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# GDNF and GFRα1 promote formation of neuronal synapses by ligand-induced cell adhesion

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**The establishment of synaptic connections requires precise alignment of pre- and postsynaptic terminals. The glial cell line–derived neurotrophic factor (GDNF) receptor GFRα1 is enriched at pre- and postsynaptic compartments in hippocampal neurons, suggesting that it has a function in synapse formation. GDNF triggered trans-homophilic binding between GFRα1 molecules and cell adhesion between GFRα1-expressing cells. This represents the first example of a cell-cell interaction being mediated by a ligand-induced cell adhesion molecule (LICAM). In the presence of GDNF, ectopic GFRα1 induced localized presynaptic differentiation in hippocampal neurons, as visualized by clustering of vesicular proteins and neurotransmitter transporters, and by activity-dependent vesicle recycling. Presynaptic differentiation induced by GDNF was markedly reduced in neurons lacking GFRα1. *Gdnf* mutant mice showed reduced synaptic localization of presynaptic proteins and a marked decrease in the density of presynaptic puncta, indicating a role for GDNF signaling in hippocampal synaptogenesis *in vivo*. We propose that GFRα1 functions as a LICAM to establish precise synaptic contacts and induce presynaptic differentiation.**

The formation of neuronal synapses involves assembly of the machinery responsible for neurotransmitter release at the presynaptic side and recruitment of neurotransmitter receptors to the postsynaptic density (PSD). Factors capable of triggering synapse formation are thought to include both membrane-bound and diffusible signals[1]. Because of the tight apposition between pre- and postsynaptic membranes, cell adhesion molecules have long been thought to have instrumental roles in holding synaptic contacts together. More recently, the participation of cell adhesion molecules in synapse development through intracellular signaling mechanisms has been widely recognized[2], and several adhesion molecules having instructive roles in presynaptic differentiation have been identified, including neurexin-neuroligin[3,4] and SynCAM[5]. Unlike membrane-bound molecules, secreted factors may regulate synapse formation in a non–cell-autonomous manner over a wide target area. Target-derived molecules—such as Wnt7A[6] and FGF22 (ref. 7)—and factors secreted by astrocytes—for example, thrombospondins[8]—have been identified as presynaptic organizers and maturation signals in several systems. Neurotrophic factors, such as BDNF, have also been implicated in synapse formation and plasticity[9,10], although it is not clear whether their effects on synapse formation are permissive or instructive. These molecules are potent regulators of neurite outgrowth and spine stability[11–13], synaptic proteins expression[14] and long-term potentiation and synaptic plasticity[15]. Although any of those processes could result in an elevated number of synapses, they are mechanistically distinct from a direct, instructive role in the formation of synapses *de novo* at arbitrary sites.

GDNF is the prototypic member of a small family of neurotrophic factors that promote the survival, neurite outgrowth and differentiation of distinct populations of central and peripheral neurons[16]. Functional receptors for GDNF ligands are formed by a subunit specialized in transmembrane signaling—that is, the RET receptor tyrosine kinase[17,18] or neural cell adhesion molecule (NCAM)[19]—and another specialized in ligand binding—glycosylphosphatidyl inositol (GPI)-anchored coreceptors, known as GFRα1–4 (refs. 16,20). An interesting feature of GFRα molecules is that they can function in both membrane-bound and soluble forms after the release of their GPI anchor from the plasma membrane. Our previous work has demonstrated that GFRα1 can be released from the surface of neuronal and glial cells, and can function in soluble form or adsorbed to the extracellular matrix to present GDNF to neuronal terminals expressing RET receptors[21,22]. Neurons plated on an extracellular matrix containing GFRα1 and stimulated with GDNF develop large growth cone expansions decorated with actin-rich filopodia[22] that resemble structures induced by synaptogenic factors[6]. There have been indications that GDNF contributes to synapse development and maturation in ventral midbrain dopaminergic neurons and spinal cord motorneurons[23,24], but the underlying mechanisms remain unknown.

In the present work, we set out to investigate the expression and function of GDNF and its receptors in hippocampal neurons, including possible roles in synapse formation. The realization that GFRα1 was localized to both pre- and postsynaptic membranes prompted us to investigate possible homophilic interactions between GFRα1 molecules, resulting in the identification of a previously unknown mechanism for regulated cell-cell interaction that combines features of both diffusible and membrane-bound synaptogenic signals.

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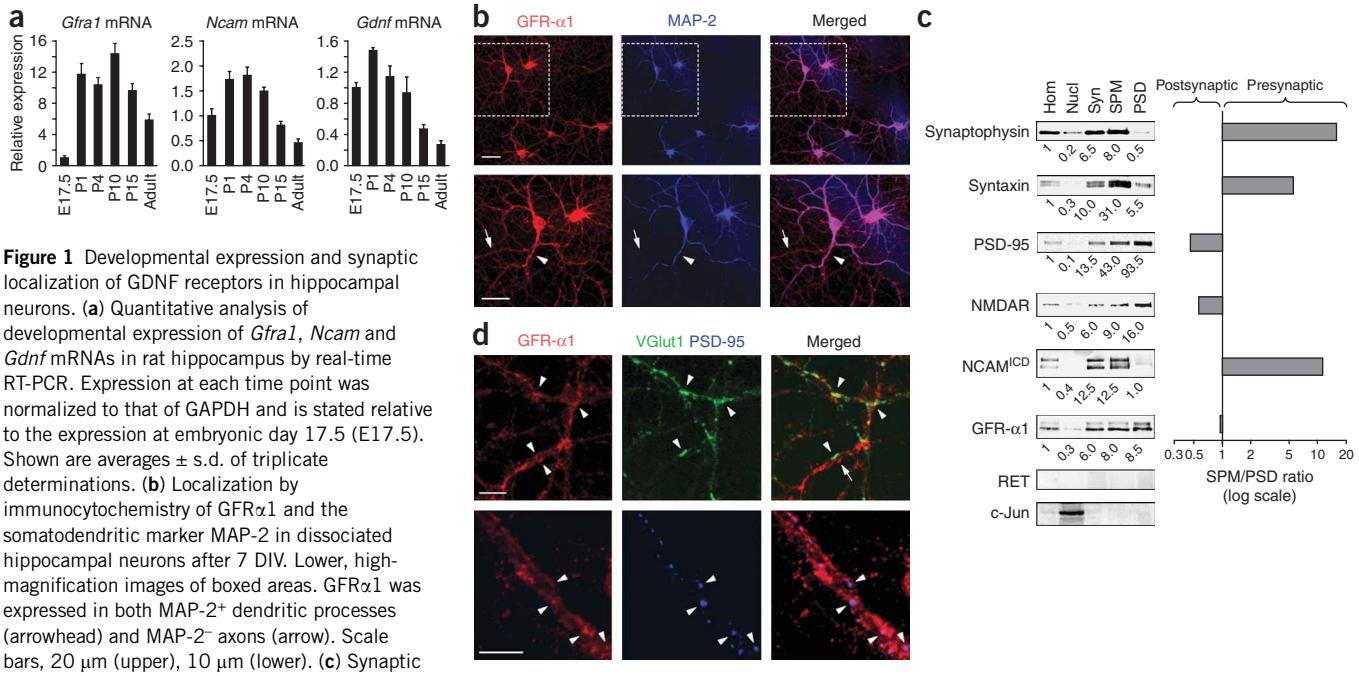



**Figure 1** Developmental expression and synaptic localization of GDNF receptors in hippocampal neurons. (**a**) Quantitative analysis of developmental expression of *Gfra1*, *Ncam* and *Gdnf* mRNAs in rat hippocampus by real-time RT-PCR. Expression at each time point was normalized to that of GAPDH and is stated relative to the expression at embryonic day 17.5 (E17.5). Shown are averages ± s.d. of triplicate determinations. (**b**) Localization by immunocytochemistry of GFRα1 and the somatodendritic marker MAP-2 in dissociated hippocampal neurons after 7 DIV. Lower, high-magnification images of boxed areas. GFRα1 was expressed in both MAP-2$^+$ dendritic processes (arrowhead) and MAP-2$^-$ axons (arrow). Scale bars, 20 μm (upper), 10 μm (lower). (**c**) Synaptic localization of GDNF receptors. Amounts of GFRα1, RET and transmembrane NCAM isoforms (NCAM$^{ICD}$) in total homogenates (Hom) and different subcellular fractions from P15 rat hippocampus were compared by western blotting (Nucl, nucleus; Syn, P2-synaptosomes). Expression relative to that of Hom is indicated. Right, log plot of SPM/PSD ratios. (**d**) Colocalization of GFRα1 with the vesicular presynaptic marker VGlut2 (green) and the postsynaptic marker PSD-95 (blue) in varicosities along processes of hippocampal neurons (arrowheads). GFRα1 was also detected in puncta that were in close apposition to VGlut2$^+$ sites (arrow). Scale bars, 5 μm.



## RESULTS

### Developmental expression and synaptic localization of GFRα1

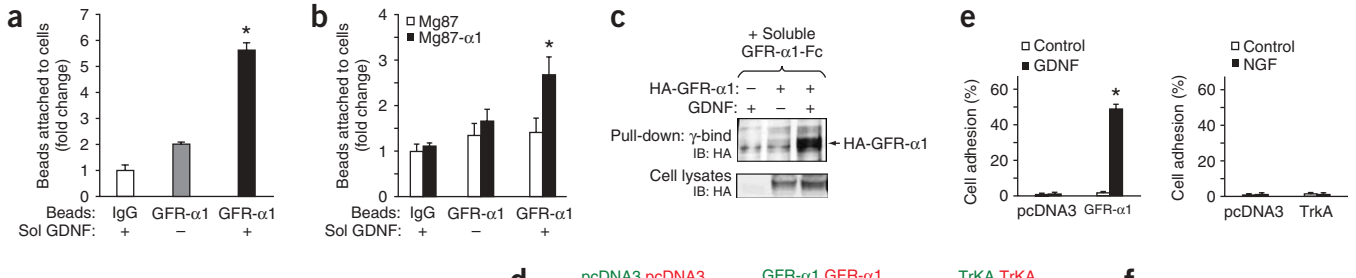
We assessed the expression of GDNF and its receptors in rat hippocampus by real-time RT-PCR during the first and second postnatal weeks, the main period of hippocampal synaptogenesis in rodents. A sharp increase in the amount of *Gfra1* mRNA was detected at birth, with a peak at postnatal day 10 (P10; **Fig. 1a**). Expression of *Ncam* and *Gdnf* mRNAs was also elevated during the same period (**Fig. 1a**). In contrast, no expression of *Ret* mRNA could be detected at the ages examined (**Supplementary Fig. 1** online). In dissociated cultures of hippocampal neurons, all cells were found to express GFRα1, with immunoreactivity localized to both cell bodies and neuritic processes (**Fig. 1b**). GFRα1 could be observed over both MAP-2$^+$ (that is, dendrite) and MAP-2$^-$ (that is, axon) processes (**Fig. 1b**, arrowhead and arrow, respectively), suggesting that GFRα1 may be localized to both pre- and postsynaptic compartments.

