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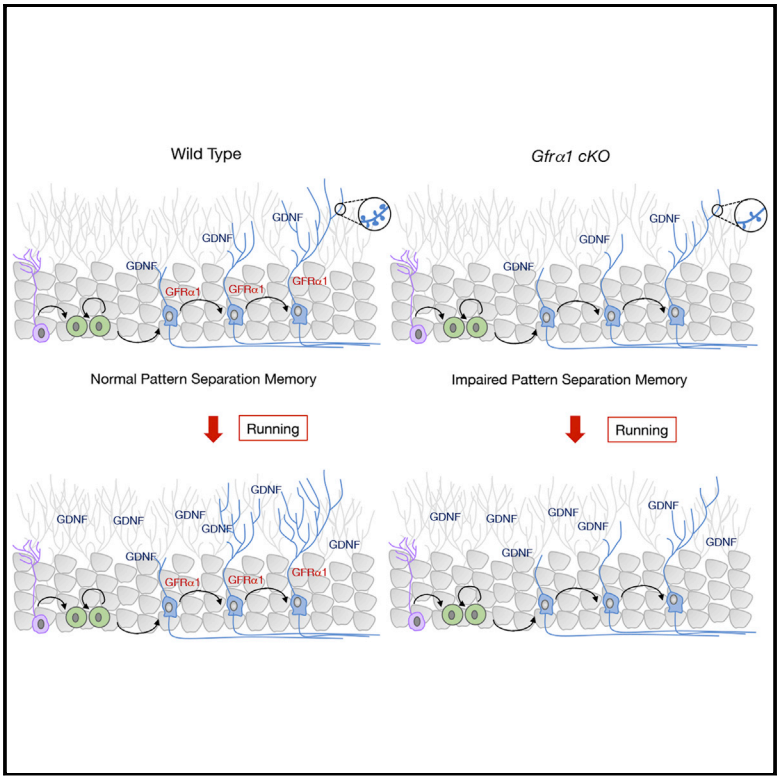
GDNF and GFRα1 Are Required for Proper Integration of Adult-Born Hippocampal Neurons

Graphical Abstract



Highlights

- The GDNF receptor GFRα1 is expressed by adult-born GCs
- GFRα1 regulates dendrite morphology and spine density of adult-born hippocampal neurons
- GFRα1 signaling in adult GCs is required for spatial memory processing
- Running increases GDNF expression in DG, promoting GFRα1-dependent dendrite development

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In Brief

Bonafina et al. demonstrate that GDNF signaling through GFRα1 is critical for morphological maturation and synaptic integration of adult-born hippocampal neurons and is required for correct spatial pattern separation memory. Voluntary exercise triggers GDNF expression and GFRα1-dependent dendritic remodeling of adult-born GCs.

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GDNF and GFRα1 Are Required for Proper Integration of Adult-Born Hippocampal Neurons

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SUMMARY

The glial cell line-derived neurotrophic factor (GDNF) is required for the survival and differentiation of diverse neuronal populations during nervous system development. Despite the high expression of GDNF and its receptor GFRα1 in the adult hippocampus, the functional role of this system remains unknown. Here, we show that GDNF, acting through its GFRα1 receptor, controls dendritic structure and spine density of adult-born granule cells, which reveals that GFRα1 is required for their integration into preexisting circuits. Moreover, conditional mutant mice for GFRα1 show deficits in behavioral pattern separation, a task in which adult neurogenesis is known to play a critical role. We also find that running increases GDNF in the dentate gyrus and promotes GFRα1-dependent CREB (cAMP response element-binding protein) activation and dendrite maturation. Together, these findings indicate that GDNF/GFRα1 signaling plays an essential role in the plasticity of adult circuits, controlling the integration of newly generated neurons.

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is the prototypic member of a small family of neurotrophic factors that promote cell survival, neurite outgrowth, and neuronal differentiation of distinct populations of central and peripheral neurons during development (Airaksinen and Saarma, 2002; Ibáñez and Andressoo, 2017; Paratcha and Ledda, 2008). While the developmental role of GDNF has been well characterized, much less is known about its function in the adult nervous system. Recently, we have described that the molecular complex GDNF/ GDNF receptor family alpha 1 (GFRα1) plays a crucial role in dendritic growth and synapse formation in hippocampal pyramidal neurons during early postnatal development (Irala et al., 2016).

The functional receptor for GDNF ligands is composed by a glycosyl-phosphatidylinositol-anchored co-receptor, specialized in ligand binding known as GFRα and a subunit specialized in transmembrane signaling, such as Ret receptor tyrosine kinase (Durbec et al., 1996; Trupp et al., 1999) or the neural cell adhesion molecule (NCAM) (Paratcha et al., 2003). Moreover, Syndecan-3 has been described as an alternative signaling partner of GDNF in the brain without the involvement of their conventional receptors (Bespalov et al., 2011).

In the adult mammalian brain, new neurons are continuously generated throughout life in discrete regions of the central nervous system, the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Gage, 2000; Ming and Song, 2005). In particular, the SGZ contains neural progenitor cells (NPCs), which give rise to astrocytes and granule cells (GCs). Cell proliferation in this region is highly controlled by different factors, which regulate the balance between quiescent and active NPCs. Once generated, new GCs are incorporated into preexisting circuitry via a stereotypical sequence of morphological transitions recapitulating what occurs during perinatal development. It has been estimated that neuronal development in the adult DG takes approximately 8 weeks (Espósito et al., 2005; Ge et al., 2006; Laplagne et al., 2006). Recent work has shown that transient functional stages that occur during development of adult-born neurons, before reaching a mature phenotype, are relevant for DG function (Kempermann et al., 2015; Kropff et al., 2015).

Adult-born GCs contribute to cognitive processes under normal physiological conditions, such as learning memory, pattern separation, and cognitive flexibility (Aimone and Gage, 2011; Bekinschtein et al., 2014; Clelland et al., 2009; Nakashiba et al., 2012; Sahay et al., 2011). In addition, there is increasing evidence indicating that neurological diseases and mood disorders have deleterious effects on adult hippocampal neurogenesis (Toda et al., 2019).

Functional integration of adult-born GCs is tightly regulated by different intrinsic and extrinsic cues, such as locally secreted neurotrophic factors. These regulators recruit

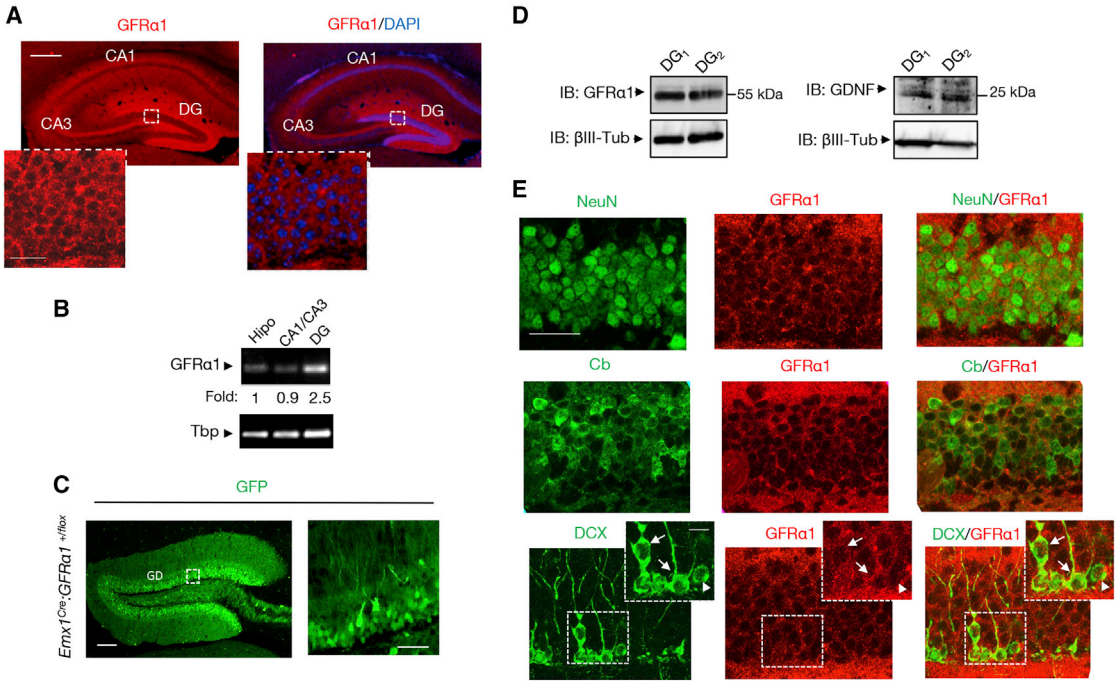

Figure 1. Expression and Localization of GFRα1 in Adult DG

(A) Localization by immunofluorescence of GFRα1 in coronal sections of 2-month-old mouse hippocampus. Expression of GFRα1 (red) is shown. Blue corresponds to DAPI-stained nuclei. Higher-magnification images showed the expression of GFRα1 on the membrane surrounding neuronal cell bodies. Scale bars: 500 and 50 μm.

