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The Neural Cell Adhesion Molecule NCAM Is an Alternative Signaling Receptor for GDNF Family Ligands

Gustavo Paratcha,[1] Fernanda Ledda,[1] and Carlos F. Ibáñez*
Division of Molecular Neurobiology
Department of Neuroscience
Karolinska Institute
Retzius väg 8, A2:2
17177 Stockholm
Sweden

Summary

Intercellular communication involves either direct cell-cell contact or release and uptake of diffusible signals, two strategies mediated by distinct and largely non-overlapping sets of molecules. Here, we show that the neural cell adhesion molecule NCAM can function as a signaling receptor for members of the GDNF ligand family. Association of NCAM with GFRα1, a GPI-anchored receptor for GDNF, downregulates NCAM-mediated cell adhesion and promotes high-affinity binding of GDNF to p140^{NCAM}, resulting in rapid activation of cytoplasmic protein tyrosine kinases Fyn and FAK in cells lacking RET, a known GDNF signaling receptor. GDNF stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons via binding to NCAM and activation of Fyn, but independently of RET. These results uncover an unexpected intersection between short- and long-range mechanisms of intercellular communication and reveal a pathway for GDNF signaling that does not require the RET receptor.

Introduction

During the development of the nervous system, short- and long-range signals cooperate to promote neuronal survival, migration, differentiation, axonal growth, guidance, and target innervation. While short-range signaling involves direct contact between cells or with the extracellular matrix, long-range signaling requires the release and diffusion of messenger molecules, which are then sensed by specific receptors in distant target cells. This subdivision implies more than a mere conceptual classification, as short- and long-range intercellular signaling also involve distinct sets of cell surface molecules coupled to different intracellular pathways.

NCAM is a prominent cell adhesion molecule in the nervous system, where it has been shown to participate in a number of developmental processes including cell migration, neurite outgrowth, and synaptic plasticity (Crossin and Krushel, 2000; Ronn et al., 2000; Schachner, 1997). The ability of NCAM to influence developmental events can result both from its adhesive as well as its signaling properties (Maness et al., 1996; Saffell et al., 1995). The cytoplasmic domain of the p140^{NCAM} isoform has been shown to associate constitutively with Fyn, a member of the Src family of cytoplasmic tyrosine kinases (Beggs et al., 1997). Fyn becomes rapidly and transiently activated upon NCAM ligation, leading to recruitment of the focal adhesion kinase FAK (Beggs et al., 1997), a nonreceptor tyrosine kinase known to participate in cytoskeletal rearrangements. Increased protein tyrosine phosphatase activity has also been observed upon NCAM activation (Klinz et al., 1995). Other studies have shown that NCAM is also able to transactivate the fibroblast growth factor receptor (FGFR) (Saffell et al., 1997; Williams et al., 1994).

The GDNF (glial cell line-derived neurotrophic factor) ligand family is a small group of neurotrophic growth factors, representative of an important class of soluble mediators of neuronal survival, neuritic growth, and differentiation (Airaksinen et al., 1999; Baloh et al., 2000). Four members are known in the family including GDNF, Neurturin (NTN), Persephin (PSP), and Artemin (ART). Intercellular signaling by members of this family is mediated by a receptor complex formed by the RET receptor tyrosine kinase and a ligand binding, GPI-anchored subunit termed GDNF family receptor α (GFRα) (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). Activation of RET by GDNF family ligands requires the presence of distinct members (GFRα1 to 4) of the GFRα family (Airaksinen et al., 1999; Baloh et al., 2000). Throughout the nervous system and, particularly in the forebrain, GFRαs are much more widely expressed than RET (Trupp et al., 1997; Yu et al., 1998), suggesting that GFRαs may signal independently of RET, presumably in collaboration with novel transmembrane proteins (Poteryaev et al., 1999; Trupp et al., 1999). Despite its potential importance, the biological significance of RET-independent signaling by GDNF family ligands has not been established, and the existence of additional GDNF receptors remains to be demonstrated.

In this work, we set out to investigate proximal events in RET-independent signaling by GDNF in neuronal and glial cells and found a striking similarity with intracellular pathways activated by the p140^{NCAM} NCAM isoform. We show that p140^{NCAM} can interact directly with GDNF family ligands and, with the aid of GFRα receptors, mediate GDNF signaling independently of the presence of RET. The ability of GDNF to promote Schwann cell migration and axonal growth in hippocampal and cortical neurons via the NCAM pathway suggests alternative mechanisms by which GDNF proteins may contribute to important biological processes in the developing and adult nervous system.

Results

Proximal Events in RET-Independent GDNF Signaling

GDNF signaling mechanisms were investigated in a line of immortalized neuronal precursors (RN33B) and in primary cultures of Schwann cells. Both cell types have been reported to express relatively high levels of GFRα1,

*Correspondence: carlos.ibanez@neuro.ki.se
[1]These authors contributed equally to this work.

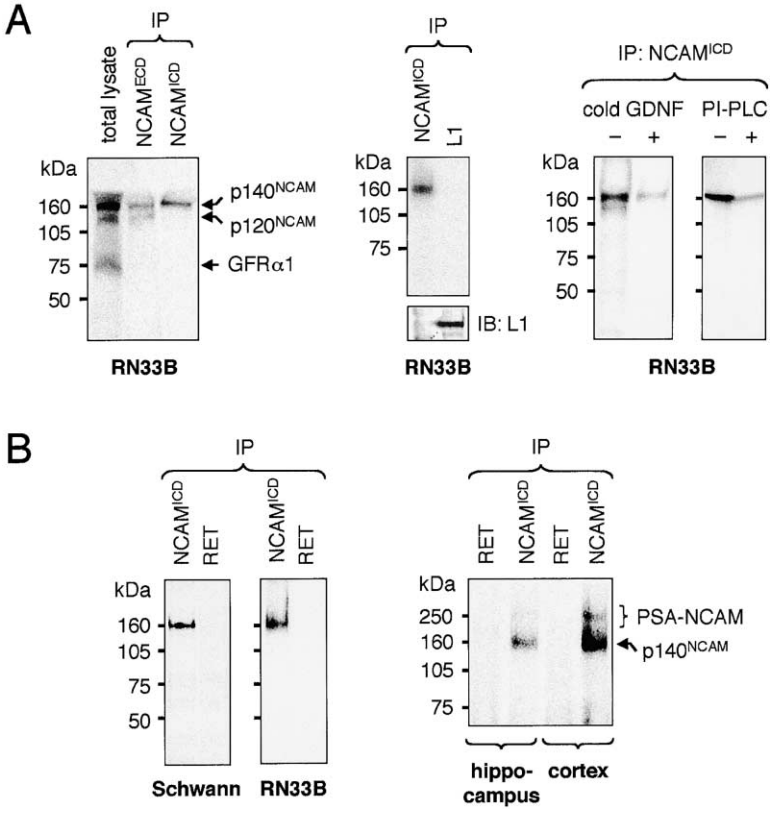

Figure 1. GDNF Interacts Directly with NCAM in Neuronal and Glial Cells

(A) Affinity labeling of RN33B cells with ^{125}I-GDNF, followed by chemical crosslinking and immunoprecipitation (IP) with the indicated antibodies. The membrane shown in the middle panel was reprobed (IB) with L1 antibodies. Cold GDNF was used at 100-fold excess.
(B) Affinity labeling of Schwann cells and embryonic hippocampal and cortical neurons with ^{125}I-GDNF, followed by immunoprecipitation with NCAM^{ICD} or RET antibodies. Complexes containing p140^{NCAM} and PSA-NCAM are indicated.

but no RET receptors (Trupp et al., 1997; Trupp et al., 1999), an observation that we could confirm by RT-PCR analysis (see Supplemental Figure S1A online at http://www.cell.com/cgi/content/full/113/7/867/DC1). GDNF induced a rapid and transient stimulation of Fyn tyrosine kinase activity in both cell types and a parallel increase in phosphorylation of FAK on Tyr-397, a residue known to be involved in the activation of this kinase (Supplemental Figure S1B). GDNF treatment also stimulated protein tyrosine phosphatase activity in lipid rafts of RN33B and Schwann cells (Supplemental Figure S1C). These proximal signaling events correlated with phosphorylation of the Erk1 and Erk2 MAP kinases (Supplemental Figure S1D). Thus, proximal events in RET-independent GDNF signaling in RN33B and Schwann cells resemble the intracellular pathways activated by p140^{NCAM}, both in its time course and in the types of molecules involved.

GDNF Binding to NCAM Activates Fyn Kinase

Affinity labeling experiments by chemical crosslinking of ^{125}I-GDNF to RN33B cells revealed, in addition to the expected GDNF/GFRα1 complex, two major receptor complexes of higher molecular weight corresponding to the binding of GDNF to receptors of about 120 and 140 kDa, respectively (Figure 1A). Immunoprecipitation of total cell lysates of affinity-labeled RN33B cells with antibodies directed against the extracellular domain of NCAM (NCAM^{ECD}) recovered the two high molecular weight complexes (Figure 1A), suggesting that they represented GDNF binding to the p120^{NCAM} and p140^{NCAM} isoforms. Only the largest complex could be recovered by immunoprecipitation with antibodies directed against the intracellular domain of NCAM (NCAM^{ICD}), in agreement with p120^{NCAM} being a GPI-anchored protein lacking an intracellular domain. The p180^{NCAM} isoform was not expressed by RN33B cells (data not shown). GDNF did not bind to the related cell adhesion molecule L1 (Figure 1A). Binding of ^{125}I-GDNF to p140^{NCAM} could be displaced with excess cold GDNF, and was greatly attenuated following treatment with phosphatidylinositol phospholipase C (PI-PLC), which removes GPI-linked proteins from the cell membrane (Figure 1A). A complex between p140^{NCAM} and ^{125}I-GDNF could also be identified in Schwann cells and in embryonic hippocampal and cortical neurons, which express high levels of NCAM and GFRα1 but undetectable levels of RET (Trupp et al., 1997; Yu et al., 1998) (Figure 1B). An additional higher molecular weight complex was also observed in neuronal cells representing GDNF binding to polysialic acid (PSA)-modified NCAM isoforms, which are highly expressed in embryonic neurons (Figure 1B). Immunoprecipitation with antibodies against RET did not bring down any affinity-labeled complex from these cells (Figure 1B). Together, these results indicated that GDNF is able to interact directly and specifically with NCAM in RN33B cells, Schwann cells, and primary neurons.