To assess the synaptic localization of GDNF receptors, we prepared fractions of synaptic plasma membrane (SPM), containing pre- and postsynaptic membrane specializations, and PSD from P15 rat hippocampus (see Methods). Enrichment in either fraction was quantified in comparison to amounts in total homogenates. A synaptosome fraction—that is, obtained before SPM isolation—and a nuclear fraction were also included as controls. As expected, the presynaptic markers synaptotagmin and syntaxin were enriched in the SPM fraction (SPM/PSD $>$ 1), whereas postsynaptic density protein of 95 kDa (PSD-95) and the 2B subunit of the NMDA receptor were enriched in the PSD fraction (SPM/PSD $<$ 1; **Fig. 1c** and **Supplementary Fig. 1**). GFRα1 was found in both SPM and PSD fractions (SPM/PSD = 1), indicating that this receptor was present in both pre- and postsynaptic compartments (**Fig. 1c**). Although GFRα1 was present throughout the cell (**Fig. 1b**), it was enriched in synaptic fractions compared with total homogenates (**Fig. 1c**). Transmembrane NCAM isoforms (NCAM$^{ICD}$) could only be detected in the SPM (**Fig. 1c**), indicating a predominant presynaptic location, whereas NCAM extracellular domain (NCAM$^{ECD}$) was found both pre- and postsynaptically (**Supplementary Fig. 1**), reflecting the presence of the GPI-anchored NCAM isoform in the PSD. RET could not be detected in hippocampal synaptic fractions (**Fig. 1c** and **Supplementary Fig. 1**).

In agreement with our fractionation experiments, GFRα1 was found colocalized with the presynaptic vesicular marker VGlut2 and the postsynaptic marker PSD-95 in varicosities present along many neuronal processes (**Fig. 1d**, arrowheads). GFRα1 was also detected in puncta that were in close apposition to presynaptic VGlut2$^+$ sites but that themselves lacked VGlut2 (**Fig. 1d**, arrow), in agreement with the presence of this receptor in apposed pre- and postsynaptic compartments.

### GDNF-dependent trans-homophilic GFRα1 interactions

The localization of GFRα1 to both pre- and postsynaptic membranes of hippocampal neurons prompted us to investigate possible functions of this protein in neuronal contact. We first assessed the attachment of microspheres coated with Fc-tagged GFRα1 (GFRα1-Fc) to hippocampal cells in the presence and absence of GDNF. We found that many more GFRα1-coated microspheres remained bound to hippocampal cells in the presence of GDNF compared with IgG-coated microspheres (**Fig. 2a**). This effect was dependent on the presence of soluble GDNF, although low binding could also be detected in its absence, presumably as a result of GDNF endogenously produced by hippocampal cells. To verify the dependence of this effect on the cellular expression of GFRα1, we tested the ability of microspheres to adhere to either control or GFRα1-expressing MG87 fibroblasts. Microspheres coated with



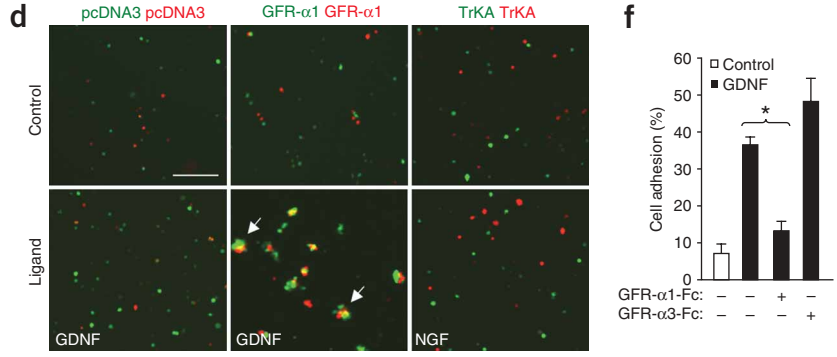




**Figure 2** GDNF-dependent trans-homophilic interactions between GFRα1 molecules promote cell adhesion. (**a**) Binding of coated beads to hippocampal neurons in the presence or absence of soluble (Sol) GDNF. The results are expressed as the average number of beads that remained bound to cells after a 3-h incubation (normalized to IgG) ± s.d. from an experiment performed in triplicate. $*P < 0.005$ versus IgG, ANOVA. (**b**) Binding of coated beads to MG87 and MG87-α1 cells in the presence or absence of GDNF. Results are expressed as the average number of beads that remained bound to cells after a 1-h incubation (normalized to IgG) ± s.d. from an experiment performed in triplicate. $*P < 0.05$ versus MG87, Student's *t*-test. (**c**) Pulldown of HA-tagged GFRα1 from living COS cells with soluble GFRα1-Fc and GDNF. IB, immunoblot. (**d**) Cell adhesion assay in transiently transfected Jurkat cells. Arrows indicate mixed cell aggregates, that is, those containing both green and red cells. Scale bar, 100 μm. (**e**) Percentage of green cells present in mixed cell aggregates under the indicated conditions ± s.d. from an experiment performed in triplicate. $*P < 0.005$, Student's *t*-test versus control. Similar results were obtained in three independent experiments. (**f**) Soluble GFRα1 (100 M excess) blocked GDNF-induced adhesion of GFRα1-expressing Jurkat cells. Soluble GFRα3 had no effect. $*P < 0.01$, Student's *t*-test.

GFRα1-Fc bound preferentially to cells expressing GFRα1 and only in the presence of exogenous GDNF (**Fig. 2b**), suggesting that GDNF was able to induce homophilic interactions between GFRα1 molecules in *trans*. Biochemical evidence in support of this concept was obtained from pull-down experiments using GFRα1 molecules that carried different epitope tags. Only in the presence of GDNF could hemagglutinin (HA)-GFRα1 be recovered from transfected COS cells after incubation of live cells with soluble GFRα1-Fc (**Fig. 2c**), demonstrating that distinct GFRα1 molecules can interact with each other in *trans* in a ligand-dependent manner.

Many synaptogenic factors, including neurexin-neuroligin, SynCAM and the Eph-ephrin ligand-receptor system, function in part by promoting cell adhesion between pre- and postsynaptic membranes[2], suggesting that this may be an intrinsic property of membrane-bound synaptogenic proteins. We therefore investigated whether the ability of GDNF to promote trans-homophilic interactions between GFRα1 molecules could support cell adhesion. To this end, we performed cell aggregation assays with Jurkat cells expressing GFRα1 together with either green or red fluorescent proteins. GFRα1-expressing cells grew as a suspension of dispersed individual cells in the absence of GDNF (**Fig. 2d**). On addition of GDNF, however, green and red GFRα1-expressing cells formed aggregates among themselves and with each other (**Fig. 2d**). GDNF had no effect on cells transfected with a control plasmid. Quantitative analysis showed that the effect of GDNF on cell adhesion was very robust, increasing the proportion of green cells present in mixed cell aggregates by 10- to 25-fold compared with control (**Fig. 2e**), an effect comparable to that of bona fide cell adhesion molecules such as NCAM (**Supplementary Fig. 2** online). Although

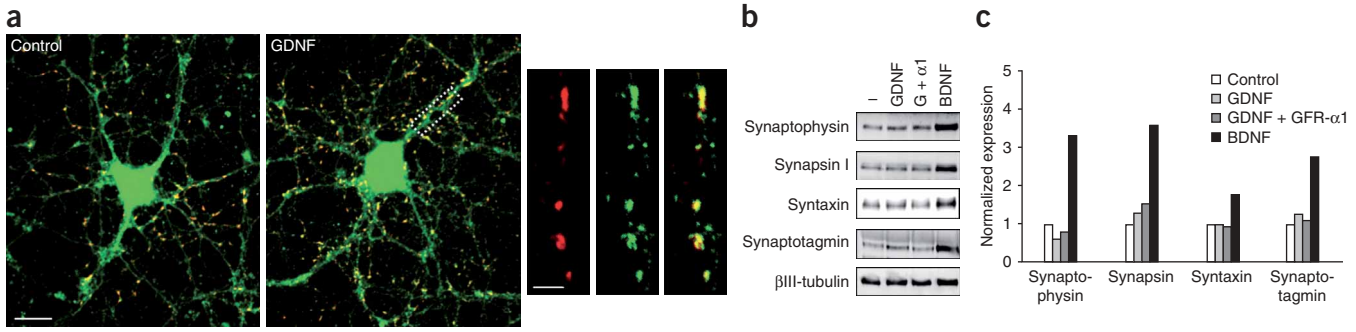



**Figure 3** GDNF increases the colocalization of pre- and postsynaptic markers, but not the expression of presynaptic proteins, in hippocampal neurons. (**a**) Colocalization of pre- and postsynaptic markers synapsin I (red) and PSD-93 (green) in hippocampal neurons treated with GDNF for 12 d. Right, high-magnification images of boxed area in GDNF panel. Scale bars, 10 μm (left), 2 μm (right). (**b**) Western blotting analysis of presynaptic protein expression in total lysates of hippocampal cells after 5 DIV with the indicated factors. βIII-tubulin was used as a loading control. (**c**) Quantification of presynaptic protein expression (from western blot data shown in **c**) normalized to βIII-tubulin.

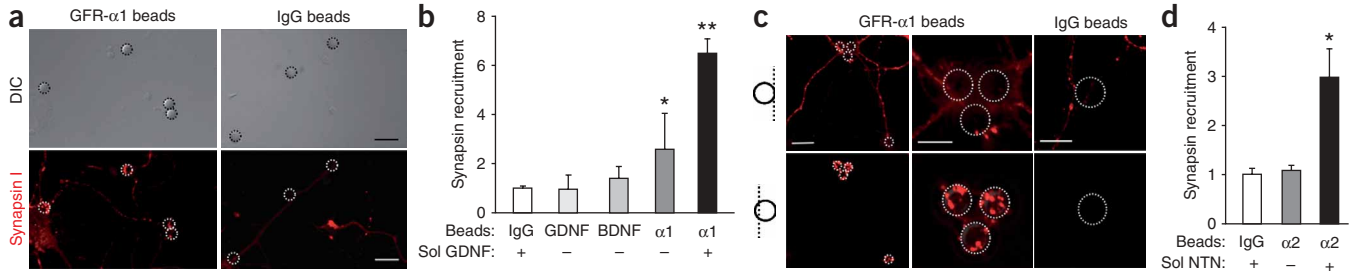



**Figure 4** Localized induction of presynaptic assembly by immobilized GFRα1 in the presence of GDNF. (**a**) Differential interference contrast (DIC) and synapsin I immunocytochemistry of neuronal hippocampal cultures 2 d after addition of polystyrene beads (dotted circles) coated with IgG or recombinant GFRα1-Fc in the presence of soluble GDNF. Scale bar, 10 μm. (**b**) Quantification of recruitment of synapsin I clusters by beads coated with different proteins in the presence or absence of soluble GDNF. α1, GFRα1. Results indicate the average intensity per bead (normalized to IgG) ± s.d. of a representative experiment performed in triplicate wells (15–20 beads analyzed per well). $^{**}P < 0.005$ versus IgG; $^{*}P < 0.05$ versus GFRα1 + GDNF, ANOVA. Similar results were obtained in three independent experiments. (**c**) High-magnification images of synapsin I staining of hippocampal neurites growing on beads coated with IgG or GFRα1-Fc (dotted circles) in the presence of GDNF. Two different confocal planes are shown. Clusters of synapsin I located above the bottom plane of the dish (lower) indicate enwrapping of GFRα1 beads by hippocampal neurites. Scale bars, 10 μm (left), 5 μm (middle and right). (**d**) Recruitment of synapsin I clusters by beads coated with GFRα2. α2, GFRα2. $^{**}P < 0.005$, ANOVA.

