thought to participate in the migration of neuronal cells mainly after they cease to divide (Delalle et al., 1997). Cdk5 plays a prominent role in the control of cell migration in the brain (Dhavan and Tsai, 2001). In the olfactory bulb, a reduced cell density in the granule cell layer has been documented in conditional Cdk5 knockout mice (Hirasawa et al., 2004), suggesting a role for this kinase in cell migration in the RMS. The fact that GDNF was able to induce Cdk5 activity and migratory responses in RMS cells – most of which are post-mitotic – but not in SVZa – most of which are still proliferating – is in agreement with this notion.

One way in which migratory neurons acquire positional information during development is by sensing the local concentration of attractant factors secreted by specific targets. However, the ways in which long-range gradients are established remain to be clarified. Recently, it has been demonstrated that some members of the TGF-β superfamily, that act as a long-range morphogens during development, could move along the surface of cells by restricted diffusion involving GPI-anchor receptors. Thus, the differential concentration of morphogen molecules on the cell surface of producing cells and receiving cells would appear to be sufficient to drive their displacement toward distant receiving cells (Belenkaya et al., 2004). In our system, immunostaining for the GPI-anchor GFRα1 receptors showed a prominent localization in the developing RMS and in the outer layers of the newborn OB (Fig. 1F), in concordance with its mRNA expression (data not shown). In the RMS, the localization of GDNF proteins was in agreement with the presence of GFRα1 – although not its own mRNA – suggesting a role for GFRα1 in the localization and presentation of GDNF molecules to RMS cells. In this respect, our previous results have demonstrated that GFRα1 can restrict the extracellular diffusion of GDNF and elicit long-range gradients of this neurotrophic factor (Ledda et al., 2002). Together with its ability to present GDNF in *trans* to receiving cells (Paratcha et al., 2001), these data suggest that the GDNF synthesized and secreted by OB cells could be transported through the RMS by a facilitated diffusion mechanism involving GPI-anchor GFRα1 receptors, and are consistent with the idea that GDNF transported along the RMS could act as a long-range chemotropic signal.

GDNF signaling and neuronal precursor migration and chemoattraction in the nervous system

PSA-NCAM and GFRα1 molecules have been found to be co-expressed in a subpopulation of RMS migratory neuroblasts (Paratcha et al., 2003). Thus, GDNF may be acting on this subpopulation of neuronal precursor cells migrating in the RMS toward the OB. Other signals are likely to act in concert with GDNF to guide cells in the RMS. In this regard, it is known that different types of OB interneurons may migrate guided by distinct attractive factors (Murase and Horwitz, 2002, 2004). In addition, functional redundancy is also likely to occur within this migratory route, creating the possibility that subpopulations of migratory neuronal cells may respond to several different motogenic and guidance factors at the same time.

Work from recent years has indicated that GDNF signaling may be more complex than previously anticipated. With respect to cell migration, RET activation by GDNF has been shown to control the differentiation and migration of enteric nervous system progenitors during gut development (Natarajan et al., 2002). More recently, GDNF was shown to regulate the differentiation and migration of cortical GABAergic neurons via GFRα1 receptors independently of RET or NCAM (Pozas and Ibáñez, 2005), suggesting the presence of additional transmembrane effectors for GDNF. In our present study, we have demonstrated that GDNF signaling through NCAM was able to evoke chemotropic effects in neuronal precursors derived from the neonatal RMS. Contrary to this chemoattractive effect obtained from a local source of GDNF, the application of a uniform concentration of this neurotrophic factor failed to induce a significant change in neuronal migration (data not shown). However, this could be explained by the fact that RMS explants contain endogenous levels of GDNF (Figs. 1D and E). Thus, local application of GDNF could create higher effective concentration of this factor in a more defined microenvironment than the uniform application to the media, presenting a more favorable scenario to detect chemoattraction. Moreover, in this particular system of extracellular cross-talk between NCAM and GDNF, short-range NCAM signaling (NCAM-NCAM interactions) could modulate migration activity, but only GDNF with the properties of a diffusible factor could, in a gradient-dependent manner, induce localized and asymmetric signaling changes required for guidance activities of RMS cells.

The lack of GFRα2 expression in the RMS (Enomoto et al., 2004) could explain why the related ligand NTN was not able to attract neuronal cells in our explant assays. Recently, mutant mice lacking GFRα1 expression in cells devoid of RET – such as those in the RMS – were generated (Enomoto et al., 2004). Although no differences were found in either the size or the general histological organization of cell layers in the adult OB of those mice (Enomoto et al., 2004), the analyses performed so far do not rule out defects in specific subpopulations of cells in this structure. A more detailed analysis of the different neuronal cell types present in the periglomerular and granule layers of the mature OB of those mice deserves further investigation.

