Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 332 (2005) 215–223
BBRC
www.elsevier.com/locate/ybbrc

Effect of neurturin on multipotent cells isolated from the adult skeletal muscle

Patrick Vourc'h [a,b], Benjamin Lacar [a,b], Laurence Mignon [a,b], Paul A. Lucas [c], Henry E. Young [d], Marie-Françoise Chesselet [a,b,*]

[a] Department of Neurology, Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[b] Department of Neurobiology, Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[c] Department of Orthopedic Surgery, New York Medical College, Valhalla, NY, USA
[d] Division of Basic Medical Science, Mercer University School of Medicine, Macon, GA, USA

Received 25 February 2005
Available online 30 April 2005

Abstract

Ligands of the glial cell line-derived neurotrophic factors (GDNF)-family are trophic factors for the development and survival of multiple cell types, however their effects on non-neuronal stem cells are unknown. We examined the action of neurturin on a candidate stem cell population isolated from adult skeletal muscles. When grown as spheres, these cells expressed mRNAs for GDNF, persephin, GFR-α2, GFR-α4 (neurturin receptor), and Ret. Exposure of these cells to neurturin significantly augmented cell numbers via increased cell proliferation. After addition of retinoic acid, the cells exited the cell cycle, developed thin processes, and became immunoreactive for βIII-tubulin, while Ret mRNA expression decreased, without changes in the level of GFR-α2 mRNA. Neurturin induced an outgrowth of processes on these βIII-tubulin positive cells. Neurturin may therefore be beneficial in the use of these multipotent cells isolated from adult muscles for autologous transplants in neurological applications.

Keywords: Stem cells; Glial cell line-derived neurotrophic factor; Retinoic acid; Neuritis

The ability of multipotent cells isolated from accessible organs such as skeletal muscles to differentiate along a neuronal lineage may lead to important advances in regenerative medicine because they could allow for autologous cell replacement in neurodegenerative diseases and after brain injury. We have previously shown that the adult skeletal muscle contains a candidate stem cell population, which may be interesting for transplantation strategies in the central nervous system (CNS) [1–5]. The nature of these cells present in the connective tissue in the skeletal muscle is unknown. They have been termed "pluripotent stem cells" (PPSCs) to indicate their ability to differentiate into cells that express markers of various cell lineages [1–3], and this term will be used for consistency in the present report. These cells can be safely and easily harvested from adult patients, making them an interesting candidate population for autologous replacement therapy. Little is known, however, about the effects of trophic factors expressed in the nervous system on multipotent cells isolated from non-neural adult tissue.

The glial cell line-derived neurotrophic factor (GDNF) family consists of GDNF [6], neurturin [7], artemin [8], and persephin [9]. This family is an important class of neurotrophic factors in regenerative medicine [10,11]. Indeed, ligands of the GDNF family are expressed in several adult CNS regions of particular interest for neurorepair strategies, such as the spinal cord, the substantia nigra, the striatum, and the cortex [12,13]. Studies have shown that the expression

* Corresponding author. Fax: +1 310 267 1786.
E-mail address: mchessel@ucla.edu (M.-F. Chesselet).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.04.104

of these molecules increases in various CNS regions after insults such as epileptic seizures, forebrain ischemia, and lesions of the nigrostriatal dopaminergic pathway [14–17]. Moreover, in vitro and in vivo studies have implicated the GDNF family in the development and survival of several subtypes of neurons both in the peripheral nervous system (PNS) and the CNS, including motor, sensory, sympathetic, cholinergic, and dopaminergic neurons [18–20]. Specifically, direct infusion of GDNF, injection into the striatum of a virus producing GDNF, or transplantation of cells expressing ligands of the GDNF family into the striatum induced partial functional recovery in animal models of Parkinson's disease [11,21–25]. Beneficial actions of GDNF have also been described in a rat model of amyotrophic lateral sclerosis [26].

Ligands of the GDNF family mediate their effects through the formation of receptor-complexes consisting of one of the GDNF receptors (GFR-α1, 2, 3 or 4) and the tyrosine kinase receptor Ret [19]. In vitro experiments have demonstrated preferred receptor–ligand interactions; GDNF, neurturin, artemin, and persephin bind preferentially to GFR-α1, GFR-α2, GFR-α3, and GFR-α4, respectively. The binding of a ligand of the GDNF family to its receptor results in the activation of Ret, and the subsequent activation of signalling pathways. The activation of Ret appears to be particularly important for neuronal differentiation [27].

In the present study, we analysed the expression of ligands of the GDNF family and their receptors by the cells termed PPSCs isolated from adult skeletal muscle that we have previously characterized [2,5]. Based on their receptor profile, we examined the effects of neurturin on these cells. We show that neurturin is a proliferative factor for these cells when grown as spheres and induces process outgrowth in those cells after pre-treatment with retinoic acid.

Materials and methods

Cell culture. Biopsy of the flexor digitorum and gastrocnemius muscles of adult Sprague–Dawley rats (6 months old; Charles River Laboratories, Wilmington, MA, USA) was performed as described previously [1,3]. Cells were cultured on gelatin-coated flasks in Opti-Mem medium (Invitrogen, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (Omega Scientific, Tarzana, CA, USA). The cells were released by trypsin and replated in OptiMem medium containing 10% horse serum (Gemini Bioproducts, Calabasas, CA, USA), 100 U/ml penicillin G, streptomycin, 250 ng/ml amphotericin B (Invitrogen), 0.028 M sodium bicarbonate, and 0.01 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA). For cryopreservation, cells were resuspended in fresh medium with 7.5% of dimethyl sulfoxide (DMSO) and placed at −80 °C for at least 1 week. Cells used in the present study were passaged 12–14 times and cryopreserved four times. Cryopreserved cultures were thawed and cells were cultured in a medium designed for the culture of "neurospheres" (sphere medium) consisting of Neurobasal A (Invitrogen), penicillin, streptomycin (both 100 U/ml, Invitrogen), 2 mM L-glutamine (BioWhittaker, Walkersville, MD, USA), B27 (Invitrogen), and 20 ng/ml epidermal growth factor (EGF, Invitrogen); 40 ng/ml basic fibroblast growth factor (bFGF, Alomone Labs, Jerusalem, Israel).