(B) Analysis of *Gfrα1* mRNA expression in total hippocampus, CA1–CA3, and DG by semiquantitative RT-PCR (27 cycles) in adult rats. Expression of the housekeeping gene *Tata binding protein* (*Tbp*) was analyzed as control. The numbers below the lanes indicate the fold of *Gfrα1* mRNA expression in the different hippocampal areas relative to total hippocampus normalized to the levels of *Tbp* mRNA.

(C) Representative confocal image of adult mouse hippocampal section from $Emx1^{Cre}$:$Gfr\alpha1^{+/flox}$ mice stained with anti-GFP. Scale bar, 100 μm. A higher-magnification image of boxed region is also shown. Scale bar: 50 μm.

(D) Expression of GDNF and GFRα1 in cell extracts obtained from DG total lysates detected by western blot. The blots were probed with anti-GDNF and anti-GFRα1 antibodies, and then reprobed with the neuronal marker βIII-Tubulin.

(E) Representative images showing co-localization of GFRα1 with different neuronal markers in 2-month-old mouse DG coronal sections. Top: Staining for NeuN and GFRα1 (red); middle: staining for Calbindin (Cb) and GFRα1 (red); bottom: staining for Doublecortin (DCX) and GFRα1 (red). Scale bar: 50 μm. Higher-magnification images of boxed areas are also shown. Scale bar: 15 μm. Arrows indicate $DCX^{+}GFR\alpha1^{+}$ cells, and arrowhead indicates $DCX^{+}GFR\alpha1^{-}$ cell.

See also Figure S1.

diverse downstream pathways to finally influence distinct aspects of neuronal maturation. Neurotrophins, like brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NTF3), have been implicated in the regulation of adult neurogenesis by contributing to the generation, maturation, and integration of newborn neurons (Bergami et al., 2008; Choi et al., 2009; Lu and Chang, 2004; Vilar and Mira, 2016). Recently, we have described that GDNF, acting through GFRα1, inhibits proliferation and promotes differentiation of glutamatergic neural progenitors during cortical development (Bonafina et al., 2018). Based on the fact that some molecules that control embryonic neural precursor development have also been implicated in adult-born neuron generation (Urbán and Guillemot, 2014) and that GFRα1 is highly expressed in neurogenic areas of postnatal brain including the DG (Irala et al., 2016; Paratcha et al., 2003, 2006), we investigated whether the GDNF/GFRα1 complex is involved in the correct development and integration of adult-born GCs into pre-existing hippocampal circuits.

RESULTS

GFRα1 and GDNF Expression in the Adult DG

The expression of endogenous GFRα1 was analyzed by immunofluorescence using specific antibodies. We observed higher expression of GFRα1 in neurons of the DG compared to other hippocampal regions such as CA1 and CA3 (Figure 1A). This observation was confirmed by reverse-transcriptase (RT)-PCR analysis of *Gfrα1* mRNA levels (Figure 1B). In agreement with other works, we did not detect expression of the GDNF canonical receptor *Ret* in hippocampus (Glazner et al., 1998; Golden et al., 1999; Lenhard and Suter-Crazzolara, 1998; Trupp et al., 1999), but we detected a high expression level of the alternative GDNF receptor *Ncam* (Figure S1A).

The expression of GFRα1 in the DG was corroborated using mice in which the function of GFRα1 can be conditionally inactivated using the Cre-LoxP system converting the floxed *Gfrα1* allele into a GFP reporter (Uesaka et al., 2007). These mice ($Gfr\alpha1^{flox/flox}$) were crossed with a Cre-Emx1 mouse line, which

excises floxed alleles in progenitors that give rise primary to glutamatergic neurons as well as astrocytes and olfactory bulb (OB) neurons in the forebrain (Figure S1B) (Bonafina et al., 2018; Gorski et al., 2002; Irala et al., 2016). The analysis of heterozygous mice *Emx1*^{Cre}*:Gfrα1*$^{+/flox}$ allowed us to visualize the expression of GFRα1 by GFP in the majority of DG neurons (Figure 1C).

We then investigated the expression GFRα1 and GDNF in the adult DG by immunoblotting. To this end, the DG region from 2-month-old wild-type mice was microdissected and homogenized for western blot analysis. Our results showed a clear expression of the mature form of GDNF and its receptor GFRα1 (Figure 1D).

To reveal which cells in the DG express GFRα1, we performed immunostainings with several cell-type-specific markers including doublecortin (DCX), NeuN, and Calbindin (Cb) (Nakashiba et al., 2012) in adult mouse hippocampal sections. Expression of GFRα1 was observed in mature neurons positive for NeuN and Cb, as well as in neurons positive for DCX, a microtubule-associated protein present during neuronal migration that is used to label immature neurons (Espósito et al., 2005) (Figure 1E). Although the majority of mature neurons express GFRα1, approximately the 50% of the total DCX$^+$ neurons expressed the GDNF receptor (52.32 ± 5.72; mean ± SEM), indicating the existence of different subpopulation of DCX cells. The co-expression of GFRα1 with immature and mature neuronal markers was also observed in DG of heterozygous mice *Emx1*^{Cre}*:Gfrα1*$^{+/flox}$ (Figures S1B and S1C). The expression of GFRα1 in DCX$^+$ cells indicated that GDNF acting through GFRα1 could play a role in the development of newborn neurons generated in the adult DG.

GFRα1 Is Involved in Adult-Born GCs Maturation

Based on the presence of GDNF in the DG and GFRα1 in immature GCs, we analyzed whether GFRα1 could play a role in hippocampal neurogenesis. To this end, we used a mouse line expressing the tamoxifen-inducible form of Cre (*Cre*^{ERT2}) in the locus of the glutamate transporter *GLAST* (Mori et al., 2006), which is expressed in GFAP$^+$ astrocytes and also in neural stem cells in the adult brain (Bonaguidi et al., 2012; DeCarolis et al., 2013). This mouse line was mated with *Gfrα1*$^{flox/flox}$ mice. Tamoxifen administration of these animals resulted in GFP labeling of a cohort of new GCs and few astrocyte-like cells in the the DG. Thus, these animals allow us to follow the development of the newborn GCs (Figures S2A–S2C) (Yang et al., 2015).

To examine whether GFRα1 ablation had any effect in the survival of newly generated GCs, we evaluated the number of GFP-expressing cells in *Gfrα1* homozygous mutant mice (*Glast*^{CreERT2}*:Gfrα1*$^{flox/flox}$), using heterozygous (*Glast*^{CreERT2}*:Gfrα*$^{+/flox}$) animals as control. Twenty-eight and 56 days after tamoxifen (TAM) injection, GFP$^+$ cells were evaluated on DG sections using an anti-GFP antibody, which allows a better visualization of the newborn cells. The vast majority of the GFP$^+$ cells had a neuronal morphology, and no differences were evident in the total number of GFP$^+$ cells between *Gfrα1*-deficient mice and control mice, suggesting that GFRα1 is not required for the survival of these cells (Figure S2D).

To analyze the contribution of GFRα1, we measured the proportion of GFP$^+$ neurons expressing DCX and Cb at 28 and 56 days. A significant increase in the density of GFP$^+$DCX$^+$ positive cells was observed in mice deficient for GFRα1 compared with control mice, while the density of GFP$^+$Cb$^+$ GCs decreased significantly. These differences were only observed at 56 days post TAM injection (dpi), indicating that GFRα1 is required for the correct maturation of adult-born GCs (Figures 2A and 2B).

In order to analyze whether the differences in the degree of neuronal maturation could be due to a delay in the time at which neuronal progenitor cells exit the cell cycle, we injected bromodeoxyuridine (BrdU) 7 days after TAM administration to label dividing cells of the SGZ, and evaluated the proportion of those cells that continued proliferating at later times. The cell cycle marker Ki67 was used to identify cells that were not in G0, 3 days after BrdU injection. No differences were evident in the density of new cells (BrdU$^+$) or in the proportion of cells that were still proliferating (BrdU$^+$/Ki67$^+$) when control animals (*Glast*^{CreERT2}*:Gfrα1*$^{+/flox}$) were compared with *Gfrα1* mutant mice (*Glast*^{CreERT2}*:Gfrα1*$^{flox/flox}$ (Figure S2E). These data indicate that neural progenitor cells exit the cell cycle at the same time in control and *Gfrα1*-deficient mice. This observation is in agreement with the absence of Ki67 observed in GFP$^+$ cells from *Glast-Cre*^{ERT2}*:Gfrα1*$^{+/flox}$ and *Glast*^{CreERT2}*:Gfrα*$^{flox/flox}$ mice (Figure S2F), suggesting that dividing cells do not express GFRα1.

As adult-born GCs migrate radially from the SGZ into the GCL (granule cell layer) (Altman and Bayer, 1990; Espósito et al., 2005; Kempermann et al., 2003), we also examined whether GFRα1 deficiency could affect GC migration. We assessed migration of GFP$^+$ neurons in control (*Glast*^{CreERT2}*:Gfrα1*$^{+/flox}$) and GFRα1 mutant (*Glast*^{CreERT2}*:Gfrα1*$^{flox/flox}$) mice by analyzing their relative position in the GCL at different neuronal ages. To this end, we divided the GCL from inner to outer layers in GCL1, GCL2, and GCL3, and each cell was assigned to one of these layers. Measurements were done at 14, 28, and 56 dpi, and no differences were found for any of the neuronal ages analyzed (Figure 2C). Overall, these observations suggest that GFRα1 is not involved in proliferation or migration of adult-born GCs but is required for their correct maturation.