Guidance of neuronal precursors in the RMS requires not only the presence of a permissive corridor toward the olfactory bulb, but also the existence of long-range diffusible attractive activities that confer directionality to the migration (Wichterle et al., 2003). It is known that the ability of NCAM to influence developmental processes can result both from its adhesive as well as its signaling properties (Maness et al., 1996). The fact that *Ncam*-deficient mice have pronounced deficits in neuronal migration in the RMS (Tomasiewicz et al., 1993; Chazal et al., 2000), has lend support to the role of short-range interactions by heterophilic and homophilic binding to NCAM molecules in this pathway. On the other hand, our present findings indicate that long-range NCAM signaling by GDNF may also be important to provide directionality to cell migration in the RMS.

Experimental methods

Recombinant proteins, rats and transgenic mice

GDNF and Neurturin (NTN) were purchased from R&D and Peprotech, respectively. Sprague–Dawley rats were obtained from Scanbur (Denmark). *Ncam*-knockout mice (Cremer et al., 1994) were obtained from the Jackson Laboratory (Maine).

Explant cultures and chemoattraction assays

Brains from newborn rat and mice were dissected in ice-cold PBS and sectioned sagittally (200–300 μm) with a tissue chopper (McIllwan). Tissue from SVZa, anterior part of RMS (RMSa) and posterior part of RMS (RMSp) were isolated and trimmed into pieces of approximately 300 μm. Explants were subsequently embedded in the mixture gel (3:2:1 of rat tail collagen:matrigel:medium) together with agarose beads (Sigma Chemical, Co) soaked in BSA, GDNF or Neurturin and cultured with DMEM-F12 medium (Invitrogen) containing 2.5% horse serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in an incubator with 5% CO_2 for 24–36 h. Then, the explants were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and stained using a monoclonal antibody anti-βIII-Tubulin (1/2000) (Tuj1/Promega) to identify neuronal precursors or DAPI (Sigma) to unambiguously identify cell bodies. For these assays, 10 μl of agarose beads was washed in PBS and incubated with 2 μg of BSA, GDNF or Neurturin overnight at 4°C in a total volume of 50 μl. After washing in PBS, the beads were inserted in the gel matrices at 200–400 μm from the explants. The quantification of these distances did not show significant differences between the different experimental groups.

In some experiments, NCAM −/− and NCAM +/+ RMS explants were embedded in a tridimensional matrix together with aggregates of COS cells expressing Slit-2N proteins.

To quantify chemoattraction, the field was divided into four orthogonal quadrants and the areas covered by DAPI nuclear staining or Tuj1-stained migratory neuroblast cells (i.e., excluding the explant) were measured in the distal (D) and proximal (P) quadrants with respect to the agarose beads, using the Axiovision software (Zeiss) version 2.01 (see also Ledda et al., 2002). For each explant, the Proximal/Distal ratio was calculated and used as a chemoattraction index. Statistical analyses were performed by one-way ANOVA and Student's *t* test using Statview program.

Dissociated primary culture

SVZa, RMSa and RMSp cells from P1 and P4 newborn rats were cultured in DMEM:F12 (Gibco) medium supplemented with B27 (Gibco) in the presence or absence of GDNF (100 ng/ml). Tissue culture dishes (48-well plate) were coated with 0.1 mg/ml of poly-D-lysine (Sigma) during 2 h at 37°C, followed by three washes with PBS. Twenty thousand cells were plated per well and cultured for 48 h at 37°C in 5% CO_2. For proliferative assays, an 8 h pulse of Bromo-deoxy-Uridine (BrdU) (10 μM) was performed 48 h after cell plating. The number of Tuj1/BrdU double positive cells was counted at 48 h and expressed as a percentage of the total number of Tuj1 positive neuroblast cells. Primary cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and stained using the following primary antibodies: BrdU (1/100; Dako), βIII Tubulin (Tuj1) (1/2000; Promega), GFRα1 (1/400; kindly provided by M. Sanicola). For binding and kinase assays, 1.5×10^6 cells were plated per P60 mm pre-coated dishes and cultured for 48 h at 37°C. In these experiments, the cultures were switched to serum-free medium before stimulation with GDNF (100 ng/ml).