Proliferation study. The fourth generation spheres were centrifuged and triturated using a polished glass pipette. Single cell suspensions were passed through a 70 μm filter (Fisher, Pittsburg, PA, USA) before cell counting with the trypan blue exclusion method. For the proliferation and differentiation experiments, the cells were cultured at 37 °C in low oxygen (1%) conditions. For the proliferation study, the cells were plated on coverslips coated with poly-ornithine/laminin (Sigma) in 24-well plates at a density of 20,000 cells/well and incubated for 2–6 days in a basal medium consisting of neurobasal A, N2 supplement (Invitrogen), 2 mM L-glutamine, 1 μg/ml laminin (Sigma), penicillin, and streptomycin (both 100 U/ml, Invitrogen) in the absence or presence of either neurturin (20 ng/ml, neurturin, PreproTech, Rocky Hill, NJ, USA) or EGF/bFGF (both at 20 ng/ml). Bromodeoxyuridine (BrdU) was used as a marker of cell division. Cells were treated with 10 μM BrdU for 1 day before fixation for 30 min with 4% paraformaldehyde at room temperature. The cells were incubated for 1 h in 2 N HCl, in borate buffer, pH 8.5, for 10 min, and then with a mouse monoclonal anti-BrdU (1:2200; Chemicon, Temecula, CA, USA) at 4 °C overnight. For detection, the cells were incubated for 2 h at room temperature with a goat anti-mouse secondary antibody labelled with Cy3 (1:200; Jackson ImmunoResearch, West Grove, PA, USA). Coverslips were mounted with Vectashield containing DAPI (1.5 μg/ml) for nuclear counterstaining (Vector Laboratories, Burlingame, CA, USA) before analysis with fluorescence microscopy (Axioskop, Zeiss, Thornwood, NY, USA).

Differentiation study. Cells were plated on coverslips coated with poly-ornithine/laminin at a density of 20,000 cells/well (24-well plates) and cultured for 2 days in basal medium containing 20 ng/ml neurturin alone. Retinoic acid (RA; Sigma, St. Louis, MO, USA), resuspended in dimethyl sulfoxide (DMSO), was then added to the culture. Control cultures were exposed to 0.01% DMSO. All experiments were performed in dark conditions because RA is light sensitive. The medium was refreshed every 2 days before fixation of the cells in 4% paraformaldehyde for 30 min at room temperature. The number of BrdU-positive cells was determined as described in the proliferation study. Cells previously cultured for 4 days in the presence of 1.5 μM RA were then transferred into media containing no growth factor, 20 ng/ml neurotrophin-3 (NT3) alone, 20 ng/ml neurturin alone, or neurturin/NT3 (both 20 ng/ml) and cultured for 4 more days. Immunocytochemistry for βIII-tubulin was performed as follows: after blocking for 45 min at room temperature in 0.1 M PBS containing 7% normal goat serum, 2% bovine serum albumin, and 0.2% Triton X-100, cells were incubated at 4 °C overnight with a rabbit antibody against βIII-tubulin (1:1500; Covance, Princeton, NJ, USA) and for 2 h at room temperature with a goat anti-rabbit secondary antibody labelled with Cy3 (1:200, Jackson Immunoresearch).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells in culture with Trizol (Invitrogen) according to the manufacturer's protocol and treated with RQ1 DNase (Promega, San Luis Obispo, CA, USA). The reverse-transcription reaction was carried out at 42 °C for 45 min in a final volume of 10 μl containing 500 ng of total RNA, 0.5 mM each of dNTP, 10 mM dithiothreitol, 1× buffer, 100 U superscript II reverse transcriptase (Invitrogen), and 250 pmol random hexamers (Promega). The reaction was followed by an incubation step at 92 °C for 5 min. PCR was performed with 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, 1.5 U *Taq* polymerase (all from Invitrogen), and 1 μM each of the forward and reverse primers in a final volume of 50 μl. The amplification protocol included an initial step at 94 °C for 5 min, 30–35 cycles of 1 min at 94 °C, 30 s at T_m (see below), and 1 min at 72 °C. The reaction was terminated by an incubation step at 72 °C for 5 min (Mastercycler gradient thermocycler, Eppendorf, Westbury, NY, USA). PCR products (5 μl) were separated on 1.5% agarose gel and visualized by staining with ethidium bromide. Reactions without

reverse-transcriptase or without RNA were done in parallel as controls. Semi-quantitative analysis was performed using the housekeeping gene β-actin. Primer sequences and size of PCR products were as follows: TrkC [28] (T_m: 55 °C, 141 bp), p75NTR [29] (T_m: 55 °C, 438 bp), GFR-α1 [30] (T_m: 68 °C, 717 bp), GFR-α2 [30] (T_m: 68 °C, 528 bp), GFR-α3 [31] (T_m: 56 °C, 667 bp), GFR-α4 (T_m: 60 °C, 301 bp), GDNF [32] (T_m: 56 °C, 660 and 590 bp), neurturin [31] (T_m: 68 °C, 482 bp), artemin [15] (T_m: 54 °C, 325 bp), persephin [31] (T_m: 70 °C, 328 and 240 bp), Ret [30] (T_m: 68 °C, 525 bp), and β-actin [5] (T_m: 56 °C, 376 bp).

Process outgrowth assay. Cells were stained for βIII-tubulin expression as described earlier. Preparations were mounted on slides with Vectashield (Vector Laboratories). Several randomly chosen fields from three separate experiments were photographed (400×) under a fluorescent microscope (Axioscop Zeiss) linked to a Spot Camera (Diagnostic Instruments, Burlingame, CA, USA). The length of the longest process was measured from a total of 50 randomly chosen cells positive for βIII-tubulin in each separate experiment ($n = 3$) using the Scion Image beta 4.0.2 software [33,34]. Cells in aggregate formation were excluded. Data from each experiment were averaged for each experimental condition.