GFRα1 Is Required for Proper Dendritic Maturation

We next investigated whether the dendritic maturation was affected by the absence of GFRα1. The ability of newborn neurons to integrate into the DG involves progression through distinct morphological and functional stages of maturation (Espósito et al., 2005; Piatti et al., 2011). Thus, *Gfrα1*-deficient (*Glast*^{CreERT2}*:Gfrα1*$^{flox/flox}$) and control (*Glast*^{CreERT2}*:Gfrα1*$^{+/flox}$) mice were treated with TAM at 2 months of age, and GFP$^+$ cells were analyzed by confocal imaging at 14, 28, and 56 dpi. GCs generated in *Gfrα1*-deficient mice displayed shorter dendritic length and reduced number of branch points than those generated in control mice, revealing a diminished complexity. This effect was observed at 14, 28, and 56 dpi (Figures 3A–3I). Three months after TAM injection (84 dpi), we were not able to detect significant differences between *Gfrα1*-deficient and control mice (Figure S3), suggesting that the absence of *Gfrα1* results in a delayed development of adult-born GCs.

Because GFRα1 is involved in the regulation of dendritic spine maturation in hippocampal pyramidal neurons (Irala et al., 2016),

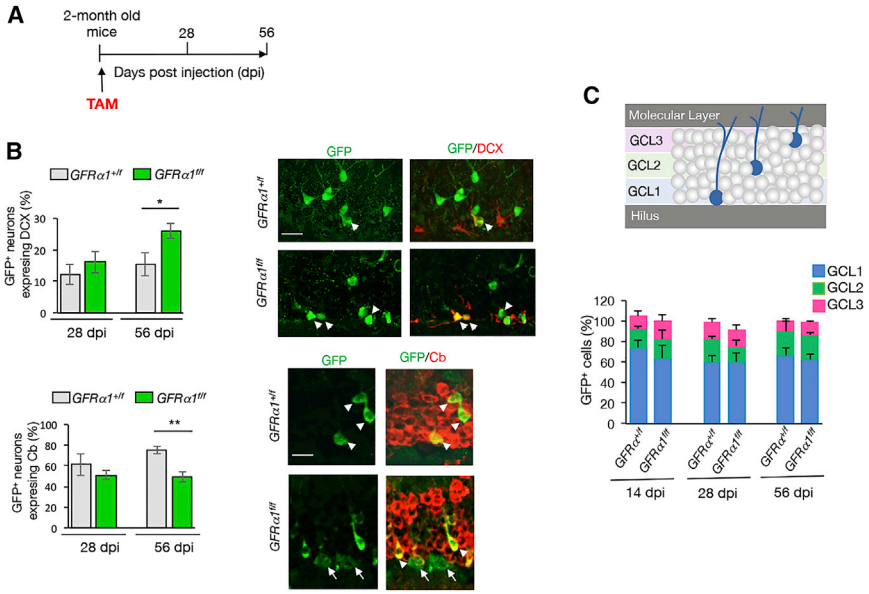

Figure 2. Deletion of GFRα1 Affects the Correct Maturation of Adult-Born GCs

(A) Schematic diagram showing the experimental design used for TAM-induced Cre recombination in *Glast*^{CreETR2}:*Gfrα1*$^{flox/flox}$ mice and control littermates, *Glast*^{CreETR2}:*Gfrα1*$^{+/flox}$. TAM was injected intraperitoneally in 2-month-old mice and analyzed at different times by immunofluorescence and confocal imaging.

(B) Bar graphs describe the proportion of GFP$^+$ neurons expressing DCX (top) or Cb (bottom) in *Gfrα1*-deficient (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$, indicated as *Gfrα1*$^{f/f}$) and control mice (*Glast*^{CreERT2}:*Gfrα1*$^{+/flox}$, indicated as *Gfrα1*$^{+/f}$). The bars denote mean ± SEM (n = 3 mice/condition). *p < 0.05; **p < 0.01 (Student's t test). Representative confocal images of 56 dpi GCs expressing the reporter marker GFP co-localized with the neuronal marker DCX or Cb. Scale bar: 50 μm. Arrowheads indicate GFP$^+$DCX$^+$ or GFP$^+$Cb$^+$ cells, and the arrows indicate GFP$^+$Cb$^-$ cells.

(C) Schematic diagram representing granule cell layer (GCL) subdivisions where newborn adult GCs are located. The GCL was divided in three sections, GCL1, 2, and 3. Graph showing the distribution of GFP$^+$ neurons in the different subdivisions from *Gfrα1*-deficient (*Gfrα1*$^{f/f}$) and control mice (*Gfrα1*$^{+/f}$). The bars indicate mean ± SEM (n = 3 mice/condition).
See also Figures S2 and S3.

we analyzed the effect of GFRα1 ablation on dendritic spine density in adult-born GCs. As the fluorescence signal from the *Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$ in which the GFP expression is under the regulation of *Gfrα1* promoter was not strong enough to visualize dendritic spines, adult-born GCs were labeled using a retroviral construct expressing the red fluorescent protein (RFP), as indicated in STAR Methods. This strategy allowed us to visualize dendritic spines of different morphologies in GFP$^+$/RFP$^+$ neurons, where mushroom-like morphology is the most mature type. Spine density was quantified in 4-week-old neurons in which extensive remodeling of input and output connections are essential to determine the role of new GCs in information processing (Ge et al., 2007; Marín-Burgin and Schinder, 2012). We were not able to detect significant differences in the total number of spines between neurons from control and GFRα1-deficient mice, 28 days post TAM injection. However, in neurons from mutant mice, we observed a significant reduction in the density of mushroom-shaped spines, which are thought to be the largest and stronger synapses (Berry and Nedivi, 2017). These results indicate that GFRα1 is important for dendritic spine maturation in adult-born GCs (Figure 3J).

Deficit of GFRα1 Results in Impaired Processing of Spatial Memory

The deficits that we found in dendritic morphology and spine development may result in altered neuronal integration and, consequently, learning defects in *Gfrα1* mutant mice. In particular, adult-born GCs are thought to contribute to spatial memory through a process called "pattern separation," required for the formation of distinct representations from similar inputs (Aimone et al., 2014; Clelland et al., 2009; Sahay et al., 2011). We thus carried out analyses to test behavioral pattern separation. The assay was done in animals after 28 days of TAM injections, as it is known that during this period newborn GCs are highly susceptible to activity-dependent synaptic modifications of input and output connections (Marín-Burgin and Schinder, 2012). First, we performed an open-field test to assess behavioral deficits and found that *Gfrα1*-deficient mice with 28 dpi exhibited similar locomotor activity (Figures 4A–4C) and similar habituation to the context as control littermates (Figures S4A–S4D). The two genotype groups spent similar amounts of time in the central region of the open field (*Gfrα1* control: 46.47 ± 0.61 s, and *Gfrα1* mutants: 45.68 ± 0.77 s; mean ± SEM), indicating that the treatment did not modify anxiety levels (Figure 4C).

We analyzed pattern separation-dependent memory by using a spontaneous location recognition (SLR) task, in which we could test whether mice could differentiate object locations in two conditions of similarity or dissimilarity as described by Bekinschtein et al. (2013). Briefly, the task consisted on a training phase in which mice were exposed to three identical objects for 10 min (A1, A2, and A3), where two of them were separated by a 50° angle (similar-SLR) or by a 120° angle (dissimilar-SLR) and the third one was further away (Figure 4D). During the test, mice explored two identical objects (A4 and A5): one in a novel location and equidistant from the two close ones explored during the training phase, and the other one in its original location. Due to the intrinsic preference of rodents for novelty, it is expected that in both conditions, the animals prefer and explore more the object in a novel position. In both the similar (s-SLR) and dissimilar (d-SLR) condition, during training the animals of both genotypes explored all the objects equally (Figures 4E, 4F, S4E, and S4F), which indicates that they did not have an initial preference for any of the positions at the beginning of the evaluation. In the testing of the dissimilar condition

