ProNGF Is a Cell-Type-Specific Mitogen for Adult Hippocampal and for Induced Neural Stem Cells

Valerio Corvaglia, a,b Domenica Cilli, b,c Chiara Scopa, b,d Rossella Brandi, b Ivan Arisi, b Francesca Malerba, a,b Federico La Regina, b Raffaella Scardigli, b,c Antonino Cattaneo, a,b

Key Words. Adult hippocampal neurogenesis • Neural stem cells • proNGF • Proliferation • Mitogenic factor • Radial glia-like stem cells • induced neural stem cells

INTRODUCTION

Understanding the cell biology of the adult neural stem cells (anSCs), in terms of their maintenance, activation, proliferation, differentiation, survival, and maturation into newborn neurons represents a challenging open question in the field of adult neurogenesis [1]. All these different steps are tightly regulated by the convergence of many signals provided by the surrounding neurogenic niche [2, 3]. Among these, signaling by members of the neurotrophin (NTs) family warrants to be investigated due to their crucial role in development of the central nervous system [4]. The neurotrophin family includes the nerve growth factor (NGF), the brain-derived neurotrophic factor, the neurotrophin-3 (NT3), and the neurotrophin-4/5 (NT4/5) [5–10]. Neurotrophins exert their biological actions by binding to two different classes of transmembrane receptors (NTRs), the Trk family of receptors and the pan-neurotrophin receptor p75 (p75NTR). Both receptors can trigger downstream signaling pathways to drive the biological effects of neurotrophins.

In the central nervous system (CNS), neurotrophins are present in equilibrium between their mature and larger precursor forms, the proneurotrophins (proNGF, proBDNF, proNT-3, proNT-4/5). Proneurotrophins preferentially bind to p75NTR (at variance with the mature forms, that are preferential Trk binders), but not all p75NTR expressing cells are sensitive to proneurotrophins;
expression of the neurotensin receptor Sortilin is apparently needed for neurotrophins to induce biological effects via p75NTR [11]. At the functional level, proneurotrophins play specific actions that are antagonist to those of the mature neurotrophins [12]. Interestingly, the antagonist effects of the proneurotrophins have pathophysiologically relevance in human neurodegenerative diseases and brain injury [13–17]. Beyond their well-established essential role during embryogenesis [4], neurotrophins play critical roles also supporting mature neurons, after development, by maintaining neuronal morphologies and functions. In the adult nervous system, NTs control the balance of survival/death response of mature cells, through the pro-survival TrkA and a pro-apoptotic p75NTR signaling, respectively [10, 18–22].

Neurotrophins have been shown to regulate neurogenesis during embryonic development [23, 24]. Instead, little is known about the relevance of NTs family members in regulating aNSCs biology [25–27], in which the cell-cycle progression or exit of the aNSCs must be tightly regulated. In addition to survival-inducing activity, NTs can be potent regulators of cell cycle [28–31], which could be potentially very relevant for their actions on aNSCs. However, the interplay between the immature or mature form of neurotrophins and cell cycle regulation has been less explored toward the understanding of aNSCs biology. Exogenous mature NGF positively affects adult hippocampal neural precursor survival and consequently hippocampal neurogenesis in physiological and neurodegenerative conditions [32, 33]. A more direct positive effect of NGF on adult subventricular zone (SVZ) neurogenesis has been recently shown by our group, as we demonstrated that NGF neutralization impairs both proliferation and neuronal differentiation of SVZ progenitors [25]. For what concerns proneurotrophins, a role in cell cycle modulation has only recently been uncovered [34, 35], but their function in this context needs further direct investigations.

In this study, we explored the role of NGF and proNGF in regulating hippocampal dentate gyrus (HP-DG) adult neurogenesis, both in vivo and in vitro. In particular, by analyzing quiescent stem cells separately from neural progenitors, we were able to demonstrate a dual role of proNGF in hippocampal neurogenesis, which is cell-stage-specific and dependent on a fine balance with its mature form NGF.

**MATERIALS AND METHODS**

**Experimental Animals**

AD11 mice expressing the functional αD11 anti-NGF antibody were obtained by intercrossing mice homozygous for the αD11 heavy chain transgene (CMV-VH-αD11 mice) and mice homozygous for the αD11 light chain transgene (CMV-VK-αD11 mice), as described [36]. The individual heavy and light chain αD11 transgenes start to be expressed at high levels in the late postnatal period, leading to the formation of functional anti-NGF antibodies at P90 [37]. TgProNGF mice constitutively express the furin-resistant mouse proNGF (proNGF-KR) in a background of normal endogenous proNGF/NGF production [38].

AD11 and ProNGF mice were used at 6 and 3 months of age, respectively. The corresponding wild-type (WT) littermates were used as control. All experiments with transgenic and control mice were conducted according to National and International Laws for Laboratory Animal Welfare and Experimentation (EEC council directive 86/609, OJ L 358, 12 December 1987; Dlgs 116/92). In detail, mice were grouped in standard cages (hardwoods bedding) in conventional animal facility (12 hour light/dark cycle). Groups included four mice per cage, balanced for genotype and mice were monitored for health and welfare for the whole duration of the experiments. Only mice without stress or discomfort signs (including hair loss, stereotyped behaviors) and weight ranging between 25 and 30 g were included in the study. Mouse genotyping of the transgenic mice is described in Supporting Information Materials and Methods.

**Brain Dissection and Tissue Processing**

Prior to brain dissection, adult mice were anesthetized with approximately 1 ml of 2,2,2-tribromoethanol (Sigma–Aldrich, St. Louis, MO) and intracardially perfused with 4% paraformaldehyde (PFA). The whole brain was, therefore, extracted and the fixation continued in 4% PFA overnight at 4°C. After cryoprotection in 30% sucrose, brains were cryosectioned at 40 μm of thickness, and slices encompassing the HP were analyzed by immunohistochemistry.

**In Vivo 5′-Bromo-2′-Deoxyuridine Labeling**

5′-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was administered to AD11 and control mice (6 months old) at 100 mg/kg by daily intraperitoneal injection for 5 days. Animals were then sacrificed either 2 (for proliferation analysis) or 28 (for differentiation analysis) days after the last injection and brains were collected and processed as described before.

**Cell Cultures**

**Hippocampal aNSCs**

Neural stem cell cultures from single HP were performed as described [25]. Six-month-old mice (WT or AD11) were anesthetized as described before and killed by decapitation. Brains were extracted out of the skull and separate in two hemispheres, then one half of the hippocampal formation was extracted from every hemisphere and was broken into 5–6 pieces. Hippocampi from each animal were processed separately to obtain cultures from single sample. Cells were isolated by enzymatic digestion (1.33 mg/ml trypsin, 0.7 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid; Sigma–Aldrich) for 30 minutes at 37°C and mechanical dissociation with small-bore Pasteur pipette. Cells were plated at 5 × 10^5 cells per cm² cells density and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with B27 (Invitrogen, San Diego, CA), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) (20 and 10 ng/ml, respectively; Peprotech, London, UK; NSC growth medium) [25] in a humidified incubator at 37°C in 5% CO₂ for 3 weeks. Growth factors were replenished weekly. By the end of the 3 weeks, primary neurospheres (21 mm in diameter) were subcultured by mechanical dissociation into single cells every fourth day.