Statistical analysis. Data from at least three separate experiments were statistically analysed using the software GB Stat (v6.5; Dynamic Microsystems, Silver Spring, MD, USA) (ANOVA with post hoc Fisher's LSD). A value of $p < 0.05$ was considered statistically significant.

Results

Expression of mRNAs for GFR-α2, GFR-α4, and Ret

Semi-quantitative RT-PCR analysis was used to study the expression of the receptors for the GDNF-family and Ret by PPSCs grown as spheres or under adherent conditions. Total RNA was prepared from cells cultured in the "sphere medium" (Fig. 1A) or plated on poly-ornithine/laminin for 2 days in a proliferation medium (Fig. 1B) (EGF 20 ng/ml, bFGF 20 ng/ml) [2]. In both conditions, cells expressed similar levels of GFR-α2 and GFR-α4 mRNAs, but did not express GFR-α1 and GFR-α3 mRNAs (Fig. 1C). Ret mRNA expression was also detected in both conditions (Fig. 1C). Using RT-PCR, we also observed the expression of mRNA for GDNF and for persephin, the ligands for GFR-α1 and GFR-α4, respectively (Fig. 1C). However, we did not detect mRNA for neurturin and artemin, the ligands for GFR-α2 and GFR-α3, respectively (Fig. 1C). The absence of endogenous neurturin but the presence of its receptor (GFR-α2) prompted us to examine the action of neurturin on the PPSCs.

Proliferative effects of neurturin

To assess whether neurturin could exert survival or proliferative effects on our cells, we compared its action to that of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cells obtained from suspensions of spheres and then plated on coverslips coated with poly-ornithine/laminin. Indeed, we have previously observed that the addition of EGF/bFGF enhanced the survival of this cell population in culture [2]. As we observed in our previous experiments, 2 days after plating, cells in cultures with EGF/bFGF (both at 20 ng/ml) did not differ from control in their morphology (Figs. 2A and B). Similarly, no change in cell mitogenesis was observed after BrdU incorporation (Fig. 2D). However, cultures with EGF/bFGF showed a higher number of DAPI positive cells (456 ± 14 cells/area) compared to control cultures (161 ± 16 cells/area) (Fig. 2E). Furthermore, we observed an increase in BrdU positive cells in

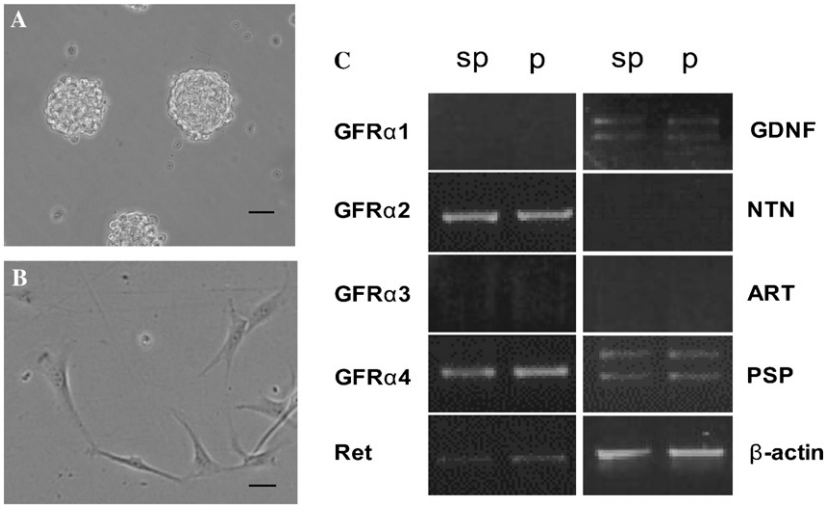

Fig. 1. Expression of mRNAs for the GDNF family receptors and ligands, and for Ret by PPSCs grown as spheres. Total RNA was extracted from cells in spheres (sp) (A) or from cells plated (p) on poly-ornithine/laminin for 2 days in a proliferation medium (B). Migration of the RT-PCR products on agarose gels. The expression of the β-actin gene was used as an internal control (C). Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), artemin (ART), and persephin (PSP). Scale bar: 100 μm (A), 30 μm (B).

