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RESEARCH ARTICLE

The GDNF-GFRα1 complex promotes the development of hippocampal dendritic arbors and spines via NCAM

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ABSTRACT

The formation of synaptic connections during nervous system development requires the precise control of dendrite growth and synapse formation. Although glial cell line-derived neurotrophic factor (GDNF) and its receptor GFRα1 are expressed in the forebrain, the role of this system in the hippocampus remains unclear. Here, we investigated the consequences of GFRα1 deficiency for the development of hippocampal connections. Analysis of conditional *Gfra1* knockout mice shows a reduction in dendritic length and complexity, as well as a decrease in postsynaptic density specializations and in the synaptic localization of postsynaptic proteins in hippocampal neurons. Gain- and loss-of-function assays demonstrate that the GDNF-GFRα1 complex promotes dendritic growth and postsynaptic differentiation in cultured hippocampal neurons. Finally, *in vitro* assays revealed that GDNF-GFRα1-induced dendrite growth and spine formation are mediated by NCAM signaling. Taken together, our results indicate that the GDNF-GFRα1 complex is essential for proper hippocampal circuit development.

KEY WORDS: GDNF, Dendrite complexity, Dendritic spines, Structural plasticity, Hippocampus, NCAM, Mouse, Rat

INTRODUCTION

The development of complex, type-specific dendrite morphology and the formation of correct synaptic connections play major roles in governing the functional properties of neurons and neural circuits (Gulledge et al., 2005; Hausser et al., 2000; Spruston, 2008). Many neurodevelopmental disorders are due to structural abnormalities of dendrites and their connections (Kaufmann and Moser, 2000; Penzes et al., 2011). The size and shape of dendritic arbors result from the interplay of intrinsic genetic programs and extrinsic signals, which determines the number and pattern of synapses received by each type of neuron. Thus, the identification of the signaling pathways triggered by extracellular cues that control neural circuit formation will be of great importance in order to decipher and understand the functioning of the mature nervous system.

Among the extrinsic cues, which modulate specific patterns of dendritic arbor growth and branching, are the neurotrophic factors (de la Torre-Ubieta and Bonni, 2011). Several studies have provided evidence showing that neurotrophic factors play important roles in regulating these processes in cortical and hippocampal neurons. Many of these studies have focused on the role of neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NTF3) and NTF4 (Huang and Reichardt, 2001; Minichiello, 2009; Reichardt, 2006; Zagrebelsky and Korte, 2014). However, little is known about the role of glial cell line-derived neurotrophic factor (GDNF) in hippocampal development.

GDNF was described as a soluble factor able to promote the survival of different neuronal populations in the central and peripheral nervous systems (Lin et al., 1993). A feature of GDNF family ligand (GFL)-receptor complexes is the requirement of two types of subunit: one specialized in transmembrane signaling, namely the receptor tyrosine kinase Ret or the neural cell adhesion molecule (NCAM); and the other involved in ligand binding, namely GDNF family receptor α (GFRα) proteins. Three close mammalian homologs of GDNF have been identified: neurturin (NRTN), persephin (PSPN) and artemin (ARTN), all of which utilize Ret or NCAM signaling receptors with the aid of different members of the GFRα family. Thus, GDNF binds preferentially to GFRα1 receptor, whereas NRTN signals through GFRα2, ARTN through GFRα3 and PSPN through GFRα4. Although each GFL member binds one preferred GFRα receptor, there is a degree of promiscuity in their ligand specificities (Airaksinen et al., 1999; Ibanez and Andressoo, 2016; Paratcha and Ledda, 2008). In recent years, syndecan 3 has been described as a novel receptor for GFLs, although its functions do not appear to require GFRα1 (Bespalov et al., 2011). Interestingly, one of the members of the sortilin receptor family, SorLA (also known as SORL1), has recently been shown to be involved in the internalization and sorting of the GDNF-GFRα1 complex to the endosomal compartment (Glerup et al., 2013).

GFRα1 and NCAM are widely expressed throughout the nervous system and particularly in the forebrain where the canonical tyrosine kinase receptor Ret is absent (Ledda et al., 2007; Paratcha et al., 2003; Trupp et al., 1997), indicating that GDNF has broader functions than initially proposed. Interestingly, physiological functions of GDNF signaling through GFRα1 in the absence of Ret have been reported (Canty et al., 2009; Marks et al., 2012; Paratcha et al., 2006; Pozas and Ibáñez, 2005). Recently, a new physiological role of GDNF has been described as a regulator of commissural axon guidance acting through GFRα1/NCAM signaling (Charoy et al., 2012).

Regarding the hippocampus and cortex, several lines of evidence indicate that GDNF and GFRα family receptors are involved in hippocampal cognitive function, but the mechanism underlying this effect is not clear (Gerlai et al., 2001; Voikar et al., 2004). In previous work we showed that, in the presence of GDNF, a localized source of exogenous GFRα1 promotes axonal

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growth and the presynaptic maturation of hippocampal and cortical developing neurons (Ledda et al., 2007; Paratcha et al., 2003). However, its role in the maturation of postsynaptic domains remains unknown. The study of the role of GDNF-GFRα1 in postnatal development was prevented by the lethality of *Gdnf* and *Gfra1* mutant mice shortly after birth due to renal agenesis. To overcome this problem we have used *Gfra1* conditional mutant mice, in which Cre-loxP-mediated targeted recombination removes the floxed *Gfra1* allele in hippocampal neurons. Using this approach, we evaluated the contribution of GFRα1 in hippocampal dendrite development and provide *in vitro* and *in vivo* evidence indicating that the GDNF-GFRα1 complex is required for the proper growth and morphology of dendritic arbors and spines through NCAM signaling.

RESULTS

GFRα1 is required for proper hippocampal dendritic arborization *in vivo*

Based on previous data indicating that GFRα1 is highly expressed in rat hippocampus during the first and second postnatal weeks, which is the main period of hippocampal dendritogenesis and synaptogenesis in rodents (Ledda et al., 2007), we investigated the role of GFRα1 in hippocampal dendrite development. First, we analyzed the expression of GFRα1 in mouse hippocampal sections at postnatal day (P) 15 by immunofluorescence. Staining was clearly evident in the pyramidal cell layers (CA1-CA3) and the granular layer of the dentate gyrus (DG) (Fig. 1A). The staining was stronger in cells from the DG and variable levels of GFRα1 expression were observed in pyramidal cells of the CA1-CA3 layers. We observed GFRα1 staining in the soma extending into the apical dendrites of CA1 hippocampal neurons (Fig. 1A).

In mature cultures of mouse hippocampal neurons, we found that GFRα1 was expressed on axons as well as along the dendritic shaft and on dendritic spines (Fig. 1B), suggesting an important role of GDNF-GFRα1 in dendrite development. In agreement with previous data (Ledda et al., 2007; Paratcha et al., 2003), we did not detect expression of *Ret* by PCR analysis from hippocampal tissue (Fig. S7A).

To investigate the physiological relevance of GDNF in hippocampal dendrite development we used mice in which the function of GFRα1 can be conditionally inactivated using the Cre-loxP system. In these mice, Cre-mediated excision converts the floxed *Gfra1* allele into a *GFP* reporter (Uesaka et al., 2007). To specifically remove GFRα1 from the hippocampus, Cre recombinase expression was driven by the *Emx1* promoter (Iwasato et al., 2000; Weisstaub et al., 2006). The *Emx1-Cre* line has been extensively used to excise floxed alleles in progenitors that give rise primarily to glutamatergic neurons as well as to astrocytes and olfactory bulb (OB) neurons in the forebrain (Gorski et al., 2002). We confirmed that *Emx1-Cre* mice efficiently recombined the *Gfra1* floxed allele by immunostaining P15 *Emx1-Cre:Gfra1*$^{flox/flox}$ hippocampal sections with an anti-GFP antibody. In agreement with the variable levels of GFRα1 expression detected in control mice with GFRα1 antibodies, we observed variable levels of GFP expression in most neurons from the DG, CA1 and CA3 hippocampal areas (Fig. S1A). A robust downregulation of GFRα1 expression was detected by immunoblot in hippocampal total lysates obtained from mutant mice as compared with controls (Fig. S1B). Moreover, GFRα1 immunostaining of dissociated hippocampal neurons from control and *Emx1-Cre:Gfra1*$^{flox/flox}$ mice provided evidence of the deletion of GFRα1 in these cells (Fig. S1C). No differences were evident in hippocampal organization between control and *Emx1-Cre:Gfra1*$^{flox/flox}$ mice. No substantial change in neuronal density, as measured by NeuN (also known as Rbfox3) staining, was observed, indicating that GFRα1 is not required for the survival of these neurons *in vivo* (Fig. S1D).