Next, we investigated NCAM-associated signaling events induced by GDNF. GFRα1 has previously been localized to lipid raft microdomains in the cell membrane (Tansey et al., 2000; Paratcha et al., 2001). By virtue of its cytoplasmic fatty acid acylation, a fraction of p140^{NCAM} molecules have also been localized to these membrane microdomains (Niethammer et al., 2002). First, we exam-

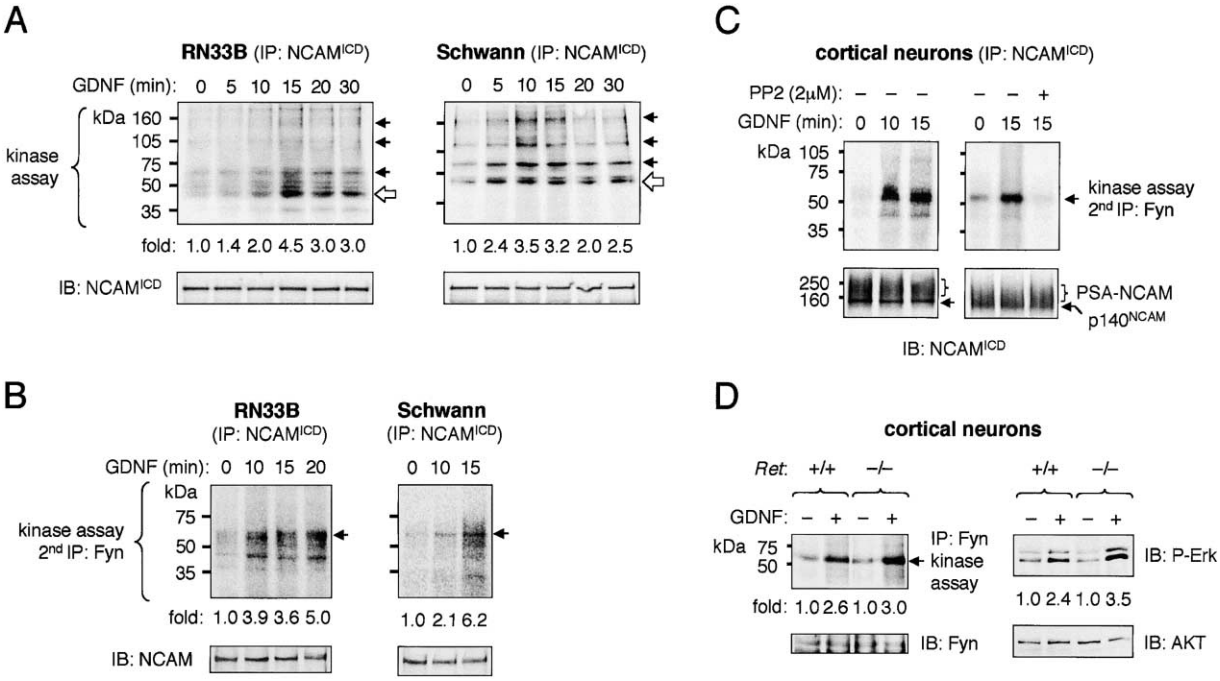

Figure 2. GDNF Stimulates NCAM-Associated Fyn Kinase Activity in Neuronal and Glial Cells

(A) In vitro kinase assays on NCAM immunoprecipitates after GDNF stimulation of RN33B and Schwann cells. Purified lipid rafts were used for RN33B cells. Phosphorylated products, including endogenous substrates (solid arrows) and exogenously added enolase (open arrows), are indicated. Reprobings with NCAM^{ICD} antibodies are shown below each panel.

(B) Following NCAM immunoprecipitation and in vitro kinase assay, samples were eluted and reimmunoprecipitated with Fyn antibodies. Phosphorylated Fyn is indicated (arrows). Supernatants were reimmunoprecipitated with NCAM^{ICD} antibodies followed by NCAM^{ICD} immunoblotting (bottom).

(C) Activation of NCAM-associated Fyn in cortical neurons. In vitro kinase assay was performed on NCAM immunoprecipitates from cortical neurons treated with GDNF (100 ng/ml) and then reimmunoprecipitated with Fyn antibodies. NCAM^{ICD} reprobing shown below.

(D) Activation of Fyn (left) and phosphorylation of Erk (right) in cortical neurons from wild-type and *Ret* knockout mice stimulated for 10 min with GDNF (100 ng/ml). Immunoprecipitation of Fyn was followed by in vitro kinase assay and autoradiography. Fyn reprobing is shown below. Erk phosphorylation was detected with anti-phospho-Erk antibodies (P-Erk) and controlled by reprobing with anti-AKT antibodies.

ined whether GDNF was able to stimulate NCAM-associated kinase activity by immunopurification of complexes containing p140^{NCAM} from lipid rafts or detergent-insoluble membrane compartments followed by in vitro kinase assay. In both RN33B and Schwann cells, GDNF treatment caused rapid stimulation of NCAM-associated kinase activity with a peak between 10 and 15 min (Figure 2A). Several endogenous substrates that coimmunoprecipitated with p140^{NCAM} were phosphorylated following GDNF stimulation (solid arrows), as well as enolase, the exogenous substrate added to the reaction (open arrows). To determine whether GDNF treatment stimulated the phosphorylation of the subpool of Fyn kinase associated with p140^{NCAM}, we performed an experiment similar to the one above except that after the in vitro kinase reaction, proteins in NCAM-immunocomplexes were solubilized in SDS and subjected to a second immunoprecipitation with anti-Fyn antibodies. In these conditions, we could detect a robust induction in the phosphorylation of NCAM-associated Fyn following GDNF treatment in both RN33B and Schwann cells (Figure 2B). GDNF also stimulated a rapid and robust increase in Fyn phosphorylation in NCAM immunoprecipitates of cortical neurons (Figure 2C), a signaling event that resembles p140^{NCAM} activation by homophilic binding (Beggs et al., 1997). We also investigated downstream signaling events in embryonic cortical neurons isolated from wild-type and *Ret* knockout mice. GDNF treatment stimulated Fyn autokinase activity in wild-type as well as *Ret* knockout neurons (Figure 2D). GDNF also stimulated Erk phosphorylation to the same extent in both types of neurons (Figure 2D), indicating that these signaling effects of GDNF were not mediated by the RET receptor.

High-Affinity GDNF Binding to NCAM and Downstream Signaling Requires Coexpression of GFRα1 Receptors

We then examined whether NCAM expression was sufficient to confer GDNF binding and signaling responses to cells lacking endogenous GDNF receptors. COS cells do not express NCAM (Figure 3A) and are unable to bind GDNF. However, following transfection of a cDNA encoding p140^{NCAM}, we could detect binding of ^{125}I-GDNF to this NCAM isoform in COS cells (Figure 3A). Radiolabeled brain-derived neurotrophic factor (BDNF) was unable to bind NCAM (Figure 3A). Cotransfection of the GFRα1 coreceptor greatly potentiated binding of GDNF to p140^{NCAM} (Figure 3A). GFRα2, another member of the GFRα family that binds GDNF with lower affinity (Klein et al., 1997), was not able to potentiate GDNF binding to NCAM (Figure 3A). However, this receptor was able to

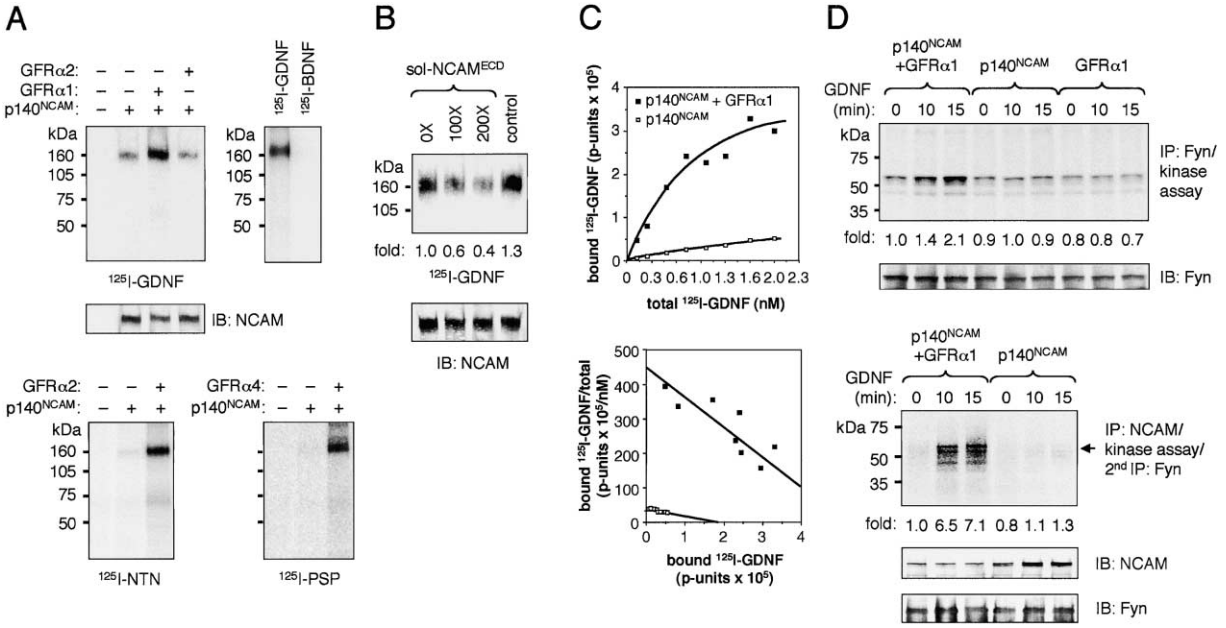

Figure 3. Reconstitution of High-Affinity GDNF Binding to NCAM and Activation of Fyn in Heterologous Cells Requires Coexpression of GFRα1 Receptors

(A) Affinity labeling of COS cells transfected with $p140^{NCAM}$ and different GFRα receptors. All panels show NCAM immunoprecipitates from cell lysates after chemical crosslinking with indicated ligands. $NCAM^{ICD}$ reprobing is shown below.

(B) Displacement of ^{125}I-GDNF binding to $p140^{NCAM}$ by soluble $NCAM^{ECD}$ (used at 100- and 200-fold molar excess). Conditioned medium from naive 293 cells was used as control.

(C) Saturation (top) and Scatchard (bottom) plots of ^{125}I-GDNF binding to $p140^{NCAM}$ in the presence or absence of GFRα1 in transfected COS cells. Binding is expressed in arbitrary phosphorimager units (p units) obtained after phosphorimaging scanning of ^{125}I-GDNF-labeled $p140^{NCAM}$ complexes.

(D) Activation of Fyn in COS cells transfected with Fyn, $p140^{NCAM}$, and GFRα1 after GDNF stimulation (100 ng/ml). Immunoprecipitation of Fyn (top) was followed by in vitro kinase assay and autoradiography. Fyn reprobing is shown below. The average increase at 15 min in four independent experiments was 1.9-fold ± 0.2. Lower panels show double immunoprecipitation/in vitro kinase assays and reprobings with NCAM and Fyn antibodies.

potentiate the binding of NTN, its high-affinity ligand, to $p140^{NCAM}$ (Figure 3A). Moreover, GFRα4 also potentiated the binding of its cognate ligand PSP to COS cells that also received the cDNA encoding $p140^{NCAM}$ (Figure 3A). Thus, NCAM is able to interact with several members of the GDNF ligand family, but not with other neurotrophic factors, and these interactions are greatly potentiated by coexpression of cognate members of the GFRα family of GPI-anchored coreceptors. Binding of GDNF to NCAM could be displaced by soluble $NCAM^{ECD}$ (Figure 3B), further attesting to the specificity of their interaction.