**Induced Neural Stem Cells**

Mouse-induced neural stem cells (iNSCs) were derived by reprogramming WT mouse embryonic fibroblasts (MEFs) with a SOX2-retroviral vector, as described earlier [39]. Reprogramming of MEFs was performed in 24 multiwell plate on a feeder of mitomycin-treated mouse stromal cell line (STO) growth on 0.1% gelatin-coated glass. MEFs were plated at 1.25 × 10^5 cells per cm² cells density. We used passage 2 STO-
MEFs for feeder cells and passage 1 MEFs for reprogramming. After infection, viral medium was replaced with NSC growth medium [25]. Growth medium was completely refreshed every day until obtaining INSCs colonies, which were subsequently subcultured as neurospheres like aNSCs.

**Cell Proliferation and Differentiation**

To quantify cell proliferation (fold increase [Fi] analysis) 2 × 10^5 cells were plated at 1 × 10^3 cells per cm^2 cell density in growing medium. After 7 days in vitro (DIV 7), the total number of viable cells was counted by trypan blue exclusion and again 2 × 10^5 viable cells were replated under the same conditions. Cell proliferation was expressed as Fi, calculated by dividing the number of cells at DIV 7 by the initial number of seeded cells. For average Fi analysis of DG-aNSCs, we grew cells at consecutive passages from p7 to p21 by dissociating and replating 2 × 10^5 cells every 7 days. For Fi analysis in the treatment experiments (purified anti-NGF mAb αD11 [40], proNGF, and NGF proteins [41]) 2 × 10^3 cells at passage 30 were seeded at 1 × 10^3 cells per cm^2 and cultured for 2 weeks. For proliferation curves, we plated 2 × 10^3 cells for DG-aNSCs and 1 × 10^4 cells for INSCs at 1 × 10^3 cells per cm^2 or at 5 × 10^3 cells per cm^2 cell density, respectively. All proliferation curves were repeated three times in independent experiments using at least three technical replicates. To assess for differentiation, neurospheres were dissociated into single cells and 1 × 10^5 cells were transferred onto matrigel-coated glass coverslips (12 mm diameter) in NSC differentiation medium (growth medium without EGF and FGF). Five days after plating, cultures were fixed in 4% PFA and processed for immunocytochemistry.

**Monolayer Cultures for Single Cell Immunofluorescence Quantification**

In order to analyze the composition of the cell populations by quantifying the immunofluorescence signals at single cell level, cultures of DG-aNSCs and INSCs were grown as monolayer by dissociating neurospheres into single cells and transferring them onto poly-l-ornithine/laminin coated glass coverslips (12 mm diameter) at 1 × 10^3 per cm^2 cells density. Cultures were left to grow until confluence. Coated glasses were prepared below. Glass coverslips were coated with 20 μg/ml of poly-l-ornithine (Sigma–Aldrich) solution in H_2O and incubated at 37°C. After 24 hours, the multiwell plate with coverslips was left to reach room temperature out of the incubator, then poly-l-ornithine was removed and coverslips were coated with 2.5 μg/ml of laminin (Invitrogen) solution in phosphate-buffered saline (PBS) and incubated at 37°C. After 24 hours, multiwell plate was left to reach room temperature and cells were plated. For analyzing cell-type composition and receptors profile, cultures were fixed with 4% PFA when confluence was reached. In proNGF-KR acute treatment experiments 50 ng/ml of proNGF-KR was added to WT cells and cultures were fixed 48 hours later. Upon immunocytochemistry and confocal analysis, quantification of fluorescence intensity was performed using Image-J software. An average number of 300 cells were analyzed for each experimental settings.

**Stem Cell Enrichment and Leukemia Inhibitory Factor Selection Method**

For putative stem cells selection (RGL/early stage), neurospheres were dissociated and plated at 4 × 10^3 per cm^2 cell density in DMEM/F12 supplemented with B27 (Invitrogen) and 10 ng/ml of leukemia inhibitory factor (LIF; human, PHC9484, Thermo Fisher Scientific, Waltham, MA; LIF medium). After 9 days of cultures, selected putative stem cells (approximately 1% of the initial number) were seeded on poly-l-ornithine/laminin coated glass and after 2 days, cells were fixed in 4% PFA to perform cell morphology analysis and marker characterization. Clonogenic assay was performed at DIV 9 by plating cells at 1 × 10^3 per cm^2 in the following experimental conditions: NSC growth medium and LIF medium (see Cell Cultures section above) with or without proNGF-KR at the indicated concentration. The total number of neurospheres forming units was counted after 5 days of culture. A minimum of four technical replicates was performed for every condition, in order to provide the statistical mean of the number of neurospheres forming units. To expand the self-renewal capacity of DG-aNSCs, cells were grown in LIF medium for 1 week, to stimulate neural stem cells renewal [42], and then replated in NSC growth medium. In this way, we could isolate few clones of transient amplifying progenitors with high self-renew capacity. This protocol was repeated by culturing LIF-selected cells in LIF medium for 3 days every time we observed a slowdown of cell growth, in order to allow a continuous expansion of the transient amplifying progenitors.

**Recombinant Proteins**

Purified recombinant NGF, proNGF-WT, and proNGF-KR proteins were prepared as described [41]. Stock solutions (1–2 mg/ml in 50 mM Na_3PO_4) were diluted in cell culture medium at the proper concentration indicated in each experimental condition. Purified recombinant αD11 anti-NGF antibody was prepared as described [41] and the stock solution (1 mg/ml in PBS) was diluted at 20 ng/ml, 100 ng/ml, or 1 μg/ml in cell culture medium.

**Immunocytochemistry on Brain Section and Cultures**

Immunohistochemistry of HP was performed on 40 μm serial free-floating sections. Prior to BrdU antibody staining, sections were exposed to 2 N HCl for 45 minutes at 37°C and then washed with 0.1 M sodium borate buffer pH 8.5 for 10 minutes. Immunostaining on cell cultures was performed after fixation in 4% PFA for 10 minutes at room temperature. Cells fixed on coverslips were permeabilized in 0.1% Triton X-100 in PBS and then incubated with the antibody of interest, as described in Supporting Information. The total number of cells in each field was determined by counterstaining cell nuclei with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Sigma–Aldrich; 50 mg/ml in PBS for 15 minutes at RT). Immunostained sections and cells were mounted in Acqua-Poly/ Mount (Polysciences, Inc., PA) and analyzed at fluorescent or confocal microscopy using a TCS SP5 microscope (Leica Microsystems, Wetzlar, Germany).

**Quantification of Cell Number**

Stereological analysis of the number of cells was performed on series of 40-μm free-floating coronal sections of the entire DG of the HP, which were analyzed by confocal microscopy to...
count cells expressing BrdU throughout the rostro-caudal extent of the granule cell layer. To obtain the average number of DG cells per section, the number of positive cells for each DG section was divided by the total number of sections counted. Three animals per group (n = 3) were analyzed. Statistical analysis was performed by unpaired Student’s t test and all experiments were expressed as mean ± SEM.