In order to analyze the contribution of GDNF-GFRα1 to dendrite development, we used *Gfra1* mutant mice, *Emx1-Cre:Gfra1*$^{flox/flox}$ (cKO) mice and heterozygous *Emx1-Cre:Gfra1*$^{+/flox}$ mice as control. The complexity of the dendritic arbors was examined on hippocampal sections using anti-GFP antibody, which allows

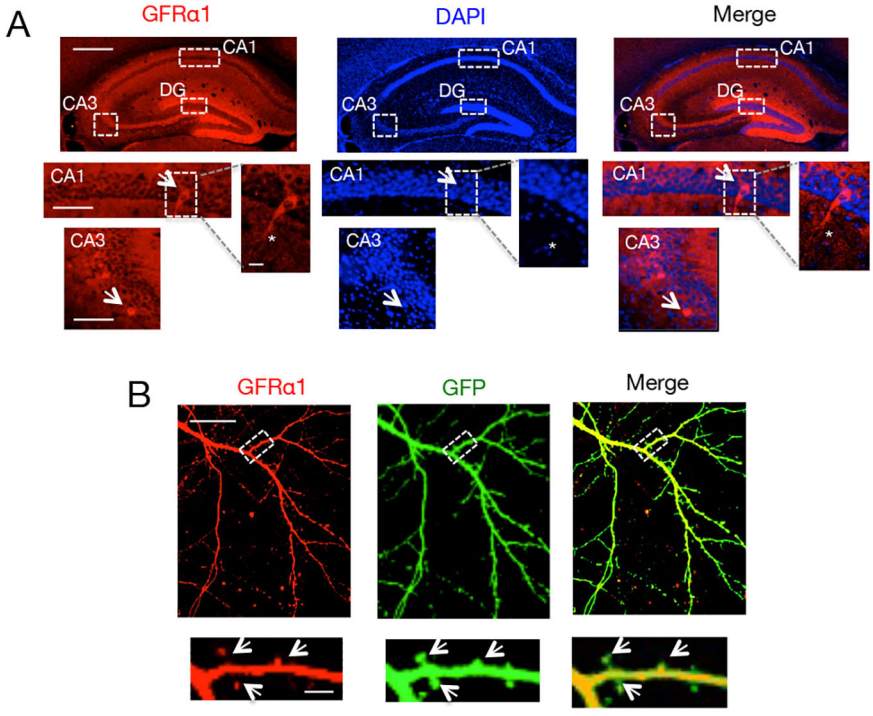

Fig. 1. GFRα1 is expressed on dendrites and dendritic spines of hippocampal neurons.
(A) Immunofluorescence of P15 mouse hippocampal sections. Staining for GFRα1 (red) and with DAPI (blue) are shown. CA1 and CA3 areas (boxed) are shown at high magnification beneath. Arrows indicate neurons with stronger GFRα1 expression. The CA1 inset shows a high-magnification image of a pyramidal neuron labeled for GFRα1. Asterisk indicates GFRα1 staining in dendrites. Scale bars: 500 µm in top row; 100 µm in CA1, CA3; 20 µm in CA1 inset.
(B) Distribution of GFRα1 along dendrites and dendritic spines. Endogenous GFRα1 expression in dissociated mouse hippocampal neurons transfected with control plasmid expressing GFP (12 DIV) was detected by immunofluorescence. Boxed regions are magnified beneath. Arrows indicate dendritic spines. Scale bars: 25 µm, top; 5 µm, bottom. DG, dentate gyrus.

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dendritic arbors to be followed in individual cells with strong GFP expression. We analyzed whether loss of GFRα1 affects dendrite complexity in GFP-expressing CA1 and CA3 pyramidal neurons by Sholl analysis, which quantifies the number of dendritic branches intersecting concentric circles of increasing radius centered on the cell body as reference point (Sholl, 1953). Morphological parameters such as total dendritic length and branching were also evaluated. A substantial decrease in apical and basal dendritic arbor complexity was found in CA1 neurons from mice lacking GFRα1 (*Emx1-Cre:Gfra1*$^{flox/flox}$) relative to heterozygous (*Emx1-Cre:Gfra1*$^{+/flox}$) controls (Fig. 2A-C,E). In addition, we observed a considerable reduction in total dendritic length and branch point number in apical and basal dendritic arbors of *Gfra1* mutant mice compared with controls (Fig. 2B). A similar decrease in dendritic growth and complexity was observed on CA3 pyramidal neurons (Fig. 2D,F and Fig. S2). Dendritic complexity could not be evaluated in neurons from the DG because we could not follow individual neurons by GFP staining. These findings indicate that GFRα1 plays a major role in regulating the elaboration of dendritic hippocampal connections *in vivo*, controlling the area for synaptic inputs.

GFRα1 promotes dendritic growth and complexity in the presence of GDNF

To analyze whether the role of GFRα1 in dendritic development is cell-autonomous, we performed *in vitro* loss-of-function experiments. We utilized a GFP-expressing plasmid that encodes a short hairpin RNA (shRNA) directed against *Gfra1* or a control nonspecific sequence. We designed different specific shRNA molecules directed against rat *Gfra1* that were able to abolish the levels of ectopically expressed rat HA-GFRα1 in heterologous cells, as analyzed by immunoblot and immunofluorescence, whereas the expression of other proteins such as βIII-tubulin remained unaltered (Fig. S3). In order to evaluate the consequences of decreasing GFRα1 levels on dendritic development, embryonic day (E) 17.5 dissociated hippocampal neurons maintained 15 days *in vitro* (DIV) were transfected with control or *Gfra1* shRNA vector. Cells were cultured in the presence of GDNF for 48 h, fixed and stained with anti-GFP. We found that neurons transfected with the control shRNA vector and maintained in the presence of GDNF (100 ng/ml) showed a significant increase in total dendritic complexity, length and branching points indicating that, in the presence of endogenous levels of GFRα1, GDNF has a crucial role in hippocampal dendrite morphology. Consistent with a role of GFRα1 in hippocampal development, we found that reducing GFRα1 levels in cultured neurons causes a reduction of dendritic tree size and complexity, relative to neurons transfected with control vector, when they were cultured in the presence of GDNF (Fig. 3A-D). Similar results were obtained with neurons from GFRα1-deficient mice.

To confirm the role of GDNF and GFRα1 in dendrite development, we examined whether increasing the levels of GFRα1 in neurons was sufficient to potentiate hippocampal dendrite development in response to GDNF. Hippocampal cultures were transfected at 16 DIV with control or HA-GFRα1 constructs in combination with GFP expression vector. Cells were maintained in the presence of GDNF for 48 h, fixed and analyzed for dendrite outgrowth. The complexity of the dendritic tree was analyzed by Sholl analysis, and total dendritic length and branching