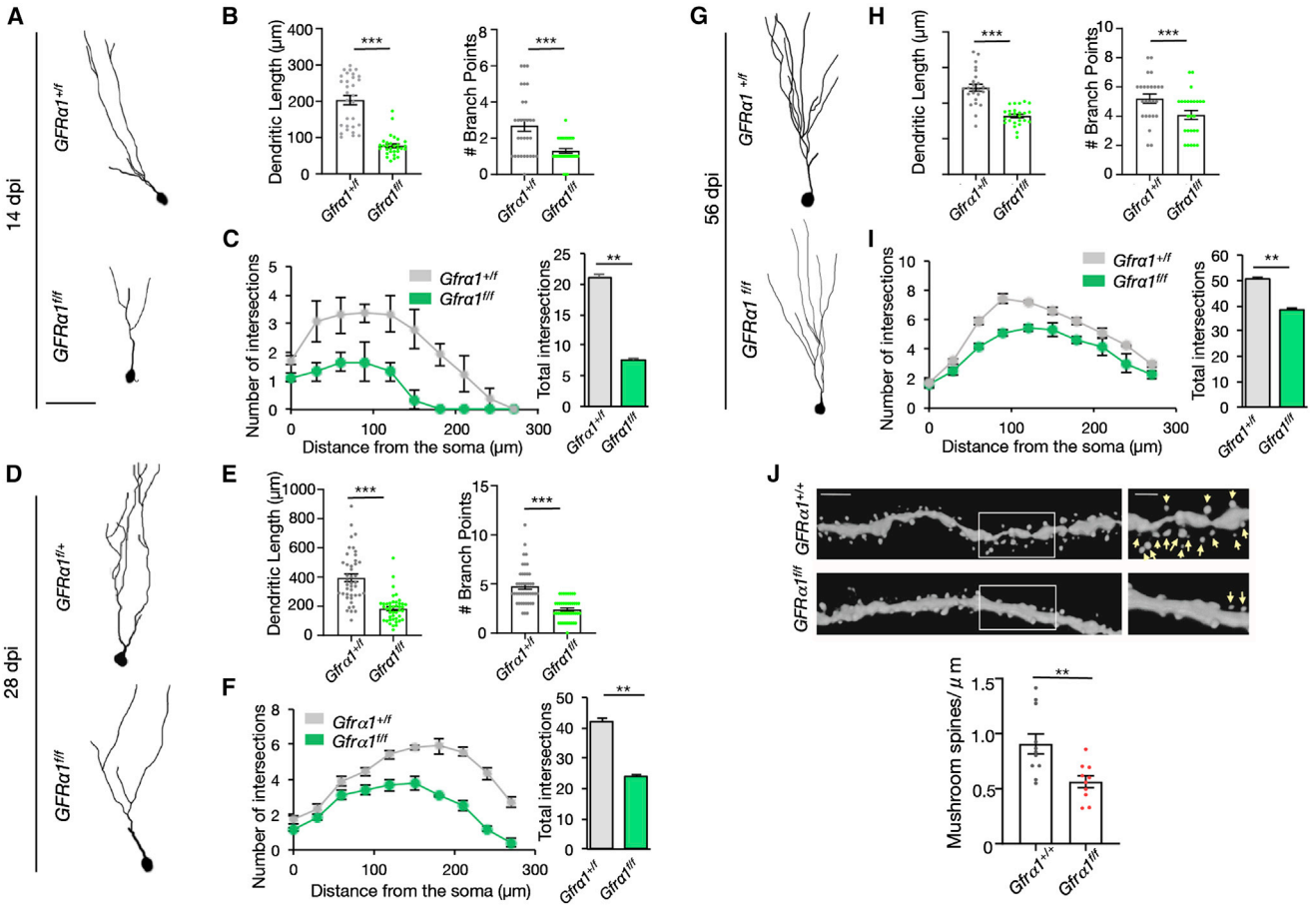

Figure 3. GFRα1 Is Required for Proper Dendritic Growth and Spine Formation in Adult-Born GCs

(A, D, and G) Representative drawings of GFP label adult-born granule GCs derived from *Gfrα1*-deficient mice (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$, indicated as *Gfrα1*$^{f/f}$) and control mice (*Glast*^{CreERT2}:*Gfrα1*$^{+/flox}$, indicated as *GFRα1*$^{+/f}$) at 14 (A), 28 (D), and 56 (G) days after TAM injection (days post-injection [dpi]). Sections were stained with anti-GFP antibodies to visualize and measure morphological parameters. Scale bar: 50 μm.

(B, E, and H) The graphs show the quantification of dendritic length and number of branch points in control (*Gfrα1*$^{+/f}$) and GFRα1 mutant (*Gfrα1*$^{f/f}$) littermates at 14 (B), 28 (E), and 56 (H) dpi. The results are shown as mean ± SEM. About 15 neurons per mouse were analyzed in 3 mice of each genotype. ***p < 0.001 by two-tailed Student's t test.

(C, F, and I) Sholl analysis of the dendritic arbors of GFP labeled cells in control (*Gfrα1*$^{+/f}$, gray lines) and *Gfrα1*-deficient mice (*Gfrα1*$^{f/f}$, green bars) at 14 (C), 28 (F), and 56 (I) dpi. The results are shown as mean ± SEM. The bar graphs show cumulative dendrite crossings obtained from Sholl analysis, which represent the summation of dendritic intersections. The results are shown as mean ± SEM of independent determinations performed in 3 mice of each genotype (n = 3). About 15 neurons per mouse were analyzed. **p < 0.01 by two-tailed Student's t test.

(J) Representative confocal images from dendritic segments from newborn GCs derived from control (*Gfrα*$^{+/+}$) and *Gfrα1*-deficient mice (*Gfrα1*$^{f/f}$) 1 month post TAM and RV-RFP injection. The graph shows mushroom-morphology spine density (spine number per micrometer), with n = 11 (*Gfrα*$^{+/+}$) and n = 10 (*Gfrα1*$^{f/f}$) GCs from 3 mice of each genotype. The results are shown as mean ± SEM. **p < 0.01 by two-tailed Student's t test. Scale bar, 5 μm.

See also Figure S3.

(d-SLR), both the control mice and the *Gfrα1*-deficient ones were able to solve the task, that is, to remember the objects exposed in the training individually, and recognize the novel position exposed in the evaluation. However, during the testing of the similar condition (s-SLR), only the control mice, but not the *Gfrα1*-deficient mice, were able to discriminate the spatial locations of the objects presented in the training as different, and to recognize the novel position (Figure 4G). This observation indicates that animals deficient in GFRα1 present impairments in the processing of spatial memory dependent on the new GCs of the DG.

GDNF/GFRα1 Complex Mediates Remodeling of Adult-Born GCs Induced by Physical Activity

In young adult mice, the generation and integration of new GCs is regulated by external factors, which include enriched environment (EE), voluntary exercise, diet, aging, and stress, among others (Alvarez et al., 2016; Bergami et al., 2015; Mirochnic et al., 2009; Piatti et al., 2011; Trinchero et al., 2017; Vadodaria and Gage, 2014). Previous studies have shown that physical exercise increases neurotrophic factor levels in the hippocampus, in particular in young rodents (Cotman and Berchtold, 2002; Farmer et al., 2004). To analyze whether physical activity

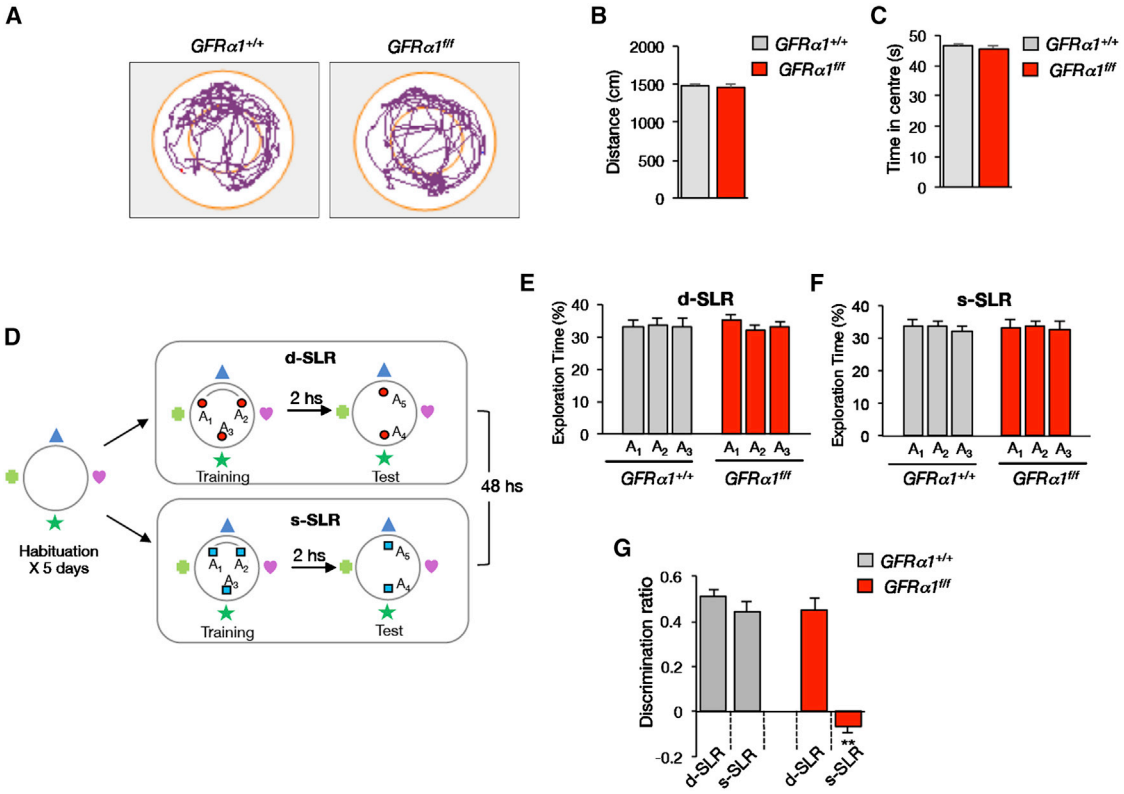

Figure 4. GFRα1-Deficient Mice Exhibit Behavioral Impairments in a Pattern Separation Task

(A–C) Locomotor activity of control (*Gfrα1*$^{+/+}$) and *Gfrα1*-deficient (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$, indicated as *Gfrα*$^{f/f}$) mice in a novel environment was assessed by a 10-min session in an open field.