RNA Isolation and Reverse Transcription-PCR
RNA was isolated from three different AD11 and three different WT neurospheres cultures. Briefly, neurospheres were lysed with trizol (Invitrogen) and DNase treated by Qiagen columns (Qiagen, Hilden, Germany). RNA quantity was determined on a NanoDrop Ultraviolet-Visible. Only samples with an absorbance ratio of 1.8, OD260/OD280, 2.0 were processed further. Each sample was then quality checked for integrity using the Agilent Bioanalyzer 2100 (Agilent G2938C, RNA 6000 nano kit; Agilent, Santa Clara, CA): samples with a RNA integrity number index lower than 8.0 were discarded. The purified RNA was used for qRT-PCR. The first strand cDNA template was synthesized from 500 ng of total RNA using random primers and superscript III reverse transcriptase (Invitrogen). All reactions were performed with SYBR Green PCR Master Mix (BioRad, Hercules, CA) and carried out in the iCycler (BioRad). Primers for quantitative PCR (QTR-PCR) analysis were designed with the assistance of Universal Probe Library Software (Roche Applied Science, Penzberg, Germany) and are described in Supporting Information. All samples were analyzed in triplicates. Relative change of mRNA amount was calculated based Dct method, as described [43].

Statistical Analysis

Animal Studies
Power analysis has been conducted to estimate the appropriate sample size by setting the probability of a type I error (α) at 0.05, power at 0.95, effect size at 0.4. To minimize the effects of subjective bias, we used randomization procedures for allocating animals to experimental groups and treatments and blind analysis of results.

In Vitro Studies
The statistical analyses were conducted by using Mann–Whitney (n ≥ 5) or by unpaired Student’s t test (n < 5) for repeated measures. Error bars on graphs are SEM. Significance markers on figures are from post hoc analysis (n.s., not significant; *, p < .05; **, p < .01; ***, p < .001).

RESULTS

Adult Hippocampal Neurogenesis Is Decreased in Anti-NGF AD11 Mice
In the adult brain, the pro- and mature forms of NGF exist in a well-defined homeostatic equilibrium [13,44], dependent on synthesis of proNGF precursor, on its cleavage by intracellular and extracellular proteases and on the degradation of mature NGF [45–49]. Adult hippocampal neurogenesis was studied in the AD11 anti-NGF transgenic mice, in which the expression of the recombinant anti-NGF antibody mAb αD11 results in a chronic postnatal interference with the activity of endogenous NGF in the brain [36]. Since the anti-NGF antibody binds mature NGF with an affinity three orders of magnitude higher than that for proNGF [41], the AD11 mice, unlike NGF knock-out mice, are a model for proNGF/NGF unbalance [50].

An important role of NGF in regulating adult mouse SVZ neurogenesis has been previously demonstrated in this AD11 model [25]. In AD11 mice, NGF neutralization led to an impairment of adult SVZ neurogenesis, in terms of reduced proliferation of neural progenitors and reduced ability to differentiate into βIII-tubulin positive neurons. In order to extend this observation to HP-DG, we analyzed the proliferative and differentiative potential of this neurogenic niche in the same animal model. We first measured the proliferative rate of HP-DG aNSCs by in vivo BrdU labeling in 6 months old AD11 and WT mice. Anti-BrdU staining, performed on brain sections encompassing the entire HP, showed that AD11 HP-DG contains twofolds more BrdU positive cells, compared with control mice (Fig. 1A, left panels), as quantified in Figure 1B (number of positive cells/DG: AD11 36.27 ± 4.31; WT 18.13 ± 3.7, p = .005). Despite this increase in proliferation, hippocampal neurogenesis was greatly affected in AD11 mice, with a significant decrease in the number of newborn neurons, as shown by immunostaining for BrdU and NeuN in Figure 1A (right panels) and quantified in Figure 1C (number of BrdU+/NeuN+ positive cells/DG: AD11 2.13 ± 0.42; WT 8.07 ± 1.79, p = .005).

Neurogenic Defects of AD11 Hippocampal Progenitors Is Maintained Also In Vitro
We analyzed in detail the biological property of hippocampal neural stem/progenitor cells of AD11 and WT mice by establishing in vitro cultures of aNSCs isolated from 6 months old animals. Differently from other aNSCs culture protocols, based on growing cells at high density, which promotes the rapid expansion of late progenitors with limited proliferative potential, we chose to establish neurosphere cultures from single individual mice, plated at low density (5 × 10⁶ cells per cm²), in order to promote the propagation of putative stem cells. With this method, we obtained three long-term (>70 passages) stable aNSCs cultures: two WT (WT1 and WT2) and two AD11 (AD3 and AD4). The average number of primary AD11 neurospheres was approximately twofold higher than WT (Fig. 2A, p value = .049). AD11 neurospheres formed in half the time of that required for WT neurospheres (Fig. 2B). This result reproduced in vitro the major proliferative rate of AD11 versus WT aNSCs observed in vivo in the DG niche. To further confirm this observation, we performed growth curves of these cultures at different passages during their expansion in vitro (see Materials and Methods for details). As shown in Figure 2C, AD11 neurospheres cultures proliferated significantly more than the control WT cultures. This difference in proliferation rate was also quantified as average FI of cell proliferation (FI), calculated between p7 and p21 (Fig. 2D), as described in Materials and Methods. To assess if this effect on proliferation was due to the persistency in vitro of the NGF neutralization occurring in vivo, by the transgenic anti-NGF antibodies still expressed in the cultures, we verified the expression of the anti-NGF antibody αD11 and of NGF in the expanded aNSCs. Double immunostaining for the human heavy and light chains of the transgenic anti-NGF antibody was performed on AD11 and WT bulk neurospheres, as described [25]. Hu-mAb αD11 expression was confirmed by demonstrating the concomitant immunoreactivity for the two antibody chains in AD11 and not in WT neurospheres (Supporting Information Fig. S1A).
Figure 1. Increased progenitor proliferation but reduced neurogenesis in AD11 dentate gyrus (DG)-hippocampus (HP). (A): Immunofluorescence staining for 5′-bromo-2′-deoxyuridine (BrdU) and for BrdU/NeuN (red and green, respectively) in adult DG of AD11 and wild-type (WT) mice. In the HP of AD11 animals, there is a higher number of BrdU+ cells (arrows in left panels) but a significant reduction of newborn neurons (arrows in right panels) compared with WT. White-squared boxes represent a ×10 magnification of the corresponding cells in the dotted line insets. Scale bar: 100 μm, ×20 magnification. (B, C): Quantification of BrdU (B) or double-positive BrdU/NeuN cells (C) in AD11 (red) and control (orange) DG. Data are mean ± SEM of five individual animals (n = 5) for each experimental group. *, p < .05, significantly different from WT, Student’s t test.

Figure 2. Increased proliferation of AD11 hippocampal progenitors is maintained also in vitro. (A): More primary neurospheres are obtained from AD11 dentate gyrus and in twofold less time compared with wild-type (WT). (B) Data are expressed as mean ± SEM of five individual animals (n = 5; A) or two (n = 2; B) for each experimental group. *, p < .05, significantly different from WT, Student’s t test. Data are mean ± SEM of three independent experiments. (C): Proliferation curve of AD11 and WT neurospheres. AD11 cells (red line) proliferated significantly more than control (green line), also quantified as average fold increase, calculated between p7 and p21 (D). Data are mean ± SEM of three independent experiments. *, p < .05 significantly different from WT, Student’s t test.
addition, αD11 expression was further confirmed at the mRNA level by real-time PCR for the heavy and light chains mRNA in both AD3 and AD4 cultures (Supporting Information Fig. S1B). The expression of the recombinant mAb αD11 results in an overall neutralization of mature NGF activity, without affecting protein expression. Immunofluorescence for NGF performed on neurospheres or freshly isolated DG-cells showed that (a) endogenous NGF was expressed in NSCs of both genotypes at similar levels (Supporting Information Fig. S1C), and (b) NGF was expressed both in type I progenitors (GFAP+/Nestin+) and neuroblasts (DCX+/Nestin+; Supporting Information Fig. S1D). Consistently, total NGF, measured by alphaLISA as previously described [51] in the medium of WT neurospheres, was 60 pg/ml/5 × 10⁶ cells.