Equilibrium binding, analyzed by chemical crosslinking and SDS-PAGE, indicated that binding of GDNF to NCAM was saturable in the low nM range (Figure 3C). Coexpression of GFRα1, increased both the binding affinity of GDNF for NCAM ($Kd_{NCAM} = 5.2 \pm 1.8$ nM versus $Kd_{NCAM+GFR\alpha1} = 1.1 \pm 0.32$ nM; $n = 3$) as well as the GDNF binding Bmax of NCAM molecules ($Bmax_{NCAM+GFR\alpha1}/Bmax_{NCAM} = 3.7$) without altering total NCAM levels in transfected cells. GDNF treatment of COS cells cotransfected with GFRα1 and $p140^{NCAM}$ stimulated Fyn kinase activity as assessed by in vitro autokinase assay in Fyn immunoprecipitates from transfected cells (Figure 3D). GDNF was unable to stimulate Fyn kinase activity in cells that received either NCAM or GFRα1 alone (Figure 3D), indicating the necessity of both receptor components for efficient intracellular signaling. Immunopurification of Fyn from solubilized in vitro kinase reactions performed on NCAM immunocomplexes showed a robust stimulation of the phosphorylation of NCAM-associated Fyn kinase following GDNF treatment of COS cells transfected with $p140^{NCAM}$ and GFRα1 (Figure 3D). The much greater increase in Fyn phosphorylation observed after a prior NCAM immunoprecipitation step indicated that the subpopulation of Fyn molecules associated with $p140^{NCAM}$ is preferentially responsive to GDNF stimulation. Together, these data indicated that NCAM is sufficient to confer GDNF binding to heterologous cells, but that high-affinity binding and ligand-dependent activation of NCAM-associated Fyn kinase requires coexpression of cognate members of the GFRα family of GPI-linked receptors.

Complex Formation between GFRα1 and NCAM Downregulates Homophilic NCAM Interactions and NCAM-Mediated Cell Adhesion

The effects of GFRα1 on the ability of NCAM to bind GDNF suggested that GFRα1 may interact directly or indirectly with NCAM. To investigate this possibility, we performed a coimmunoprecipitation assay in COS cells transfected with $p140^{NCAM}$ together with increasing amounts of GFRα1. Cell surface molecules were labeled by whole-cell biotinylation prior to immunoprecipitation with $NCAM^{ICD}$ antibodies. High levels of cell surface

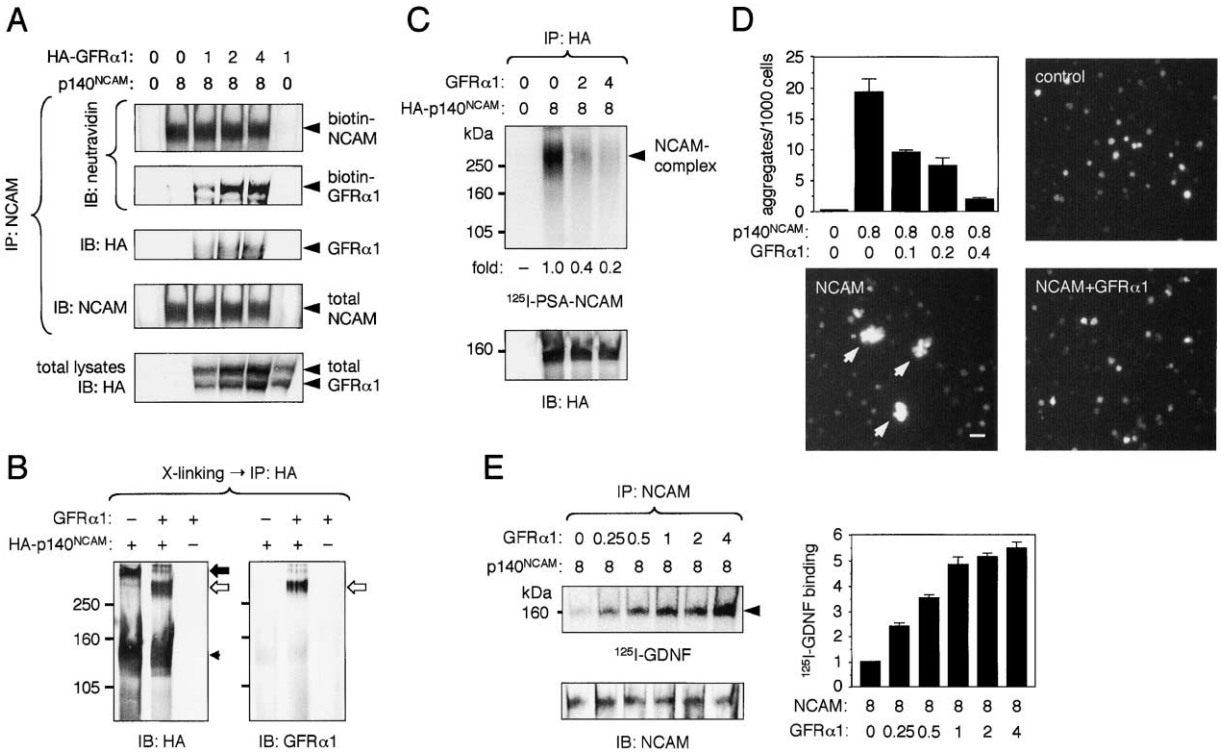

Figure 4. Complex Formation between GFRα1 and NCAM Inhibits Homophilic NCAM Interactions and NCAM-Mediated Cell Adhesion

(A) Coimmunoprecipitation of hemagglutinin (HA)-tagged GFRα1 and NCAM in the surface of transfected COS cells. Biotinylated NCAM and GFRα1 were detected by NCAM immunoprecipitation and probing with neutravidin. Reprobings with anti-HA and NCAM antibodies are shown.

(B) Direct physical interaction between p140^{NCAM} and GFRα1 in transfected COS cells analyzed by chemical crosslinking and immunoprecipitation. Monomeric p140^{NCAM} (arrowhead), oligomeric NCAM complexes (solid arrows), and complexes containing both NCAM and GFRα1 (open arrows) are indicated.

(C) Chemical crosslinking of radiolabeled PSA-NCAM to COS cells expressing HA-tagged p140^{NCAM} in the presence or absence of different amounts of transfected GFRα1 (in μg plasmid DNA). Reprobing with anti-HA antibodies is shown below.

(D) Cell adhesion assay in Jurkat cells transiently transfected with p140^{NCAM} and increasing amounts of GFRα1 (in μg plasmid DNA). Only cell aggregates containing more than five cells were counted (arrows). Results are expressed as number of aggregates per 1000 GFP-positive cells ± SD (n = 4). Similar results were obtained in three independent experiments. Scale bar, 50 μm.

(E) Chemical crosslinking of ^{125}I-GDNF to COS cells transfected with p140^{NCAM} and increasing amounts of GFRα1 (in μg plasmid DNA). Quantitative results are expressed as ^{125}I-GDNF binding ± SD (n = 3) relative to p140^{NCAM} alone and normalized to the levels of p140^{NCAM} in each lane as determined by immunoblotting.

p140^{NCAM} and GFRα1 could be detected in NCAM immunoprecipitates (Figure 4A), indicating that the two receptors associate in the same molecular complex at the cell surface. No GFRα1 could be recovered in the absence of NCAM (Figure 4A). Coimmunoprecipitation of GFRα1 with endogenous NCAM could also be detected in RN33B cells (data not shown). In cells expressing endogenous NCAM, overexpression of GFRα1 resulted in an increase in the level of p140^{NCAM} localized to lipid rafts (see Supplemental Figure S2 online at http://www.cell.com/cgi/content/full/113/7/867/DC1), suggesting stabilization or recruitment of p140^{NCAM} in this compartment by interaction with GFRα1.

Chemical crosslinking of monolayers of cells transfected with p140^{NCAM} allowed the detection of a high molecular weight complex (> 300 kDa), likely representing oligomerization of NCAM molecules (Figure 4B, solid arrow). Cotransfection with GFRα1 inhibited the formation of this complex and resulted in the appearance of a lower molecular weight complex containing both p140^{NCAM} and GFRα1 (Figure 4B, open arrows). This result indicated that the association between NCAM and GFRα1 is direct and suggested that this interaction may be accompanied by alterations in the ability of NCAM to interact with other NCAM molecules. We therefore assayed homophilic NCAM binding by chemical crosslinking of a radiolabeled preparation of purified PSA-NCAM to cells transfected with p140^{NCAM} in the presence or absence of GFRα1. Following immunoprecipitation of epitope-tagged p140^{NCAM}, a broad band of high molecular weight (≥300 kDa) corresponding to NCAM-NCAM complexes could be detected in cells expressing p140^{NCAM} (Figure 4C). Interestingly, coexpression of GFRα1 reduced the formation of this complex in a dose-dependent manner without affecting the total levels of p140^{NCAM} expressed in the cells (Figure 4C), indicating that the interaction between GFRα1 and NCAM downregulates NCAM homophilic binding.

The effects of GFRα1 expression on NCAM function were further tested in a cell adhesion assay performed in transiently transfected Jurkat cells. Control Jurkat cells expressing green fluorescence protein (GFP) grew

as a suspension of dispersed individual cells (Figure 4D). Upon transfection with $p140^{NCAM}$, GFP-expressing Jurkat cells formed cell aggregates of variable sizes, including 2 to about 20 cells, indicating NCAM-mediated cell adhesion (Figure 4D). Coexpression of GFRα1 reduced the formation of cell aggregates mediated by NCAM in a dose-dependent manner (Figure 4D), without altering the intensity or the number of GFP-positive cells or the levels of $p140^{NCAM}$ at the cell surface (see Supplemental Figure S3 online at http://www.cell.com/cgi/content/full/113/7/867/DC1). Even low relative levels of GFRα1 had a significant effect on NCAM-mediated cell adhesion. Addition of GDNF had no detectable effect on the adhesion of NCAM-expressing cells (see Supplemental Figures S4A and S4B online at http://www.cell.com/cgi/content/full/113/7/867/DC1). On the other hand, a soluble GFRα1-Fc fusion protein reduced the formation of cell aggregates in NCAM-transfected cells in a dose-dependent manner (Supplemental Figure S4A). At the same ratios of coexpression, a reciprocal increase in GDNF binding to $p140^{NCAM}$ was observed in COS cells cotransfected with increasing doses of GFRα1 (Figure 4E). Together, these data indicated that GFRα1 can form a complex with NCAM at the cell surface, resulting in increased GDNF binding and decreased homophilic interactions between NCAM molecules.

GDNF Stimulates Schwann Cell Migration via NCAM but Independently of RET

To address the physiological significance of the role of NCAM as an alternative signaling receptor for GDNF, we examined biological responses in primary glial cells and neurons predominantly expressing GFRα1 and NCAM but little or no RET. Previous work had implicated NCAM in cell migration, and a recent study demonstrated a specific role of NCAM in the migration of Schwann cells from peripheral nerve explants (Thomaidou et al., 2001). GDNF is highly expressed in developing and regenerating peripheral nerves, suggesting a role for GDNF in the control of Schwann cell migration. To investigate whether GDNF was able to stimulate Schwann cell migration via the NCAM pathway, explants of newborn rat sciatic nerve were cultured on confluent cell monolayers of Fisher rat 3T3 (FR3T3) fibroblasts genetically engineered to stably express GDNF (Arenas et al., 1995). Control cultures were established on monolayers of naive FR3T3 fibroblasts. After 3 days in culture, explants grown on fibroblasts expressing GDNF displayed a large halo of exiting Schwann cells (Figure 5A). Many more cells were observed leaving the explants stimulated with GDNF than those grown on control fibroblasts (Figure 5A). Independent experiments demonstrated that GDNF does not promote Schwann cell proliferation (data not shown), indicating that GDNF stimulates robust Schwann cell migration from sciatic nerve explants. The distance covered by the leading migrating Schwann cell was on average 60% longer in explants cultured on GDNF-expressing fibroblasts than in controls (Figure 5A). The effects of GDNF could be suppressed by low doses of the Fyn kinase inhibitor PP2 (1 μM) or by anti-$NCAM^{ECD}$ and anti-GDNF blocking antibodies (Figure 5A). Control antibodies had no effect on Schwann cell migration, and neither PP2 or anti-$NCAM^{ECD}$ treatments affected cell viability or the motility of Schwann cells grown on control fibroblasts (data not shown). A comparable effect of GDNF on Schwann cell motility could be observed in sciatic nerve explants isolated from *Ret* knockout mice (Figure 5B), indicating that this activity was not mediated by the RET receptor. In contrast, GDNF-mediated Schwann cell migration was abolished in explants isolated from *Ncam* mutant mice (Figure 5C), supporting the participation of NCAM in this biological activity.