(A) Representative traces of mice pattern activity in the first 2 min of the open field. Each panel depicts the activity of one individual.

(B) Bar graph shows the total distance traveled during the first 5 min in the open field.

(C) Bar graph shows the total time (in seconds) spent in the center area of the open field by control and mutant animals. ns, not significant (Student's t test). Number of animals analyzed of each genotype, n = 8. Data represent mean ± SEM.

(D) Schematic representation of the SLR task for the similar (s-SLR) and dissimilar (d-SLR) conditions is shown.

(E and F) The percentage of time spent by the control and GFRα1 mutant animals exploring each of the locations during the training phase of the s-SLR (E) and the d-SLR (F). Two-way repeated-measures ANOVA, no significant differences were found.

(G) Bar graph represents the object preference index for s-SLR and d-SLR conditions. The results are expressed as mean ± SEM. **p < 0.01. Two-way ANOVA followed by Bonferroni post-test. Number of animals analyzed of each genotype, n = 8–10.

See also Figure S4.

modifies the expression of GDNF, protein levels of this neurotrophic factor were analyzed by immunoblot in total lysates obtained from DG from sedentary mice or from mice with access to a running wheel for 14 days. After running, we observed a significant increase in the levels of GDNF, which was accompanied by an increase in BDNF expression (Figures 5A and 5B). These results suggest that the regulation exerted by running on adult neurogenesis could be partially mediated by these neurotrophic factors and the signaling through their receptors.

To analyze whether developing GCs deficient in GFRα1 respond to physiological stimuli, we studied the effects of running on neuronal integration. Two-month-old control (*Glast*^{CreERT2}:*Gfrα1*$^{+/flox}$) or GFRα1 mutant (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$) mice were treated with TAM, and 14 days post injection, mice were maintained in sedentary or running conditions for 14 days. In control animals, running increased the complexity of dendritic arborization compared with that of sedentary mice. We observed an increase in total neurite length as well as in dendrite branching. Notably, GFRα1 defects abolished the dendritic growth induced by running. No significant differences were observed in dendrite length or in branching between sedentary or running conditions in GFRα1-deficient mice (Figures 5C–5G). These results demonstrate that GDNF acting through GFRα1 mediates the integration of new GCs induced by physical exercise.

In order to explore the signaling pathway activated by GDNF/GFRα1 complex in newborn GCs, we focused on the activation of the transcription factor, CREB (cAMP response element-binding protein), which has been described to be activated by GDNF/GFRα1 signaling in different neuronal cell lines (Trupp et al., 1999). It is known that CREB is activated in response to neuronal activity (Greenberg et al., 1990) and that is involved in the dendritic development of adult newborn GCs (Jagasia et al., 2009). Based on this evidence, we

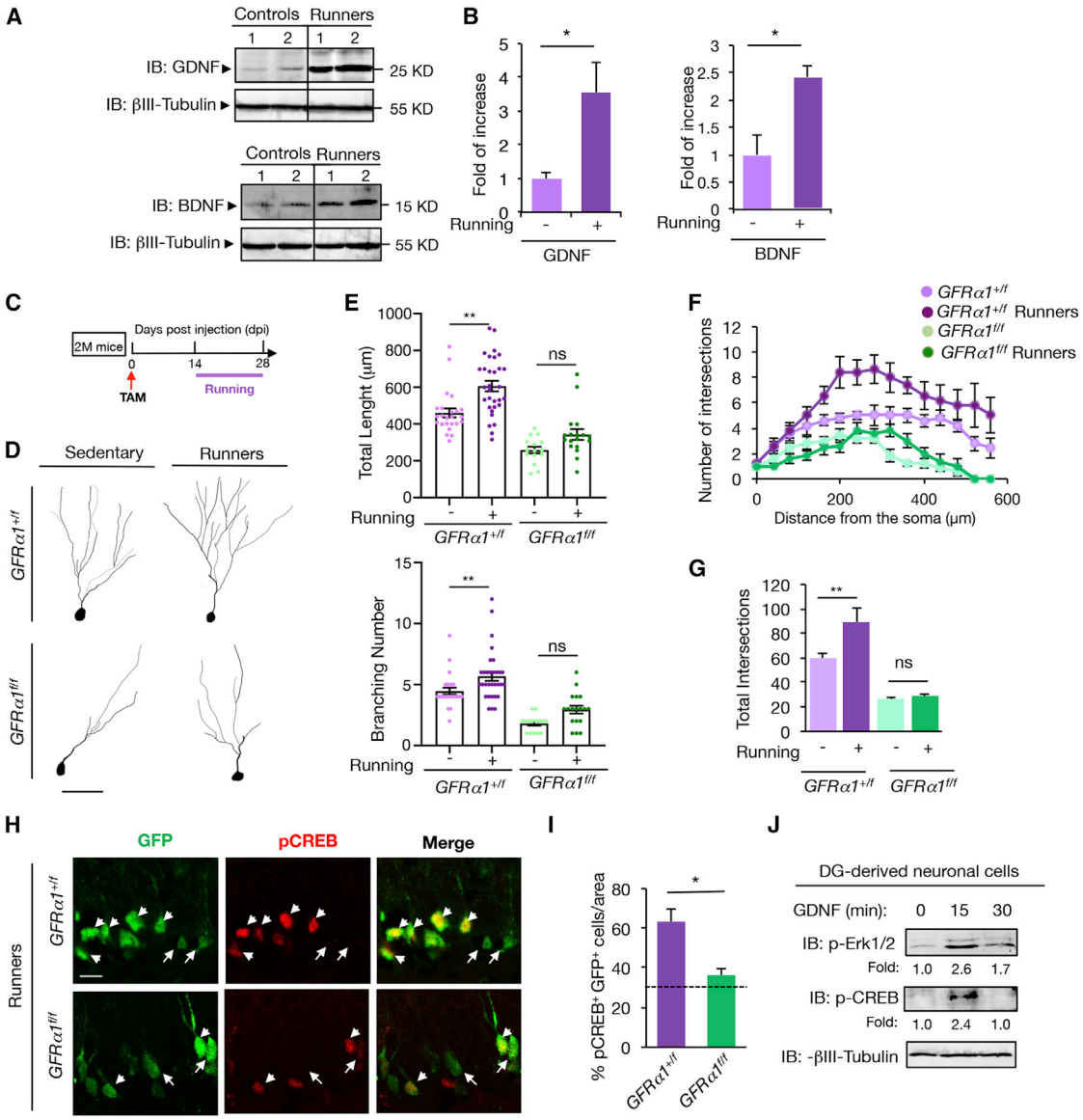

Figure 5. Running Increases GDNF Expression in DG and GFRα1 Is Required for Correct Arborization of Immature Adult-Born Granule Cells

(A) Analysis by immunoblotting of GDNF and BDNF expression in DG homogenates obtained from 2-month-old control and runner mice. The blot was re-probed with anti-βIII-Tubulin.

(B) The graphs show GDNF and BDNF content in DG total extracts relative to βIII-Tubulin; n = 3 mice per group were measured. The results are expressed as mean ± SEM. *p < 0.05 (Student's t test).

(C) Experimental design used for TAM-induced Cre recombination in *Gfrα1* mutant, *Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$ (*Gfrα1*$^{f/f}$) mice, and control littermates, *Glast*^{CreERT2}:*Gfrα*$^{+/flox}$ (*Gfrα1*$^{+/f}$). TAM was injected i.p. in 2-month-old mice (2M). After 14 dpi, mice were maintained under sedentary or running conditions for 14 days. Morphological analysis was done at 28 dpi by immunofluorescence and confocal imaging.

(D) Representative drawings of 28 dpi GFP$^+$ GCs from control (*Gfrα1*$^{+/f}$) and *Gfrα1* mutant (*Gfrα1*$^{f/f}$) sedentary and runner mice. Scale bar, 50 μm.

(E) Quantification of dendritic length and branching of GFP granule cells. The graphs show the measurement of n = 15–30 neurons/condition from 3 control and 3 runner mice from each genotype. The bars denote means ± SEM. **p < 0.01 (ANOVA followed by Bonferroni); ns, not significant.

(F) Sholl analysis of the dendritic arbors of GFP labeled cells from the animals analyzed in (E).

(G) The graph shows cumulative dendrite crossings obtained from Sholl analysis, which represent the summation of dendritic intersections (n = 3 animals of each genotype per condition). **p < 0.01 (ANOVA followed by Bonferroni multiple-comparison test). ns, not significant.

(H) Representative confocal images showing newborn GCs co-expressing GFP and pCREB from control (*Gfrα*$^{+/f}$) and *Gfrα1*-deficient mice (*Gfrα1*$^{f/f}$) that were maintained in running conditions. Scale bar: 20 μm. Arrowheads indicate GFP$^+$ pCREB$^+$ neurons; arrows indicate GFP$^+$ pCREB$^-$ neurons.