Finally, we tested the capacity of WT and AD11 neurospheres to differentiate into mature neurons by mitogens withdrawal for 5 days and labeling with anti-βIII-tubulin antibody (Tuj1). Although the percentage of Tuj1⁺ cells was not significantly changed between WT and AD11 cultures, AD11 newborn neurons were poorly differentiated, displaying atrophic neurites (Supporting Information Fig. S2: percentage of atrophic Tuj1⁺ cells/tot Tuj1⁺ cells: AD11 80 ± 19; WT 30 ± 11, p = .02). This defect of neuronal maturation, accompanying the increased proliferation rate, recapitulated in vitro the defective neurogenesis observed in vivo in AD11 adult HP.

NGF and proNGF Differentially Affect Proliferation of WT Hippocampal Progenitors

The concomitant expression of NGF and of αD11 anti-NGF antibody in AD11 cultures strongly suggested that NGF neutralization is occurring also in vitro and might be responsible for the increased proliferation of AD3 and AD4 cultures. To verify this hypothesis, we tested if anti-NGF mAb αD11 antibody could increase the proliferation of WT aNSCs in vitro.

Figure 3. Effects of in vitro nerve growth factor (NGF) neutralization on the proliferation of wild-type (WT) adult neural stem cells (aNSCs) and of proNGF or NGF treatment. (A): Fold increase of proliferation of WT progenitors in the presence of mAb αD11. Progenitors treated with mAb αD11 at the lower concentration of 20 ng/ml (NGF binding) and 100 ng/ml (NGF binding and partially proNGF binding) proliferate more than those treated with the higher concentration of 1,000 ng/ml (NGF+ ProNGF binding). Zero nanogram per milliliter corresponds to vehicle-treated cells. (B): Fold increase of WT progenitors in the presence of NGF (red line) or proNGF-KR (light blue line) at equimolar increasing concentrations. ProNGF-KR increases cell proliferation at low concentration but reduce it at higher concentration, whereas NGF reduces the proliferative rate regardless its concentration. Data are mean ± SEM of three independent experiments. *, p < .05 and **, p < .01 significantly different from WT, Student’s t test.

©2019 The Authors. STEM CELLS published by Wiley Periodicals, Inc. on behalf of AlphaMed Press 2019

ProNGF Is a Cell-Type-Specific Mitogen
based also on the concentration of NGF measured in our experimental conditions. After 2 weeks of treatment with 20 ng/ml anti-NGF mAb αD11, a concentration that ensures the neutralization of the mature form only, proliferation of WT progenitors greatly increased, with respect to the vehicle-treated culture (Fig. 3A, FI WT = 3.5/C6 0.95; WT + αD11 = 47.8/C6 3.1). At the intermediate concentration of 100 ng/ml, αD11 antibody continued to induce a fivefold increase of the proliferative rate of WT aNSCs, but when we used the saturating concentration of 1,000 ng/ml of the antibody, at which both NGF and proNGF are neutralized, the effect on the proliferative potential disappeared completely (Fig. 3A), and the cultures proliferated at the same rate as untreated cultures. Thus, increased proliferation of AD11 cultures with respect to WT cultures can be reproduced by inhibiting selectively mature NGF in WT cultures, but not when both NGF and proNGF are neutralized. The increased proliferation can, therefore, be ascribed to proNGF. The selective neutralization of endogenously expressed mature NGF obtained in vitro accounts for the increased proliferative capacity of AD11 cultures respect the control WT cells, and suggests that the residual proNGF might play a role in controlling the proliferative rate of adult hippocampal stem/progenitor cells.

To directly evaluate the effects of mature NGF on aNSCs proliferation versus those of the precursor proNGF, we treated WT aNSCs with NGF or proNGF at equimolar concentrations (0.4 nM, 0.8 nM, and 2 nM dose range). We used a recombinant furin-cleavage resistant form of proNGF (proNGF-KR) [52]. The results (Fig. 3B) showed that pro and mature NGF had opposite effects on aNSCs proliferation. At the lowest neurotrophins concentration of 0.4 nM, while NGF treatment (red line in Fig. 3B) significantly (p value = .001) reduced WT DG cells proliferation, proNGF-KR (blue line in Fig. 3B) had a positive effect on proliferation. At increasing concentrations (>0.4 nM), NGF trend to inhibit proliferation remained confirmed, while the proliferation inducing effect of proNGF-KR decreased in a dose-dependent manner. Indeed, while at the lowest concentration of 0.4 nM (10 ng/ml), proNGF-KR-treated cells proliferate three times more than control (FI of cell proliferation, WT = 11.9 ± 1.3, WT + proNGF-KR = 36.7 ± 12.2, p value = .02), at higher concentrations proNGF-KR mitigated its positive effect on proliferation.

(Supporting Information Materials and Methods and Fig. S3A)
lowering the Fl of cell proliferation to 21.2 ± 3.9 at 0.8 nM (20.8 ng/ml), while at 2 nM (50 ng/ml), the effect was totally abolished. Even though the presence of other cleavage enzymes, as serum metalloproteases, was limited by the fact that the neurospheres were cultured in serum free conditions, we could not exclude that proNGF-KR might be cleaved by other endogenous proteases. Thus, one possible explanation of the dose-dependence of proNGF on proliferation might be that at high proNGF concentrations the released mature NGF might counterbalance the effects on proliferation. To test this possibility, the time course of proNGF cleavage to NGF was evaluated in conditioned medium from primary hippocampal neurons cultured without serum for 12 days. Compared with proNGF WT processing, which released ~44% of mature NGF over this time period, the degradation of proNGF-KR was negligible (released mature NGF was 13%, Supporting Information Fig. S3B). In this scenario, the possibility that at higher concentrations proNGF-KR action is counterbalanced by mature NGF is unlikely to occur.

Cell-Type Composition and Neurotrophin Receptors Expression Pattern of Hippocampal aNSC Cultures

Based on the previous results, we then hypothesized that increasing concentrations of proNGF might have differential effects on cells at different neurogenic stages (i.e., early versus late progenitors). Indeed, WT1 cultures are heterogeneous in terms of progenitor maturation, as demonstrated by immunostaining analysis performed with different cell-type-specific markers (Fig. 4A). Table 1 shows the different subpopulations identified in WT cultures based on the concomitant expression of Nestin/Msi1/DCX or GFAP/Nestin/Sox2 markers. As represented in the diagram of Figure 4B, the major portion of WT cultures were GFAP+/Nestin+ multipotent progenitors (66%), that we further subdivided in early multipotent (early type-2, in green, 32.3% ± 2.6%) and middle multipotent (middle type-2a, in yellow, 33.7% ± 5.1%), by the fact that they were, respectively, negative or positive for the marker Musashi-1 (Msi1). Neural committed progenitors were less represented (approximately 2% ± 0.1%). Of note, our cultures maintained a subpopulation of cells with the proper stem cells feature of quiescence, that is, the RGLs (4.6% ± 0.9%, in orange) that allow the long-term expansion potential of the culture. In this view, the different progenitors could differentially respond to proNGF based on distinct differential expression of proNGF receptors p75NTR and TrkA. In order to investigate the neurotrophin receptor profile of our WT cultures, we performed immunostaining for p75NTR or TrkA in combination with GFAP, Nestin, and DCX markers. Immunostaining analysis on freshly isolated cells from WT HP (ex vivo analysis) showed that RGLs (GFAP+/Nestin+) expressed more p75NTR than the late Nest+/DCX+ progenitors, whereas TrkA was equally expressed at low level among the different populations (Fig. 5A). Thus, the observed difference in cell proliferation in response to proNGF concentration, previously observed (Fig. 3A), might be related to difference in p75NTR expression among early and middle/late progenitors in our WT culture. To further investigate this hypothesis, we analyzed the distribution of p75NTR in WT progenitors (Fig. 5B). RGLs (GFAP+/Nestin+/DCX+) expressed more p75NTR than middle/late progenitors (GFAP+/Nestin+/DCX−; Fig. 5C), confirming also for DG what was previously reported in SVZ [26]. The two distributions, evaluated by measuring the p75NTR fluorescence intensity among 300 events, were significantly different, as calculated by two independent statistical tests (Wilcoxon–Mann–Whitney test, p value <1×10−5; and Kolmogorov–Smirnov test, p value <1×10−3).