GFRα1 can interact with NCAM in *cis*[19], we found no evidence that the two molecules interacted in *trans* to mediate ligand-induced cell adhesion (**Supplementary Fig. 3** online). Addition of soluble GFRα1 blocked GDNF-induced adhesion of GFRα1-expressing Jurkat cells, whereas soluble GFRα3, which does not bind GDNF[25], had no effect (**Fig. 2f**). On the other hand, cell adhesion could not be prevented by treatment with a battery of small-molecule signaling inhibitors, including blockers of the Src, MAP kinase and PI3K pathways (data not shown). Together, the data from microsphere binding, biochemical and cell aggregation assays are consistent with the hypothesis that GDNF-induced cell adhesion involves physical contact between GDNF-bound GFRα1 molecules in opposite cells, indicating the ability of GFRα1 to function as a LICAM.

GDNF is a dimeric molecule with twofold symmetry[26], and cross-linking experiments have shown that it promotes dimerization of GFRα1 on cell membranes[27]. However, a mere capacity to dimerize on ligand binding is not sufficient for a molecule to function as a LICAM, as Jurkat cells transfected with the nerve growth factor (NGF) receptor TrkA failed to form cell aggregates in either the presence or absence of NGF (**Fig. 2d,e**), which is known to induce receptor dimers on binding[28]. To identify the domains of the GFRα1 molecule underlying its LICAM activity, we performed adhesion assays between cells expressing different deletion constructs. GFRα1-ΔN144 lacks the first globular domain[29] but retains ligand binding[25], whereas GFRα1-ΔN161 also lacks the first α-helix of the second domain and is therefore unable to interact with GDNF[25]. GFRα1-ΔN144 could function as a LICAM to the same extent as wild-type GFRα1, but GFRα1-ΔN161 was completely inactive (**Supplementary Fig. 4** online), indicating that the ability of GFRα1 to mediate cell adhesion requires ligand binding. Moreover, cells expressing GFRα1-ΔN161 did not interact with cells that received wild-type GFRα1 (**Supplementary Fig. 4**), indicating that GFRα1-mediated cell adhesion requires the presence of an intact ligand binding domain in both interacting partners.

## GDNF increases colocalization of pre- and postsynaptic markers

Together, the temporal expression pattern of GDNF and GFRα1 in the developing hippocampus, the localization of GFRα1 to pre- and postsynaptic membranes, and the ability of this receptor to function as a LICAM in the presence of GDNF suggest that GDNF and GFRα1 could act as synaptogenic factors in hippocampal neurons. A known property of synaptogenic molecules is their ability to induce the clustering of synaptic vesicles at sites of contact with target cells and the colocalization of pre- and postsynaptic markers[3]. The number of synaptophysin puncta colocalizing with MAP-2 over a fixed dendritic length was increased in hippocampal cultures treated with GDNF (**Supplementary Fig. 5** online). Notably, GDNF treatment augmented

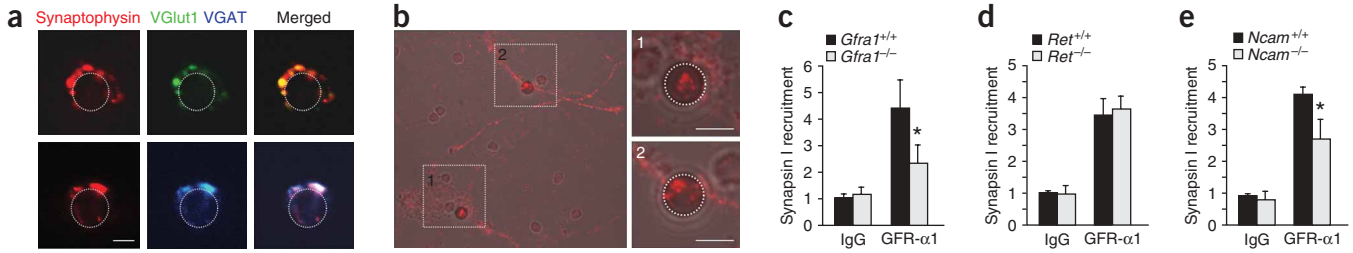



**Figure 5** Excitatory and inhibitory presynaptic differentiation, activity-dependent vesicle recycling, and requirement of GFRα1 and NCAM but not RET. (**a**) Colocalization of synaptophysin and VGlut1 (green) or VGAT (blue) in presynaptic clusters induced by GFRα1-coated beads (dotted circles) in the presence of soluble GDNF. Scale bar, 2.5 μm. (**b**) Activity-dependent uptake of antibodies against the luminal domain of synaptotagmin. Hippocampal cultures were incubated with IgG- or GFRα1-coated beads in the presence of soluble GDNF and depolarized in the presence of antibodies. Micrographs show superimposed DIC and synaptotagmin immunocytochemistry of hippocampal axons in contact with polystyrene beads coated with GFRα1 in the presence of soluble GDNF. Right, high-magnification images of areas boxed in left panel. Scale bar, 5 μm. (**c**–**e**) Synapsin I recruitment by beads coated with IgG or GFRα1-Fc in the presence of GDNF in wild-type hippocampal neurons and in neurons lacking GFRα1 (**c**), RET (**d**) or NCAM (**e**). Histograms show averages ± s.d. of triplicate measurements. For each condition, cultures obtained from 3–4 animals from each genotype were evaluated. $^{*}P < 0.05$ versus wild type, Student's *t*-test.

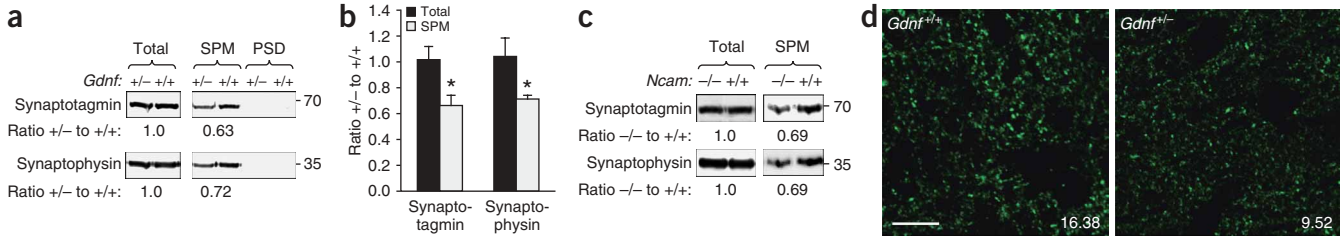



**Figure 6** Requirement of GDNF signaling for hippocampal presynaptic assembly and maturation *in vivo*. (**a**) Localization of presynaptic proteins to hippocampal synapses in P15 *Gdnf* heterozygous mice. Total hippocampal homogenates (15 μg), SPM and PSD fractions (3 μg) were analyzed by western blotting with antibodies to synaptotagmin and synaptophysin. (**b**) Synaptotagmin and synaptophysin content in hippocampal total extracts and SPM fractions of P15 *Gdnf* $^{+/-}$ mice relative to wild type. Results are expressed as average ± s.d. from three independent experiments, each involving 3–4 animals from each genotype. $^{*}P < 0.01$ versus Total, Student's *t*-test. (**c**) Recruitment of presynaptic proteins to hippocampal synapses in P15 *Ncam* knockout mice. Ratios between mutant and wild type from a representative experiment are indicated. (**d**) Confocal sections of P15 stratum radiatum of CA1 immunostained for synaptophysin in wild-type (*Gdnf* $^{+/+}$) and heterozygous mutant (*Gdnf* $^{+/-}$) mice. Densities of synaptophysin puncta per 100 μm$^2$ are indicated. Scale bar, 10 μm.

the number of puncta showing colocalization of synapsin I and PSD-93 (10.33 ± 0.68 versus 15.61 ± 1.24 colocalized puncta per neuron, $P < 0.02$; **Fig. 3a**), indicating an increase in synapse formation in hippocampal neurons.

These effects were not due to enhanced expression of presynaptic proteins, as neither GDNF nor GDNF combined with soluble GFRα1, which is known to potentiate some of the effects of this factor[22], had any effect on the expression of presynaptic proteins (**Fig. 3b,c** and **Supplementary Fig. 5**). In contrast, BDNF increased the expression of all proteins tested (**Fig. 3b,c**), in agreement with previous observations[14].

### Localized induction of presynaptic assembly by GFRα1

Next, we tested whether a localized source of exogenous GFRα1, mimicking its postsynaptic localization, could induce presynaptic assembly and differentiation in the presence of GDNF. To this end, we supplied recombinant GFRα1-Fc immobilized on the surface of polystyrene microspheres to hippocampal cultures in the presence or absence of soluble GDNF. Microspheres coated with GFRα1-Fc induced a robust recruitment of synapsin I at contact sites with hippocampal axons (**Fig. 4a,b**). This activity was dependent on the presence of soluble GDNF, although a small effect could also be observed in the absence of added factor, presumably resulting from endogenously produced GDNF. We did not observe any effect of control microspheres coated with IgG or BSA (**Fig. 4a,b** and data not shown). Clusters of synapsin I were localized at different planes around GFRα1-coated microspheres (**Fig. 4c**), which appeared enwrapped by hippocampal neurites as shown by phalloidin staining (data not shown). Similar effects were observed with other presynaptic proteins, including synaptotagmin and synaptophysin (**Supplementary Fig. 6** online). Presynaptic differentiation could also be induced in cortical neurons (**Supplementary Fig. 6**). Despite BDNF's ability to upregulate presynaptic protein synthesis, BDNF-coated microspheres were ineffective at inducing clustering of presynaptic proteins (**Fig. 4b**). Microspheres that were directly coated with GDNF had no effect (**Fig. 4b**), suggesting that exogenous GFRα1 is required for the synaptogenic effects of this factor. Microspheres coated with GFRα2-Fc were also able to recruit aggregates of synapsin I in hippocampal neurons treated with the GDNF homolog neurturin (NTN; **Fig. 4d** and **Supplementary Fig. 6**). Finally, synaptophysin aggregates induced by soluble GDNF and GFRα1-coated microspheres colocalized with either VGlut1 or VGAT (**Fig. 5a**), the vesicular transporters of glutamic acid and GABA, respectively, indicating that GFRα1 and GDNF can induce both excitatory and inhibitory presynaptic differentiation.

We then tested whether presynaptic vesicles recruited to sites of contact with GFRα1-coated beads were competent for vesicle recycling by monitoring activity-dependent uptake of antibodies against the luminal domain of synaptotagmin (see Methods). A robust induction of antibody uptake was observed at the sites of contact of GFRα1-coated beads in the presence of soluble GDNF, compared with control-coated beads (1.0 ± 0.27 versus 5.68 ± 0.16 normalized bead intensity, $P < 0.005$; **Fig. 5b**), indicative of functional presynaptic differentiation. No or weak antibody labeling was observed in the absence of depolarization and no increase over control was observed in the absence of soluble GDNF (data not shown).