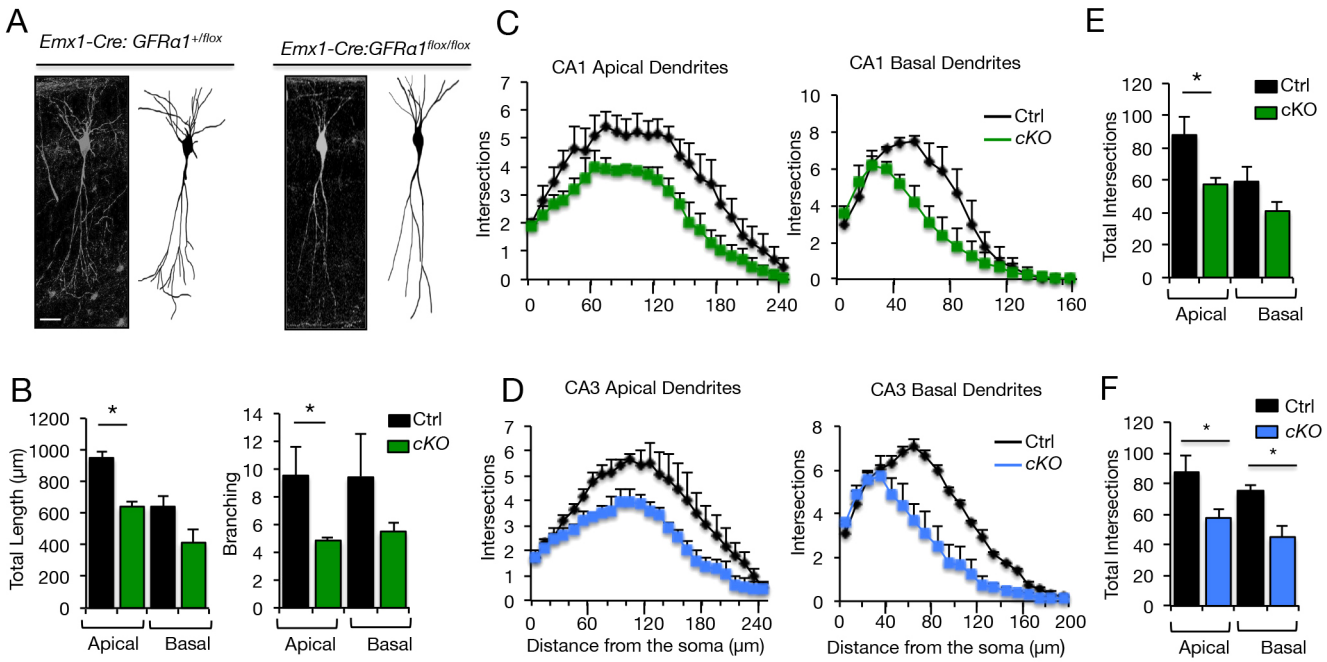

Fig. 2. Downregulation of GFRα1 reduces hippocampal dendrite complexity *in vivo*. (A) Representative confocal images and drawings from the CA1 region of the hippocampus of P15 *Emx1-Cre:Gfra1*$^{flox/flox}$ (*Emx1-Cre:Gfra1*$^{GFP/GFP}$ or *Gfra1* cKO) mutants and *Emx1-Cre:Gfra1*$^{+/flox}$ (*Emx1-Cre:Gfra1*$^{+/GFP}$ or Ctrl) control mice. Hippocampal sections were stained with anti-GFP antibodies. Strong GFP expression in isolated pyramidal CA1 neurons allowed us to visualize and measure morphological parameters. Scale bar: 15 µm. (B) Quantification of total dendritic length and number of branches of apical and basal dendritic arbors of hippocampal CA1 pyramidal neurons from P15 control (Ctrl) and *Gfra1* cKO littermate mice. Mean±s.e.m. of independent determinations performed in three separate mice of each genotype (*n*=30-45 neurons/genotype). **P*<0.05, two-tailed Student's *t*-test. (C,D) Sholl analysis of apical and basal dendritic arbors of hippocampal CA1 (C) and CA3 (D) pyramidal neurons from P15 control and *Gfra1* cKO mice. Mean±s.e.m. (E,F) Cumulative dendrite crossings obtained from Sholl analysis, which represent the sum of the dendritic intersections shown in C and D. Mean±s.e.m of independent determinations performed in three separate mice of each genotype. **P*<0.05, two-tailed Student's *t*-test. The dendrites of 30-45 neurons from three separate mice of each genotype were analyzed. See also Figs S1 and S2.

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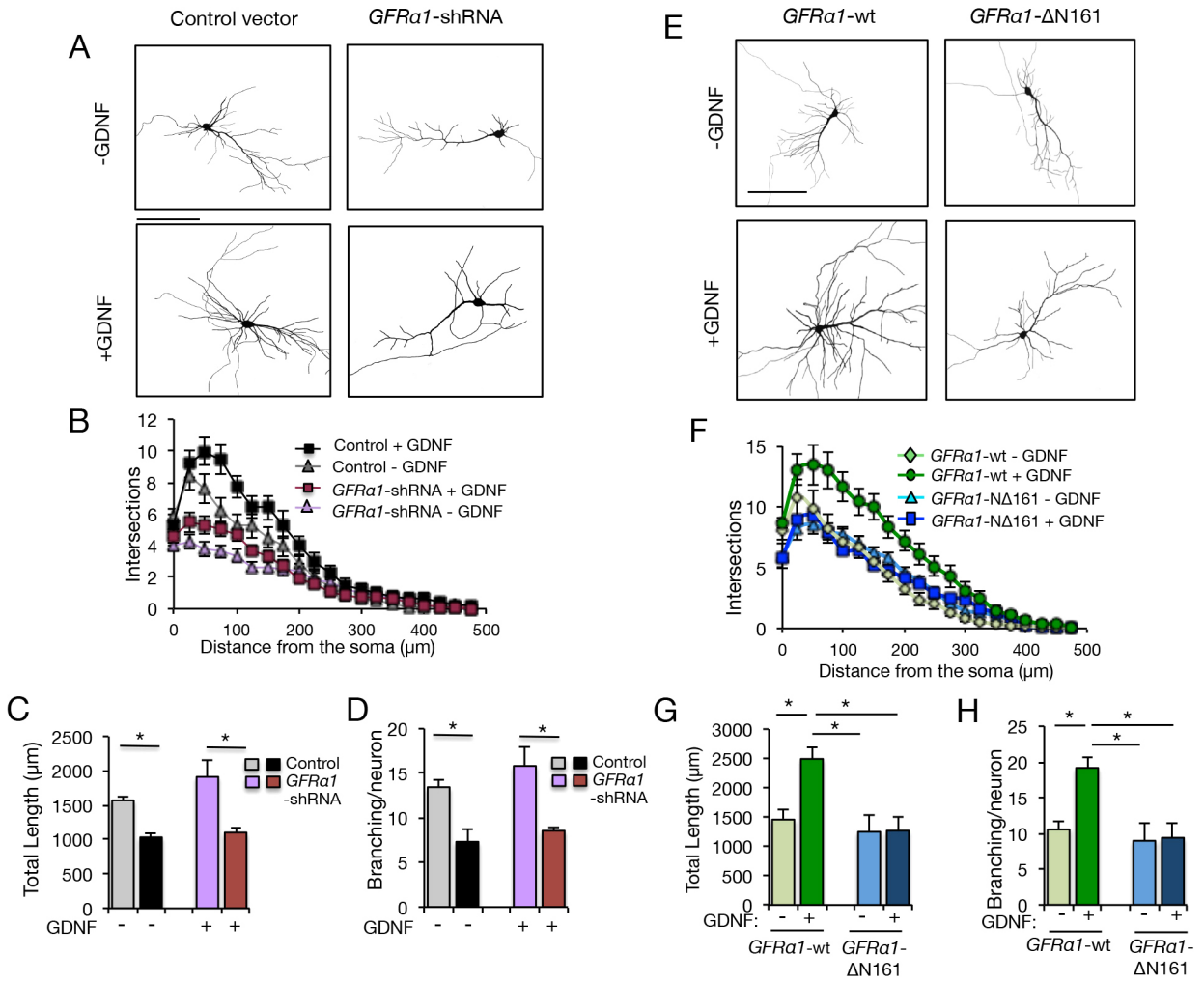

Fig. 3. In the presence of GDNF, GFRα1 induces hippocampal dendrite growth and complexity. (A) Representative drawings of rat hippocampal neurons transfected (15 DIV) with control shRNA or *Gfra1* shRNA GFP-expressing plasmid. At 17 DIV the neurons were fixed, subjected to immunofluorescence with anti-GFP antibodies and analyzed for dendritic arbor length and complexity. (B) Quantification of dendritic complexity by Sholl analysis (mean±s.e.m.) of neurons expressing control or *Gfra1* shRNA in the presence or absence of GDNF (100 ng/ml). Data are from a representative experiment of n=3 independent experiments. (C,D) Quantification of dendritic length (C) and branching (D) of neurons expressing control or *Gfra1* shRNA in the presence or absence of GDNF (100 ng/ml). Mean±s.e.m. from three independent experiments; 20-30 neurons were analyzed per condition in each experiment. *P<0.05, ANOVA followed by Newman-Keuls multiple comparison test. (E) Representative drawings of hippocampal neurons transfected (16 DIV) with GFP-expressing plasmid together with HA-tagged wild-type (GFRα1-wt) or mutant (GFRα1-ΔN161) GFRα1 and maintained in the absence or presence of GDNF (100 ng/ml). After 48 h in culture, neurons were fixed and analyzed. (F) Quantification of dendritic complexity by Sholl analysis (mean±s.e.m.) of neurons overexpressing GFRα1-wt or GFRα1-ΔN161 in the presence or absence of GDNF (100 ng/ml). Data are from a representative experiment of n=3 independent experiments. (G,H) Quantification of dendritic length (G) and branching (H) in the indicated conditions. Mean±s.e.m. from three independent experiments; 15-20 neurons were analyzed per condition in each experiment. *P<0.05, ANOVA followed by Newman-Keuls multiple comparison test. Scale bars: 200 μm. See also Fig. S3.

were also evaluated. We found that hippocampal cells treated with GDNF and overexpressing GFRα1 showed a significant increase in dendritic tree development in terms of dendritic length and branching compared with neurons that had not been treated with exogenous GDNF (Fig. 3E,H). In order to evaluate whether GFRα1-induced dendritic development requires the presence of GDNF, we analyzed a *Gfra1* mutant that lacks the first globular domain and the first α-helix of the second domain of GFRα1 and is therefore unable to interact with GDNF (HA-GFRα1-ΔN161). This construct, which localizes correctly in the plasma membrane at levels comparable to the wild-type GFRα1 protein (Ledda et al., 2007), was co-expressed in hippocampal neurons together with a GFP-expressing plasmid. As expected, cells expressing the mutated form of GFRα1 were not able to promote dendritic complexity. No difference was observed in dendritic length nor in the number of dendritic branch points between neurons overexpressing the mutated GFRα1 when exposed or otherwise to GDNF. This indicates that lack of the GDNF-binding site on GFRα1 abrogates its ability to respond to GDNF with regard to dendritic growth and branching (Fig. 3E-H). Taken together, these findings provide evidence that, in the presence of GDNF, GFRα1 promotes dendritic growth in a neuronal autonomous manner, increasing the area for potential synaptic contacts.