(I) The bars show the proportion of GFP$^+$ cells that display pCREB expression/area (10,000 μm^2) in control and *Gfrα1*-deficient mice that were maintained in running conditions. The bars denote means ± SEM. *p < 0.05 (Student's t test); n = 3 mice per group. Dotted line indicates the proportion of pCREB$^+$ GFP$^+$ neurons in sedentary control mice.

(legend continued on next page)

analyzed by immunostaining the activation of CREB in control (*Glast*^{CreERT2}:*Gfrα1*$^{+/flox}$) and *Gfrα1*-deficient mutant (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$) animals treated with TAM and maintained under running conditions for 14 days. Our results indicate that TAM-induced *Gfrα1* mutants (*Glast*^{CreERT2}:*Gfrα1*$^{flox/flox}$) exposed to voluntary exercise exhibited a significant reduction in the proportion of GFP$^+$ cells containing pCREB$^+$ nuclei compared to control animals (*Glast*^{CreERT2}:*Gfrα1*$^{+/flox}$), revealing that GFRα1 is required for CREB activation in newborn GCs (Figures 5H and 5I).

Finally, we examined the activation of CREB in response to GDNF in postnatal DG-derived neural stem cell cultures. To this end, neural stem cells isolated from DG were expanded by growing them as neurospheres in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF). After amplification, the spheres were dissociated and cultured in differentiative conditions as it was previously described (Rodrigues et al., 2017). After confirming the expression of GFRα1 in mature (βIII-Tubulin$^+$) and immature (DCX$^+$) neurons by immunofluorescence (Figure S5B), the cells were starved and then exposed to GDNF for different times. Immunoblot analysis revealed that GDNF stimulation resulted in phosphorylation of Erk1/2 and CREB (Figure 5J) in postnatal DG-derived neurons.

DISCUSSION

Integration of newborn GCs in the mature hippocampal network constitutes an important form of structural plasticity that contributes to regulate brain functions such as spatial learning and mood. Defects in neurogenesis have been associated with several human neurological and psychiatric diseases (Choi et al., 2018; Duan et al., 2007; Gonçalves et al., 2016; Miller and Hen, 2015), and consequently, there is a strong interest to understand the molecular signals that control neuronal generation, dendrite maturation, and synaptic integration of these new neurons in the adult hippocampus.

Here, we report that GFRα1 expression is essential for proper morphological maturation and synaptic integration of adult-born hippocampal neurons. The effects of GDNF/GFRα1 on dendritic arborization take place immediately after the GCs express the immature neuronal marker DCX and the GDNF signaling receptor PSA-NCAM (Zhang and Jiao, 2015; Zhao et al., 2008). Thus, this observation is in line with the putative non-involvement of GFRα1 in adult hippocampal neural precursor cell (NPC) proliferation described in the present work.

The data shown here reveal that voluntary running triggers GDNF expression and promotes GFRα1-dependent dendritic remodeling of newborn GCs in the adult hippocampus, demonstrating that structural plasticity of adult DG is potentiated by physical exercise and is mediated by GFRα1. In agreement with this, we showed that the activation of the transcription factor CREB, which has been involved in dendrite development of adult-born GCs (Jagasia et al., 2009), requires GFRα1 expression (see Figure 5). Thus, our findings suggest that upregulation of GDNF after running triggers CREB activation in GFRα1-expressing adult-born GCs promoting dendritic development.

In the present study, we also found that mice specifically lacking GFRα1 in the newborn neuron population of 4 weeks of age, exhibited impairment in a behavioral pattern separation task, evidenced by their defects to discriminate subtle differences rather than general processing of spatial information. Thus, GFRα1 regulation of newborn neuron integration into the pre-existing hippocampal circuitry could represent an event of plasticity critically required for learning and memory.

GFRα1 Is Required for Proper Maturation of Adult-Born Dentate Gyrus GCs

A large body of evidence has identified key factors for the control of neural precursor cell proliferation in the SGZ, but the molecular signals that regulate the early development of dendritic arbors and spines in adult-born GCs are less well understood (Gonçalves et al., 2016). Various neurotrophic factors have been implicated in the processes of adult neurogenesis and neuronal integration by promoting dendrite development and survival (Vilar and Mira, 2016). The role of NTF3 in adult hippocampal neurogenesis was analyzed in conditional mutant mice in which the *Ntf3* gene was deleted in the brain. This study shows normal proliferation in the SGZ and a reduction in the number of newly generated NeuN$^+$ granule neurons, indicating a role of NTF3 regulating the number of newly differentiated neurons in the adult DG (Shimazu et al., 2006). Several studies have described the importance of BDNF on adult neurogenesis.

BDNF has been described to be relevant for proliferation of SGZ progenitor cells, as well as for dendrite development and synaptic maturation of newborn SGZ neurons. Conditional *TrkB*-knockout mice in which the gene encoding TrkB is deleted specifically in adult-born neurons shows that dendrite and spine growth is markedly altered in adult-born GCs of *TrkB*$^{flox/flox}$ mice (Bergami et al., 2008). In agreement with these findings, a significant reduction in dendritic development, synaptic formation, and maturation has been observed in postnatal-born granule neurons in different BDNF conditional knockout mice (Chan et al., 2008; Gao and Chen, 2009). Interestingly, Wang et al. (2015) described that BDNF secreted by newborn GCs acts as an autocrine factor for dendrite development and synaptic maturation.

During the last years, the neurotrophic factor GDNF acting through its receptor GFRα1 has emerged as an important molecular system controlling structural plasticity and synapse formation in postnatal pyramidal hippocampal neurons (Irala et al., 2016; Ledda et al., 2007); however, nothing was known about its role in the maturation of adult born DG neurons. GDNF has been described to be expressed by astrocytes and mature neurons in the forebrain, but it is not clear which is the source of

(J) Immunoblot showing phosphorylation of CREB in Ser-133 (pCREB) and activation of Erk1/2 (pErk1/2) in postnatal neural stem cells stimulated with GDNF (100 ng/mL) for 15 and 30 min. Reprobing of the same blot with anti-βIII-Tubulin is shown as loading control. The numbers below the lanes indicate the fold induction of CREB and Erk1/2 phosphorylation relative to control normalized to the levels of Tubulin. The experiment was repeated two more times with similar results.

See also Figure S5.

GDNF that act on newborn GCs. Interestingly, a recent study in which the authors perform single-cell RNA sequencing of adult DG (Hochgerner et al., 2018) indicates that GDNF is expressed at low levels by few mature and immature GCs.

Previously, we have described that GDNF acting through GFRα1 and NCAM, but independently of its canonical Ret tyrosine kinase receptor, promotes the growth and complexity of dendritic arbors as well as the establishment of excitatory synaptic contacts of postnatal pyramidal hippocampal neurons (Irala et al., 2016; Ledda et al., 2007). In line with the role of this neurotrophic factor in neural dendrite remodeling, the present work provides evidence indicating that GFRα1 is required for proper dendritic maturation and synaptic integration of adult-born granule hippocampal neurons. The absence of Ret expression in hippocampus (Irala et al., 2016) and the expression of NCAM in adult-born GCs suggest that this cell adhesion molecule could act as GFRα1 co-receptor in the integration of these neurons in the DG. However, the transmembrane molecular partner through which GFRα1 regulates the maturation of these cells deserves further investigation. A role of GDNF/GFRα1, in dendrite remodeling, has also been described by our group in cortical progenitors (Bonafina et al., 2018). During corticogenesis, GDNF, acting through GFRα1, has a dual role by promoting neuronal differentiation and inhibiting self-renewal capacity of mouse cortical neural precursors induced by the mitogenic factor FGF2 (Bonafina et al., 2018). Our present data indicate that, in adult-born GCs, GDNF/GFRα1 modulates dendrite complexity but does not have an effect on NPC proliferation.

Functional Role of GDNF/GFRα1 Complex in Adult-Born GCs

Adult-born neurons in the DG have been described to be functionally important in different aspects of hippocampus-dependent functions, such as memory formation, flexibility of learning strategies, as well as pattern separation (Toda et al., 2019). Interestingly, the neurotrophin BDNF has been described to be part of an essential mechanism underlying the consolidation of pattern-separation memories (Bekinschtein et al., 2013, 2014). In the present work, we present evidence indicating that another neurotrophic factor such as GDNF acting through GFRα1 has an essential role in a pattern-separation paradigm of spatial memory. We found that mice lacking GFRα1 in the newborn GC population exhibit significant behavioral deficits compared to control ones.

This behavioral impairment could be explained by the deficits observed in dendrite outgrowth and the lack of mature spines, which would lead to an altered integration of newborn hippocampal cells.