### Requirement of GFRα1 and GDNF in presynaptic differentiation

GFRα1-deficient neurons showed a marked decrease in the recruitment of synapsin I by GFRα1-coated microspheres (**Fig. 5c**), indicating a cell-autonomous requirement of GFRα1 for presynaptic differentiation. The residual recruitment of synapsin I observed in knockout neurons could be due to the presence of GFRα2 in these cells, to which GDNF can also bind[30]. In contrast, RET-deficient neurons responded normally to GFRα1-coated beads in the presence of GDNF (**Fig. 5d**), whereas those lacking NCAM showed a significant, but milder, impairment (**Fig. 5e**). In agreement with this, $^{125}$I-labeled GDNF binding to NCAM, but not to RET, could be detected in hippocampal synaptosomes (**Supplementary Fig. 7** online). Together, these data indicated that presynaptic differentiation induced by GDNF and exogenous GFRα1 requires presynaptic GFRα1 receptors and is independent of RET, but only partially dependent on NCAM, suggesting that additional signaling molecules are involved.

To evaluate the contribution of GDNF signaling to hippocampal synaptogenesis *in vivo*, we investigated the levels of presynaptic proteins in SPM fractions isolated from hippocampi of *Gdnf* mutant mice. Because homozygous *Gdnf* mutants do not survive after birth, heterozygous mutants were used for these experiments. Real-time RT-PCR analysis confirmed that hippocampi from P15 *Gdnf* $^{+/-}$ mice express reduced amounts of *Gdnf* mRNA compared to wild type (**Supplementary Fig. 7**). Total homogenates of P15 wild-type and mutant hippocampi contained identical amounts of synaptotagmin and synaptophysin (**Fig. 6a,b**), confirming that GDNF does not affect the overall expression of these molecules *in vivo*. In contrast, presynaptic protein amounts were reduced by 30% in SPM fractions isolated from P15 *Gdnf* $^{+/-}$ hippocampi compared to wild type (**Fig. 6a,b**), indicating reduced synaptic incorporation of these proteins in the mutants. A similar reduction was observed in SPM fractions from the

hippocampus of P15 *Ncam* knockout mice (**Fig. 6c**). Four weeks later, synaptophysin concentrations in SPM fractions of *Gdnf* $^{+/-}$ hippocampi were not significantly different from wild type ($P = 0.256$, **Supplementary Fig. 8** online), indicating that *Gdnf* haploinsufficiency causes a transient impairment during the synaptogenic period that recovers at maturity. Reduced synaptic localization of presynaptic proteins could reflect a deficit in either synapse maturation or number. We therefore examined the number of presynaptic terminals at P15 in brain cryosections prepared from wild-type and heterozygous *Gdnf* mutants by immunostaining with antibodies to synaptophysin, followed by confocal imaging. The density of synaptophysin puncta in the stratum radiatum of CA1 was markedly reduced in the hippocampus of heterozygous *Gdnf* mutants compared to wild-type mice (9.80 ± 1.48 versus 17.70 ± 1.13 puncta per 100 μm$^2$, $P < 0.001$; **Fig. 6d**). No difference in the density of dendritic fields in synaptic areas of the hippocampus was found between wild-type and *Gdnf* mutant mice (**Supplementary Fig. 8**), ruling out indirect effects due to morphological alterations. Together, these data indicate that reduced amounts of GDNF result in a decrease in presynaptic maturation and the number of presynaptic sites formed during hippocampal synaptogenesis, supporting a role for GDNF signaling in hippocampal presynaptic assembly *in vivo*.

## DISCUSSION

The results of the present study suggest an instructive role for GDNF and its receptor GFRα1 in presynaptic differentiation. This conclusion is supported by several observations: (i) the localization of GFRα1 at pre- and postsynaptic specializations in the developing hippocampus, (ii) the ability of GDNF to increase the association of pre- and postsynaptic markers in hippocampal neurons independently of changes in neurite length or marker expression, (iii) the ability of an ectopic source of immobilized GFRα1 to induce the recruitment of clusters of vesicular presynaptic proteins and neurotransmitter transporters to sites of contact on hippocampal axons in a GDNF-dependent manner, (iv) the requirement of presynaptic GFRα1 for the induction of presynaptic differentiation by ectopic GFRα1 and GDNF and (v) the reduced number of presynaptic sites formed during hippocampal synaptogenesis in *Gdnf* $^{+/-}$ mice. Although several other factors have previously been implicated in synapse formation, the mechanism by which GDNF and GFRα1 contribute to this process is unique, as it combines features of both membrane-bound and soluble signals.

GFRα1 and GFRα2 are broadly expressed in many areas of the mammalian forebrain, particularly the cerebral cortex and hippocampus[30,31], where they are thought to contribute to neuronal differentiation and migration in a cell-autonomous manner together with transmembrane effector proteins[32]. Non–cell-autonomous functions have also been suggested, on the basis of the ability of GFRα molecules to present GDNF ligands in *trans*[21,22]. The newly discovered functions of GDNF and GFRα1 in presynaptic differentiation proposed here would seem to make use of both types of mechanisms: (i) postsynaptic GFRα1 may act non–cell-autonomously to present GDNF to presynaptic receptors and to facilitate the alignment of pre- and postsynaptic membranes through ligand-induced trans-homophilic interactions, and (ii) presynaptic GFRα1 may act cell-autonomously in ligand-mediated cell adhesion and induction of presynaptic differentiation in collaboration with transmembrane effector molecules such as NCAM. Whether postsynaptic GFRα1 molecules may also contribute cell-autonomously to the differentiation of the PSD remains to be explored.

The ability of microspheres coated with GFRα1 to induce the formation of clusters of presynaptic proteins *de novo*, at arbitrary contact sites with axons, and in the presence—but not in the absence—of GDNF would support an instructive role for GDNF signaling in hippocampal synaptogenesis. Notably, its dependence on soluble GDNF distinguishes the activity of GFRα1-coated microspheres from the effects of beads coated with positively charged substances and other factors reported in earlier studies[33]. Moreover, the fact that microspheres coated with GDNF alone had no effect indicates that the ability of ectopic GFRα1 to induce presynaptic differentiation entails more than mere ligand clustering or presentation, and suggests an active role of trans-homophilic GFRα1 interactions in synapse induction. In agreement with this notion, presynaptic differentiation required neuronal expression of GFRα1, but was independent of RET and partially dependent on NCAM, suggesting the participation of additional transmembrane effectors.

*Gdnf* haploinsufficiency caused a reduction in the synaptic localization of presynaptic proteins at P15, but not at 6 weeks of age, indicating a transient impairment of presynaptic differentiation in these mutants. This transient defect could be due either to compensatory mechanisms similar to those affecting Wnt-7a function in the cerebellum[6] or, perhaps more likely, to the partial reduction in the amount of GDNF that is achieved in heterozygous mutants. At P15, even a partial decrease in *Gdnf* expression resulted in a marked reduction in the density of hippocampal presynaptic sites, in agreement with a role for GDNF signaling in presynaptic assembly. It remains possible that complete ablation of GDNF expression may cause more pronounced and long-lasting defects. Regardless, our present results demonstrate a requirement for GDNF signaling in presynaptic differentiation and maturation *in vivo* and are consistent with an important function for this pathway in hippocampal development and function. Several previous studies have described GDNF expression in neuronal cells of the developing rodent and human hippocampus[31,34]. Likewise, neuronal activity has been shown to upregulate GDNF expression in hippocampal neurons[31,35], although whether GDNF is released only tonically or through a regulated pathway is not known at present. Notably, learning defects and cognitive impairments have been reported in *Gdnf* and *Gfra2* mutant mice[36,37], and the importance of NCAM for synapse formation and function has been demonstrated in several studies[38–41].

The majority of cell adhesion mechanisms known to date involve interactions that occur by default, triggered by the mere encounter of cell adhesion molecules with their ligands. Notable exceptions include some members of the integrin family whose adhesive properties can be activated through inside-out signaling in response to external stimuli[42], allowing the regulation of cell-cell interactions over shorter time-scales. The ability of GDNF to trigger trans-homophilic interactions between GFRα1 molecules represents a previously unknown mechanism for regulated cell-cell interactions in which the receptor for the triggering ligand is the actual mediator of intercellular contact (**Supplementary Fig. 9** online). We have named this type of receptor a ligand-induced cell adhesion molecule or LICAM. In principle, GDNF could promote trans-homophilic interactions between GFRα1 molecules by acting as a physical bridge between them. However, other ligands known to induce receptor dimerization, such as NGF, were unable to induce adhesion between receptor-expressing cells. In addition, the fact that GFRα1-Fc fusion proteins, which are preformed covalent dimers, were also able to mediate ligand-dependent trans-homophilic interactions would argue against trans-homodimerization as the underlying mechanism. Another possibility is that GDNF acts through an allosteric mechanism by inducing a conformational change in GFRα1 that exposes determinants responsible for trans-homophilic binding. Distinguishing between these mechanisms may require high-resolution imaging of complexes of GFRα1 and GDNF.

Several cell adhesion molecules have been reported to participate in synapse development, including cadherins, protocadherins, integrins, NCAM, L1, fasciclin, Syg, Sidekicks, SynCam and neurexin-neuroligin[2]. Unlike those examples, cell adhesion mediated by GFRα1 was dependent on GDNF, and thus combines features of synaptogenic mechanisms mediated by both soluble and membrane-bound mediators. The formation of specific synaptic contacts is likely governed by the cooperative participation of several different classes of synaptogenic factors. By stimulating contact between pre- and postsynaptic membranes, GDNF may also trigger interactions between other pre- and postsynaptic ligand-receptor systems and thereby indirectly elicit signaling events that could also contribute to synapse formation and development. This newly discovered mechanism considerably expands the functional repertoire of the GDNF and GFRα1 signaling system, and represents a new way to regulate intercellular interactions that may have broad implications for the development of the vertebrate nervous system and possibly other tissues and organs.

## METHODS

**Materials, RT-PCR, neuronal cultures, biochemistry and immunohistochemistry.** Description of recombinant proteins, primers, antibodies, cell lines, mouse lines, and methods for RT-PCR, neuronal culture, western blotting, pull-down and cross-linking assays, colocalization of pre- and postsynaptic markers, immunohistochemistry and microscopy can be found in the **Supplementary Methods** online. Animal experiments were approved by Stockholms Norra djurförsöksetiska nämnd.

**Synaptic plasma membrane and postsynaptic density fractionation.** Hippocampi were homogenized in ice-cold 25 mM Tris-HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA and proteinase inhibitors. Tissue homogenization was performed by 40 strokes in a glass homogenizer. After centrifugation at 1,000*g* for 10 min, the pellets were washed twice in homogenization buffer and then discarded or else processed for extraction of nuclear proteins. Combined supernatants were centrifuged again at 13,000*g* for 20 min to obtain a synaptosomal-mitochondrial pellet. After two washes, the pellets were resuspended in double-distilled water, pH 6.8 (osmotic shock), and centrifuged at 20,000*g* for 30 min. The resulting pellets were then resuspended in homogenization buffer, loaded in a discontinuous sucrose gradient (0.8–1.2 M) and centrifuged at 100,000*g* for 90 min. The material floating at 1.2 M sucrose was collected, washed in the same buffer, re-pelleted by centrifugation at 150,000*g* for 90 min and finally resuspended in 25 mM Tris-HCl, pH 7.4, to obtain the SPM fraction. PSD fractions were prepared by extraction of SPM with 1% Triton X-100 as previously described[43]. Although the PSD is included in the SPM, the former represents only a small fraction of the latter, so that the SPM appears enriched in presynaptic components when equal amounts of protein are compared. Nuclear proteins were extracted from the first pellet in 20 mM HEPES (pH 7.9), 0.4 M KCl, 1.5 mM $Cl_2Mg$, 0.5 mM EDTA, 1 mM DTT and 1% Nonidet P-40 plus proteinase inhibitors for 30 min at 4 °C, and then centrifuged at 14,000*g* for 30 min. Twenty-five micrograms of protein from homogenate and nuclear fractions and 5 μg of protein from synaptosomal, SPM and PSD fractions were loaded for analysis by SDS-PAGE and immunoblotting.