Based on this result, we predict that RGLs (that expressed high p75NTR) might be more prone to respond to proNGF by re-entering cell cycle and starting to proliferate. Indeed, acute treatment of WT cells with proNGF-KR (50 ng/ml for 48 hours) induced a significant increase in the expression levels of cyclin D1 in Nestin+/DCX− cells, that correspond to RGLs, early and middle multipotent progenitors (Fig. 5D). Cyclin D1 is involved in G1 phase progression and thus plays an important role to induce quiescent cells to reenter the cell cycle [53]. The distribution of cyclin D1 signal in the Nestin+/DCX− population (Fig. 5E) showed that, in the presence of proNGF-KR, a small fraction (6%) of RGLs/early-middle multipotent progenitors expressed cyclin D1 at high level (intensity value = 260). This fraction could thus correspond to quiescent/early progenitors that respond to proNGF by increasing cyclin D1 expression and probably reactivating cell cycle.

It has been recently reported that the mitogenic induction of cyclin D1 expression in neural stem cells is driven by the phosphorylation of c-Jun protein [54], which is downstream of the p75NTR signaling pathway [9]. This pathway is usually involved in promoting the apoptotic effect of p75NTR, even though there are cumulative evidences showing a potential link between p75NTR signaling and cell-cycle progression [28]. Interestingly, upon acute treatment of WT cells with proNGF-KR, we showed an increased amount of the phosphorylated c-Jun protein by Western blot analysis (Supporting Information Fig. S4), together with an increased level of cyclin D1, whereas both activated cleaved caspase-3 and phospho-AKT (TrkA signaling) levels remained unchanged between proNGF-KR-treated and untreated cells. This data confirms that proNGF stimulates early progenitors proliferation through the activation of p75NTR signaling pathway and the induction of cyclin D1.

Mitogenic Effects of proNGF on Pure Cultures of RGLs Selected by LIF Treatment

Due to the high heterogeneity of WT neurospheres cultures, we decided to better address this selective, hitherto not
Figure 5. p75NTR-expressing progenitors are reactivated by proNGF-KR treatment. (A): Ex vivo expression of p75NTR and TrkA in stem cells and neuroblasts. Immunofluorescence staining for p75NTR or TrkA (red) in GFAP/nestin double-positive radial glia-like stem cells (RGLs; green and blue signal, respectively, early progenitor panels) and in DCX/nestin double-positive neuroblasts (green and blue signal, late progenitor panels). (Figure legend continues on next page.)
respectively, late progenitor panels) shows that p75NTR is more expressed in RGLs than in neuroblasts, whereas TrkA is equally expressed at low level in all populations. Scale bar: 10 \( \mu m \), \( \times 40 \) magnification.

(B): Immunofluorescence staining for nestin (green signal), GFAP (red signal), and p75NTR (blue signal) in WT cells shows that p75NTR is expressed in both RGLs and multipotent early and middle progenitors at different intensity levels as quantified in the graphic on the right. White square boxes in each panel represent \( \times 2 \) magnification of the corresponding smaller insets. Scale bar: 100 \( \mu m \), \( \times 40 \) magnification.

(C): Distribution of the fluorescent intensity of p75NTR. Two independent statistical tests, Wilcoxon–Mann–Whitney and Kolmogorov–Smirnov test, respectively, indicate that the two distribution, that of p75NTR in the RGLs and that of p75NTR in early/middle progenitors, are actually two distinct distributions. Data are mean of three independent experiments. \( p < 1 \times 10^{-5} \) and \( p < 1 \times 10^{-3} \) significantly different from control, Student’s t test.

(D): In the presence of mitogens, proNGF-KR increases the number of neurospheres in a concentration-dependent manner (upper panels), as quantified in the upper histogram on the right. ProNGF-KR induces the formation of neurospheres in combination with LIF in nonmitogenic conditions (lower panels), as quantified in the bottom histogram. Scale bar: 200 \( \mu m \), \( \times 5 \) magnification. Data are mean \( \pm \) SEM of three independent experiments. *, \( p < .05 \); **, \( p < .01 \); ***, \( p < .001 \) significantly different from control, Student’s t test.

Figure 6. ProNGF-KR acts as mitogenic factor in a concentration-dependent manner on radial glia-like stem cells (RGLs). (A): Phase-contrast micrograph of wild-type progenitors upon leukemia inhibitory factor (LIF) selection shows that cells have a radial morphology typical of RGLs, as indicated by the black arrows. Scale bar: 50 \( \mu m \), \( \times 40 \) magnification. These cells express high level of both RGL markers (nestin in green and GFAP in red in panel (B]), as well as of early type-2a progenitors markers (nestin in green and Mash1 in red in panel \( \text{[C]} \)), and p75NTR (blue signal). Scale bar: 100 \( \mu m \), \( \times 40 \) magnification.

(Figure legend continued from previous page.)
described, biological property of proNGF of inducing proliferation of quiescent stem cells, by testing its effect on proliferation specifically on a homogenous population of RGLs. To this aim, these putative stem cells were selected in vitro from the original WT culture by treating cells with leukemia inhibitory factor (LIF), as described (see Materials and Methods). This method, based on the property of LIF to block differentiation pathway, increases GFAP expression and promotes symmetrical division during proliferation, allowing a reliable enrichment in quiescent/early progenitors. LIF-treated progenitors were grown on glass slides double-coated with laminin and poly-ornithine. In this way, we were able to obtain a homogenous population of cells with a radial elongation, resembling the typical radial glia morphology of RGLs observed in vivo (Fig. 6A). These selected cells are GFAP+/Nestin+ and Mash-1+/Nestin+, thus representing early type-1 and type-2a progenitors, and expressed high level