Chemical cross-linking and binding assay

GDNF was iodinated by the lactoperoxidase method and chemical cross-linking was performed with ethyl–dimethyl–aminopropyl–carbodimide (EDAC) supplemented with sulfo-NHS (Pierce). Affinity labeling of RMS-derived cells was performed during 4 h incubation at 4°C in phosphate buffer saline (PBS) supplemented with Ca^{2+} and BSA. This was followed by chemical cross-linking, EDAC neutralization with 500 mM glycine and two washes in PBS. Then, the cells were lysed in buffer containing 0.5% Triton X-100 and 1% β-octyl-D-glucopyranoside (Pierce) to ensure complete solubilization of membrane lipid rafts. Lysates were immunoprecipitated with anti-RET (C20 and T20) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-$NCAM^{icd}$ (12F11) (BD PharMingen) antibodies, separated by SDS-PAGE, blotted onto PVDF membranes and exposed to phosphorscreens. The screens were developed using Storm 840 phosphorimager and imageQuant software (Molecular Dynamics).

Immunoprecipitation, Western blotting and kinase assay

Cells were lysed at 4°C in buffer containing 0.5% Triton X-100, 1% β-octyl-d-glucopyranoside plus protease and phosphatase inhibitors. Protein lysates were clarified and analyzed by immunoprecipitation and Western blotting as previously described (Ledda et al., 2002). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics).

For kinase assay, endogenous Cdk5 was immunoprecipitated from cell lysates by incubation with 1.5 μg anti-Cdk5 for 16 h at 4°C and then 2 h with protein G Sepharose beads (Pharmacia). The beads were precipitated, washed and then mixed with 10 μg of histone H1 in kinase reaction buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 1 mM sodium vanadate, 20 mM sodium fluoride and protease inhibitors) with 100 μM (γ-^{32}P) ATP (2 μCi). The reaction was performed at room temperature during 20 min and stopped by addition of sample buffer. After SDS-PAGE, radioactivity was analyzed in a Storm 840 phosphorimager as we described before. Numbers below the lanes indicate fold of induction relative to control normalized to total levels of target or control protein. In some experiments, the Cdk5 inhibitor, Roscovitine (Calbiochem), was used at the specified concentration.

Real-time PCR

The expression of GDNF and GAPDH was analyzed by RT-PCR. Total RNA was isolated from SVZa, RMSa, RMSp and OB during embryonic and postnatal development using Quiagen columns (Invitrogen) according to the manufacturer's instructions. Single stranded cDNA was synthesized using multiscribe reverse transcriptase and random hexamers (Perkin Elmer). The cDNA was amplified using the following primer sets: GAPDH: forward, 5′ TGG GTG TGA ACC ACG AGA AAT A 3′; reverse, 5′ GCT AAG CAG TTG GTG GTG CAG 3′. GDNF: forward, 5′ GGT GCG TTT TAA CTG CCA TAC A 3′; reverse, 5′ AAG ATC AGT TCC TCC TTG GTT TCA 3′.

Real-time was performed using LightCycler rapid thermal cycler system (Perkin Elmer) according to the manufacturer′s instructions. Reactions were performed in 25 μl volume and measured by triplicates. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix (Perkin Elmer).

Slice blotting and immunofluorescence

Slice blotting technique was performed as described (Lowe, 1999). Sagittal sections of P4 rat brain (300 μm) were incubated for 30 min in control artificial cerebrospinal fluid (ACSF) consisting of: 122 mM NaCl; 3.75 mM KCl; 26 mM $NaHCO_3$; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 0.5 mM NaH_2PO_4; 10 mM glucose, pH 7.4, bubbled with 95% O_2 and 5% CO_2 and then placed directly onto 1.2 × 1.5 cm rectangles of PVDF membranes (Hybond C Super, Amersham; 0.45 μm pore size, 100 μm thickness). The remaining thin layer of solution was removed, causing the slice to adhere tightly to the filters. The slices on PVDF were then incubated for 2–3 h in ACSF. During blotting, membranes were held by their edges and submerged in a 500 ml bath, bubbled continuously with 95% O_2 and 5% CO_2. All blotting was performed at room temperature. After blotting, slices were lifted off the filters with a gentle pulse of ACSF, placed on blocking solution (5% BSA in TTBS (0.9% NaCl, 100 mM Tris–HCl (pH 7.4) and 0.1%

Tween 20) and immunostained using affinity-purified goat-polyclonal antibodies against GDNF (1/2500; R&D), biotinylated anti-goat IgG and developed with DAB using the Vectastain Elite ABC-peroxidase kit (Vector).

Cryostat sections (20 μm) of P0 newborn mice brain were blocked with 5% donkey serum and incubated with affinity-purified rabbit polyclonal anti-GFRα1 (1/400, provided by M Sanicola). Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) were from Jackson Immunoresearch Lab. Photographs were obtained using a Zeiss LSM 510 confocal microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mcn.2005.11.007.

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