**Synaptic differentiation and vesicle recycling.** For presynaptic differentiation assays, 6-μm polystyrene microspheres (Polybeads, Polysciences) were coated with 25 μg $ml^{-1}$ of recombinant GFRα1-Fc, GFRα2-Fc, GDNF, BDNF, IgG or BSA in borate buffer (pH 8.0) overnight at 4 °C, and then subjected to several washes in PBS. Microspheres were added to 7-d-old dissociated neuronal cultures in the presence or absence of soluble GDNF. Recruitment of presynaptic protein clusters at the sites of contact between axons and microspheres was scored 2 d later by immunocytochemistry. Images were acquired with a Zeiss LSM 510 confocal microscope in ten different confocal planes using a 63× objective. The average intensity of presynaptic protein staining, integrated across all confocal planes, was measured on the bead (B) and in a neighboring radial area (NA) one bead diameter away, using OpenLab software (Improvision). The B/NA intensity ratio was then calculated, averaged across a minimum of 45 beads from three different wells and normalized to the value obtained with IgG beads.

Depolarization with KCl in the presence of antibodies to the luminal domain of synaptotagmin causes fusion of presynaptic vesicles with the plasma membrane, allowing binding of antibodies against the luminal domain of synaptotagmin. After vesicle retrieval by endocytosis, antibodies become internalized along with presynaptic vesicles[44,45]. We used this assay to test whether presynaptic vesicles recruited to sites of contact with GFRα1-coated beads were competent for activity-dependent membrane fusion and endocytosis, a sign of their functionality. Hippocampal neurons exposed to GFRα1-coated or control beads in the presence of GDNF (for 48–72 h) were incubated for 5 min in a depolarization solution (90 mM KCl) in the presence of antibody to synaptotagmin luminal domain (1:50, Synaptic Systems). After a 5-min incubation, the cells were washed five times in medium, fixed, permeabilized and stained with fluorescence-conjugated secondary antibody (Jackson ImmunoResearch).

**Bead binding and cell adhesion assays.** After 7 (for hippocampal cells) or 2 (for fibroblasts) days *in vitro* (DIV), polystyrene microspheres coated with IgG or GFRα1-Fc (see above) were added to cell cultures in the presence or absence of GDNF (100 ng $ml^{-1}$). After a 1–3-h incubation in serum-free medium, the cells were washed with DMEM to remove unbound beads and the number of beads that remained adhered to the cells was counted. For cell adhesion assays, Jurkat cells were transfected in 12-well plates with GFRα1 or NCAM constructs together with either GFP- or dsRED-encoding plasmids using Fugene-6 (Roche) in 2 ml complete medium with 10% FCS. On the following day, 100 μl of each GFP- and dsRED-transfected cells were combined and mixed with 100 μl of serum-free medium in 48-well plates in the presence or absence of GDNF. After a 48-h incubation, green cells, red cells and cell aggregates were quantified under green and red fluorescence illumination on a motorized Axiovert 200 microscope controlled by OpenLab software (Improvision). Cell adhesion was expressed as the percentage of green cells present in clusters that also contained red cells. Receptor expression in Jurkat cells was confirmed by western blotting (**Supplementary Fig. 9**) and immunocytochemistry (**Supplementary Fig. 10** online).

**Statistical analyses.** Data were analyzed with the program Statview (SAS Institute Inc) using the Student's *t*-test for bead binding assay (**Fig. 2b**), cell adhesion assay (**Figs. 2e,f** and **Supplementary Fig. 4**), quantification of synaptic puncta (**Figs. 3a** and **6d** and **Supplementary Figs. 5** and **8**), antibody uptake (**Fig. 5b**), recruitment of synaptic clusters (**Figs. 5c–e**), quantification of synaptic proteins in synaptic extracts (**Fig. 6b**) and *Gdnf* mRNA (**Supplementary Fig. 7**), and ANOVA for bead binding assay (**Fig. 2a**) and recruitment of synaptic clusters (**Figs. 4b,d** and **Supplementary Fig. 6**).

*Note: Supplementary information is available on the Nature Neuroscience website.*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### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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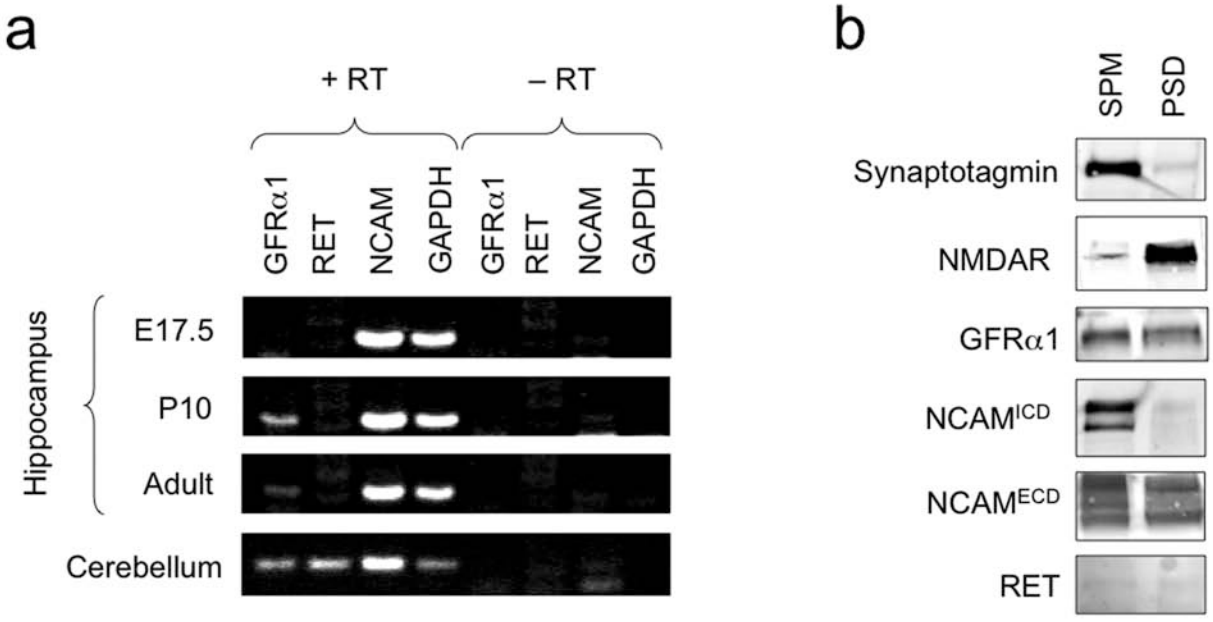


**Figure S1 Expression of GDNF receptors in hippocampus.**
(**a**) RT-PCR analysis of GDNF receptor expression during rat hippocampal development. GAPDH and cerebellar samples were used as controls. RT, reverse transcriptase.
(**b**) Expression of GDNF receptors GFRα1, RET, transmembrane NCAM isoforms ($NCAM^{ICD}$) and all NCAM isoforms ($NCAM^{ECD}$) in synaptic plasma membrane (SPM) and post-synaptic density (PSD) of P15 rat hippocampus analyzed by Western blotting. Synaptotagmin and NMDAR were used as pre- and post-synaptic markers, respectively, to control for enrichment in the corresponding fractions.

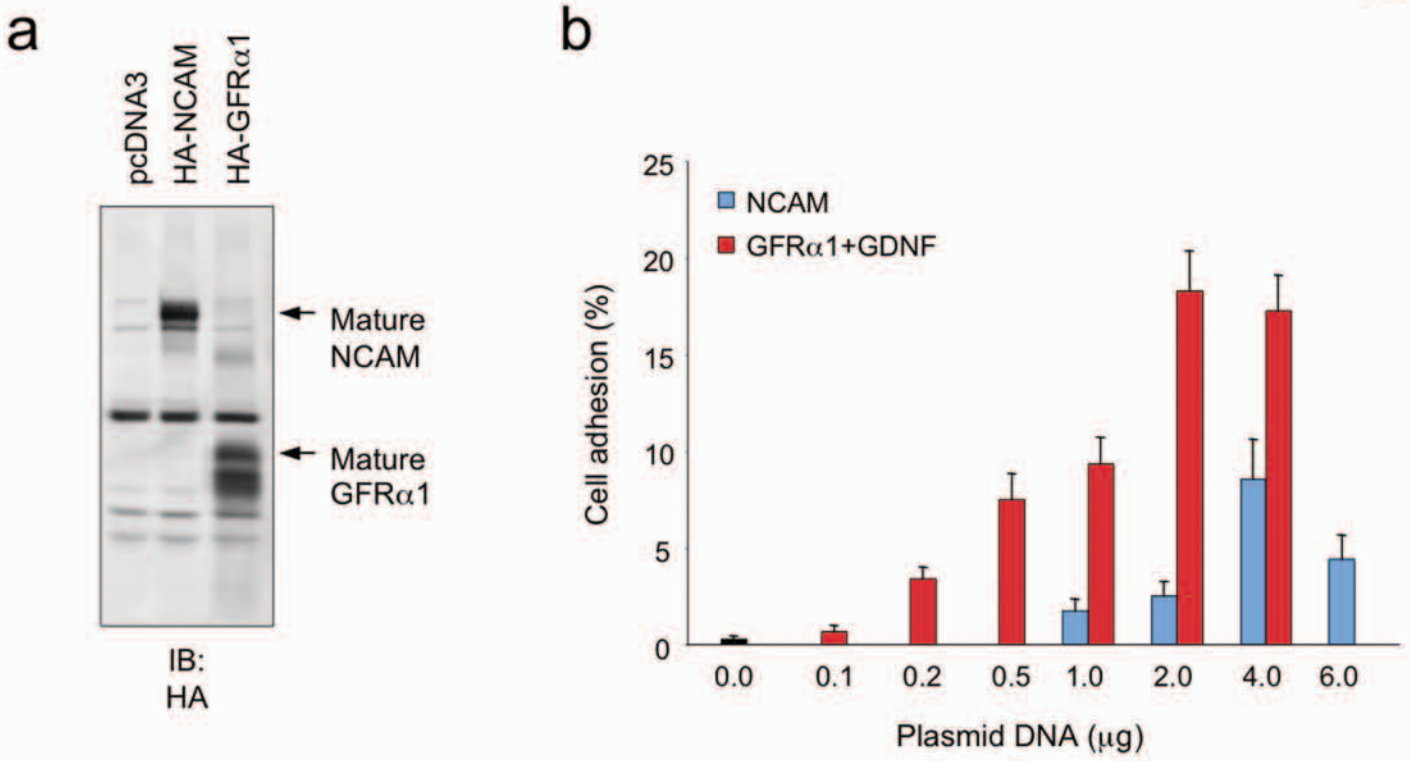


**Figure S2 Comparison between the adhesive activities of GFRα1 and NCAM.**
(**a**) Western blot analysis of HA-tagged NCAM and GFRα1 expression in COS cells transfected with equal amounts (5 μg) of plasmid DNA. The migration of mature (i.e. plasma membrane) NCAM and GFRα1 are indicated. Note comparable levels of expression of the two molecules. (**b**) Comparative analysis of cell adhesion induced by NCAM or by GFRα1 in the presence of GDNF in Jurkat cells transfected with different amounts of plasmids encoding HA-tagged NCAM or GFRα1. Note that lower levels of GFRα1 plasmid DNA compared to NCAM are required for comparable cell adhesion levels. Results are presented as average ± SD of quadruplicate determinations.