GDNF Stimulates Axonal Growth in Primary Neurons via NCAM and Fyn Kinase but Independently of RET

Previous work had shown that hippocampal neurons plated on a monolayer of fibroblasts expressing NCAM on their surface develop longer neuritic extensions than control cultures grown on naive cells (Doherty and Walsh, 1994). We therefore investigated whether GDNF, via its interaction with NCAM and activation of NCAM-associated pathways, could stimulate neurite outgrowth in primary hippocampal and cortical neurons isolated from the embryonic rat brain. We examined the length of the longest neurite in neuronal cultures plated on different substrates, including plain tissue-culture plastic, bovine serum albumin (BSA), cytochrome C (cyto-C), poly-D-lysine (PDL), IgG, and GDNF. Although neurons plated on PDL developed many more processes than those plated on plastic or on any of the control proteins, the length of the longest neurite was not significantly different between PDL and control treatments, reaching on average 150–200 μm after 2 days in culture (Figure 6A). However, hippocampal and cortical neurons plated on GDNF developed neurites that were at least twice as long, reaching on average 400 μm (Figure 6A). The combination PDL+GDNF was also able to promote axonal growth beyond the effect of PDL alone (data not shown). Denatured GDNF had no effect on neurite outgrowth (Figure 6A), indicating the requirement of a native three-dimensional conformation. A significant increase in neurite length could also be observed in hippocampal and cortical neurons growing on a monolayer of fibroblasts expressing GDNF compared to control fibroblasts (see Supplemental Figure S5A online at http://www.cell.com/cgi/content/full/113/7/867/DC1). Neurons plated on PDL presented a greater number of primary neurites than those plated on GDNF, which instead displayed one major long process (Figure 6A), suggesting that GDNF primarily stimulated axonal growth in these cells. Interestingly, this response was qualitatively different from that elicited by BDNF, an established and potent neurotrophic factor for these neuronal subpopulations, which was characterized by a greater number of shorter primary neurites and a more profuse neuritic branching (Supplemental Figure S5B).

The effects of GDNF on axonal growth could be eliminated by treatment with PP2 but were not affected by the FGFR kinase inhibitor SU5402 (Figure 6A). PP2 had no effect on the viability of the cells. Importantly, GDNF-mediated outgrowth could be reduced using anti-$NCAM^{ECD}$ function-blocking antibodies (Figure 6B), indicating the involvement of NCAM in this activity. The effect of GDNF could also be partially attenuated by

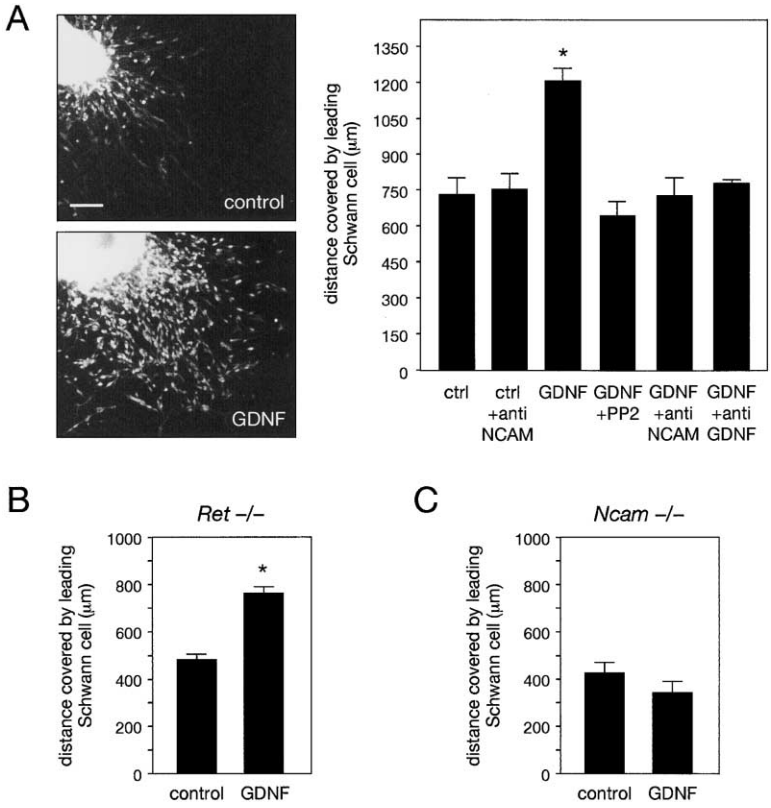

Figure 5. GDNF Promotes Schwann Cell Migration via NCAM but Independently of RET

(A) Migration of Schwann cells (S-100 staining) from newborn rat sciatic nerve explants plated on naive (control) or GDNF-expressing FR3T3 fibroblasts (GDNF). Scale bar, 200 μm. The results correspond to the averages ± SEM of the means of four independent experiments, each including measurements from 5 to 12 different explants. *$p \leq 0.001$.

(B) Migration of Schwann cells from newborn mouse sciatic nerve explants isolated from *Ret* knockout mice. Shown is the mean of at least four different explants ± SD from three animals in each condition. *$p \leq 0.001$.

(C) Migration of Schwann cells from newborn mouse sciatic nerve explants isolated from *Ncam* knockout mice. Shown is the mean of at least five different explants ± SD from three animals in each condition.

function-blocking antibodies against GFRα1 (Supplemental Figure S5C). Antibodies against the NCAM intracellular domain (Figure 6B) or RET (Supplemental Figure S5C) had no effect. Neither PP2 or anti-NCAM^{ECD} treatments had any significant effect on the neurite outgrowth of cells plated on PDL (Figures 6A and 6B). GDNF was able to stimulate neurite outgrowth in embryonic hippocampal neurons isolated from *Ret* knockout mice to a similar extent than in wild-type neurons (Figure 6C), confirming that this activity was mediated independently of the RET receptor. In contrast, GDNF-mediated neurite outgrowth in embryonic cortical and hippocampal neurons isolated from *Ncam* knockout mice was severely reduced compared to wild-type (Figure 6D), providing genetic evidence for the participation of NCAM in the biological response of these cells to GDNF. Of note, lack of NCAM did not affect the response of hippocampal neurons to BDNF (Figure 6E). Together, these results demonstrated a neurite outgrowth-promoting activity of GDNF on hippocampal and cortical neurons that was dependent on NCAM expression and function and on the activity of Fyn kinase but that was independent of signaling by the RET receptor tyrosine kinase.

GFRα1 and NCAM in the Development of the Rostral Migratory Stream

In this last section, we turn our attention to some of the possible implications of the interactions between GDNF, GFRα1, and NCAM for development in vivo. The most distinctive abnormality of the central nervous system of mice lacking NCAM is a dramatic reduction of the size of the adult olfactory bulbs (Cremer et al., 1994). This is caused by defects in the migration of neuronal precursors in the rostral migratory stream (RMS), a migratory pathway that delivers cells from the subventricular zone to the olfactory bulb during postnatal and adult life (Garcia-Verdugo et al., 1998). NCAM is essential for correct chain migration of neuronal precursors in the RMS, a process whereby cells use each other as the migratory substrate (Lois et al., 1996). In the absence of NCAM, neuronal progenitors 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). The ability of GFRα1 to modulate NCAM-mediated cell adhesion and migration prompted us to investigate the expression of GFRα1 in the RMS. Analysis of sagittal sections of adult brain by immunofluorescence revealed a striking juxtaposition of GFRα1 and PSA-NCAM expression along the entire RMS (Figure 7A). RET immunoreactivity could not be detected in the pathway (Figure 7A), although cells in the facial motor nucleus were readily labeled (Figure 7A, inset). As expected, no signal for PSA-NCAM could be detected in sections from adult *Ncam* mutant mice (Figure 7A). On the other hand, staining for GFRα1 revealed the characteristic enlargement of the mutant RMS at its exit in the anterior horn of the lateral ventricle and in its ventral projection (Figure 7A), indicating that cells affected in the RMS of *Ncam* mutant mice express GFRα1.

The premature death of mice lacking GFRα1 during the first hours after birth prevented us from studying the adult RMS in these animals. We therefore examined PSA-NCAM and GFRα1 expression in wild-type and mu-

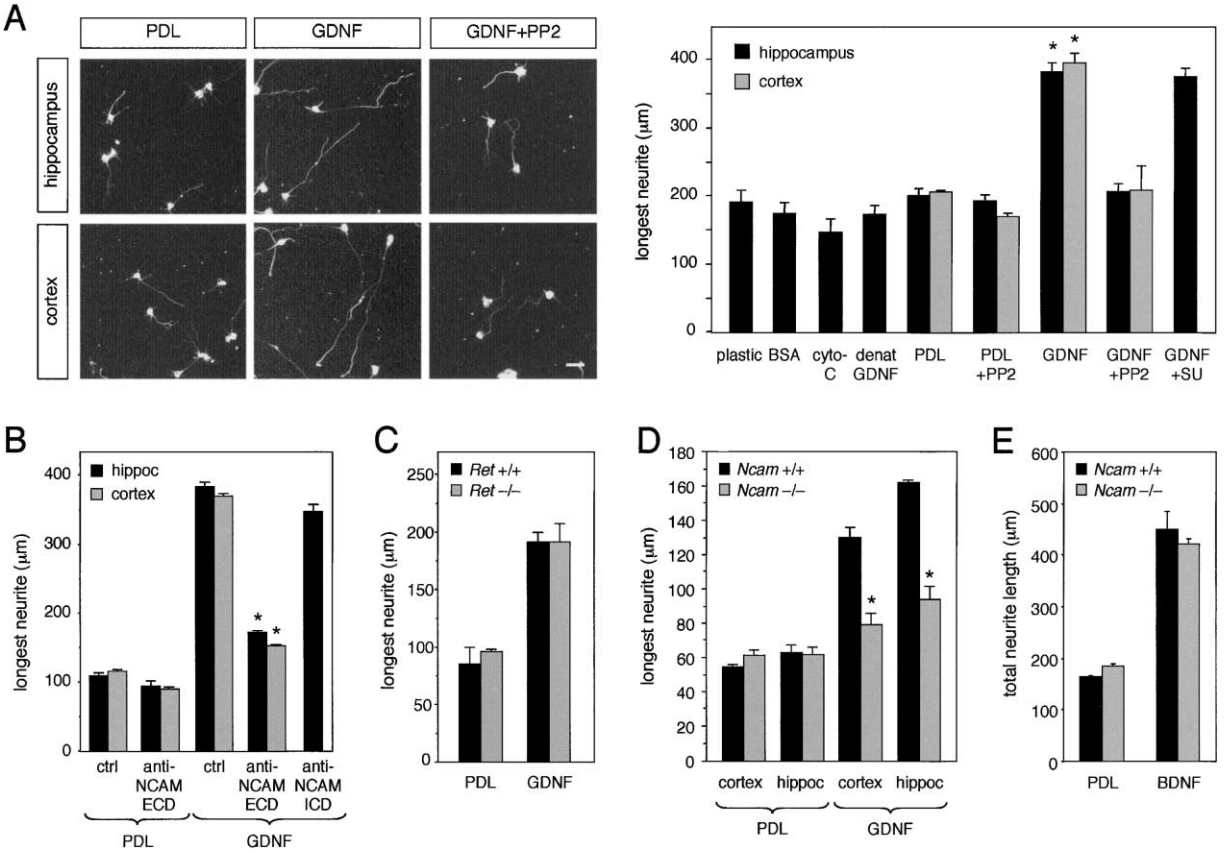

Figure 6. GDNF Promotes Axonal Growth in Hippocampal and Cortical Neurons via Fyn and NCAM but Independently of RET

(A) Representative photomicrographs of embryonic rat hippocampal and cortical neurons grown on PDL or GDNF (β III tubulin staining). Scale bar, 50 μm. Results are mean ± SEM from a representative experiment performed in triplicate. *$p < 0.001$ versus PDL or GDNF+PP2 (ANOVA). Similar results were obtained in four independent experiments.