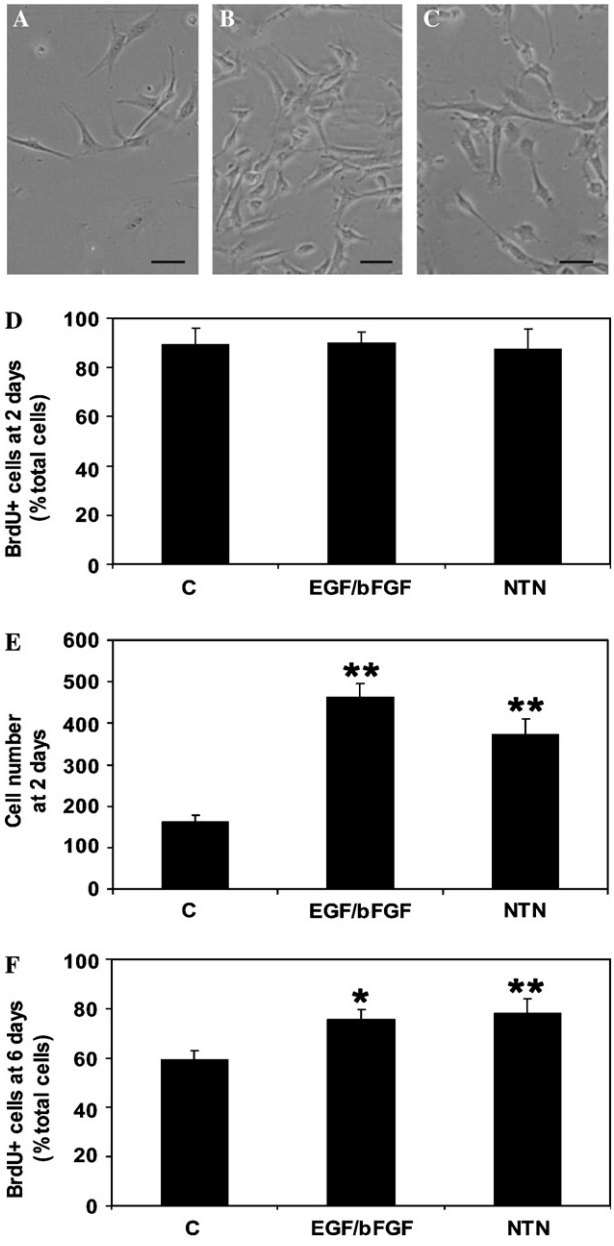

Fig. 2. Proliferative effect of neurturin on PPSCs. PPSCs after 2 days in control medium (A), in medium containing EGF/bFGF (both 20 ng/ml) (B) or neurturin (20 ng/ml) (C). Scale bars: 40 μm. Percentage of proliferating cells after 2 days (D) and 6 days (F) of culture in the absence or presence of either EGF/bFGF or neurturin (NTN). Proliferating cells were labelled with 10 μM bromodeoxyuridine for 1 day before fixation and immunocytochemistry for BrdU ($n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control). (E) Number of cells after 2 days in the three different media. The number of cells corresponds to the total number of DAPI stained nuclei in five fields (100×) across one diagonal of coverslip. Two coverslips were analyzed in three separate experiments ($n = 3$; $^{**}p < 0.01$ compared to control).

cultures after 6 days with EGF/bFGF (75.67 ± 4.04%) compared to control cultures (59.33 ± 3.79%; Fig. 2F).

After 2 days in the presence of 20 ng/ml neurturin, the cell morphology was similar to controls and to cells treated with EGF/bFGF (Figs. 2A and C). The number of cells was also significantly higher (372 ± 24 cells/field) in cultures containing neurturin compared to control cultures (161 ± 16 cells/area) (Fig. 2E). The increase in cell number seen at 2 days was not associated with a variation in BrdU incorporation (Fig. 2D), which is similar to the results obtained with EGF/bFGF. However, we observed an increase in BrdU positive cells after treatment for 6 days with neurturin (78.00 ± 6.08%) compared to control (59.33 ± 3.79%; Fig. 2F). We did not detect expression of MyoD or myogenin, two markers of myogenic lineage, in any culture condition by RT-PCR analysis (data not shown). Thus, the results show that neurturin exerts a survival effect after plating and a mitogenic effect on PPSCs, resulting in increased cell number.

Induction of growth arrest by retinoic acid

We tested the effects of retinoic acid (RA) on PPSCs that were previously cultured for 2 days with 20 ng/ml neurturin. RA concentrations were chosen based on

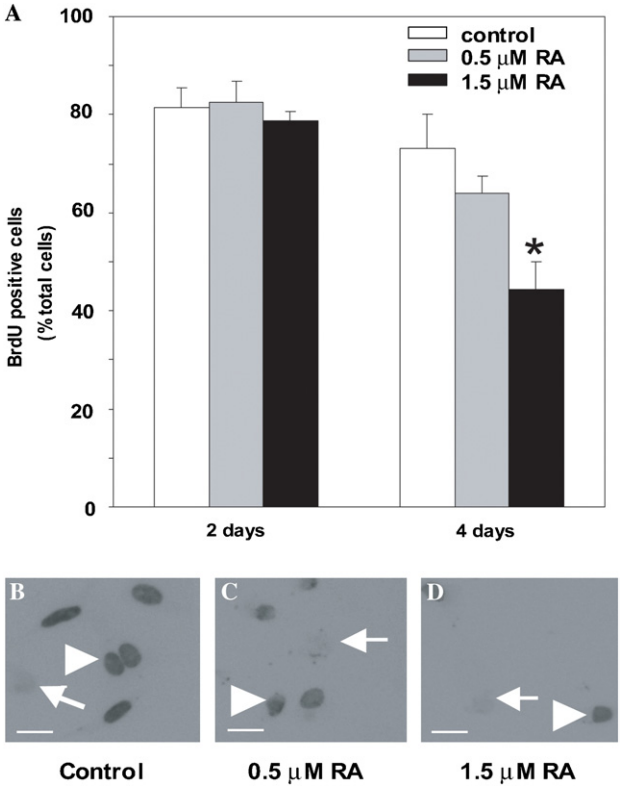

Fig. 3. Retinoic acid (RA) induces growth arrest of PPSCs. (A) Percentage of proliferating cells after 2 and 4 days in control medium (DMSO) or in media containing 0.5 μM RA or 1.5 μM RA. RA was resuspended in DMSO before addition to culture. Proliferating cells were labelled with 10 μM BrdU for 1 day before fixation and BrdU immunocytochemistry ($n = 6$; $^{*}p < 0.05$ compared to control medium). (B–D) Representative photomicrographs showing that addition of RA is associated with a decrease in the number of Brdu labelled nuclei (arrowheads) (arrows: non-labelled nuclei). Scale bar: 20 μm.

experiments performed on neural stem cells [35]. The effect of addition of 0.5 or 1.5 μM RA on cell proliferation was measured with BrdU incorporation. As shown in Fig. 3A, no difference in the number of BrdU positive cells was observed after 2 days of RA treatment. After 4 days, cells cultured with 0.5 μM RA showed a slight but not significant reduction in BrdU incorporation ($66.71 \pm 1.13\%$) compared to control ($78.38 \pm 4.22\%$). However, a significant reduction in BrdU incorporation, and consequently in the number of cycling cells, was obtained with 1.5 μM RA ($47.81 \pm 5.79\%$) after 4 days (Figs. 3A–D). This concentration of 1.5 μM RA was also associated with a significant increase in cells positive for the immature neuronal marker βIII-tubulin ($13.36 \pm 1.81\%$) compared to control ($4.36 \pm 1.62\%$) (Fig. 4A). Semi-quantitative RT-PCR analysis showed that RA induced a decrease in Ret mRNA expression, but did not affect the level of expression of mRNA encoding GFR-α2, the receptor for neurturin (Fig. 4C). We also analyzed the expression of TrkC and p75NTR, two receptors for neurotrophin-3 (NT3), an important trophic factor in the CNS [36]. TrkC mRNA expression was significantly increased after treatment with 1.5 μM RA. We did not detect mRNA encoding for p75NTR, a low affinity binding receptor for NT3 (Fig. 4C).