GFRα1 is required for proper synapse formation *in vivo*

Dendrite differentiation during brain development involves not only the growth of the dendritic arbor but also the establishment of specific synaptic connections. In order to explore the role of postsynaptic GFRα1 in the hippocampus, we analyzed the localization of GFRα1 in the core of postsynaptic density (PSD)

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fractions by biochemical fractionation studies. PSD fractions were prepared from P15 mouse forebrain using standardized subcellular fractionation and Triton/sarkosyl extraction. PSD fractions were highly enriched in characteristic PSD proteins such as N-methyl-D-aspartate receptor 1 (NMDAR1; also known as GRIN1) (Fig. 4A). Immunoblot assays revealed enrichment of GFRα1 in the Triton X-100-resistant PSD fractions to an extent comparable to NMDAR1 (Fig. 4A), indicating that GFRα1 is tightly associated with the PSD.

In order to address the contribution of GFRα1 to synaptic development *in vivo*, we investigated the levels of postsynaptic proteins in PSD fractions isolated from hippocampus of *Gfra1* mutant mice (*Emx1-Cre:Gfra1*$^{flox/flox}$, referred to as cKO). Total homogenates of P15 control (*Gfra1*$^{flox/flox}$) and mutant hippocampi contained similar levels of postsynaptic proteins. By contrast, the levels of postsynaptic proteins were reduced by ∼30% in PSD fractions isolated from P15 *Gfra1* cKO mice (Fig. 4B,C).

To extend this analysis using a different approach, we performed electron microscopy to visualize excitatory synapses in the proximal dendritic region of CA1, CA3 and DG. Quantification of the excitatory synapses in photomicrographs of P15 mice revealed that GFRα1-deficient mice had a significant decrease in the density of PSDs relative to control mice (Fig. 4D,E). From these results we conclude that the *in vivo* loss of GFRα1 results in a decrease in postsynaptic specializations.

GFRα1 promotes GDNF-mediated postsynaptic differentiation

In previous work we described that a localized source of exogenous GFRα1, mimicking its postsynaptic localization, was able to induce presynaptic differentiation (Ledda et al., 2007). In order to address whether postsynaptic GFRα1 could affect postsynaptic differentiation, we overexpressed HA-GFRα1 in hippocampal neurons and examined the ability to form dendritic spines, where the majority of excitatory synapses are established. Hippocampal neurons were cotransfected with either a control plasmid or a plasmid encoding HA-GFRα1, together with a plasmid encoding GFP, and were maintained in the presence of GDNF. A significant increase in the density of spine protrusions was observed in GFRα1-overexpressing neurons relative to the control (Fig. 5A,B). The increase was observed in all major morphological types of dendritic spines (stubby, thin, mushroom and cup; Fig. 5C).

It is well known that the formation of dendritic spines involves not only changes in the structure and dynamics of the cytoskeleton, but also the recruitment of postsynaptic proteins, such as glutamate receptors, through interaction with scaffolding proteins. For this reason, we analyzed the recruitment of specific excitatory postsynaptic proteins in spines. Our results indicated that the density of dendritic spines containing the scaffolding protein Homer1 was significantly increased in GFRα1-overexpressing neurons relative to control neurons (Fig. 5D,E). Consistently, the number of dendritic spines containing Homer1 puncta in apposition with the presynaptic protein synapsin 1 was also increased (Fig. 5D,F). In addition, staining for NMDAR1 revealed that, in the presence of GDNF, GFRα1 promotes its recruitment to dendritic spines (Fig. 5G,H). Results from immunoblot assays indicated that these effects were not due to enhanced expression of postsynaptic markers, as GDNF had no effect on the expression levels of postsynaptic proteins (data not shown).

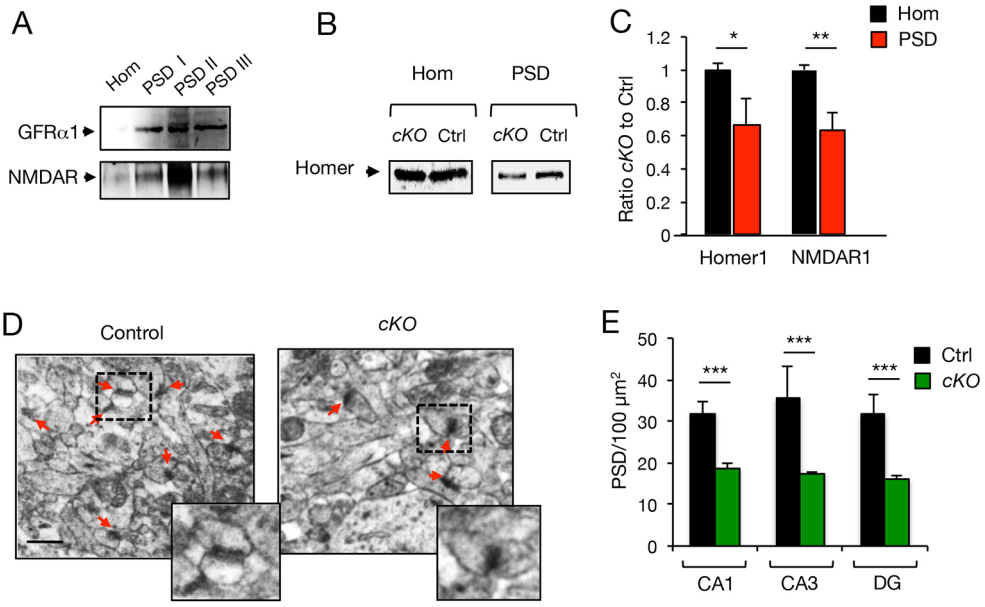

Fig. 4. *Gfra1* conditional knockout mice show reduced hippocampal synaptic development. (A) Postsynaptic localization of GFRα1. Enrichment of GFRα1 proteins in mouse postsynaptic density (PSD) fractions extracted with Triton X-100 once (PSD I) or twice (PSD II), or with Triton X-100 plus 3% sarkosyl (PSD III). A total of 10 µg homogenate (Hom) and 3 µg PSD fraction samples was loaded. The blot was probed with anti-GFRα1 antibodies and NMDAR1 was used as a positive control. (B) Localization of postsynaptic proteins to hippocampal synapses in P15 mice deficient in hippocampal GFRα1, comparing *Emx1-Cre:Gfra1*$^{flox/flox}$ (cKO) with *Gfra1*$^{flox/flox}$ (Ctrl) mice. Total hippocampal homogenates (Hom, 5 µg) and PSD fractions (3 µg) were analyzed by western blotting with antibodies to PSD proteins. A representative immunoblot is shown probed with anti-Homer1. (C) Homer1 and NMDAR1 content in hippocampal total extracts (Hom) and PSD fractions of P15 *Gfra1*$^{flox/flox}$ (Ctrl) relative to *Emx1-Cre:Gfra1*$^{flox/flox}$ (cKO) mice. Results are expressed as average±s.d. from three animals of each genotype. **P*<0.05, ***P*<0.01, versus total (Student's *t*-test). (D,E) Hippocampi of P15 control or cKO mice were analyzed by electron microscopy. (D) Representative transmission electron micrographs from CA1. Arrows mark examples of excitatory PSDs. An example PSD (boxed) is magnified beneath. Scale bar: 300 nm. (E) Quantification of asymmetric PSDs, showing the average number of PSDs in 100 µm^2 CA1, CA3 and DG from control or cKO mice. ****P*<0.0001, Student's *t*-test. Data are mean±s.e.m. from three littermate pairs analyzing ten regions of 100 µm^2 per animal for each hippocampal area.