(B) Blockade of GDNF outgrowth activity by $NCAM^{ECD}$ antibodies. Scale bar, 50 μm. *$p \leq 0.001$ (ANOVA). Four independent experiments were performed with similar results.

(C) Axonal outgrowth in hippocampal neurons from wild-type and *Ret* knockout mice. Scale bar, 25 μm. Similar results were obtained in three independent experiments.

(D) Axonal outgrowth in cortical and hippocampal neurons from wild-type and *Ncam* knockout mice. *$p < 0.01$, compared to wild-type (Student's t test). Similar results were obtained in three independent experiments.

(E) Neurite outgrowth of hippocampal neurons from *Ncam* heterozygous and knockout mice grown on BDNF. The length of the complete dendritic arbor was measured in these experiments.

tant newborn mice. As previously reported, staining for PSA-NCAM in the newborn mouse brain was broadly distributed and included the developing RMS, which at these early stages comprises a broad band of migrating cells (Figure 7B). At this stage, GFRα1 expression was more restricted than that of PSA-NCAM and localized prominently in the developing RMS, the subventricular zone and the olfactory bulb (Figure 7B). Many cells in the middle portion of the RMS appeared to coexpress PSA-NCAM and GFRα1 at this stage (Figure 7C). As expected, no staining for GFRα1 was observed in the brain of newborn *Gfra1* mutants (Figure 7B), confirming the specificity of the patterns observed in wild-type animals. Staining for PSA-NCAM revealed essentially the same pattern as in newborn wild-type animals; no significant difference in the overall size of the olfactory bulb of *Gfra1* mutants could be recognized at this early stage of development (Figure 7B). We examined whether early signs of RMS enlargement could be discerned in newborn mice lacking GFRα1. To this end, we measured the width of the caudal portion of the RMS after this turns ventrally from the lateral ventricle in equivalent sagittal sections of wild-type and *Gfra1* mutant brains stained with anti-PSA-NCAM antibodies. This analysis revealed a statistically significant increase in RMS width in the brain of newborn mice lacking GFRα1 (Figure 7D). No such difference could be observed in the RMS of *Ret* mutant animals (Figure 7D), suggesting a RET-independent role for GFRα1 in RMS development.

Discussion

The presence of GFRα receptors in many regions of the central and peripheral nervous systems in the absence of RET has suggested the existence of alternative, RET-independent signaling mechanisms for GDNF family ligands, presumably mediated by the collaboration of GFRαs with unknown transmembrane receptors (Trupp

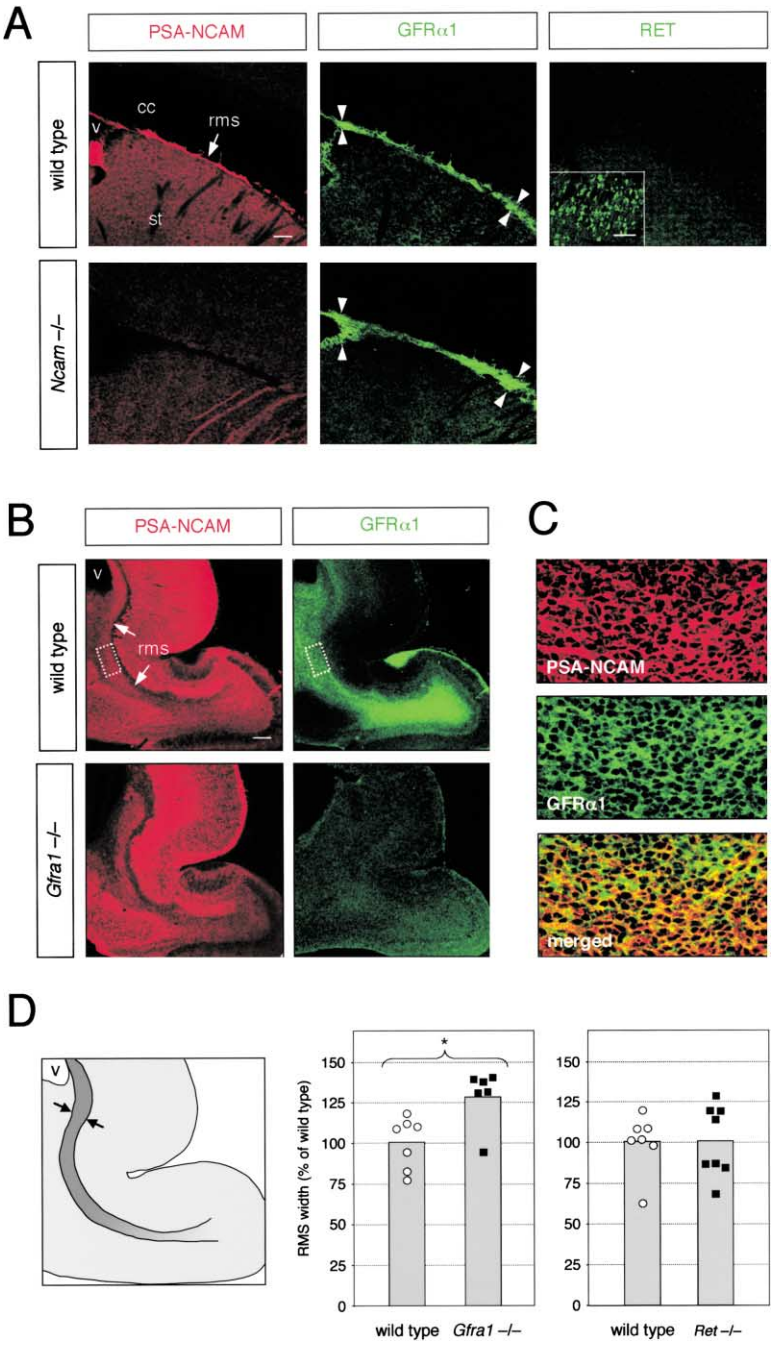

Figure 7. GFRα1 and NCAM in the Development of the Rostral Migratory Stream

(A) Expression of PSA-NCAM and GFRα1 in sagittal sections of the RMS of adult mice. The inset shows prominent RET staining in cells of the adult facial motor nucleus. Arrowheads delineate the RMS stained with GFRα1 antibodies. Abbreviations: cc, corpus callosum; rms, rostral migratory stream; st, striatum; v, ventricle. Scale bars, 100 μm.

(B and C) Expression of PSA-NCAM and GFRα1 in the RMS of newborn mice. Arrows indicate the developing RMS. Boxed areas are shown at higher magnification in (C). Scale bar, 200 μm.

(D) Morphometric analysis of the RMS in newborn wild-type, *Gfra1*, and *Ret* mutant mice. The region measured in equivalent sections of wild-type and mutant animals is indicated in diagram form. The scatter graphs show measurements of RMS width for each individual animal normalized to the mean value in wild-type mice. Bars indicate the mean value in each group. *p = 0.0136, Student's t test.

et al., 1997, 1999). Here, we have shown that the neural cell adhesion molecule NCAM, in collaboration with GFRα receptors, can function as a signaling receptor for members of the GDNF ligand family independently of the presence of RET, thereby providing an explanation for the tissue distribution of GFRα proteins. NCAM is abundantly expressed in cell types that also express high levels of GFRα molecules, but not RET, including Schwann cells and hippocampal and cortical neurons (Crossin and Krushel, 2000). In vivo, all these cell types are normally exposed to GDNF in a paracrine or autocrine fashion (Trupp et al., 1997), suggesting that the signaling pathways and biological effects described here are physiologically relevant for these cells. Our results demonstrate that NCAM and GFRα1 associate in the same molecular complex and reveal an unexpected ability of GFRα1 to gate NCAM function. The dose-dependent, reciprocal effects of GFRα1 on NCAM-mediated cell adhesion and GDNF binding suggest that interactions between GFRα1 and NCAM molecules may result in conformational changes in NCAM that favor GDNF binding over homophilic interactions, thereby regulating the ability of NCAM to interact with different ligands.

Our findings suggest that a number of functions previously ascribed to short-range signaling by homophilic interactions between NCAM molecules, including regu-

lation of axonal growth, cell migration, and synaptic plasticity, could also be mediated by the interaction of long-range signaling messengers, such as GDNF family ligands, with NCAM receptors. Schwann cell migration is an important developmental process for the formation of peripheral nerves and plays a critical role during nerve regeneration. Schwann cells express high levels of NCAM, GFRα1, and GDNF, all of which become upregulated following nerve injury and Schwann cell activation. Mechanisms controlling the balance between adhesion and motility are likely to be of importance for the regulation of cell migration. Thus, through their ability to regulate both NCAM-mediated cell adhesion and motility, GDNF and GFRα1 may also contribute to the control of Schwann cell migration during peripheral nerve development and regeneration.

Our results also indicate that GDNF can utilize NCAM signaling pathways to promote axonal growth in hippocampal and cortical neurons. The lack of involvement of the FGFR in GDNF-mediated outgrowth is a notable difference with NCAM activation by homophilic binding (Doherty and Walsh, 1996). The possibility that GDNF family ligands may participate in the regulation of synaptic plasticity is particularly interesting in view of the marked impairments in spatial learning and induction of long-term potentiation (LTP) observed after loss of NCAM function (Cremer et al., 2000, 1994; Doherty et al., 1995; Luthl et al., 1994). Intriguingly, seizure activity is known to regulate the expression of GDNF family ligands and GFRα receptors in limbic structures (Kokaia et al., 1999), and development of hippocampal kindling epilepsy, a form of synaptic plasticity, is markedly reduced in mice lacking GFRα2 (Nanobashvili et al., 2000). Our results suggest that GDNF family ligands may contribute to the regulation of synaptic plasticity in the brain via activation of NCAM signaling pathways.