Induction of process outgrowth by neurturin

Because RA induced the expression of TrkC mRNA, we considered the possibility that neurotrophin-3 (NT3) could act on PPSCs that had been previously treated for 4 days with 1.5 μM RA. To examine this possibility, we determined the number of cells positive for βIII-tubulin after treatment for 4 days in the presence or absence of 20 ng/ml of NT3. Addition of NT3 did not affect the number of βIII-tubulin positive cells (control: 60.00 ± 12.29 cells, NT3: 58.33 ± 17.01 cells). We then asked whether neurturin could have an effect on cells cultured for 4 days with 1.5 μM RA, as these cells expressed GFR-α2 mRNA and low levels of Ret mRNA (Fig. 4C). Neurturin did not increase the number of cells

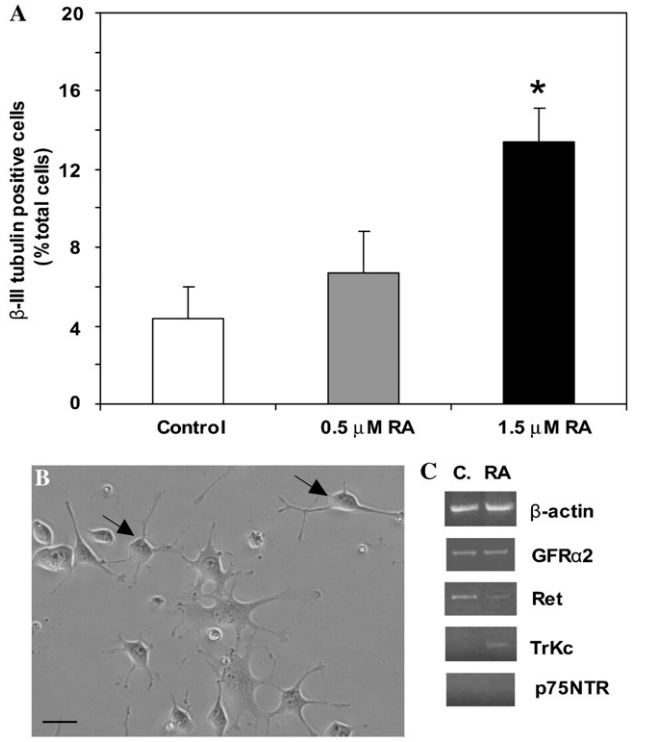

Fig. 4. RA induces the differentiation of PPSCs into βIII-positive cells. (A) Percentage of βIII-positive cells in the presence of RA ($n = 3$; $^*p < 0.05$ compared to control medium). (B) Photomicrograph of a culture of PPSCs after 4 days in the presence of 1.5 μM RA. The arrows indicate cells with thin process, a refractile cell body, and positive for βIII-tubulin after immunocytochemistry. Scale bar: 40 μm. (C) Semi-quantitative RT-PCR analysis of the expression of the genes for GFR-α2, Ret, TrKc, and p75NTR. Total RNA was extracted from cells cultured for 4 days in control medium (C) or in presence of 1.5 μM RA (RA). The expression of the β-actin gene was used as an internal control.

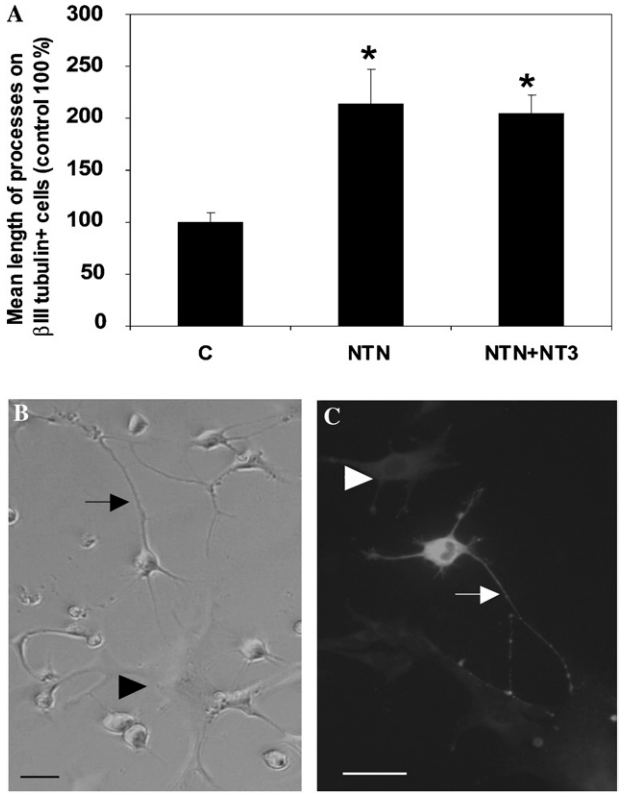

Fig. 5. Neurturin induces process outgrowth on βIII-tubulin positive cells. (A) Mean length of the longest process on βIII-tubulin positive cells and in media containing 20 ng/ml neurturin or 20 ng/ml of both neurturin and NT3 compared to cultures in control medium C ($n = 3$; $^*p < 0.05$ compared to control medium). (B,C) Photomicrograph of PPSCs after 4 days in the presence of neurturin. βIII-tubulin negative cells (arrowhead in C) displayed a large and flat morphology (arrowhead in B,C), whereas βIII-tubulin positive cells (arrow in C) showed round and refractile cell bodies with long processes (arrow in B,C). Scale bar: 40 μm.

positive for βIII-tubulin (control: 60.00 ± 12.29 cells, neurturin: 63.33 ± 13.05) and did not improve the survival of the cells in culture (control: 101.20 ± 12.39 cells, neurturin: 95.26 ± 3.30 cells; DAPI positive cells/field, 100×). Using RT-PCR analysis we observed that none of our cells expressed markers for the myogenic lineage (MyoD and myogenin; data not shown) suggesting that neurturin did not induce the differentiation of the PPSCs into myoblasts or myocytes under our culture conditions. However, we observed that the cells positive for βIII-tubulin expression displayed long and thin processes when cultured in the presence of neurturin. Quantification analysis indicated that the mean length of the longest process of the cells positive for βIII-tubulin incubated with neurturin was 114% longer than in control cultures (Figs. 5A–C). Thus, neurturin induced process outgrowth on βIII-tubulin positive PPSCs. The presence of NT3 did not increase the effect of neurturin on process outgrowth (Fig. 5B).