Several studies have shown that external factors can positively or negatively impact the levels of neurogenesis throughout the life of mammals (Aimone et al., 2014). Thus, factors such as running and EE are considered as positive stage-specific modulators of hippocampal adult neurogenesis (Alvarez et al., 2016; Kempermann et al., 1998; Kuipers et al., 2015; Marlatt et al., 2012; Morgenstern et al., 2008; Piatti et al., 2011; van Praag et al., 1999; Vivar et al., 2013), while others such as stress and aging have been proposed as negative regulators of this process (Kempermann et al., 1998; Kuipers et al., 2015; Morgenstern et al., 2008). Many different neurotrophic factors link running activity with neurogenesis, suggesting that functional redundancy is likely to occur in the adult hippocampus. Previous work has shown that running induces growth factors such as insulin growth factor 1 (IGF-1) (Trejo et al., 2001), VEGF (Fabel et al., 2003), FGF-2 (Campuzano et al., 2002), and BDNF (Scharfman et al., 2005; Vivar et al., 2013), which have been shown to influence hippocampal neurogenesis. Although running has been associated with proliferation of NPCs, it is now accepted that physical exercise also has relevant effects on the development and integration of adult-born GCs (Alvarez et al., 2016; Bergami et al., 2015; Wang et al., 2015). Interestingly, a recent publication described that induction of adult hippocampal neurogenesis combined with overexpression of BDNF could mimic exercise-induced improvements in cognition in an Alzheimer's mouse model (Choi et al., 2018).

Although there is evidence indicating that GDNF levels in the spinal cord can be modulated by running (McCullough et al., 2013) or by EE in the substantia nigra (Faherty et al., 2005), our findings show that GDNF levels are increased after running in the adult DG. Moreover, we provide evidence indicating that voluntary running, which triggers the expression of GDNF as well as other neurotrophins, cannot compensate the reduced dendrite arborization detected in adult-born GCs lacking GFRα1. This result suggests that subpopulations of adult-born GCs may respond to different neurotrophic factors. The fact that GFRα1 is expressed in approximately 50% of DCX^+ GCs supports this idea.

In this regard, it is known that NPCs of the adult SGZ present transcriptional heterogeneity that could explain the effects described on adult-born neurogenesis for different running-induced trophic factors (Shin et al., 2015). However, the expression of different arrays of neurotrophic factor receptors in the adult-born GCs should be analyzed by additional approaches.

Altogether, our data show that GFRα1 is required for proper maturation of newborn GCs in the DG, which is essential for spatial memories and that voluntary running triggers endogenous GDNF expression, which contributes to GFRα1-dependent dendritic arborization of newborn GCs in the adult hippocampus. Thus, GDNF/GFRα1 complex represents a key mediator linking running activity with the control of the structural plasticity and synaptic integration required for spatial memories.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Confocal Microscopy

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.11.100.

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AUTHOR CONTRIBUTIONS

A.B. designed and performed immunofluorescence, confocal image data collection, western blot, and behavioral assays and analyzed the data. M.F.T. designed and performed the analysis of spine development by confocal imaging. A.S.R. contributed to experiments involving running and analyzed data. P.B. contributed to the performance and analysis of behavioral assays. A.F.S. contributed to the data analysis and provided insightful ideas. G.P. and F.L. contributed to the concept, designed the experiments, analyzed the data, wrote the manuscript, and provided financial support.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-GFRα1	R&D Systems	Cat# AF560; RRID: AB_2110307
α-Doublecortin	Santa Cruz Biotechnology	Cat# sc-8066 ;RRID: AB_2088494
α-Doublecortin	Piatti et al., 2011	N/A
α-Calbindin (26D12)	Cell Signaling Technology	Cat# 2173; RRID: AB_2183553
α-Ki67	Leica Biosystems	Cat# NCL-Ki67p; RRID: AB_442102
α-GFP	Aves	Cat# GFP-1010; RRID: AB_2307313
ABα-pCREB-Ser133 (87G3)	Cell Signaling Technology	Cat# 9198; RRID: AB_2561044
α-BrdU (BU20a)	Agilent	Cat#M0744; RRID: AB_10013660
α-GDNF	R&D Systems	Cat# AF-212; RRID: AB_211398
α-BDNF	Santa Cruz Biotechnology	Cat# sc-546 ; RRID: AB_630940
α-p44/42 MAPK (Erk1/2)(137F5)	Cell Signaling Technology	Cat# 4695; RRID: AB_390779
α-βIII-Tubulin	Promega	Cat# G7121; RRID: AB_430874
Cy2-Donkey anti Chicken IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 703-225-155; RRID: AB_2340370
Cy3-Donkey anti Goat IgG (H+L)	Jackson ImmunoResearch Labs	Cat#705-165-147; RRID: AB_2307351
Cy2-Donkey anti-Goat IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 705-225-147: RRID: AB_2307341
Cy3-Donkey anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 711-165-152; RRID: AB_2307443
Cy2-Donkey anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 711-225-152; RRID: AB_2340612
Cy2-Donkey anti-Mouse IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 715-225-150; RRID: AB_2340826
Alkaline Phosphatase-Donkey anti Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 711-055-152; RRID: AB_2340591
Alkaline Phosphatase-Donkey anti Mouse IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 715-055-150; RRID: AB_2340777
Bacterial and Virus Strains		
RV-RFP	Trinchero et al., 2017	N/A
Chemicals, Peptides, and Recombinant Proteins		
Tamoxifen	Sigma-Aldrich	T5648
Corn Oil	Sigma-Aldrich	C8267
BrdU	Sigma-Aldrich	BS002
Poly-D-Lysine	Sigma-Aldrich	P0899
GDNF	R&D	212-GD
BDNF	R&D	248-BD
FGF-2	R&D	233-FB
EGF	R&D	2028-EG
B27	ThermoFisher Scientific	17504-044
Critical Commercial Assays		
RNeasy Mini Kit	Quiagen	Cat# 74104
Multiscribe Reverse Transcriptase	ThermoFisher Scientific	Cat# 4366596
Experimental Models: Organisms/Strains		
Mouse: *Gfrα*$^{f/f}$	Uesaka et al., 2007	N/A
Mouse: *Glast*^{CreERT2}	Mori et al., 2006	N/A
Mouse: *Emx1*^{Cre}	Iwasato et al., 2004	N/A
Mouse:*CAG*^{floxStopTom}	Madisen et al., 2010	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
NCAM F: **5′ TCATGGACATCACCTGCTAC 3′,** NCAM R: **5′ GGTTCTTTTGACTCATCTTTCG 3′**	Ledda et al., 2007	N/A
GFRα1 F: **5′ CTGAGAATGAGATCCCCACAC 3′,** GFRα1 R: **5′ CGACACATTGGATTTCAGCTT 3′**	Ledda et al., 2007	N/A
Ret F: **5′ ATGATGATGAAGACGACTCCCC 3′,** Ret R: **5′C GCTTAAACTCCACCACAGCA 3′**	Ledda et al., 2007	N/A
TBP F: **5′ GCCTTCCACCTTATGCTCAG 3′,** TBP R: **5′ CCGTAAGGCATCATTGGACT 3′**	Ledda et al., 2007	N/A
Software and Algorithms		
Image Quant Software	GE Healthcare Life Science	N/A
Graph Pad Prism	Graph Pad Software Company	N/A
Any-Maze	Stoelting	N/A
ImageJ	Schneider et al., 2012	https://imagej.nih.gov
Critical Commercial assays		
RNeasy Mini Kit	Quiagen	N/A
Multiscribe Reverse Transcriptase	ThermoFisher Scientific	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed and will be fulfilled by Fernanda Ledda (fledda@leloir.org.ar).

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Gfrα1$^{flox/flox}$ mice were generously provided by Dr J. Milbrandt (Washington University School of Medicine, St Louis, MO, USA). *Gfrα1*$^{flox/flox}$ mice were mated with *Emx1*^{Cre} mice generously provided by Dr. N Weisstaub (Universidad Favaloro, INECO-CONICET, Argentina) (Iwasato et al., 2004; Weisstaub et al., 2006), or with *Glast*^{CreERT2} *CAG*^{floxStopTom} mice (Madisen et al., 2010; Mori et al., 2006), kindly provided by Dr. G. Lanuza (Leloir Institue, IIBBA-CONICET, Argentina) All transgenic strains were genotyped using PCR-based strategy. PCR primer sequences are available upon request. Tamoxifen (TAM, Sigma-Aldrich) was delivered intraperitoneally at 150 μg/g/d for 2 consecutive days to achieve appreciable expression of GFP in adult-born GCs (Yang et al., 2015). Mice were killed at the indicated times after TAM induction. The use of animals was approved by the Animal Care and Use Committee (CICUAL) of the School of Medicine, University of Buenos Aires and Instituto Leloir according to the Principles for Biomedical Research involving animals of the Council for International Organizations for Medical Sciences and provisions stated in the Guide for the Care and Use of Laboratory Animals.