Despite NCAM's broad expression and widely documented roles in adhesion, migration, and growth, mice lacking NCAM are viable and fertile (Cremer et al., 1994). Moreover, all phenotypes so far reported in these animals, which include deficits in neuronal migration, synaptic organization, plasticity, and spatial learning, do not appear until later in postnatal and adult life (Chazal et al., 2000; Cremer et al., 1994; Esni et al., 1999; Holst et al., 1998; Muller et al., 1996; Polo-Parada et al., 2001; Stork et al., 1997; Tomasiewicz et al., 1993). On the other hand, the analysis of mice lacking GDNF has so far been focused on two main phenotypes, i.e., renal agenesis and absence of enteric neurons (Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996), which appear early during embryonic development and lead to the premature death of these animals a few hours after birth. Both these phenotypes are also found in mice lacking RET (Schuchardt et al., 1994) and are therefore likely mediated by this receptor. It is at present unclear to which extent lack of GDNF, or RET, affects the development of the central nervous system, and direct comparisons between the two mutants are yet to be performed. In addition, the early lethality of these animals has precluded investigation of the roles of GDNF in brain maturation and function that can be anticipated from its pattern of expression in the postnatal and adult nervous system. Intriguingly, heterozygote *Gdnf* knockout mice, which survive to adulthood, present deficiencies in spatial learning similar to those observed in *Ncam* mutants (Gerlai et al., 2001). In addition, a recent study reported that effects of GDNF on survival, growth, and function of midbrain dopaminergic neurons could be antagonized with anti-NCAM function blocking antibodies both in vitro and in vivo (Chao et al., 2003), an observation that supports the physiological relevance of GDNF/NCAM interactions in this system.

The ability of GFRα1 to modulate NCAM-mediated cell adhesion, even in the absence of GDNF, together with its prominent localization in the developing and adult RMS, suggests that GFRα1/NCAM interactions may play a role in the migration of neuronal progenitors along this pathway and in the development of the olfactory bulb. In agreement with this notion, the developing RMS of newborn *Gfra1* mutants appeared moderately enlarged, a phenotype that was absent in newborn *Ret* mutants but that resembled the enlargement of the RMS that is characteristic in adult mice lacking NCAM (Chazal et al., 2000). In future studies aimed to address the role of GDNF/ and GFRα1/NCAM interactions in vivo, it will be necessary to employ strategies designed to disrupt GDNF and GFRα1 function specifically in postnatal and adult stages and to dissect the specific contributions of RET and NCAM to the observed phenotypes.

In conclusion, our findings indicate that crosstalk between different systems of cell communication may be more prevalent than previously thought. Crosstalk between extracellular components could reflect a level of signal integration required for the coordination of multicellular responses. The interactions we describe here between NCAM and GDNF family ligands and GFRα receptors reveal NCAM as a surprisingly versatile transmembrane receptor capable of mediating both short- and long-range intercellular communication via distinct ligand systems.

Experimental Procedures

Recombinant Proteins, Cell Lines, and Mice

GDNF, GFRα1-Fc, and ALK4-Fc were purchased from R&D, NTN was purchased from Peprotech, and recombinant Persephin (PSP) was a gift from Miroslav Cik and Bob Gordon, Jannssen Research Foundation (Belgium). Purified PSA-NCAM was obtained from Chemicon (cat. no. AG265). RN33B is an immortalized neuronal precursor cell line isolated from embryonic raphe nucleus (Trupp et al., 1999). Fisher rat 3T3 fibroblasts producing GDNF have been described previously (Arenas et al., 1995). *Ret* knockout mice (Schuchardt et al., 1994) were provided by Judith Crusells and Mart Saarma with the kind permission of Vassilis Pachnis. *Ncam* knockout mice (Cremer et al., 1994) were obtained from the Jackson Laboratory (Maine). *Gfra1* mutant mice (Cacalano et al., 1998) were kindly provided by Arnon Rosenthal.

RET and GFRα1 RT-PCR

Total RNA was isolated from RN33, Schwann cells, or adult rat cerebellum using SNAP 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 specific primers: rat RET forward primer, 5′-CCCGTGTGTACTTCTCCTT CAT-3′; reverse primer, 5′-GCACTCAGCCTCCAGCAC-3′; rat GFRα1 forward primer, 5′-GCGTGCACCGAGCGGCGCCGACAGACTATCG-3′; reverse primer, 5′-TTCCAGGTCATTTCCGGAGTTGCTGCAGTC-3′.

Cell Transfection and Plasmids

Transient transfection of COS cells was performed using the calcium phosphate method. Plasmid cDNA encoding $p140^{NCAM}$ (without VASE

sequence) was kindly provided by Patricia Maness. cDNAs for rat GFRα1 and GFRα2 were described previously (Trupp et al., 1998). Chicken GFRα4 cDNA was a gift from Alun Davies. Human Fyn cDNA was kindly provided by Tohru Tezuka. The GFP plasmid was from Clontech.

Cell Adhesion Assay

Jurkat cells were transfected in 24-well plates (1.3 μg total DNA per well) using the Fugene-6 reagent (Roche) in 0.5 ml complete medium with 10% serum. 2 days after transfection, they were transferred to 12-well plates and 0.5 ml serum-free medium was added. On the third day, cell aggregates larger than five cells were counted in each well under green fluorescence illumination. The total number of green cells was also counted for normalization. NCAM immunofluorescence was performed on cells fixed and permeabilized as indicated below using anti-NCAM^{ICD} antibodies (Developmental Studies Hybridoma Bank).

Schwann Cell Preparation and Migration Assays

Schwann cells were extracted from the newborn rat sciatic nerve by collagenase treatment and cultured serum-containing medium supplemented with bFGF and forskolin. At confluence, cultures were switched to serum-free medium before stimulation with GDNF. For Schwann cell migration studies, sciatic nerves were dissected out from newborn rats or mice, subjected to collagenase treatment, and placed onto confluent monolayers of either control or GDNF-expressing FR3T3 fibroblasts in 24-well plates. Cultures were allowed to grow in complete medium (5% serum) for an additional 2–3 days, after which they were permeabilized, fixed, and stained with antibodies against S-100 (rat) or p75 (mouse). In each explant, Schwann cell migration was quantified as the distance from the border of the explant to the leading migrating cell.

Chemical Crosslinking and Binding Assays

Ligands were iodinated by the lactoperoxidase method and chemical crosslining was performed with ethyl-dimethyl-aminopropyl-carbodimide (EDAC) supplemented with sulfo-NHS (Pierce). Cells were lysed in buffer containing 0.5% Triton X-100 and 60 mM β-octyl-D-glucopyranoside (Pierce) to ensure complete solubilization of membrane lipid rafts. For PI-PLC treatments, cell monolayers were washed and then incubated with 1 U/ml PI-PLC (Sigma) in cell culture medium for 60 min at 37°C followed by affinity labeling as above. Soluble NCAM extracellular domain (NCAM^{ECD}) was produced in the supernatant of 293 cells stably transfected with a cDNA encoding N terminally, hemagglutinin (HA)-tagged rat NCAM^{ECD}, concentrated by ultrafiltration and quantified by immunoblotting. Purified PSA-NCAM was iodinated by the lactoperoxidase method. Following chemical crosslinking with ^{125}I-PSA-NCAM (performed as above), an acid wash was made to remove radiolabeled probe unspecifically adsorbed to the cells, followed by several washes with PBS and cell lysis as above.

For saturation binding assays, increasing concentrations of ^{125}I-GDNF were incubated with COS cells monolayers transfected with p140^{NCAM} alone or together with GFRα1. Equilibrium was reached after a 4 hr incubation at 4°C in phosphate buffer saline (PBS) supplemented with Ca^{2+} and BSA. This was followed by chemical crosslinking, EDAC neutralization with 500 mM glycine, two washes in PBS, and cell lysis as above. Cell lysates were immunoprecipitated with anti-NCAM^{ICD} antibodies, separated by SDS-PAGE, and blotted onto PVDF membranes. After autoradiographic exposure to phosphorscreens, the band corresponding to the complex between ^{125}I-GDNF and p140^{NCAM} was quantified using a Storm 840 phosphorimager and ImageQuant software (Molecular Dynamics). For each ligand concentration, the level of ^{125}I-GDNF bound to p140^{NCAM} was normalized to the total levels of p140^{NCAM} expressed (determined by NCAM immunoblotting of the same membranes) and then plotted as a function of the total amount of ^{125}I-GDNF added. A linear transformation of these data was made in a Scatchard plot analysis.

Lipid Rafts and PTP Activity

Preparation of rafts was performed as previously described (Paratcha et al., 2001). Protein tyrosine phosphatase activity was evaluated in duplicates of lipid raft fractions (8–10 μg of protein) using a nonradioactive phosphatase assay system (Promega) based on the substrate DADE(pY)LIPQQG according to the manufacturer's instructions. Protein concentrations were evaluated by the micro-BCA method (Pierce).

Total Cell Lysates, Immunoprecipitation, and Western Blotting

For total cell lysates, cells were lysed for 60 min at 4°C in buffer containing 0.5% Triton X-100, 1% β-octylglucoside plus protease and phosphatase inhibitors. Protein lysates were clarified and analyzed by immunoprecipitation and Western blotting as previously described (Paratcha et al., 2001). All blots were scanned in a Storm 840 fluorimager and quantifications were done with ImageQuant software (Molecular Dynamics). The antibodies were obtained from various sources as follows: anti-Fyn and anti-FAK from Upstate Biotechnology (Lake Placid, NY); anti-phospho-FAK (Tyr-397) from Biosource (Camarillo, CA); anti-AKT and anti-Phospho-ERK (Thr-202/Tyr-204) from New England Biolabs; monoclonal anti-HA from Covance; anti-RET and anti L1 from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibodies anti-NCAM^{ICD} (12F11) and anti-NCAM^{ECD} (N-CAM13) from BD PharMingen. Whole-cell biotinylation was performed with the EZ-link reagent (Pierce), following the manufacturer's instructions.

In Vitro Kinase Assay

Fyn or NCAM immunoprecipitates were assayed by in vitro kinase assay as previously described (Trupp et al., 1999). In some experiments, the NCAM immunocomplexes were eluted by 10 min incubation at 100°C in 2% SDS, and supernatants were then diluted 20-fold in lysis buffer and reimmunoprecipitated with anti-Fyn antibodies. As loading control, the supernatant of the Fyn immunoprecipitation was reprecipitated and immunoblotted with anti-NCAM^{ICD} antibodies.

Neurite Outgrowth Assay and Pharmacological Treatments

Rat hippocampal and cortical cells from embryonic day (E)17.5 and mouse cortical cells from E15.5 embryos were cultured in Neurobasal (Gibco) supplemented with B27 (Gibco). Mouse hippocampal cells (E15.5) were cultured in N2 medium. Tissue culture dishes (48-well) were coated with the indicated proteins at 10 μg/ml followed by three washes with PBS. Three thousand cells were plated per well and cultured for 48 hr. The cells were then fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and stained with anti-β III tubulin (Promega). For blocking experiments, PP2 (Alexis) was used at 1 μM, SU5402 (Calbiochem) at 10 μM, and the monoclonal anti-NCAM^{ECD} (MAB310, Chemicon) was utilized at 5 μg/ml. Function-blocking anti-GFRα1 antibodies or anti-RET antibodies (R&D Systems) were added at 12 μg/ml. GDNF-expressing fibroblasts for coculture experiments were obtained by transient transfection of MG87 cells (a derivative of NIH3T3 fibroblasts) as previously described (Arenas et al., 1995). Quantification of neurite length was done with the IP-Lab Spectrum software package. At least 200 neurons were evaluated in each well; three wells were counted per experiment; each experiment was repeated four times.