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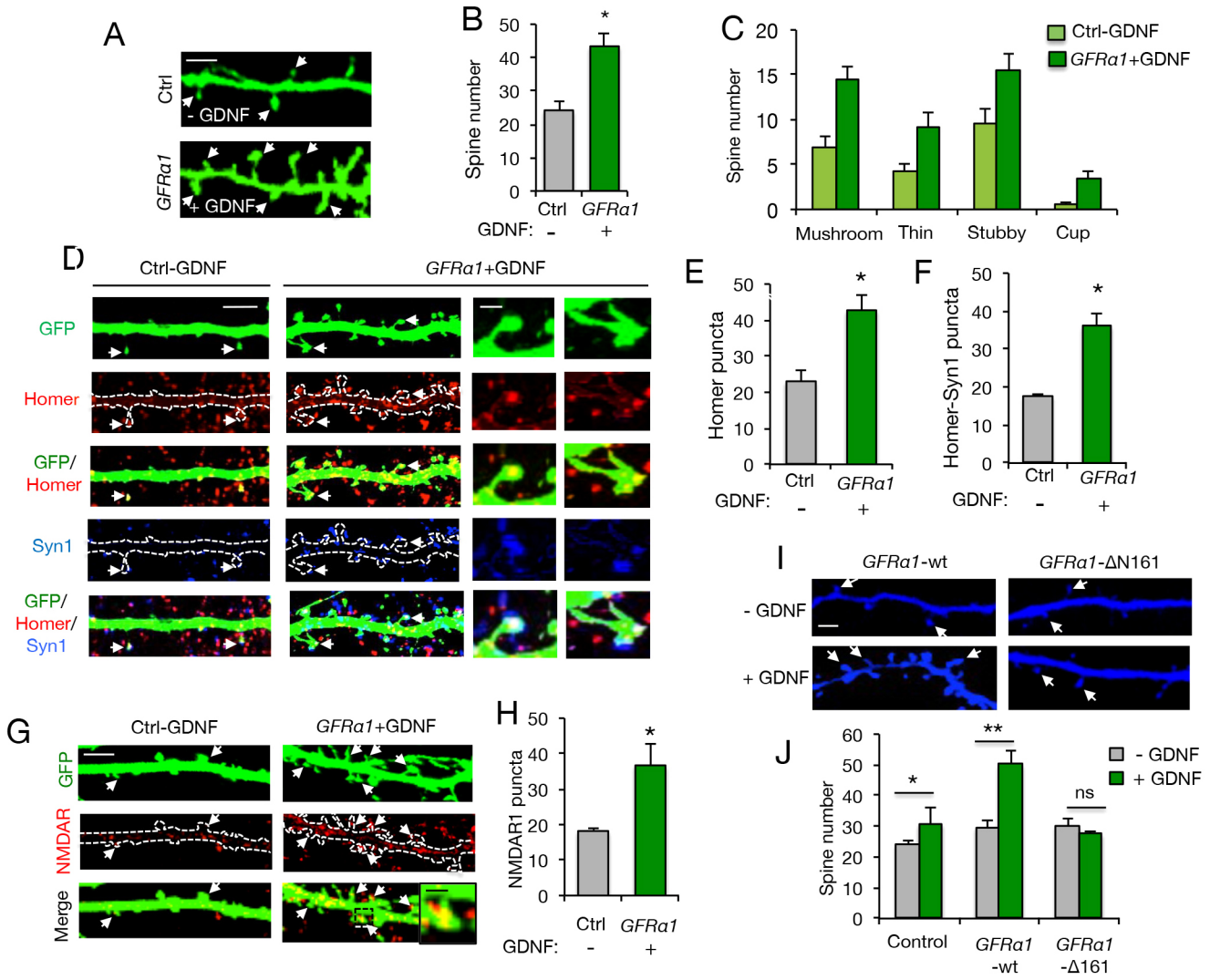

Fig. 5. In the presence of GDNF, GFRα1 promotes spine formation and postsynaptic differentiation of dissociated hippocampal neurons. (A) Representative images of hippocampal neurons transfected with GFP-expressing plasmid in combination with control or HA-tagged GFRα1 vector (16 DIV) and in the absence or presence of GDNF for 48 h. Arrows indicate dendritic spines. (B) Quantification of total spine number along 100 µm of dendrite length of neurons treated as indicated in A. Mean±s.e.m. from three independent experiments (n=20 neurons/condition/experiment). *P<0.05, two-tailed Student's t-test. (C) Quantification of the four main types of dendritic spines along 100 µm of dendritic length of neurons treated as indicated in A from a representative experiment. (D) Representative images of control or HA-GFRα1-wt-transfected neurons immunostained for the excitatory postsynaptic marker Homer1 and the presynaptic marker synapsin 1 (Syn1). Higher magnification of dendritic spines indicated by arrows are shown to the right. (E,F) Quantification of the number of dendritic spines containing Homer1 (E) or Homer1 in apposition with Syn1 (F) along 100 µm of dendrite length of hippocampal neurons cotransfected with GFP-expressing plasmid and control or HA-GFRα1-wt. Mean±s.e.m. from three independent experiments. *P<0.05, two-tailed Student's t-test. (G) Representative images of control or HA-GFRα1-wt-transfected hippocampal neurons immunostained for the excitatory postsynaptic receptor NMDAR1. Arrows indicate postsynaptic protein puncta on dendritic spines. Inset shows higher magnification of a spine stained for NMDAR1. (H) The number of spines containing NMDAR1 puncta along 100 µm. Mean±s.e.m. from three independent experiments. *P<0.05, two-tailed Student's t-test. (I) Representative images of hippocampal neurons co-expressing GFP and HA-GFRα1-wt or HA-GFRα1-ΔN161 treated or not with GDNF. Arrows indicate dendritic spines. (J) Quantification of spine number along dendrites (100 µm) of transfected hippocampal neurons co-expressing GFP and control, HA-GFRα1-wt or HA-GFRα1-ΔN161 treated or not with GDNF. Mean± s.e.m. from three independent experiments (n=15 neurons/condition/experiment). *P<0.05, **P<0.005, ANOVA followed by Newman-Keuls multiple comparison test. ns, not significant. Scale bars: 2 µm in A; 5 µm in D left and G,I; 1 µm in D right and G inset. See also Figs S4 and S5.

Similar assays were performed in cortical neurons, which normally express low levels of GFRα1 (Trupp et al., 1997). When GFRα1 was exogenously expressed in these neurons and they were cultured in the presence of GDNF, a significant increase in postsynaptic parameters, such as spine density and the number of Homer1-positive puncta, was observed on dendritic spines compared with the control (Fig. S4).

Altogether, these data suggest that, in the presence of GDNF, exogenous GFRα1 expression induces the formation of dendritic spines and the recruitment of the excitatory postsynaptic machinery to the synapses.

In order to analyze whether GFRα1-induced postsynaptic differentiation requires the presence of GDNF, we co-expressed the HA-GFRα1-ΔN161 mutant in hippocampal neurons together with a GFP-expressing plasmid. The addition of GDNF to hippocampal neurons transfected with control vector was sufficient to induce a significant increase in dendritic spines, whereas neurons expressing HA-GFRα1-ΔN161 showed no enhancement in the

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number of dendritic spines even in the presence of GDNF. These results indicated that the GDNF-binding site is necessary for GFRα1-induced excitatory synapses in hippocampal neurons (Fig. 5I,J and Fig. S5). Moreover, the overexpression of wild-type GFRα1 resulted in a stronger effect on the development of dendritic spines in the presence of the GDNF (Fig. 5I,J).

These results support the conclusion that GFRα1-mediated excitatory synapse formation is dependent on the presence of an intact GDNF-binding domain in GFRα1.