Figure 7. Mitogenic effect of proNGF on induced neural stem cells (iNSCs) and dentate gyrus-derived primary neurospheres. (A): Immunofluorescence staining for different cellular subtype markers in iNSCs cultures treated with proNGF-KR. ProNGF-KR induces an enrichment in middle multipotent progenitors (nestin+/Msi1+/DCX−, yellow cells) and a reduction of late neural progenitors (Msi1+/nestin+/DCX+, cyan cells) and late multipotent progenitors (nestin+/Msi1+/DCX−, red cells), as quantified in the histogram on the right. Data are mean ± SEM of three independent experiments. *, p < .05 significantly different from control, Student’s t test. Scale bar: 100 μm, ×40 magnification. (B): Phase-contrast micrographs of iNSCs neurospheres. ProNGF-KR-treated iNSCs are bigger in size compared with control, as quantified in the histogram on the right. Data are mean ± SEM of three independent experiments. **, p < .01 significantly different from control, Student’s t test. Scale bar: 200 μm, ×20 magnification. (C): The expansion capacity of proNGF-KR-treated iNSCs (red line) continued to grow exponentially, whereas that of control iNSCs (blue line) is gradually lost over time. Data are mean ± SEM of three independent experiments. *, p < .05 and **, p < .001 significantly different from control, Student’s t test. Mit = mitogens; MPk = mitogens + proNGF-KR; LPk = LIF + proNGF-KR; DIV = days in vitro. (E): Proliferation curve of SVZ neurospheres derived from proNGF-KR transgenic and wild-type (WT) mice. proNGF-KR cells (green line) proliferated significantly more than control (red line). Data are mean ± SEM of three independent experiments. *, p < .05 significantly different from WT, Student’s t test.
of p75<sub>NTR</sub> respect to the untreated heterogeneous population (Fig. 6B, 6C). We then tested the effect of proNGF-KR on the proliferation of LIF-selected RGL cells, by a clonal assay (see Materials and Methods) in which we counted the number of neurospheres forming from 300 starting RGLs in the following conditions: mitogens (EGF + bFGF) or LIF in combination or not with proNGF-KR. The results shown in Figure 6D demonstrated that, in the presence of mitogens, proNGF-KR increases the number of neurospheres in a concentration dependent manner (upper panels). Strikingly, proNGF-KR was also able to induce the formation of neurospheres in the absence of added mitogens, that is, in nonmitogenic conditions (lower panels). This demonstrates that proNGF-KR acts as mitogenic factor in a concentration dependent manner on a homogenous population of putative stem cells.

**Mitogenic Effect of proNGF on iNSCs and Primary NSC Cultures**

We took advantage of this new biological property of proNGF-KR to improve the expansion capacity of induced neural stem cells (iNSCs; see Materials and Methods). iNSCs are a promising reprogramming technology for future application in cell therapy [39]. We derived iNSCs cultures by infecting MEF with the p-Sox-2 retrovirus [39]. One limitation of current iNSCs reprogramming protocols is that they produce mainly late Nestin<sup>−</sup>/DCX<sup>−</sup> progenitors, with restricted propagation potential (8-9 passages [55]; this article). To explore the possibility of overcoming this limitation, we chronically exposed mouse iNSCs to 0.4 nM proNGF-KR from passage 0 to passage 6 (before the culture undergoes senescence to the active state, while at later stages it affects neural differentiation or the overall proliferation of the NSCs culture, probably acting on the late progenitors that undertake neural commitment or become mature glia. We base this hypothesis on the fact that proNGF induces cell death of the mature cells of CNS [15, 17, 56]. It is also plausible that RGLs are more sensitive to proNGF than their progeny, due to the higher expression of p75<sub>NTR</sub> in the stem cell compartment. So, at low concentration, proNGF would bind and activate only RGLs without affecting their progeny while, when present at high concentration, proNGF would arrest the proliferation of late progenitors. Interestingly, cyclin E has been recently proposed as a specific marker of quiescence, since its expression is required to maintain the quiescent state of hippocampal RGLs [29]. In this view, we can speculate that proNGF, through cyclin E downregulation [35] and cyclin D1 upregulation (our article), switches the RGLs from quiescence to the active state, while at later stages it affects neural maturation, probably by inducing apoptosis in neuroblasts [57].

The differential expression of p75<sub>NTR</sub> among the different stages of maturation is of considerable importance. A previous study on SVZ niche [26] showed that p75<sub>NTR</sub> expression defines a population of stem or precursor cells that persist from development to adulthood and is able to respond to neurotrophin stimulation. In our study, we found that p75<sub>NTR</sub> is highly...
expressed in the RGLs subpopulation of the HP-DG. These cells, selected in vitro by the “LIF method” show a specific mitogenic responsiveness to proNGF even in absence of other mitogens. In this view, p75NTR is emerging as a marker of “stemness” in both neurogenic niches and other tissues. For instance, several tissues originating from migratory Neural Crest Stem Cells (NCSs) have been shown to maintain a number of multipotent/bipotent undifferentiated cells that express p75NTR [58–60]. In the eient nervous system, differentiation of these cells is driven by a combination of NT-3 and other neurotrophic factors, trough upregulation of TrkC and the concomitant downregulation of p75NTR [61], suggesting that p75NTR is required to maintain the undifferentiated phenotype and survival of stem cells [62]. Thus, p75NTR expression identifies cells that are Ki67-negative or slowly cycling in vivo, but retain high clonal potential in vitro, highlighting the importance of this receptor for the maintenance of a stem cells pool through the preservation of their quiescent state [63]. In this framework, our results show for the first time that the proNGF stimulation reactivates the cell cycle of a specific type of these p75NTR-positive quiescent stem cells, that is the RGLs, in the hippocampal neurogenic niche.

The effect of proNGF-KR on proliferation, which is opposite to that of NGF and is cell-type-specific, is also dose-dependent. This underscores the importance that in vivo proNGF and mature NGF coexist and their ratios are subject to a complex homeostatic regulation [44, 64]. Moreover, proNGF and NGF mixtures can exert actions that neither exerts alone [65]. WT hippocampal NSCs proliferate less than AD11 but, unlike the latter, produce mature differentiated new βIII-Tub+ neurons. Accordingly, we propose a model for the modulation of adult hippocampal neurogenesis by the NGF system (see graphical abstract), whereby proNGF locally produced in the neurogenic niche stimulates cell cycle activation in the quiescent stem cells and slowly dividing early Type-2a through p75NTR+, whereas the mature counterpart (NGF) is required to modulate cell proliferation of late progenitors and to drive their final neural maturaion by binding to its high affinity receptor TrkA. The differential expression of p75NTR and TrkA in our hippocampal progenitor culture (p75NTR high/TrkA low in early and p75NTR low/TrkA high in late progenitors) is in favor of our model. Our results highlight the overall importance of an adequate proNGF cleavage, in which the mature NGF acts as “calibrator” of the proNGF effect for a functional neurogenesis in the DG of HP.

The interpretation of studies investigating signals involved in the proliferation of NSC is sometime hampered by the cellular heterogeneity of the cultures [66, 67]. The different cell types in the NSCs population have different responsiveness to stimuli depending on their receptor expression [67]. By selecting in vitro the neural stem cells with the “LIF method,” we could demonstrate the existence of a population of truly quiescent stem cells derived from the DG and prove that proNGF signaling is very specific for those cells. According to some authors [68, 69], in the adult hippocampal neurogenic niche there are not indefinitely self-renewable stem cells but only neurogenic precursors. Instead, our results demonstrate the existence of a long-term expandable and self-renewal quiescent subpopulation of stem cells, in line with the original classification of Palmer in 1997 [70]. The ability of these cells to survive in the absence of mitogens (likely entering in the quiescent, G0 phase) and to re-enter the cell cycle when mitogens are readded to the culture is clearly a feature of quiescent stem cell. Moreover, the coexpression of GFAP and Nestin and the morphological analysis strengthen this concept by identifying them as radial glia.