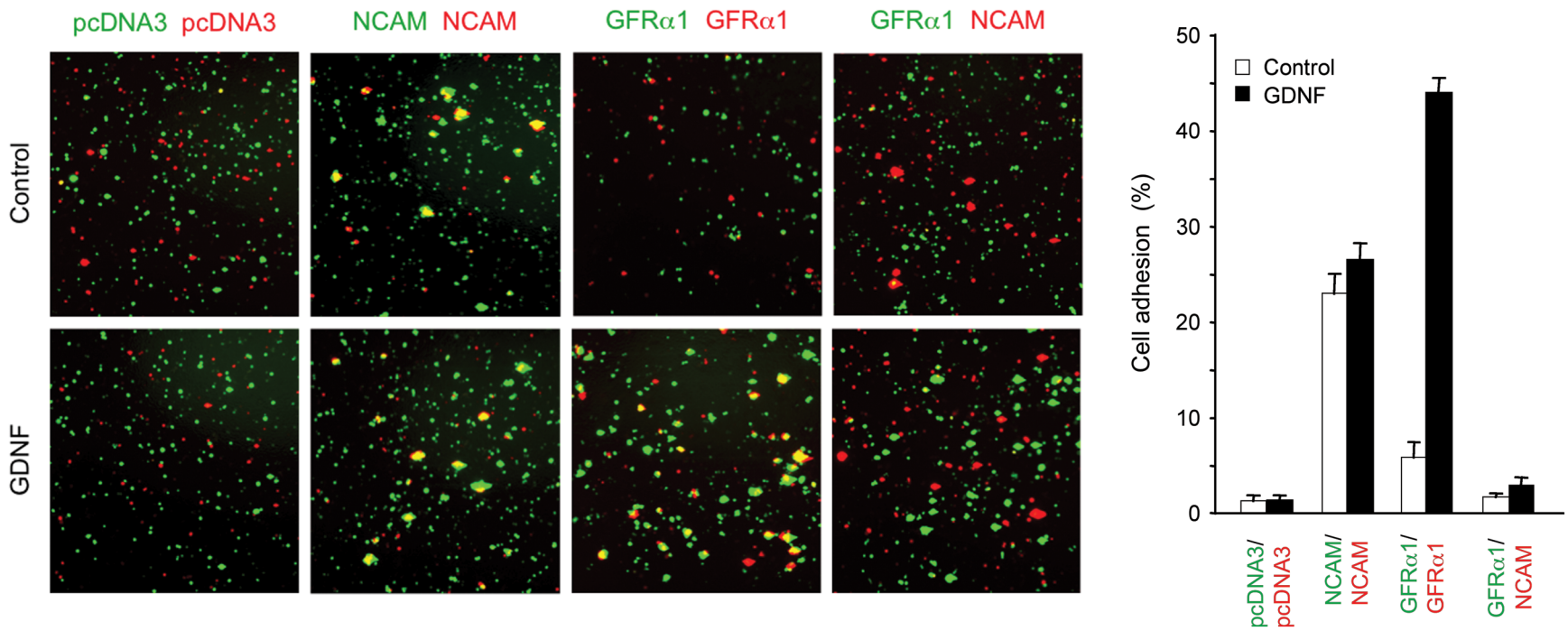


**Figure S3 Lack of heterophilic interactions in *trans* between GFRα1 and NCAM**
Adhesion between Jurkat cells expressing GFRα1 (green) and cells expressing NCAM (red) was tested. Homophilic configurations (i.e. NCAM/NCAM and GFRα1/GFRα1) were used as controls. Clusters of cells expressing NCAM were observed both without or with GDNF. Clusters of GFRα1-expressing cells were only seen in the presence of GDNF. No mixed cell aggregates containing both GFRα1- and NCAM-expressing cells could be observed above background in the presence or absence of ligand. Results are presented as average ± SD of quadruplicate determinations.

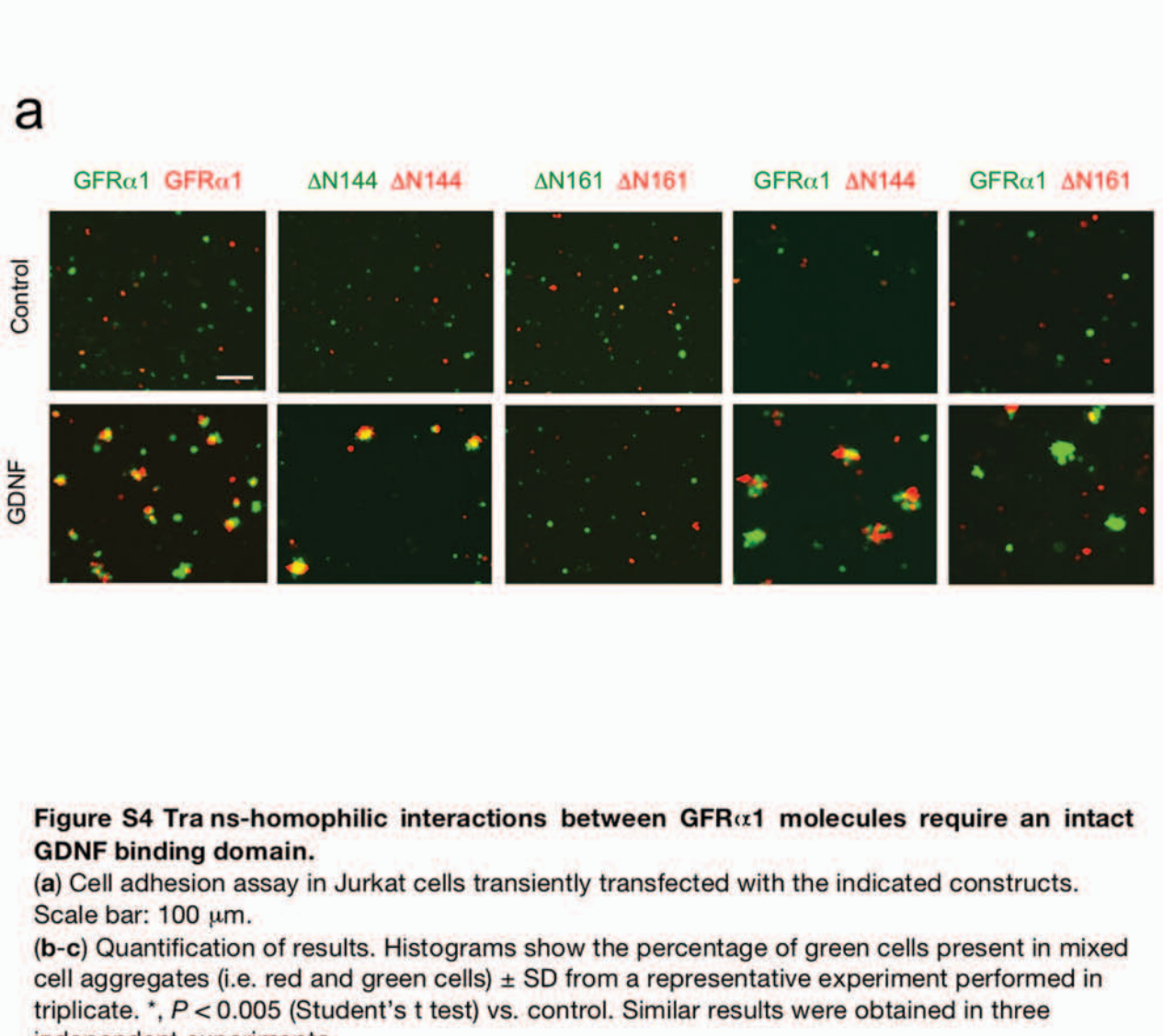


**Figure S4 Tra ns-homophilic interactions between GFRα1 molecules require an intact GDNF binding domain.**

(**a**) Cell adhesion assay in Jurkat cells transiently transfected with the indicated constructs. Scale bar: 100 μm.

(**b-c**) Quantification of results. Histograms show the percentage of green cells present in mixed cell aggregates (i.e. red and green cells) ± SD from a representative experiment performed in triplicate. *, $P < 0.005$ (Student's t test) vs. control. Similar results were obtained in three independent experiments.