Knockdown of GFRα1 in hippocampal neurons restricts postsynaptic assembly

In order to confirm the involvement of GFRα1 in hippocampal postsynaptic maturation, we evaluated the consequences of reduced GFRα1 using an RNA interference approach. Hippocampal neurons were cultured and transfected at 15 DIV with vectors expressing GFP, *Gfra1* shRNA or control shRNA and fixed 48 h later. Knockdown of GFRα1 expression in hippocampal cells inhibited postsynaptic maturation, as indicated by a significant reduction in the density of dendritic spines relative to controls (Fig. 6A,B). A similar pattern was observed in total NMDAR1 recruitment to dendritic spines, indicating a loss of postsynaptic specializations (data not shown). The effect of GFRα1 downregulation on postsynaptic assembly was observed in the presence and absence of exogenous GDNF, which is in agreement with the presence of endogenous levels of GDNF secreted by the cultures. Moreover, hippocampal neurons derived from GFRα1-deficient animals exhibited a significant decrease in the ability to develop spines in the presence of GDNF compared with cells derived from control animals (Fig. 6C,D). Interestingly, this effect could be reverted by the overexpression of HA-GFRα1 in cultured neurons derived from *Gfra1* mutant mice in the presence of GDNF (Fig. 6C,D).

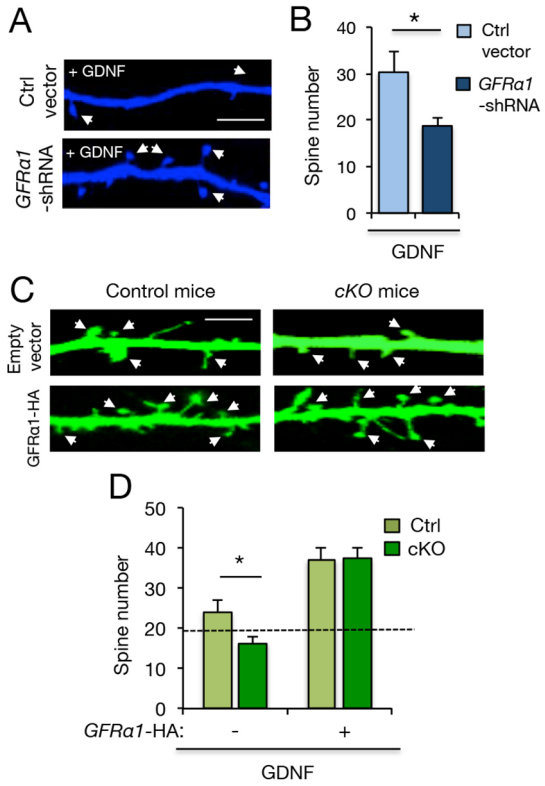

Fig. 6. GFRα1 knockdown reduces spine development in response to GDNF. (A) Representative microphotographs of rat hippocampal neurons transfected with control shRNA or *Gfra1* shRNA, GFP-expressing constructs at 15 DIV and treated with GDNF (100 ng/ml). Two days later, neurons were fixed and subjected to immunofluorescence with antibody against GFP. Scale bar: 5 µm. Arrows indicate spines. (B) Quantification of the effect of reduced GFRα1 expression on spine density. The number of spines along 100 µm dendrite length is indicated. Mean±s.e.m. from three independent experiments; n=20 neurons/condition/experiment. *P<0.05, Student's t-test. (C) Representative microphotographs of neurons derived from control or *Gfra1* cKO mice, transfected with GFP-expressing vector or empty vector and HA-GFRα1-containing plasmid and maintained in the presence of GDNF (100 ng/ml). Arrows indicate spines. Scale bar: 5 µm. (D) The number of spines along 100 µm dendrite length from the experiment described in C. Mean±s.e.m. of triplicate measurements. For each condition, cultures obtained from three animals from each genotype were evaluated. *P<0.05, Student's t-test. Dashed line indicates the number of dendritic spines on neurons from control mice not treated with GDNF.

Dendrite development induced by GDNF-GFRα1 is mediated by NCAM

GDNF promotes its effect by interacting with GFRα1, which lacks an intracellular signaling domain. Previously, NCAM has been reported as an alternative signaling receptor for GDNF (Charoy et al., 2012; Ledda et al., 2007; Nielsen et al., 2009; Paratcha et al., 2003). Whereas the GDNF tyrosine kinase receptor Ret is not expressed in hippocampal neurons, NCAM is highly expressed in this brain area. Therefore, we analyzed whether GDNF-GFRα1 can exert postsynaptic effects by signaling through NCAM. As in previous work (Persohn et al., 1989; Persohn and Schachner, 1990), the NCAM180 isoform was detected in the PSD, and we investigated whether this isoform is required for postsynaptic maturation triggered by GDNF and GFRα1. As shown in Fig. 7A, the overexpression of NCAM180 induced a significant increase in spine density relative to control-transfected neurons in the absence of GDNF (Fig. 7A,B). However, the addition of GDNF resulted in a significant enhancement in dendritic spine development relative to neurons overexpressing NCAM, indicating that postsynaptic differentiation mediated by NCAM180 is potentiated in the presence of soluble GDNF.

To further analyze whether NCAM mediates the effect of GDNF-GFRα1 on hippocampal postsynaptic maturation, we knocked down NCAM by shRNA in cells overexpressing GFRα1 that were maintained in the presence or absence of GDNF. Different shRNAs to *NCAM* (*Ncam1*) were validated in heterologous cells (Fig. S6A). As shown in Fig. 7C,D, interference with NCAM expression abrogates dendritic spine formation induced by GDNF-GFRα1. A similar experiment was performed using a *Ret*-specific shRNA, and we did not observe a significant reduction in the dendritic spine density induced by GFRα1 and GDNF. Together, these data indicated that postsynaptic differentiation induced by GFRα1 and GDNF requires postsynaptic NCAM but not Ret (Fig. 7D, Fig. S7C). Moreover, we analyzed morphometric parameters to ascertain whether NCAM could also mediate the GDNF-GFRα1-induced dendritic growth and observed a significant reduction in GDNF-GFRα1-dependent dendritic length and complexity (number of branch points) (Fig. 7E,F) when NCAM expression was disrupted.

In agreement with a role of NCAM as a mediator of GFRα1-GDNF signaling in hippocampal neurons, treatment of hippocampal cultures with GDNF triggered FAK, Src and MAPK, but not Akt, activation – a feature of GDNF signaling mediated by NCAM (Fig. S7D).

Together, these data indicate that a reduction in the levels of NCAM results in a decrease in postsynaptic maturation and dendritic outgrowth induced by GDNF-GFRα1, supporting a role

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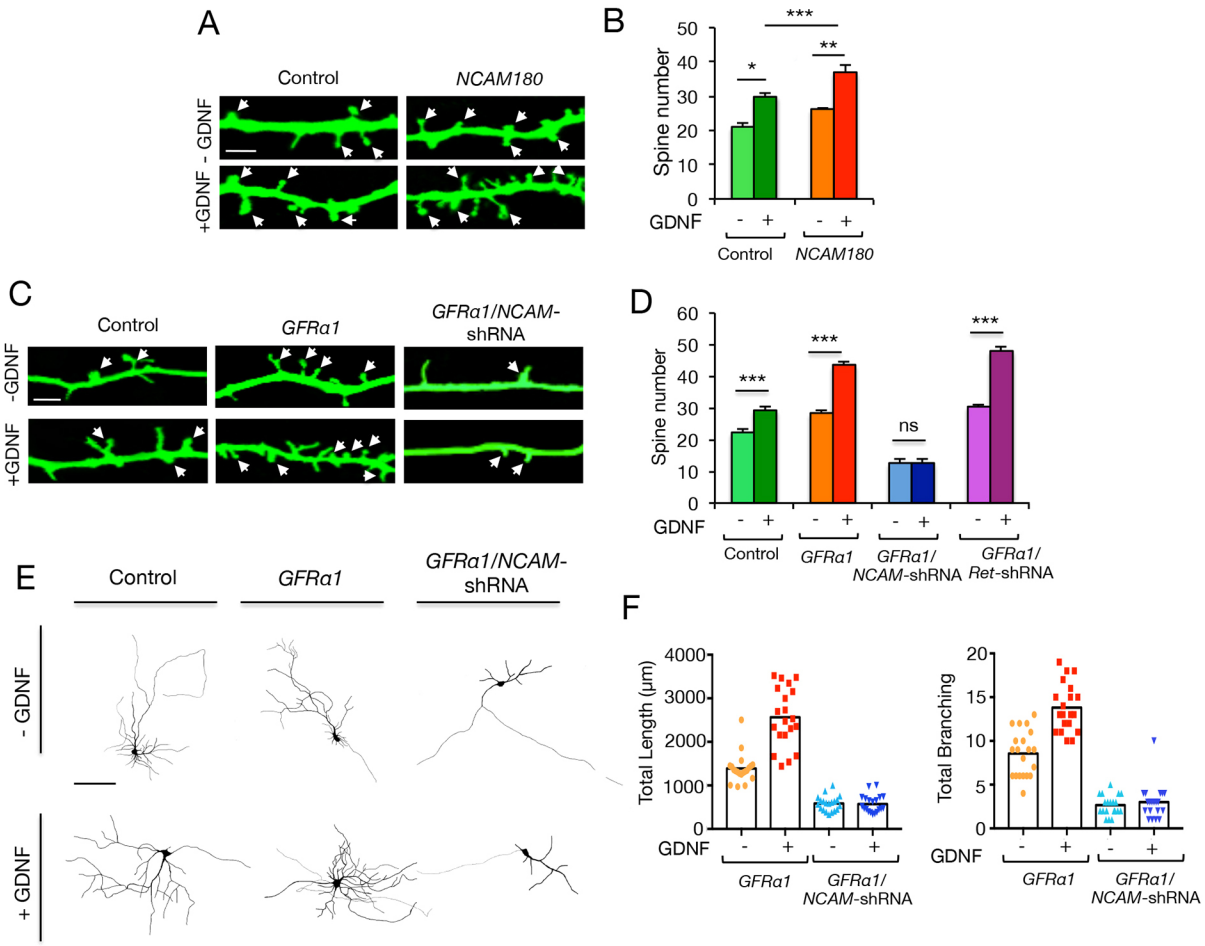