METHOD DETAILS

Stereotaxic Surgery for Retroviral Delivery

Tamoxifen administration was carried out in 2 month-old deficient *Glast*^{CreERT2}*:Gfrα1*$^{flox/flox}$ and control *Glast*^{CreERT2}*:Gfrα1*$^{flox/+}$ mice as previously described. 12 hours after the last TAM injection, mice were anesthetized (150 mg ketamine/15 mg xylazine in 10 mL saline per gram), and retrovirus (RV) were infused into the septal region of the right DG (1.5 mL at 0.15 ml/min) using sterile calibrated microcapillary pipettes through stereotaxic surgery coordinates from bregma (in millimeters): −2 anteroposterior, −1.5 lateral, and −1.9 ventral. Brain sections were obtained 4 weeks later for confocal imaging. Only neurons in the septal dentate gyrus were included in the analysis, corresponding to sections containing the septal region of the hippocampus (−0.96 to −2.30 mm from the bregma) according to Paxinos and Franklin's mouse brain atlas (Temprana et al., 2015). Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Fundación Instituto Leloir according to the Principles for Biomedical Research involving animals of the Council for International Organizations for Medical Sciences and provisions stated in the Guide for the Care and Use of Laboratory Animals

Immunofluorescence

Immunostaining was done on 55 μm floating coronal adult brain sections. Antibodies were applied in Phosphate Buffer (PBS) containing 0.3% Triton X-100, previously blocked with 10% Normal donkey serum (Jackson ImmunoResearch Laboratories). Immunofluorescence was performed using the following primary antibodies: anti-GFRα1 (1:300, R&D), anti-Dcx (1:350, Santa Cruz), anti-Cb (1:1000, Cell Signaling Technology), anti Ki67 (1:1000, Leica Biosystems), anti-GFP (1:1000, Aves), anti-pCREB (1:100, Cell Signaling Technology). The secondary antibodies were from Jackson ImmunoResearch Laboratories (1:300). For BrdU detection, DNA was denatured with 2N HCl at 37°C for 30 min and washed in 0.1 M boric acid, pH 8.5, for 2 min. For double labeling with the cell cycle marker Ki67 and BrdU, we used a serial protocol. Sections were first incubated with BrdU antibody (DAKO, 4°C, overnight), and followed by Ki67 antibody incubation (4°C, overnight) (Wojtowicz and Kee, 2006).

Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdU) (Sigma-Aldrich) was dissolved in 0.9% NaCl and was delivered intraperitoneally at a dose of 50 μg/g to mice housed under standard conditions to label dividing cells. To measure the proportion of cells that continue to proliferate after labeling, the mice were injected 3 times in a single day (Once every 6 h) to label different pools of neural progenitor cells. Mice were killed 2 or 4 d later to asses co-labeling with Ki67.

Production of Viral Vectors

A replication-deficient retroviral vector based on the Moloney murine leukemia virus was used to specifically transduce adult-born GCs as done previously (Temprana et al., 2015). Retroviral particles were assembled using three separate plasmids containing the capside (CMV-vsvg), viral proteins (CMV-gag/pol), and the transgene CAG-RFP retroviral plasmid. Plasmids were transfected onto HEK293T cells using deacylated polyethylenimine. HEK293T cells were cultured in DMEM with high glucose, supplemented with 10% fetal calf serum and 2 mM glutamine. Virus-containing supernatant was harvested 48 hr. after transfection and concentrated by two rounds of ultracentrifugation. Virus titer was typically 10^5 particles per microliter.

Confocal Microscopy

Images were acquired using an Olympus FV-1000 confocal microscope and an LSM 880 with Ayrscan, using identical settings between control and experimental images. For dendritic complexity analysis, images were acquired (60 X) from 55 μm thick sections taking Z stacks including 25-60 optical slices, 0.9 μm intervals. Dendritic length and branching then measured from projections of three-dimensional reconstructions onto a single plane in GCs of septal DG, expressing GFP. For spine analysis, images were acquired using the 710 Zeiss confocal microscope (63 x; NA, 1.4; oil-immersion) from 60-μm-thick sections taking *z*-stacks including 50–140 optical slices, airy unit = 1 at 0.1 μm intervals. Three-dimensional reconstruction of dendritic segments was performed as previously described (Morgenstern et al., 2008). Spines were counted manually from dendritic fragments of > 40 μm located in the middle third of the molecular layer. Mushrooms spines were identified as long necked spines with large head.

For all analysis, only neurons localized in the septal portion of DG were analyzed, because the rate of maturation of GCs varies along the septo-temporal axis of the hippocampus (Piatti et al., 2011).

PCR and Western Blot analysis

For mRNA analysis, total RNA was isolated from total hippocampus or dissected in different areas (CA1/CA3) and DG under a stereomicroscope, using RNA-easy columns (Quiagen) according to manufacturer's instructions and cDNA was synthesized using Multiscribe reverse transcriptase (ThermoFisher Scientific). The cDNA was amplified using primers directed to rat *Ret*, *GFRα1*, *NCAM* and *Tata binding protein* (*Tbp*) mRNA sequence previously described (Ledda et al., 2007).

Western blot analysis was performed as previously described (Paratcha et al., 2003). Briefly, dentate gyrus tissue from adult sedentary and running mice was dissected under the microscope and homogenized (10% w/v) in ice-cold 25 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose, 1 mM EDTA and protease inhibitors. After centrifugation at 1,000 x g for 10 min, the supernatant was analyzed by western blot to evaluate the protein levels of GDNF with anti-GDNF antibody (1:1000, BD), anti-BDNF (1:1000, Santa Cruz), anti-pErk1/2 (1:1000, Cell Signaling Technology), anti-pCREB (1:1000, Cell Signaling Technology) and anti-βIII tubulin (1:5000, Promega). Immunoblots were scanned in a Storm 845 PhosphorImager (GE Healthcare Life Sciences) and quantifications were done with ImageQuant software (GE Healthcare Life Sciences).

Running

Mice were housed in a cage with a running wheel for 14 days, where they ran about 5-10 km/night. DG was then dissected for immunofluorescence or western blotting analysis. Sedentary mice were left in a regular cage without running wheel.

Behavioral Procedures

For behavioral testing, we used male mice to avoid hormonal effects. Open field activity was measured in a circular arena (35 cm of diameter x 15 cm high). Four external spatial keys were placed 10 cm outside and above the arena; separated from each other by an angular difference of 90°. At the beginning of a session, mice were placed in different positions of the arena at random and allowed to explore the apparatus and the activity was recorded for 10 min with a computer-linked video camera mounted above the testing box.

Mouse position was determined by automatic video tracing (ANY-maze, Stoelting). The ANY-maze software was used to quantify the position and distance traveled by the animal. The measures of locomotor activity (distance traveled across the time) and anxiety level (time spent in the central area) were analyzed (Alfieri et al., 2014). The pattern separation behavioral assay was performed as previously described by Bekinschtein et al. (2013). Briefly, the mice were habituated to the circular arena during five days, 10 min each session. The assay began 24 h after the fifth habituation session. For the SLR task, mice were exposed to three identical objects (A1, A2 and A3) during training phase that lasted for 10 min. For the s-SLR, objects A2 and A3 were placed 50° apart and object A1 at an equal distance of the other two. For the d-SLR, objects A1, A2 and A3 were equidistant, 120°apart from each other. Two hours later the training phase, during the testing phase the mice were exposed to two new identical copies of the objects, named A4 and A5 objects for 5 min. During the testing phase, object A4 was placed in the familiar location (same position as A1 in the training phase) and object A5 was placed in a novel location defined as a position exactly in between the ones in which objects A2 and A3 were located during the sample phase (See schemes in Figure 5D). Results were expressed as a discrimination ratio that was calculated as the time exploring the object in the novel location minus the time exploring the object in the familiar location over total exploration time $[(t_{novel}-t_{familiar})/t_{total}]$. All sessions were video recorded through a camera mounted above the maze and mouse position was tracked of an object was defined as pointing the nose to the object at a distance of < 1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration. Mice were food deprived to 85%–90% of their free feeding during the entire behavior paradigm. Water remained available *ad libitum* throughout.

Dentate Gyrus Cell Culture

DG neurospheres were obtained from early postnatal Wistar rats (P1-P5) as previously described (Rodrigues et al., 2017). Briefly, the DGs were dissected from brain under a stereomicroscope, digested with 0.05% Trypsin (ThermoFisher Scientific) in DMEM and mechanically dissociated until obtaining a cell suspension. The cells were diluted in DMEM:F12 (1:1, ThermoFisher Scientific) with Glutamax (2mM, ThermoFisher Scientific) supplemented with 100 U/ml penicillin and 100mg/ml streptomycin (ThermoFisher Scientific), 1% B27 (ThermoFisher Scientific) in the presence of mitotic factors: EGF (20 ng/ml, R&D) and FGF-2 (10 ng/ml, R&D). Cells were plated on 24 well plates at a density of 60,000 cells/well and incubated for 10 days. After this period, the neurospheres generated from DG were dissociated and plated into 24 well plates coated with poly-D-Lysine (PDL, 0.1 mg/ml, Sigma-Aldrich) and grown in serum free medium without growth factors for 6-7 days.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics used throughout the paper are described in the figure legends and in the text. Data were analyzed using GraphPhad, and are expressed as mean ± SEM. The n of each experiment is indicated in the figure legends and the significance is shown as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data distribution was assumed to be normal. Two-tailed unpaired Student's t test was performed to assess statistical significance between two independent groups. One-way ANOVA followed by a post hoc test was used to assess statistical significance between three or more groups, as indicated in legends.

DATA AND CODE AVAILABILITY

This study did not generate any dataset or code.