Discussion

We examined the expression of ligands and receptors of the GDNF family by a candidate stem cell population isolated from adult skeletal muscle (PPSCs). We found that these cells, when grown as spheres, expressed mRNA for GDNF, persephin, GFR-α2, and GFR-α4. Addition of neurturin, the ligand for GFR-α2, increased proliferation of the PPSCs in culture. Their expression of the immature neuronal marker βIII-tubulin was obtained by adding RA, a vitamin A derivative. When cultured in the presence of neurturin, these cells positive for βIII-tubulin displayed longer processes. This is the first time that a ligand from the GDNF-family has been observed to act on multipotent cells isolated from adult non-neuronal tissue in ways similar to what has been observed for some neuronal precursors. In addition, the data suggest that PPSCs may combine useful features for cell replacement therapies in the CNS.

PPSCs grown as spheres express GDNF and persephin mRNAs

The PPSCs are uncommitted cells present in the connective tissue of adult skeletal muscle [3,4]. Although the embryonic origin of these cells is not clear, they can be safely and easily isolated from the skeletal muscles of adult patients, which make them an attractive source of cells for autologous replacement therapies. Our previous studies showed a rapid proliferation of these cells and their ability to express neural markers in vitro [2,5]. We report here that these cells express mRNA for GDNF and persephin when cultured as free-floating spheres and after treatment with retinoic acid. A recent study from Garcia et al. [37] showed similar expression of GDNF mRNA by undifferentiated bone marrow stromal cells, however we have shown previously by cell surface marker analysis that the cells we are studying are not derived from bone marrow [5].

Current cell replacement protocols with stem cells in the CNS are based on two main strategies. The transplanted cells are either used to replace the endogenous damaged or lost cells and/or they are expected to secrete neurotrophic factors supporting the survival and the regeneration of surviving endogenous cells. This second strategy, using stem cells as chaperones for degenerating neurons [38], gave promising results in models of Parkinson's disease [39] and amyotrophic lateral sclerosis [26]. Interestingly, in both studies the authors suggested that the observed regenerative events might be due to the release of ligands from the GDNF family by the transplanted cells. This idea is strongly supported in Parkinson's disease by results from studies using neural stem cells over-expressing the ligands of the GDNF family, GDNF or persephin [25,34], a virus producing GDNF [22,40], or direct infusion of GDNF into the CNS [24]. Further studies will be necessary to show if the transplantation of non-neural adult stem cells, such as the PPSCs we have examined, may also lead to the protective and regenerative effects observed after the transplantation of neural or embryonic stem cells in neurodegenerative diseases. Our results suggest that PPSCs, which can be easily harvested, may represent an alternative source of GDNF-producing cells for in vivo transplantation.

PPSCs grown as spheres express mRNAs for the neurturin receptor and Ret

Our results show that the PPSCs express mRNAs for GFR-α2 and GFR-α4, the receptors for the GDNF family ligands neurturin and persephin, respectively. We also observed that PPSCs grown in culture as spheres expressed mRNA for Ret, a member of the receptor tyrosine kinase superfamily which can transduce signalling by the ligands of the GDNF family [19]. Several CNS regions, which suffer cell loss during degenerative diseases or after brain injuries, express ligands of the GDNF family [14–17]. These molecules are important for both the development and the survival of several types of neurons in vivo [18]. The fact that the PPSCs express GFR-α2 and GFR-α4 mRNA suggests that they might respond to neurturin and persephin in vivo, after transplantation, in ways that could affect their proliferation and/or differentiation.

Neurturin is a proliferative factor for PPSCs

Here we report that neurturin increases the proliferation of PPSCs in culture. A previous study showed similar proliferative effects for neurturin on spermatogonial

stem cells expressing Ret [41]. Neurturin also has a proliferative effect on Ret-positive multipotential progenitors extracted from the enteric nervous system [42]. In contrast to neurturin, RA, a vitamin A derivative expressed both in the developing and adult brain, induced a decrease in the proliferation of PPSCs that were previously incubated in the presence of neurturin and their exit from the cell cycle. These results are in accordance with other studies showing that RA induces growth arrest in neuroblastoma cell lines [43] and leads to an exit from the cell cycle in bone marrow mesenchymal stem cells and neural stem cells [35,44].

Neurturin induces process outgrowth on cells expressing βIII-tubulin

We have previously reported that PPSCs can give rise to cells expressing βIII-tubulin, neuronal specific enolase, neurofilament MW68, and NeuN, in vitro when exposed to neurotrophin-3 or brain-derived neurotrophic factor [2,5]. In the present study, we observed that RA also induced the expression of the immature neuronal marker βIII-tubulin. A similar observation has been previously made on neural stem cells, in which RA induced both growth arrest and differentiation into cells expressing markers of neurons [35]. This differentiation of PPSCs by RA was associated with an increased expression of TrkC mRNA, a receptor for neurotrophin-3 (NT3). Studies on neural stem cells have reported that RA induced a similar increase in TrkC expression during the early steps of the differentiation along the neuronal lineage [35]. However, in contrast to previous observations [45], we did not observe an effect of RA on the expression of p75NTR, a membrane protein that modulates the response of neurons to neurotrophins.