Fig. 7. NCAM is required for hippocampal postsynaptic assembly induced by GDNF-GFRα1. (A) Representative confocal images of neurons transfected with control or NCAM180-expressing plasmid together with a GFP-expressing vector and maintained in the presence or absence of GDNF (100 ng/ml) for 48 h. Arrows indicate spines. (B) Quantification of spine density along 100 µm of dendritic length in neurons described in A. Mean±s.e.m. from three independent experiments. **P*<0.05, ***P*<0.005, ****P*<0.001, ANOVA followed by Newman-Keuls multiple comparison test. (C) Representative confocal images of neurons transfected with control plasmid or HA-GFRα1-expressing plasmid together with a GFP-expressing vector or with HA-GFRα1-expressing plasmid and *NCAM* shRNA-expressing GFP construct and maintained in the presence or absence of GDNF for 48 h. (D) Quantification of spine density along 100 µm dendrite length of neurons transfected as indicated in C. Mean±s.e.m. from three independent experiments. ****P*<0.001, ANOVA followed by Newman-Keuls multiple comparison test; ns, not significant. (E) Representative drawings of neurons transfected as indicated described in C, maintained in the presence or absence of GDNF. (F) Quantification of dendritic length and branching of neurons treated as indicated in C. Results show a representative experiment, *n*=20 neurons/condition. Similar results were obtained in two independent experiments. Scale bars: 2 µm in A,C; 200 µm in E. See also Fig. S6.

for NCAM in hippocampal structural plasticity triggered by GDNF and GFRα1.

DISCUSSION

In the present work, we provide *in vivo* evidence indicating that the expression of GFRα1 in hippocampal neurons plays crucial roles in the development of dendritic arbors and the establishment of excitatory synaptic contacts. Ultrastructural analysis of synapses from *Gfra1* mutant mice revealed that, in the absence of GFRα1, there is a reduction in hippocampal PSDs. In accordance with the *in vivo* data, a decrease in dendritic spine formation and dendrite arborization was observed in cultured hippocampal neurons lacking GFRα1 expression. Overexpression of GFRα1 in the presence of GDNF was sufficient to induce recruitment of the postsynaptic machinery to the synaptic sites as well as for dendrite outgrowth. We provide evidence indicating that these processes triggered by GDNF are mediated through GFRα1 and the neural cell adhesion molecule NCAM (Figs 7 and 8).

Further behavioral analysis in different *Gfra1* conditional mutant strains will be required to more fully characterize the effect of GFRα1 in hippocampal development and function. Unfortunately, although mice deficient in GFRα1 (*Emx1-Cre:Gfra1*$^{flox/flox}$) were born in Mendelian proportions, they died around P20. This precluded any behavioral analysis that might have revealed effects of deficit in hippocampal function in these mice.

GFRα1 as a bidirectional synaptic organizing protein

Previously, we proposed a novel mechanism of ligand-induced cell adhesion (Ledda et al., 2007). We have described that GDNF, as a ligand, is able to mediate transhomophilic cell adhesion between neurons expressing its receptor GFRα1 in pre- and postsynaptic compartments. We observed that in the presence of GDNF, ectopic GFRα1 induces localized presynaptic differentiation in hippocampal neurons via NCAM, but in that study the contribution of GFRα1 to differentiation of the PSD was not analyzed (Ledda et al., 2007). In the present work we describe the role of GDNF and GFRα1 in the

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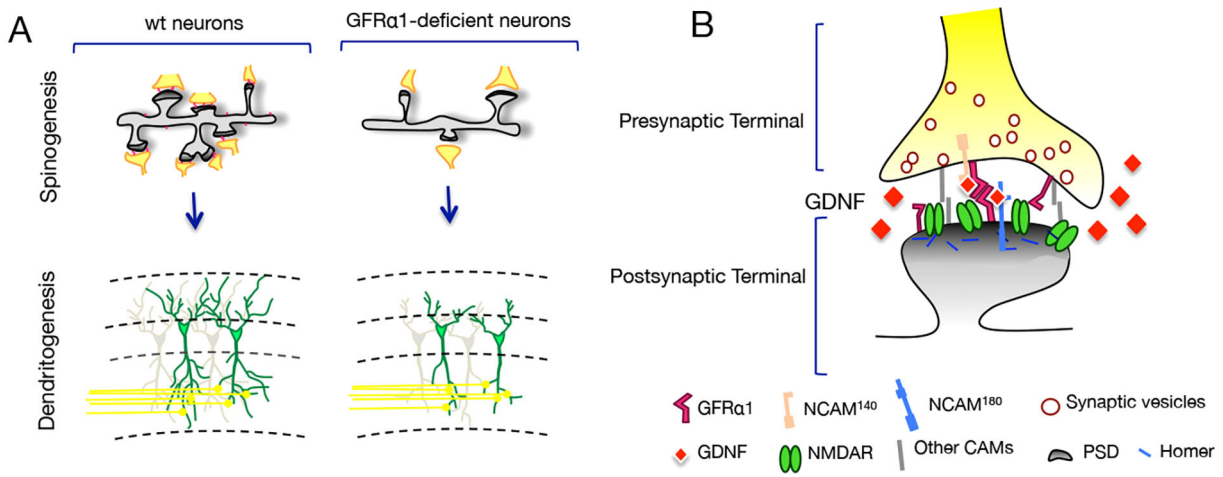

Fig. 8. Model describing the role of GDNF and GFRα1 in hippocampal spinogenesis and dendritogenesis. (A) When the hippocampus is devoid of GFRα1, both the number of synaptic spines and dendritic growth are reduced. The model proposes that GFRα1-mediated trans-synaptic signaling triggered by GDNF confers local stabilization of the cytoskeleton and promotion of dendritogenesis. (B) At synaptic sites, GDNF engagement with GFRα1 promotes synaptic contacts, inducing the recruitment of pre- and postsynaptic machinery and enhancing dendritic spine density through NCAM.

organization of postsynaptic specializations and we provide evidence that this effect is also mediated by NCAM – presumably the NCAM180 isoform, which has been described to be enriched in the PSD (Persohn et al., 1989; Persohn and Schachner, 1990) (Fig. 7). Uemura et al. (2010) described another example of ligand-induced trans-synaptic adhesion interaction, in which the postsynaptic glutamate receptor GluRδ2 interacts with presynaptic β-Nrnx through the presynaptically secreted glycoprotein cerebellin 1 precursor protein (Cbln1). Unlike other cell adhesion systems, which involve direct interaction between membrane-associated proteins in *trans*, the ligand-induced cell adhesion molecule (LICAM) mechanism depends on the presence of a soluble ligand, which triggers the interaction of membrane-associated proteins. This system combines the features of trans-synaptic cell adhesion molecules and soluble synaptogenic factors (Ledda, 2007).