Immunofluorescence and Microscopy

Cryostat sections (20 μm) of adult newborn mouse brains were generated as previously described (Trupp et al., 1997, 1998). Sections were blocked with 5% donkey serum and incubated with primary antibodies: mouse monoclonal anti-PSA-NCAM (Chemicon), affinity-purified rabbit polyclonal anti-GFRα1 (provided by Michele Sanicola), and hamster monoclonal anti-RET (provided by David Anderson). Secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or rhodamine (TRITC) were from Jackson Immunoresearch Lab. Confocal microscopy was performed in a Zeiss LSM 510 confocal microscope. Morphometric analysis of the RMS in newborn mice was performed on sagittal sections stained with PSA-NCAM, where the RMS could be seen as a stream of cells extending from the anterior limit of the lateral ventricle to the caudal boundary of the olfactory bulb. We measured the width of the caudal portion of the RMS after it turns ventrally from the lateral ventricle in equivalent sagittal sections of wild-type and mutant mice using the OpenLab software package.

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Supplementary Figure legends

Figure S1. Proximal events in RET-independent GDNF signaling resemble intracellular pathways activated by NCAM.

(A) RT-PCR analysis (35 cycles) shows lack of RET expression (left), but abundant GFR1 expression (right), in RN33B and Schwann cells.

(B) Activation of Fyn and FAK kinases in RN33B (left) and Schwann (right) cells. Immunoprecipitation of Fyn (upper panels) was followed by *in vitro* kinase assay and autoradiography. Reprobings of the same blots with Fyn antibodies are shown below. Immunoprecipitation of FAK (lower panels) was followed by immunoblotting with antibodies recognizing phosphorylation of Tyr-397 in FAK. Reprobings with FAK antibodies are shown below.

(C) Tyrosine phosphatase activity in lipid rafts of RN33B (left) and Schwann (right) cells. *, $p<0.01$; **, $p<0.001$ (n=3, ANOVA).

(D) Activation of Erk MAP kinases in total lysates of RN33B (left) and Schwann (right) cells. Immunoblotting with anti-phospho-Erk antibodies (upper panels) and reprobing with anti-AKT antibodies (lower panels) are shown.

Figure S2. GFR1 expression stabilizes or recruits NCAM in lipid rafts. PC12 cells expressing endogenous levels of NCAM were mock-transfected (control) or transfected with HA-tagged GFR1; lipid rafts were then isolated as described above. Twenty micrograms of lipid raft protein were immunoblotted with anti $NCAM^{ICD}$ antibodies (upper panel). The filter was then reprobed with anti-HA antibodies, detecting the transfected GFR1 (middle panel). The lower panel shows equal levels of NCAM in total cell lysates.

Figure S3. Immunofluorescence analysis of cell surface NCAM expression in transfected Jurkat cells. Jurkat cells were transfected with GFP alone (control) or GFP plus NCAM (NCAM) or GFP plus NCAM plus GFR1 (NCAM+GFR1), permeabilized, stained with anti-$NCAM^{ICD}$ antibodies (Rhodamine) and analyzed in

a confocal microscope for green (GFP) and red (NCAM) fluorescence. As it can be seen in the central column, the effect of GFR1 expression on the formation of cell aggregates by NCAM-expressing cells was not due to a reduction in the levels of NCAM expression at the surface of transfected cells.

Figure S4. Effects of GDNF on NCAM-mediated cell-adhesion.

(A) Cell adhesion of NCAM-overexpressing Jurkat cells in the presence of GDNF, soluble GFR1-Fc and a control Fc fusion protein (ALK4-Fc). Only GFR1-Fc had a significant effect on NCAM-mediated cell adhesion in this assay. n=4.

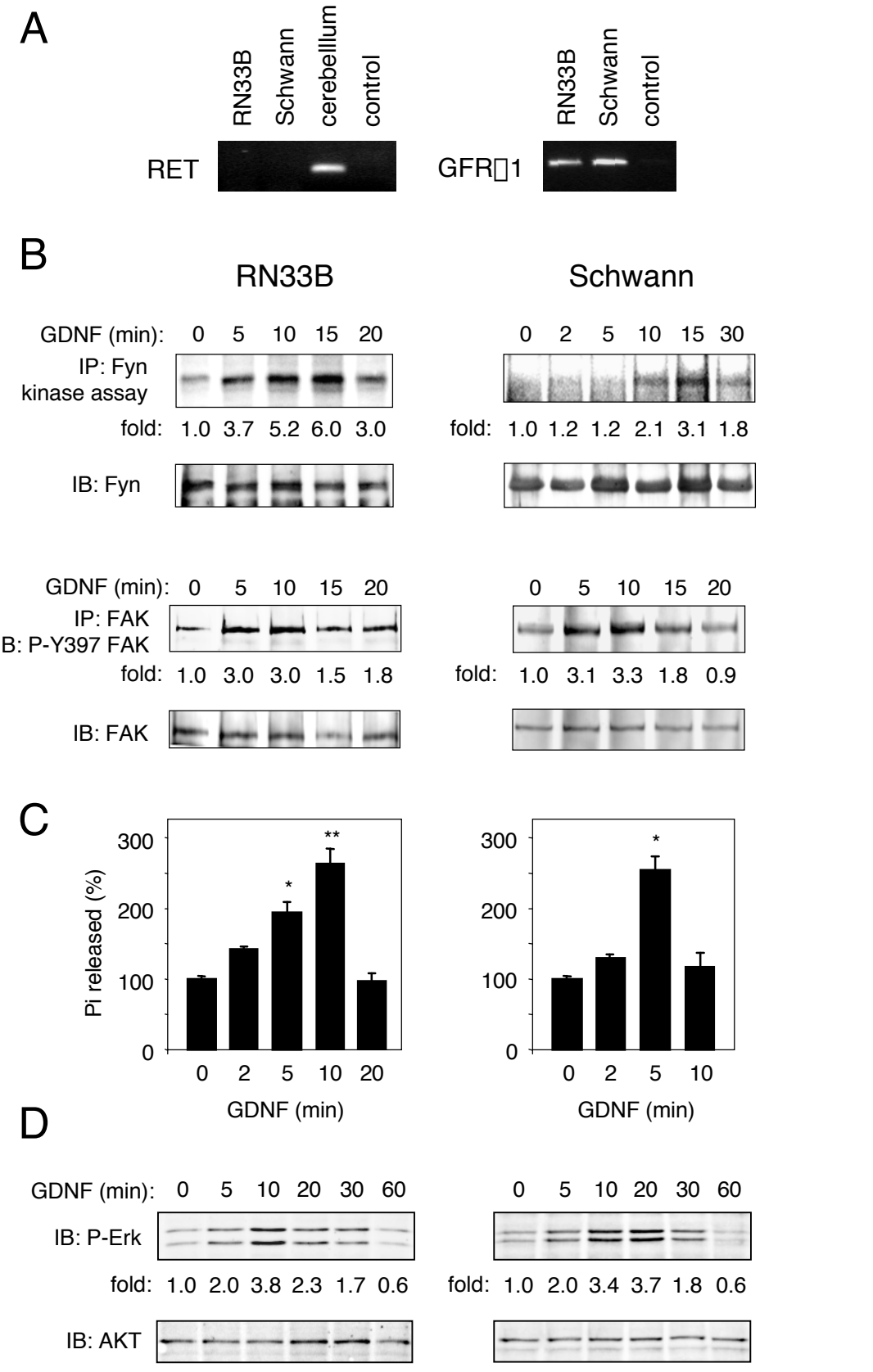
(B) Cell adhesion of NCAM- and GFR1-overexpressig Jurkat cells in the presence of GDNF. Addition of GDNF to cell aggregates formed by cells expressing NCAM or NCAM plus GFR1 does not result in any further reduction in NCAM-mediated cell adhesion. n=4.

Figure S5. Neurite outgrowth in hippocampal and cortical neurons.

(A) Neurite outgrowth of grown on monolayers of mock-transfected fibroblasts (control-MG87) or fibroblasts expressing GDNF (GDNF-MG87). GDNF-MG87 cells expressed approximately 100 ng/ml GDNF per million cells per day. Hippocampus, n=4, $p<0.005$ (Student's t-test). Cortex, n=4, $p<0.001$ (Student's t-test).

(B) Representative photomicrographs of embryonic mouse hippocampal neurons grown on PDL, BDNF or GDNF for 48 h and stained with III tubulin antibodies.

(C) Neurite outgrowth of hippocampal neurons stimulated by GDNF can be partially blocked by function-blocking anti-GFR1 antibodies. Antibodies against RET had no effect. *, n=4, $p<0.01$ (ANOVA).