LIF receptor is known to be expressed in NSCs during development [71, 72] and previous data reported that LIF modulates NSCs self-renewal through the possible transition from late to early stage of progenitors [42]. Thus, it might be argued that the RGLs obtained in vitro after the LIF selection would not reflect a similar counterpart in vivo in the DG, but rather represent an in vitro artifact due to the ability of LIF to directly drive some cells at later stage to regress to the quiescent and long-term self-renewable state. Indeed, we demonstrate the presence of GFAP+ /Nestin+ cells expressing high levels of p75NTR (and with the same morphological features of our LIF-selected cells) in the DG-HP of WT animals by ex vivo immunolabeling of freshly dissociated tissue. Of note, the existence of an in vivo widespread LIF signaling in the adult nervous system has recently emerged [73], supporting the relevance of our finding.

Given the difficulty to produce NSCs from single adult HP, the positive effect of proNGF on primary neurospheres formation is very important. As known for the SVZ, mitogens in the neurospheres protocol (EGF and bFGF) do not support the proliferation of the quiescent stem cells, as these do not express EGFRI [67] and because bFGF is more effective on late neural progenitors. This could explain the difficulty in isolating NSCs cultures from single adult HP with current methods, since quiescent stem cells would not be stimulated by EGF and bFGF, and because type-2 cells of HP have a more limited expansion potential than their SVZ counterpart. So, the identification of a factor, like proNGF, that specifically stimulates the quiescent stem cells is very important. However, further investigations will be required, in terms of timing of treatment and proNGF concentration, for a better optimization of this protocol.

We demonstrate the mitogenic role of proNGF also for the inducible NSCs (iNSCs). iNSCs technology is a powerful tool for studying neural development and neurological disorders, both in vitro and in animal model [39, 74]. One main obstacle for an efficient use of iNSCs is their limited expansion potential, being composed mainly of late multipotent and late neural precursors that soon reach senescence [55], while earlier progenitors are poorly represented in the culture. In this view, our results on the mitogenic effect of proNGF-KR on these latter cells are important, as this allows expanding this population of cells prior to their differentiation into neurons. The differentiation capacity of iNSCs after proNGF-KR treatment remains to be investigated in detail. For instance, we do not know whether the combination of LIF-selection and proNGF treatment can further improve the enrichment in the early, expandable progenitors, as we did with DG-aNSCs. Also, we do not know whether we could obtain with this method an in vitro model of quiescent cells with NSC features (Astrocytic Like), in order to better control the maintenance and the expansion of INSCs, with the perspective to be exploited in regenerative medicine studies.

**CONCLUSION**

This work demonstrates that proNGF plays a critical role in hippocampal neurogenesis. It specifically acts as mitogen on RGLs (resident and tissue-derived), which express high levels of...
p75NTR and respond to proNGF re-entering cell cycle by increasing the cyclin D1 expression. The mitogenic effect of proNGF needs to be counteracted by mature NGF, which, conversely, is required for neuroblasts survival and differentiation. Thus, a fine balance between proNGF/NGF signaling is critical for a correct hippocampal neurogenesis. In addition, we have further demonstrated the mitogenic property of proNGF in another cell system, the induced Neural Stem cells (iNSCs), which opens new perspectives for the implementation of cell-reprogramming protocols. Our results warrant further investigations into the role of proNGF/NGF signaling in neural stem cells biology, in the view of developing future therapeutic approaches based on the stimulation of endogenous adult neurogenesis or on cell-reprogramming protocols.

ACKNOWLEDGMENTS

This work was funded by MIUR (framework agreement EBRI-CNR 2015-2017) and institutional funds from Scuola Normale Superiore. We thank Dr. Michele Sommessa for his contribution to the statistical analyses.

REFERENCES

1. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–1710.
2. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. Nature 2001;414:98–104.
3. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: Stem cells and their niche. Cell 2004;116:769–778.
4. Huang EJ, Reichardt LF. Neurotrophins: Roles in neuronal development and function. Annu Rev Neurosci 2001;24:677–736.
5. Levi-Montalcini R. The nerve growth factor 35 years later. Science 1987;237:1154–1162.
6. Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. EMBO J 1982;1:549–553.
7. Ibanez CF. Neurotrophic factors: From structure-function studies to designing effective therapeutics. Trends Biotechnol 1995;13:217–227.
8. Bothwell M. NGF, BDNF, NT3, and NT4. Handb Exp Pharmacol 2014;220:3–15.
9. Skaper SD. The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS Neurol Disord Drug Targets 2008;7:46–62.
10. Skaper SD. The neurotrophin family of neurotrophic factors: An overview. Methods Mol Biol 2012;846:1–12.
11. Nykjaer A, Lee R, Teng KK et al. Sortilin is essential for proNGF-induced neuronal cell death. Nature 2004;427:843–848.
12. Hempstead BL. Dissecting the diverse actions of pro- and mature neurotrophins. Curr Alzheimer Res 2006;3:19–24.
13. Fahnestock M, Michalski B, Xu B et al. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer’s disease. Mol Cell Neurosci 2001;18:210–220.
14. Peng S, Wuu J, Mufson EJ et al. Increased proNGF levels in subjects with mild cognitive impairment and mild Alzheimer dis- ease. J Neuropathol Exp Neurol 2004;63:641–649.
15. Harrington AW, Leiner B, Blechschmidt C et al. Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. Proc Natl Acad Sci USA 2004;101:6226–6230.
16. Teng HK, Teng KK, Lee R et al. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 2005;25:5455–5463.
17. Beattie MS, Harrington AW, Lee R et al. ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neu- ron 2002;36:375–386.
18. Barde YA. Trophic factors and neuronal survival. Neuron 1989;2:1525–1534.
19. Bothwell M. Functional interactions of neurotrophins and neurophin receptors. Annu Rev Neurosci 1995;18:223–253.
20. Lewin GR, Barde YA. Physiology of the neurotrophins. Annu Rev Neurosci 1996;19:289–317.
21. Kew JN, Smith DW, Sofroniew MV. Nerve growth factor withdrawal induces the apopto- tic death of developing septal cholinergic neurons in vitro: Protection by cyclic AMP analogue and high potassium. Neuroscience 1996;70:329–339.
22. Chen K, Nishimura MC, Armanini MP et al. Disruption of a single allele of the nerve growth factor gene results in atrophy of basal forebrain cholinergic neurons and memory deficits. J Neurosci 1997;17:7288–7296.
23. Cattaneo E, McKay R. Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 1990;347:762–765.
24. Farinas I, Jones KR, Backus C et al. Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 1994;369:658–661.
25. Scardigli R, Capelli P, Vignone D et al. Neutralization of nerve growth factor impairs proliferation and differentiation of adult neu- ral progenitors in the subventricular zone. Stem Cells 2014;32:2516–2528.
26. Young KM, Merson TD, Sothibundhau A et al. p75 neurotrophin receptor expression defines a population of BDNF-responsive neurogenic precursor cells. J Neurosci 2007;27:5146–5155.
27. Bibel M, Barde YA. Neurotrophins: Key regulators of cell fate and cell shape in the vertebrate nervous system. Genes Dev 2000;14:2919–2937.
28. Lopez-Sanchez N, Frade JM. Control of the cell cycle by neurotrophins: Lessons from the p75 neurotrophin receptor. Histol Histopathol 2002;17:1227–1237.
29. Ikeda T, Puro DG. Nerve growth factor: A mitogenic signal for retinal Muller glial cells. Brain Res 1994;649:260–264.
30. Marshall CJ. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–185.
31. Patapoutian A, Reichardt LF. Trk receptors: Mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272–280.
32. Zhang H, Petit GH, Gaughwin PM et al. NGF rescues hippocampal cholinergic neuronal markers, restores neurogenesis, and improves the spatial working memory in a mouse model of Huntington’s disease. J Huntington Dis 2013;2:69–82.
33. Frielingsdorf H, Simpson DR, Thal LJ et al. Nerve growth factor promotes survival of new neurons in the adult hippocampus. Neurobiol Dis 2007;26:47–55.
34. Zanin JP, Abercrombie E, Friedman WJ. Proneurotrophin-3 promotes cell cycle withdrawal of developing cerebellar granule cell