Postsynaptic GFRα1 promotes dendritic spine formation through NCAM

We and others have previously shown that Ret is not detected in the hippocampus, whereas NCAM is distributed throughout the different areas of this structure. Of the three major isoforms of NCAM, NCAM120 is not detectable in synaptosomal membranes, NCAM140 is mainly expressed presynaptically, and NCAM180 is restricted mostly to postsynaptic sites (Persohn et al., 1989; Persohn and Schachner, 1990). In this work, we propose that GDNF-GFRα1-induced hippocampal postsynaptic maturation is mediated by NCAM180. Our data demonstrate that, in the presence of GFRα1, GDNF potentiates the effects of postsynaptic NCAM180 on hippocampal dendrite development. The abrogation of postsynaptic expression of NCAM abolishes the ability of GDNF and GFRa1 to induce dendritic spine formation and dendrite development. Interestingly, NCAM directly interacts with βI-spectrin (Leshchyns'ka et al., 2003; Puchkov et al., 2011; Sytnyk et al., 2006), one of the major components of the PSD (Baines et al., 2001; Malchiodi-Albedi et al., 1993), organizing the postsynaptic NCAM-spectrin adhesion complex and recruiting NMDA receptors and CAMKIIα. Disruption of the NCAM180-spectrin complex affects the morphology of the PSDs and reduces synaptic strength (Sytnyk et al., 2002). Therefore, we may assume that this intracellular mechanism could regulate the dendritic growth and morphology induced by GDNF-GFRα1 and NCAM180 in hippocampal neurons.

Together with our previous results, these data indicate that GDNF has a crucial role in the establishment of hippocampal circuitry acting bidirectionally through the complex GFRα1-NCAM. We propose that, in the presence of GDNF, presynaptic GFRα1 interaction with NCAM140 and another unknown partner leads to presynaptic maturation (Ledda et al., 2007), while interaction with NCAM180 in dendrites promotes postsynaptic assembly (Fig. 8).

NCAM has been shown to be important in hippocampal synaptogenesis and synaptic plasticity (Cremer et al., 1997; Dityatev et al., 2000; Seki and Rutishauser, 1998). Mice lacking the NCAM gene (*Ncam1*) exhibit impaired spatial memory (Cremer et al., 1994) as well as reduced long-term potentiation in the CA1 and CA3 regions of the hippocampus *in vitro* (Cremer et al., 1998; Muller et al., 1996). In humans, mutation in NCAM (*NCAM1*) and its abnormal expression and processing are associated with bipolar affective disorders and schizophrenia (Brennaman and Maness, 2010; Sullivan et al., 2007; Vawter et al., 2000a,b, 1999).

Synapse formation requires the cooperative participation of multiple synaptogenic factors (Siddiqui and Craig, 2011) and it is still unknown whether separate cell adhesion molecule (CAM) systems act in a parallel or in a hierarchical manner. In this context, we propose that GDNF induces GFRα1 trans-synaptic interactions between pre- and postsynaptic membranes, triggering interactions between other pre- and postsynaptic CAMs, such as NCAM, contributing to synapse consolidation. Thus, GDNF-GFRα1 can work in collaboration with other synaptogenic molecules to orchestrate synapse development and the maturation of hippocampal neurons, dependent on the availability of ligand.

Hippocampal dendrite growth is induced by GDNF and GFRα1

Several lines of evidence have indicated a close relationship between synaptogenesis and dendrite morphogenesis. While the size and shape of dendritic arbors influence the number and types of synaptic contacts, synapse formation imparts structural stabilization to growing dendritic processes (Cline and Haas, 2008; Wong and Ghosh, 2002). Recently, the neurexin-neuroligin cell adhesion complex has been described as contributing to dendritogenesis

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through growth stabilization mechanisms (Chen et al., 2010). Our findings indicating that GDNF-GFRα1 is involved in synapse maturation and the promotion of dendrite elongation and branching are in agreement with the synaptotrophic hypothesis, which in general terms states that synaptic inputs control the elaboration of dendritic and axonal arbors (Vaughn, 1989).

Cognitive impairments have been reported in mice heterozygous for *Gdnf* (Gerlai et al., 2001) and in mice lacking GDNF family receptor α2 (GFRα2) (Voikar et al., 2004). Furthermore, expression of the *Gdnf* transgene in astrocytes has been reported to improve cognitive deficits in aged rats (Pertusa et al., 2008). Our findings suggest the existence of an underlying mechanism that might explain these observations and predict cognitive impairments in mouse strains lacking hippocampal GFRα1. The availability of conditional *Gfra1* strains permits the generation of viable mutants, enabling the study of postnatal consequences of GFRα1 absence in hippocampal maturation and consolidation. The contribution of CAMs to neurodevelopmental disorders such as schizophrenia and mental retardation is well documented (Brennaman and Maness, 2010; Sullivan et al., 2007; Vawter, 2000; Vawter et al., 1999). Thus, it will be interesting to explore whether the improper assembly of synaptic connections due to mutations affecting GDNF or GFRα1 might have implications for neurodevelopmental disease characterized by cognitive impairments. Interestingly, it was recently reported that cultured cortical neurons and post-mortem brain tissue from patients with Alzheimer's disease (AD) have lower levels of GFRα1 receptors compared with neurons derived from normal brains (Konishi et al., 2014). In addition, it was recently shown that the NCAM homolog NCAM2 is markedly reduced in hippocampal synapses from AD patients (Leshchyns'ka et al., 2015). It remains to be investigated whether the expression of NCAM is altered in AD patients. Together, these observations support the relevance of understanding the contribution of GDNF-GFRα1-NCAM signaling in hippocampal and cortical neurons that are affected in AD.

MATERIALS AND METHODS

Mice

Gfra1$^{flox/flox}$ mice were generously provided by Dr J. Milbrandt (Washington University School of Medicine, St Louis, MO, USA). *Gfra1*$^{flox/flox}$ mice were mated with *Emx1-Cre* mice generously provided by Dr N. Weisstaub (School of Medicine, University of Buenos Aires, Argentina) (Iwasato et al., 2004; Weisstaub et al., 2006). All transgenic strains were genotyped by PCR. The use of animals was approved by the Animal Care and Use Committee of the School of Medicine, University of Buenos Aires (CICUAL-UBA), ethical permit number: 67341/2013.

DNA constructs and cell transfection

Details of GFRα1 and control constructs used in overexpression and knockdown experiments and methods of neuronal cell transformation are described in the supplementary Materials and Methods.

PSD fractionation, western blotting and PCR

PSD fractions were prepared from mouse hippocampi as described in the supplementary Materials and Methods. Protein lysates were analyzed by western blot using the antibodies described in the supplementary Materials and Methods. cDNA prepared from total hippocampal mRNA was analyzed by PCR as described in the supplementary Materials and Methods.

Primary neuronal cultures

Rat and mouse hippocampal and cortical neurons were isolated from E17.5 Wistar rats and P0 mice, respectively (School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina), as previously described (Ledda et al., 2007; Otero et al., 2013). Hippocampal and cortical neurons were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions. For morphometric analysis, the cells were stained with anti-GFP and anti-MAP2 (see the supplementary Materials and Methods).

Immunostaining, confocal and electron microscopy

For immunofluorescence, cells were washed, fixed with 4% paraformaldehyde (PFA) in PBS, permeabilized with 0.3% Triton X-100, blocked with normal serum (Jackson ImmunoResearch) and incubated overnight at 4°C with the indicated antibodies. Mouse brains were isolated from animals perfused with 4% PFA, maintained in sucrose 30% in PBS overnight and then embedded in OCT (Tissue-Tek) and sectioned at 50 µm. Cryostat sections were permeabilized with 0.3% Triton X-100, blocked with normal serum and incubated overnight at 4°C with primary antibodies (see the supplementary Materials and Methods).

After immunostaining, confocal microscopy was performed using an Olympus IX-81 inverted microscope or an Olympus FV-1000 confocal microscope using identical settings between control and experimental images. Image analysis is described in the supplementary Materials and Methods.

cKO (*Emx1-Cre:Gfra1*$^{flox/flox}$) and control (*Gfra1*$^{flox/flox}$) mice were perfused transcardially with 4% PFA and 0.25% glutaraldehyde in 0.9% NaCl. Brain tissue was collected and postfixed with 4% PFA and 0.25% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) overnight. The hippocampal CA1 and CA3 region was blocked and fixed with osmium oxide, dehydrated, embedded in epoxy Durcupan resin, and 70 nm sections were prepared and stained with 2% uranyl acetate and lead citrate. Images were obtained on a Zeiss EM 109T transmission electron microscope. Synapses, which had clear synaptic vesicles and PSD, were visually identified and counted in 12,000× magnification images. Every synapse with clear presynaptic and postsynaptic membranes was manually analyzed using ImageJ. Three animals per genotype were analyzed.

Quantification and statistical analysis

Statistical analysis was performed using GraphPad Prism. Details are provided in each figure legend and in the supplementary Materials and Methods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.I., G.P. and F.L. designed the experiments. D.I., A.B., P.A.F. and F.C.A. performed the experiments. D.I., A.B. and F.L. analyzed the experiments. G.P. and F.L. wrote the manuscript.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.140350.supplemental

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