A
RN33B
Schwann
cerebelllum
control
RET
RN33B
Schwann
control
GFR1
B
RN33B
Schwann
GDNF (min): 0 5 10 15 20
IP: Fyn
kinase assay
fold: 1.0 3.7 5.2 6.0 3.0
IB: Fyn
0 2 5 10 15 30
fold: 1.0 1.2 1.2 2.1 3.1 1.8
GDNF (min): 0 5 10 15 20
IP: FAK
IB: P-Y397 FAK
fold: 1.0 3.0 3.0 1.5 1.8
IB: FAK
0 5 10 15 20
fold: 1.0 3.1 3.3 1.8 0.9
C
Pi released (%)
300
200
100
0
0 2 5 10 20
GDNF (min)
0 2 5 10
GDNF (min)
D
GDNF (min): 0 5 10 20 30 60
IB: P-Erk
fold: 1.0 2.0 3.8 2.3 1.7 0.6
IB: AKT
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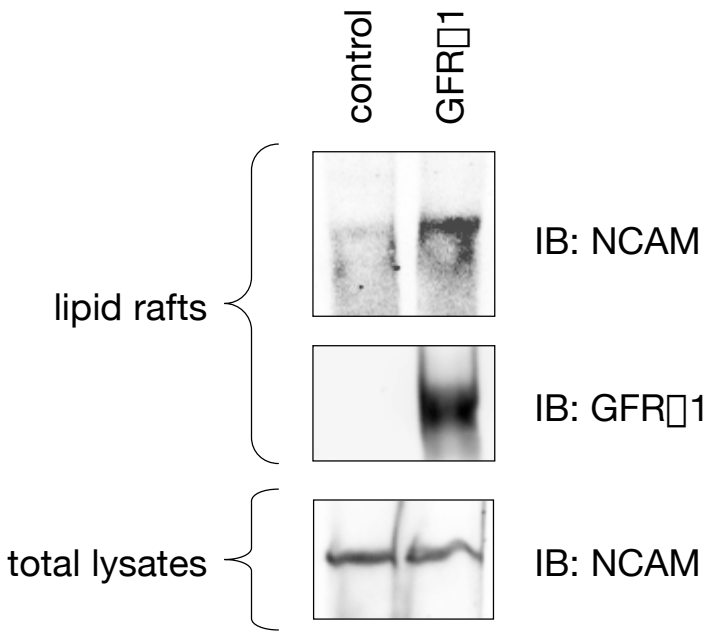
fold: 1.0 2.0 3.4 3.7 1.8 0.6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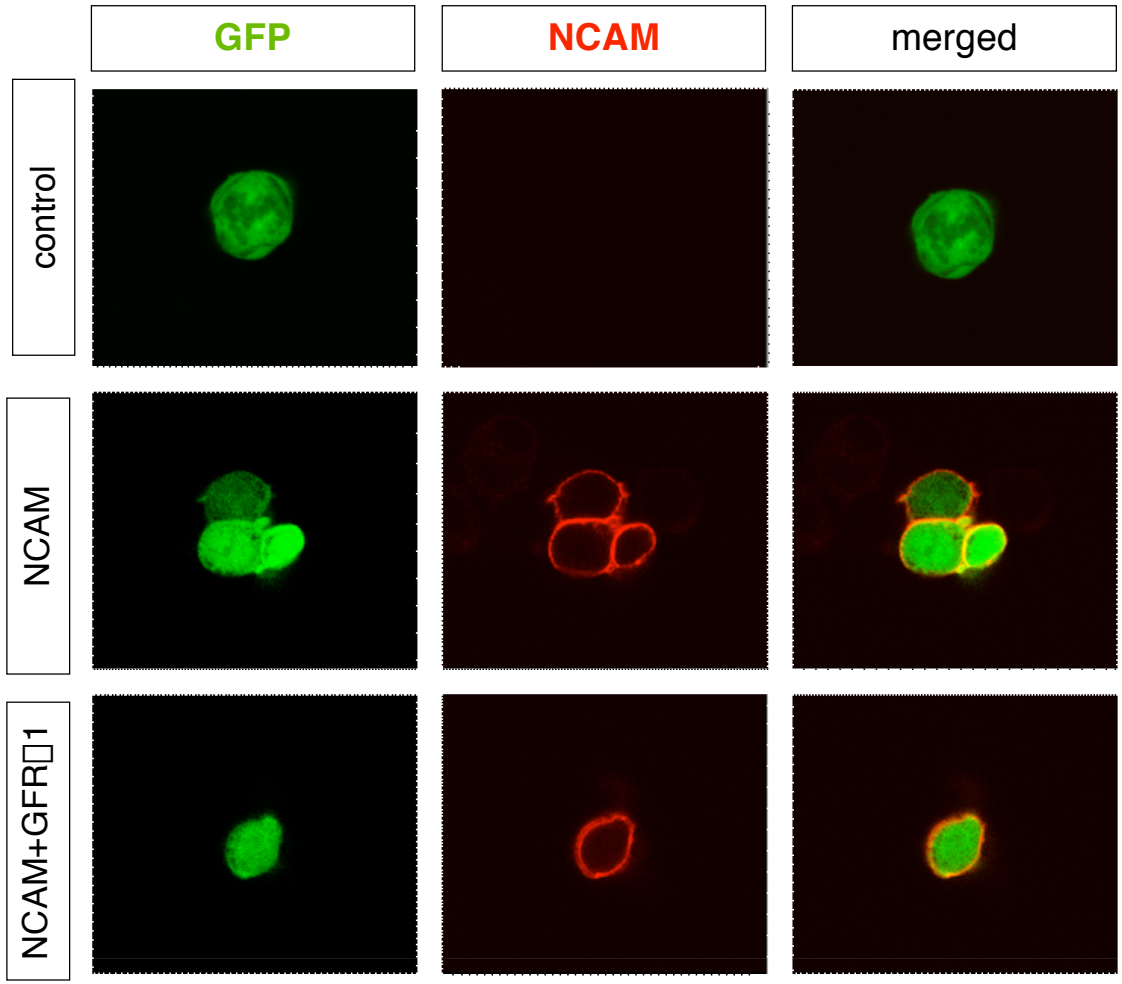
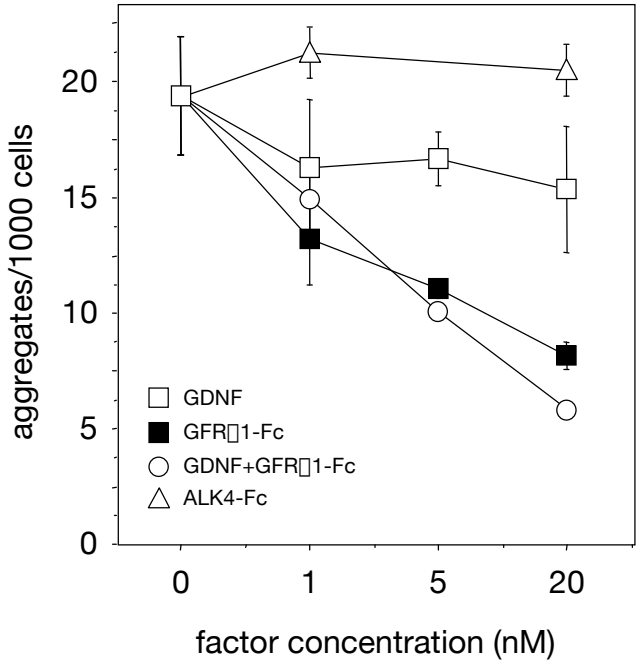
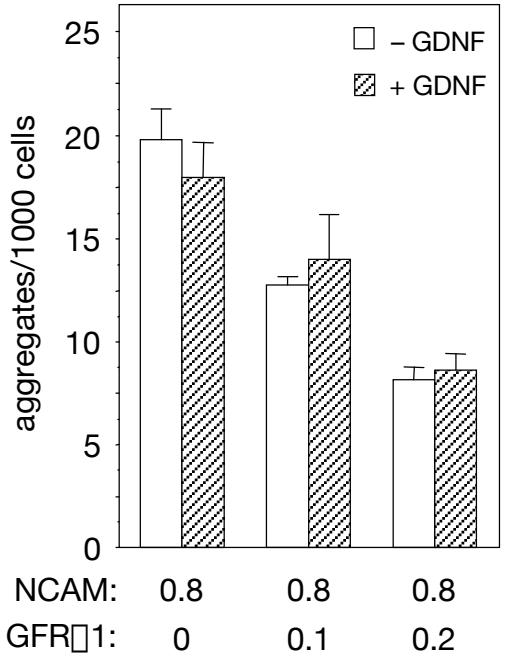
GFR□1
lipid rafts
IB: NCAM
IB: GFR□1
total lysates
IB: NCAM


GFP
NCAM
merged
control
NCAM
NCAM+GFR1

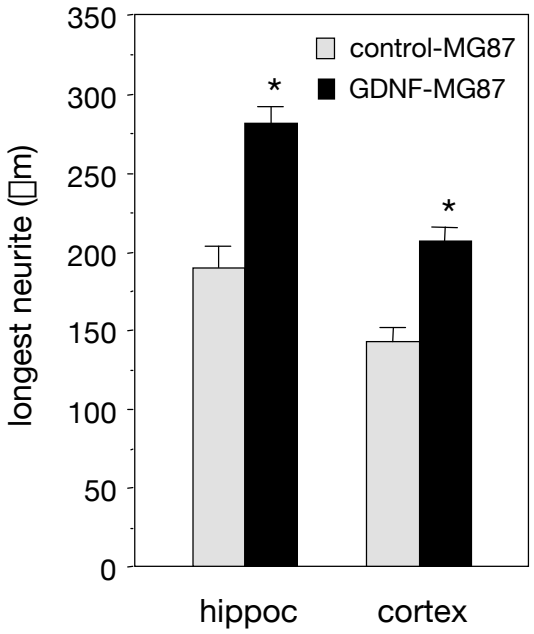
A


aggregates/1000 cells
0
5
10
15
20
0
1
5
20
factor concentration (nM)
GDNF
GFRα1-Fc
GDNF+GFRα1-Fc
ALK4-Fc

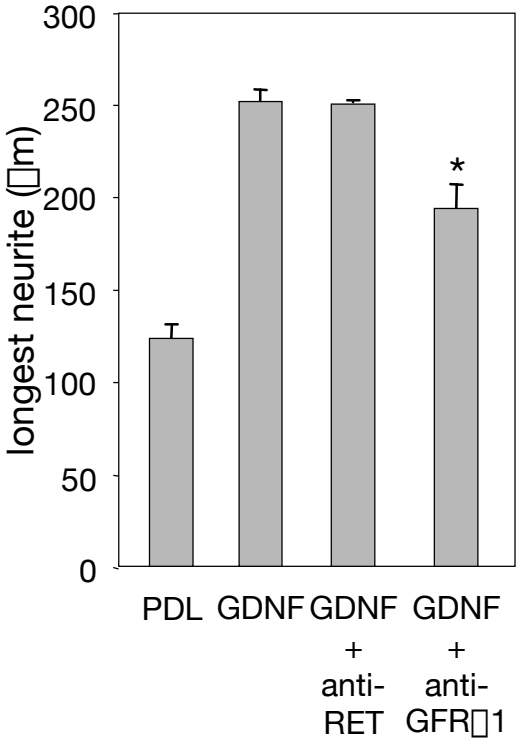
B


aggregates/1000 cells
0
5
10
15
20
25
– GDNF
+ GDNF
NCAM: 0.8 0.8 0.8
GFRα1: 0 0.1 0.2

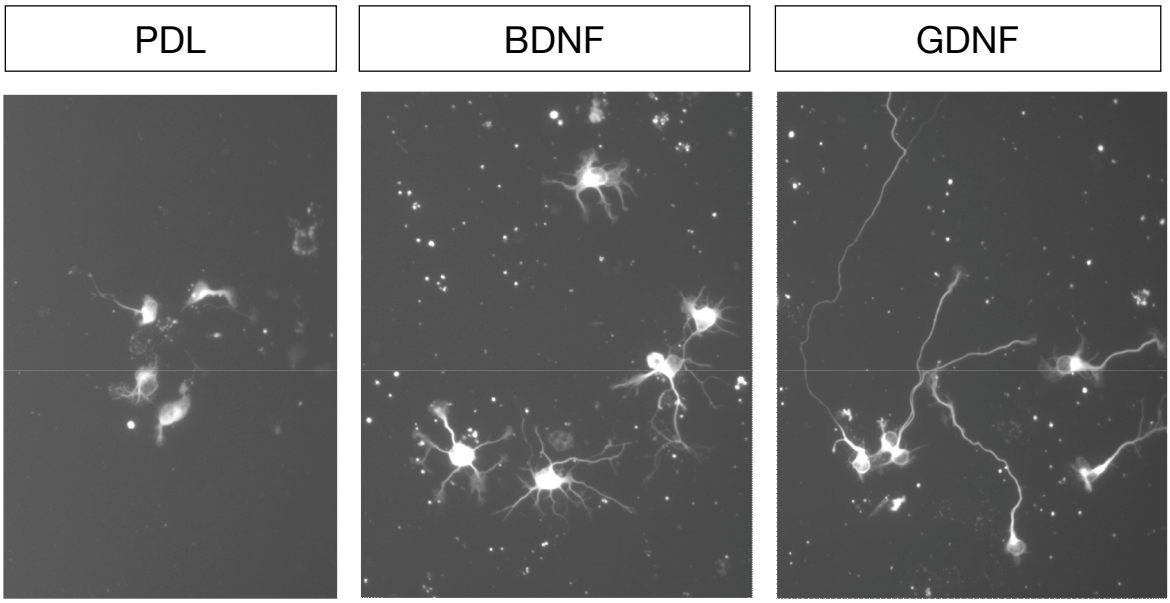
A


longest neurite (□m)
350
300
250
200
150
100
50
0
control-MG87
GDNF-MG87
*
*
hippoc
cortex

C


longest neurite (□m)
300
250
200
150
100
50
0
*
PDL
GDNF
GDNF + anti-RET
GDNF + anti-GFR□1

B


PDL
BDNF
GDNF