Based on the observation that PPSCs expressed mRNA for GFR-α2 following treatment with RA, we analyzed the effect of the addition of neurturin to the culture. Neurturin significantly induced process outgrowth on PPSCs expressing βIII-tubulin. Similar actions of neurturin have been reported on several subtypes of neurons including dorsal root ganglion neurons and sympathetic neurons [20,33]. Moreover, Holm et al. [46] have shown that grafting fibroblasts over-expressing neurturin into the locus coeruleus in rats with a 6-OHDA lesion promotes the sprouting of noradrenergic neurons. Here we demonstrate for the first time a similar effect of neurturin on process outgrowth in cultured cells isolated from non-neural adult tissue.

The expression of Ret mRNA was reduced but still present after treatment of PPSCs with RA. These results contrast with observations made by others on more differentiated cells such as sympathetic neurons where an increase in Ret mRNA expression was seen after treatment with RA [47]. However, several studies have suggested the possibility of Ret-independent signalling pathways for neurturin [48]. Further studies will be necessary to demonstrate if the effects of neurturin on process outgrowth in PPSCs-derived cells may be mediated by Ret-independent pathways activating Akt, MAPK, PLCγ or Src-family kinases [19,33].

Conclusion

In summary, we have shown that a candidate stem cell population, extracted from the adult skeletal muscle (PPSCs), expresses mRNA for the GDNF family members GDNF and persephin, and responds to neurturin in ways similar to that reported for some precursors of the neuronal lineage and neurons from the CNS and PNS. Neurturin acts as a proliferative factor on PPSCs and induces process outgrowth on cells expressing the immature neuronal marker βIII-tubulin. Non-neural adult stem cells represent a particularly attractive source of cells for regenerative medicine in the nervous system because they can be safely isolated from accessible peripheral tissue. Our results suggest that PPSCs, which are easy to extract and proliferate, may combine interesting features for autologous CNS transplantation. Grafted cells releasing trophic factors like GDNF and persephin could induce protection and regeneration of the endogenous cells damaged in CNS regions during neurodegenerative diseases or after injuries. They could also respond to trophic factors present in the CNS, such as the ligands of the GDNF family, which may help them to better integrate, differentiate, and survive in the host tissue.

Acknowledgments

This work was supported by Cybios, LLC, a Bio-STAR Grant (01-10195A), and the UCLA Morris K. Udall Parkinson's Disease Research Center of Excellence at UCLA (P50NS38367).

References

[1] P.A. Lucas, A.F. Calcutt, S.S. Southerland, D. Warejcka, H.E. Young, A population of cells resident within embryonic and newborn rat skeletal muscle is capable of differentiating onto multiple mesodermal phenotypes, Wound Repair Regen. 3 (1995) 457–468.

[2] M. Romero-Ramos, P. Vourc'h, H.E. Young, P.A. Lucas, Y. Wu, O. Chivatakarn, R. Zaman, N. Dunkelman, M.A. el-Kalay, M.F. Chesselet, Neuronal differentiation of stem cells isolated from adult muscle, J. Neurosci. Res. 69 (2002) 894–907.

[3] H.E. Young, T.A. Steele, R.A. Bray, J. Hudson, J.A. Floyd, K. Hawkins, K. Thomas, T. Austin, C. Edwards, J. Cuzzourt, M. Duenzl, P.A. Lucas, A.C. Black Jr., Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors, Anat. Rec. 264 (2001) 51–62.

[4] H.E. Young, C. Duplaa, M. Romero-Ramos, M.F. Chesselet, P. Vourc'h, M.J. Yost, K. Ericson, L. Terracio, T. Asahara, H. Masuda, S. Tamura-Ninomiya, K. Detmer, R.A. Bray, T.A. Steele, D. Hixson, M. el-Kalay, B.W. Tobin, R.D. Russ, M.N. Horst, J.A. Floyd, N.L. Henson, K.C. Hawkins, J. Groom, A. Parikh, L. Blake, L.J. Bland, A.J. Thompson, A. Kirincich, C. Moreau, J. Hudson, F.P. Bowyer 3rd, T.J. Lin, A.C. Black Jr., Adult reserve stem cells and their potential for tissue engineering, Cell. Biochem. Biophys. 40 (2004) 1–80.

[5] P. Vourc'h, M. Romero-Ramos, O. Chivatakarn, H.E. Young, P.A. Lucas, M. El-Kalay, M.F. Chesselet, Isolation and characterization of cells with neurogenic potential from adult skeletal muscle, Biochem. Biophys. Res. Commun. 317 (2004) 893–901.

[6] L.F. Lin, D.H. Doherty, J.D. Lile, S. Bektesh, F. Collins, GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons, Science 260 (1993) 1130–1132.

[7] P.T. Kotzbauer, P.A. Lampe, R.O. Heuckeroth, J.P. Golden, D.J. Creedon, E.M. Johnson Jr., J. Milbrandt, Neurturin, a relative of glial-cell-line-derived neurotrophic factor, Nature 384 (1996) 467–470.

[8] R.H. Baloh, M.G. Tansey, P.A. Lampe, T.J. Fahrner, H. Enomoto, K.S. Simburger, M.L. Leitner, T. Araki, E.M. Johnson Jr., J. Milbrandt, Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex, Neuron 21 (1998) 1291–1302.

[9] J. Milbrandt, F.J. de Sauvage, T.J. Fahrner, R.H. Baloh, M.L. Leitner, M.G. Tansey, P.A. Lampe, R.O. Heuckeroth, P.T. Kotzbauer, K.S. Simburger, J.P. Golden, J.A. Davies, R. Vejsada, A.C. Kato, M. Hynes, D. Sherman, M. Nishimura, L.C. Wang, R. Vandlen, B. Moffat, R.D. Klein, K. Poulsen, C. Gray, A. Garces, E.M. Johnson Jr., et al., Persephin, a novel neurotrophic factor related to GDNF and neurturin, Neuron 20 (1998) 245–253.