AUTHOR CONTRIBUTIONS

V.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; D.C., C.S.: collection and assembly of data; R.B., I.A., F.M.: collection and assembly of data, data analysis and interpretation; F.L.R.: collection of data; R.S.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; A.C.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
progenitors via the p75 neurotrophin receptor. Elife 2016;5:1–21.
35 Guo J, Wang J, Liang C et al. proNGF inhibits proliferation and oligodendrogenesis of postnatal hippocampal neural stem/progenitor cells through p75NTR in vitro. Stem Cell Res 2013;11:874–887.
36 Ruberti F, Capsoni S, Comparini A et al. Phenotypic knockout of nerve growth factor in adult transgenic mice reveals severe deficits in basal forebrain cholinergic neurons, cell death in the spleen, and skeletal muscle dystrophy. J Neurosci 2000;20:2589–2601.
37 Capsoni S, Ugolini G, Comparini A et al. Alzheimer-like neurodegeneration in aged antineuriteregent factor transgenic mice. Proc Natl Acad Sci USA 2000;97:6826–6831.
38 Tiveron C, Fasulo L, Capsoni S et al. ProNGF/NGF imbalance triggers learning and memory deficits, neurodegeneration and spontaneous epileptic-like discharges in transgenic mice. Cell Death Differ 2013;20:1017–1030.
39 Ring KL, Tong LM, Balestra ME et al. Direct reprogramming of mouse and human fibroblasts into multipotent neural stem cells with a single factor. Cell Stem Cell 2012;11:100–109.
40 Cattaneo A, Rapposelli B, Calissano P. Three distinct types of monoclonal antibodies after long-term immunization of rats with mouse nerve growth factor. J Neurochem 1988;50:1003–1010.
41 Paletti F, Covaceusch S, Koneare PV et al. Intrinsic structural disorder of mouse proNGF. Proteins 2003;57:990–1009.
42 Pitman M, Emery B, Binder M et al. LIF receptor signaling modulates neural stem cell renewal. Mol Cell Neurosci 2004;27:255–266.
43 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(- delta delta C(T)) method. Methods 2001;25:402–408.
44 Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat Rev Neurosci 2005;6:603–614.
45 Seidah NG, Benjannet S, Pareek S et al. Processing and secretion of nerve growth factor: Expression in mammalian cells with a vaccinia virus vector. Mol Cell Biol 1998;8:2456–2464.
46 Capsoni S, Tiveron C, Vignone D et al. Dissecting the involvement of tropomyosin-related kinase A and p75 neurotrophin receptor signaling in NGF deficit-induced neurodegeneration. Proc Natl Acad Sci USA 2010;107:12299–12304.
47 Malerba F, Paletti F, Cattaneo A. NGF and proNGF reciprocal interference in immunoassays: Open questions, criticalities, and ways forward. Front Mol Neurosci 2016;9:63.
48 D’Onofrio M, Paletti F, Arisi I et al. NGF and proNGF regulate functionally distinct mRNAs in PC12 cells: An early gene expression profiling. PLoS One 2011;6:e20839.
49 Stacey DW. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr Opin Cell Biol 2003;15:158–163.
50 Chen X, Tian Y, Yao L et al. Hypoxia stimulates proliferation of rat neural stem cells with influence on the expression of cyclin D1 and c-jun N-terminal protein kinase signaling pathway in vitro. Neuroscience 2010;165:705–714.
51 Winiecka-Klimek M, Smolarz M, Walczak MP et al. SOX2 and SOX2-MYC reprogramming process of fibroblasts to the neural stem cells compromised by senescence. PLoS One 2015;10:e0141688.
52 Wang YJ, Valadares D, Sun Y et al. Effects of proNGF on neuronal viability, neurite growth and amyloid-beta metabolism. Neurotox Res 2010;17:257–267.
53 Copani A, Caraci F, Hoozemans JJ et al. The nature of the cell cycle in neurons: Focus on a “non-canonical” pathway of DNA replication causally related to death. Biochim Biophys Acta 2007;1772:409–412.
54 Morrison SJ, White PM, Zock C et al. Prospective identification, isolation, and flow cytometry, and in vivo self-renewal of multipotent mammalian neural stem cells. Cell 1999;96:737–749.
55 Li HY, Say EH, Zhou XF. Isolation and characterization of neural crest progenitors from adult dorsal root ganglia. Stem Cells 2007;25:2053–2065.
56 Kruger GM, Mosher JT, Bixby S et al. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 2002;35:657–669.
57 Chalazonitis A. Neurotrophin-3 in the development of the enteric nervous system. Prog Brain Res 2004;146:243–263.
58 Tomellini E, Lagadec C, Polakowska R et al. Role of p75 neurotrophin receptor in stem cell biology: More than just a marker. Cell Mol Life Sci 2014;71:2467–2481.
59 Nakamura T, Endo K, Kinoshi S. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. Stem Cells 2007;25:628–638.
60 Julita MF, Caraci F, Cuello AC. A link between nerve growth factor metabolic deregulation and amyloid-beta-driven inflammation in Down syndrome. CNS Neurosci Ther 2016;22:1534–447.
61 Chaker Z, Codiga P, Doetsch F. A mosaic world: Puzzles revealed by adult neural stem cell heterogeneity. Wiley Interdiscip Rev Dev Biol 2016;5:640–658.
62 Dulkien BW, Leeman DS, Boutet SC et al. Single-cell transcriptional analysis defines heterogeneity and transcriptional dynamics in the adult neural stem cell lineage. Cell Rep 2017;18:777–790.
63 Bull ND, Bartlett PF. The adult mouse hippocampal progenitor is neurogenic but not a stem cell. J Neurosci 2005;25:10815–10821.
64 Cheng Y, Black IB, DiCicco-Bloom E. Hip- pocampal granule neuron production and population size are regulated by levels of bFGF. Eur J Neurosci 2002;15:3–12.
65 Palmer TD, Takahashi J, Gage FH. The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci 1997;8:389–404.
66 Oshima K, Teo DT, Senn P et al. LIF promotes neurogenesis and maintains neural precursors in cell populations derived from spinal ganglion stem cells. BMC Dev Biol 2007;7:112.
67 Onishi K, Zandstra PW. LIF signaling in stem cells and development. Development 2015;142:2230–2236.
68 Scott RL, Gursusinge AD, Rudovsky AA et al. Expression of leukemia inhibitory factor receptor mRNA in sensory dorsal root ganglion and spinal motor neurons of the neonatal rat. Neurosci Lett 2000;295:49–53.
69 Liu GH, Yi F, Suzuki K et al. Induced neu- ronal stem cells: A new tool for studying neural development and neurological disorders. Cell Res 2012;22:1087–1091.