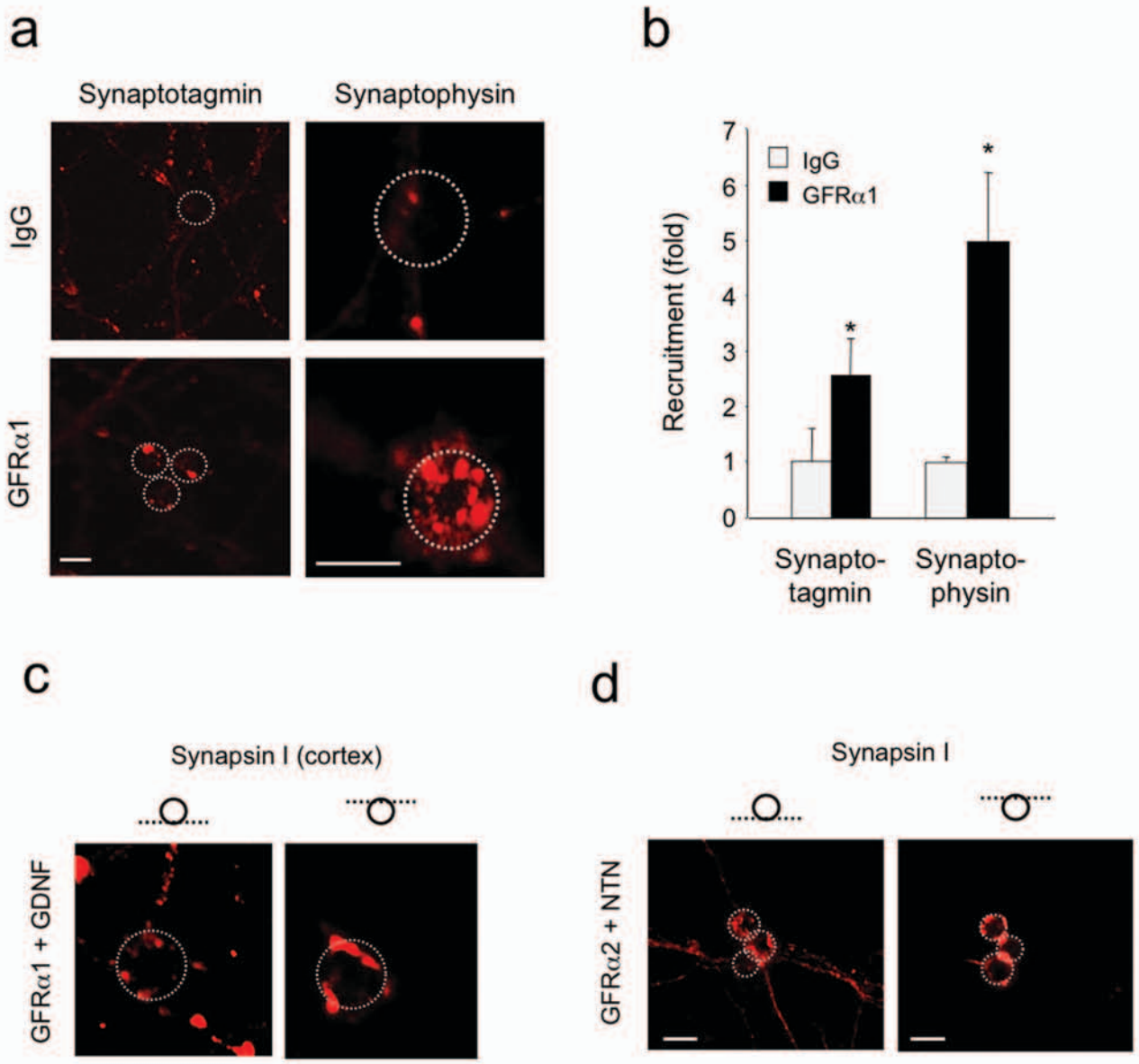


**Figure S6 Localized induction of pre-synaptic differentiation by immobilized GFRα proteins in the presence of GDNF ligands.**

(**a**) Recruitment of synaptotagmin and synaptophysin by beads coated with GFRα1-Fc in the presence of GDNF to axons of rat hippocampal neurons. Scale bar: 5 μm.

(**b**) Quantification of synaptotagmin and synaptophysin recruitment. Histogram shows average intensity per bead (normalized to IgG) ± SD of a representative experiment performed in triplicate wells (15-20 beads analyzed per well). *, $p<0.05$ (Student's t test).

(**c**) Recruitment of synapsin I by beads coated with GFRα1-Fc in the presence of GDNF to axons of cortical neurons. Two different confocal planes are shown. Bead coated in GFRα1 is indicated with a dotted line.

(**d**) Recruitment of synapsin I by beads coated with GFRα2-Fc in the presence of NTN to axons of rat hippocampal neurons. Two different confocal planes are shown. Beads coated in GFRα1 are indicated with a dotted line. Scale bar: 5 μm.

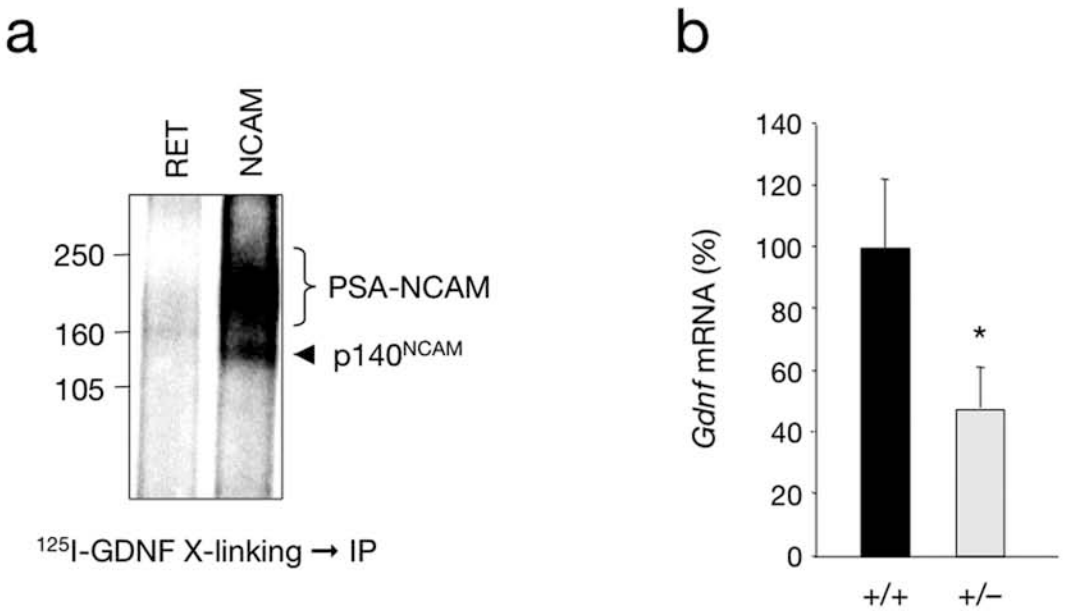


**Figure S7 GDNF binding to NCAM in hippocampal synaptosomes and *Gdnf* mRNA expression in P15 *Gdnf*$^{+/-}$ hippocampus.**
(**a**) Following $^{125}$I-GDNF binding and chemical cross-linking, lysed synaptosomal fractions were immuno-precipitated with antibodies against NCAM or RET as indicated and analyzed by SDS/PAGE and autoradiography.
(**b**) Quantitative analysis of *Gdnf* mRNA expression by real-time RT-PCR in hippocampus of P15 wild type and *Gdnf* heterozygous mutant mice. *, $P < 0.05$ (Student's t test).

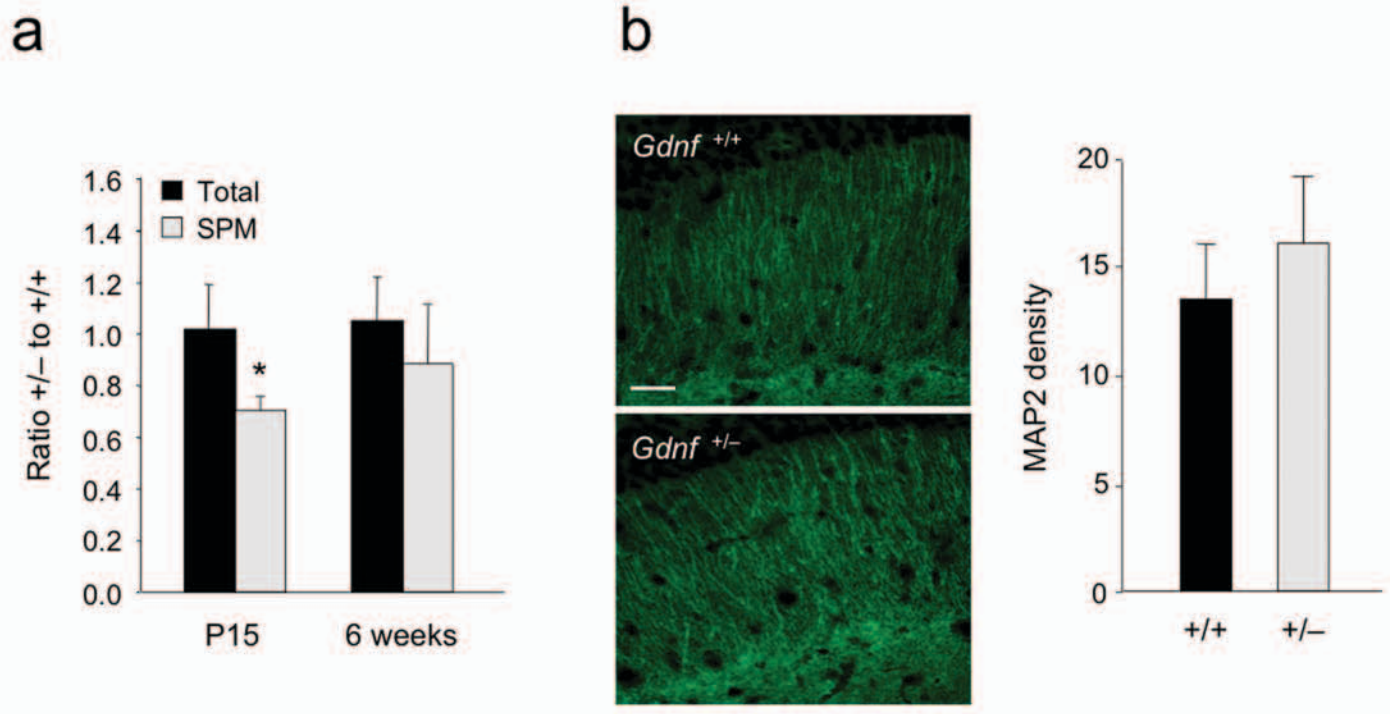


**Figure S8 Synaptophysin content in synapses of P15 and 6 week *Gdnf*$^{+/-}$ mice.**
(**a**) Synaptophysin content in total extracts and SPM fractions of P15 and 6 week *Gdnf*$^{+/-}$ mice relative to wild type. *, $P < 0.01$ vs. total (n = 6 mice per genotype and stage). (**b**) Confocal sections of P15 stratum radiatum of CA1 immunostained for MAP-2 in wild type (*Gdnf*$^{+/+}$) and heterozygous mutant (*Gdnf*$^{+/-}$) mice. Scale bar, 35 μm. The histogram shows average density of MAP-2 staining in arbitrary units ± SEM (n = 7).

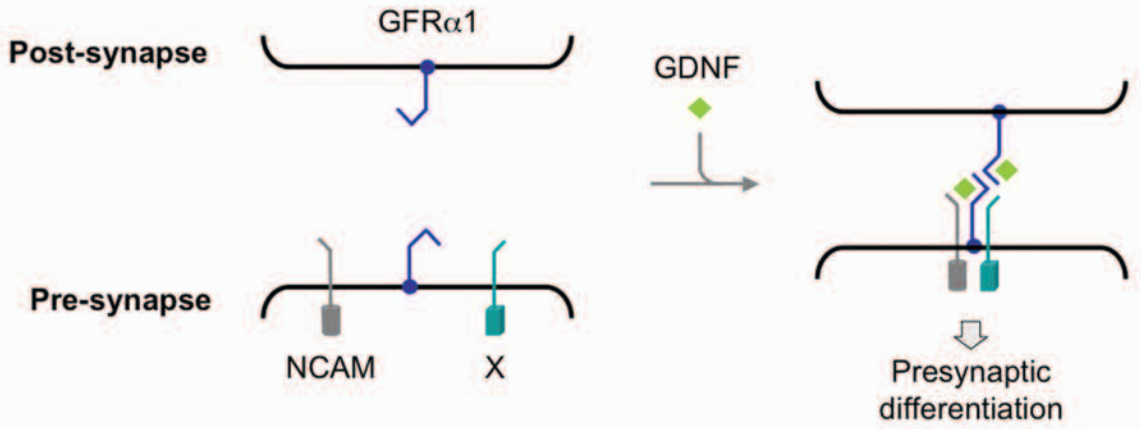


**Figure S9 Schematic of synapse formation by ligand-induced cell adhesion.**
Model of the proposed role of GDNF-mediated *trans*-homophilic GFRα1 interactions in pre-synaptic differentiation. For simplicity, receptors are depicted in monomeric form. The partial requirement of NCAM (grey) suggests the participation of additional transmembrane mediators (light blue).

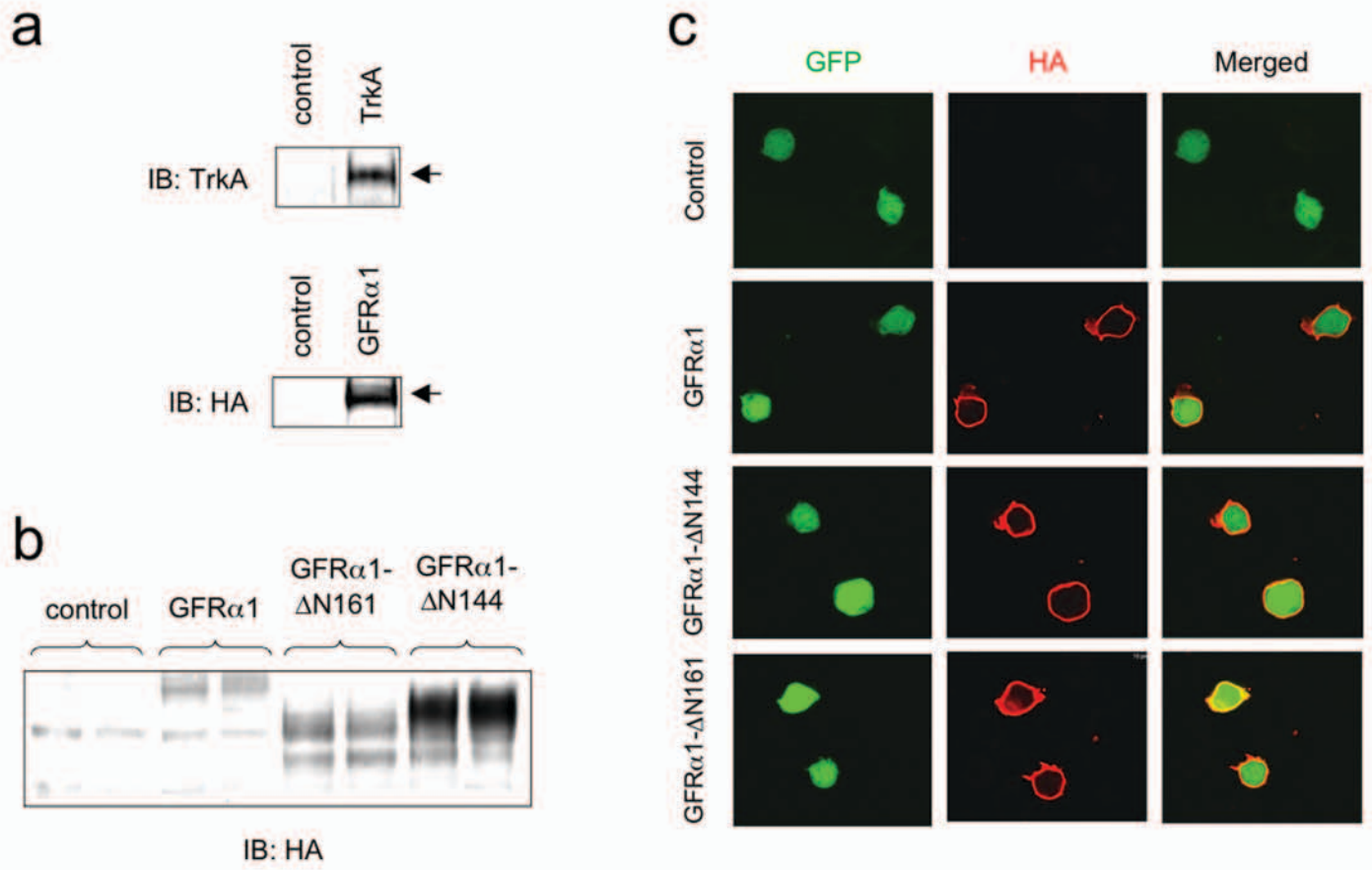


**Figure S10 Controls for Jurkat cell adhesion experiments.**
(**a**) Expression of HA-tagged GFRα1 and TrkA in transfected Jurkat cells was detected by Western blotting using anti-HA and anti-TrkA antibodies, respectively.
(**b**) Analysis of the expression of different HA-tagged GFRα1 constructs in Jurkat cells by Western blotting. The same sample was run in duplicate.
(**c**) Analysis of surface expression of different GFRα1 mutants by immunofluorescence. The staining was done on fixed, non-permeabilized cells using anti-HA antibodies. Scale bar: 10 μm.

## SUPPLEMENTARY METHODS

**Recombinant proteins, cell lines and mice**
GDNF, GFRα1-Fc and GFRα2-Fc were purchased from R&D, NTN from Peprotech, and NGF from Promega. BDNF was provided by Regeneron Pharmaceuticals. Soluble GDNF, NTN or BDNF were used at 100 ng $ml^{-1}$. MG87 fibroblasts expressing GFRα1 have been described previously[43]. *Ret* knock-out mice[44] were obtained from Vassilis Pachnis; *Gfrα1* mutant mice[45] from Arnon Rosenthal; and *Ncam* knockout mice[46] from the Jackson Laboratory (Maine).

**RT-PCR**
Total RNA was isolated from hippocampus using RNA-easy columns (Qiagen) 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: GFRα1: forward, 5'-GAC CGT CTG GAC TGT GTG AAA G-3'; reverse, 5'-TTA GTG TGC GGT ACT TGG TGC T-3'; RET: forward, 5'-ATG ATG ATG AAG ACG ACT CCC C-3'; reverse, 5'-CGC TTA AAC TCC ACC ACA GCA-3'; NCAM: forward, 5'-CCT AGA CTG GAA CGC CGA GTA C-3'; reverse, 5'-GAA GTG AGC TGC CTT GGA TTT T-3'; GAPDH: forward, 5'-TGG GTG TGA ACC ACG AGA AAT A-3'; reverse, 5'-GCT AAG CAG TTG GTG GTG CAG-3'; mouse GDNF: forward, 5'-GGT GCG TTT TAA CTG CCA TAC A-3'; reverse, 5'-AAG ATC AGT TCC TCC TTG GTT TCA-3'; rat GDNF: forward, 5'- ATG TCA CTG ACT TGG GTT TGG G-3'; reverse, 5'-GCT TCA CAG GAA CCG CTA CAA-3'. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Perkin Elmer) according to manufacturer's instructions using Master SYBR Green I mix (Perkin Elmer).

**Primary neuronal cultures**
Rat hippocampal and cortical neurons from embryonic day (E)18.5 and mouse hippocampal neurons from E16.5 embryos were dissociated by trituration and cultured in Neurobasal medium (Gibco) supplemented with B27 (Gibco). Twenty thousand cells were plated on poly-lysine coated glass coverslips and cultured in 24 well plates. Immunolocalization of synaptic markers and GFRα1 was done 48-72 hs after plating.

**Colocalization of pre- and post-synaptic markers**
Colocalization of pre- and post-synaptic markers was analysed in hippocampal neurons cultured for 12 days in the presence or absence of GDNF (added every 4 days at 100 ng/ml). Quantification of colocalization of Synapsin I and PSD-93 was done by counting the number of double-labeled puncta in 4 different 50 μm dendritic segments per neuron. Puncta were defined as distinct spots of high intensity visualized at high magnification by confocal microscopy. At least ten neurons were analyzed per well in triplicate wells. Quantification of synaptophysin clustering on proximal dendrites of hippocampal neurons was done by counting the number of synaptophysin puncta in the most proximal 50 μm segment of individual dendrites as stained with anti-MAP-2 antibodies (Sigma). Restricting these measurements to a fixed dendritic length made it independent of possible changes in neurite outgrowth.

**Total cell lysates, Western blotting, pull-down and cross-linking assays**
For total lysates, cells were lysed 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 Western blotting as previously described[22]. All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics) and analyzed with ImageQuant software (Molecular Dynamics). Antibodies were obtained from various sources as follows: anti-GFRα1 rabbit anti-serum (#1371) was kindly provided by Michele Sanicola (Biogen-Idec); anti-$NCAM^{ICD}$ (12F11) and anti-$NCAM^{ECD}$ (N-CAM13) were purchased from BD PharmMingen; anti-RET from Santa Cruz Biotechnology, anti-synapsin I, anti-synaptophysin, anti-synaptotagmin, anti-syntaxin and anti-GAP-43 from Chemicon; anti-PSD-95 from Affinity BioReagents; anti-NMDA 2A and 2B from Affinity BioReagents; monoclonal anti-HA from Covance; and anti-TrkA was kindly provided by D. Kaplan. For

pull-down assays, COS cells were transfected with pcDNA3 or HA-tagged GFRα1. After 48 h, the cells were incubated with recombinant GFRα1-Fc (R&D, 300 ng/ml) in the presence or absence of GDNF (100 ng $ml^{-1}$). After 20 min at 37°C, the cells were lysed at 4°C in buffer containing 0.5% Triton X-100, 1% β-octylglucoside plus protease and phosphatase inhibitors. Samples were incubated with gentle rocking for 2 h at 4°C with protein G Sepharose beads. After washing, the beads were resuspended in loading buffer and separated by SDS-PAGE. Bound protein was detected on immunoblots using a mouse monoclonal anti-HA (Covance). Cross-linking assays to SPM fractions were performed with EDAC (Pierce) using GDNF iodinated by the lactoperoxidase method as previously described[50].

**Immunofluorescence and microscopy**

For immunoytochemistry, cells were washed, permeabilized with 0.3% Triton X-100 at room temperature, fixed in 4% paraformaldehyde, and stained with the indicated antibodies by overnight incubation at 4° C. An affinity purified anti-GFRα1 rabbit anti-serum (#1371) provided by Michele Sanicola (Biogen-Idec) was used at 1:300 dilution. MAP-2 antibody (Sigma) was used 1:750; VGlut2 and VGAT antibodies (Synaptic Systems) 1:1000; antibodies to synapsin I, synaptophysin and synaptotagmin (Chemicon) 1:1000; to PSD-95 (Affinity BioReagents) 1:100; and to PSD-93 (Synaptic Systems) 1:500. Anti-HA staining was done on non-permeabilized, PFA-fixed Jurkat cells with 1:100 dilution of the anti-HA (COVANCE) for 2 h at room temperature. Hippocampal cryostat sections (14 μm) were obtained from PBS-perfused P15 mice and probed with antibodies against synaptophysin (Chemicon) at 1:1000 dilution. Synaptophysin puncta were quantified in 3 different fields of the stratum radiatum of 5 animals of each genotype. Density of dendritic fields was assessed with antibodies against MAP-2 (Sigma). In all cases, secondary antibodies conjugated to the fluorescein isothiocyanate (FITC) or rhodamine (TRITC) were from Jackson Immunoresearch Lab. After immunostaining, confocal microscopy was performed in a Zeiss LSM 510 confocal microscope using laser excitation wavelengths 488 and 543 nm. Quantification of synaptophysin puncta in hippocampal sections was performed in 48 x 48 μm micrographs of the stratum radiatum of CA1 (three fields per animal in 4-5 animals of each genotype) using OpenLab software (Improvision).