[10] A.C. Tomac, A.D. Agulnick, N. Haughey, C.F. Chang, Y. Zhang, C. Backman, M. Morales, M.P. Mattson, Y. Wang, H. Westphal, B.J. Hoffer, Effects of cerebral ischemia in mice deficient in Persephin, Proc. Natl. Acad. Sci. USA 99 (2002) 9521–9526, Epub 2002 July, 9521.

[11] H. Li, Z. He, T. Su, Y. Ma, S. Lu, C. Dai, M. Sun, Protective action of recombinant neurturin on dopaminergic neurons in substantia nigra in a rhesus monkey model of Parkinson's disease, Neurol. Res. 25 (2003) 263–267.

[12] J. Widenfalk, C. Nosrat, A. Tomac, H. Westphal, B. Hoffer, L. Olson, Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs, J. Neurosci. 17 (1997) 8506–8519.

[13] J.P. Golden, J.A. DeMaro, P.A. Osborne, J. Milbrandt, E.M. Johnson Jr., Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse, Exp. Neurol. 158 (1999) 504–528.

[14] Z. Kokaia, M.S. Airaksinen, A. Nanobashvili, E. Larsson, E. Kujamaki, O. Lindvall, M. Saarma, GDNF family ligands and receptors are differentially regulated after brain insults in the rat, Eur. J. Neurosci. 11 (1999) 1202–1216.

[15] J. Zhou, Y. Yu, Z. Tang, Y. Shen, L. Xu, Differential expression of mRNAs of GDNF family in the striatum following 6-OHDA-induced lesion, Neuroreport 11 (2000) 3289–3293.

[16] H. Miyazaki, K. Nagashima, Y. Okuma, Y. Nomura, Expression of glial cell line-derived neurotrophic factor induced by transient forebrain ischemia in rats, Brain Res. 922 (2001) 165–172.

[17] J. Tokumine, K. Sugahara, O. Kakinohana, M. Marsala, The spinal GDNF level is increased after transient spinal cord ischemia in the rat, Acta Neurochir. Suppl. 86 (2003) 231–234.

[18] R.H. Baloh, H. Enomoto, E.M. Johnson Jr., J. Milbrandt, The GDNF family ligands and receptors—implications for neural development, Curr. Opin. Neurobiol. 10 (2000) 103–110.

[19] M.S. Airaksinen, M. Saarma, The GDNF family: signalling, biological functions and therapeutic value, Nat. Rev. Neurosci. 3 (2002) 383–394.

[20] H. Yan, D.F. Newgreen, H.M. Young, Developmental changes in neurite outgrowth responses of dorsal root and sympathetic ganglia to GDNF, neurturin, and artemin, Dev. Dyn. 227 (2003) 395–401.

[21] D.M. Gash, Z. Zhang, A. Ovadia, W.A. Cass, A. Yi, L. Simmerman, D. Russell, D. Martin, P.A. Lapchak, F. Collins, B.J. Hoffer, G.A. Gerhardt, Functional recovery in parkinsonian monkeys treated with GDNF, Nature 380 (1996) 252–255.

[22] D.L. Choi-Lundberg, Q. Lin, Y.N. Chang, Y.L. Chiang, C.M. Hay, H. Mohajeri, B.L. Davidson, M.C. Bohn, Dopaminergic neurons protected from degeneration by GDNF gene therapy, Science 275 (1997) 838–841.

[23] C. Rosenblad, D. Kirik, B. Devaux, B. Moffat, H.S. Phillips, A. Bjorklund, Protection and regeneration of nigral dopaminergic neurons by neurturin or GDNF in a partial lesion model of Parkinson's disease after administration into the striatum or the lateral ventricle, Eur. J. Neurosci. 11 (1999) 1554–1566.

[24] S.S. Gill, N.K. Patel, G.R. Hotton, K. O'Sullivan, R. McCarter, M. Bunnage, D.J. Brooks, C.N. Svendsen, P. Heywood, Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease, Nat. Med. 9 (2003) 589–595.

[25] P. Akerud, J.M. Canals, E.Y. Snyder, E. Arenas, Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease, J. Neurosci. 21 (2001) 8108–8118.

[26] D.A. Kerr, J. Llado, M.J. Shamblott, N.J. Maragakis, D.N. Irani, T.O. Crawford, C. Krishnan, S. Dike, J.D. Gearhart, J.D. Rothstein, Human embryonic germ cell derivatives facilitate motor recovery of rats with diffuse motor neuron injury, J. Neurosci. 23 (2003) 5131–5140.

[27] M.G. Tansey, R.H. Baloh, J. Milbrandt, E.M. Johnson Jr., GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival, Neuron 25 (2000) 611–623.

[28] J.P. Merlio, P. Ernfors, M. Jaber, H. Persson, Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system, Neuroscience 51 (1992) 513–532.

[29] N.J. Pantazis, A. Zaheer, D. Dai, S. Zaheer, S.H. Green, R. Lim, Transfection of C6 glioma cells with glia maturation factor upregulates brain-derived neurotrophic factor and nerve growth factor: trophic effects and protection against ethanol toxicity in cerebellar granule cells, Brain Res. 865 (2000) 59–76.

[30] T. Lenhard, C. Suter-Crazzolara, Developmental expression of GDNF, neurturin and their receptors in rat hippocampus, Neuroreport 9 (1998) 2927–2931.

[31] T. Stover, T.L. Gong, Y. Cho, R.A. Altschuler, M.I. Lomax, Expression of the GDNF family members and their receptors in the mature rat cochlea, Brain Res. Mol. Brain Res. 76 (2000) 25–35.

[32] C. Suter-Crazzolara, K. Unsicker, GDNF mRNA levels are induced by FGF-2 in rat C6 glioblastoma cells, Brain Res. Mol. Brain Res. 41 (1996) 175–182.

[33] M. Paveliev, M.S. Airaksinen, M. Saarma, GDNF family ligands activate multiple events during axonal growth in mature sensory neurons, Mol. Cell. Neurosci. 25 (2004) 453–459.

[34] P. Akerud, P.C. Holm, G. Castelo-Branco, K. Sousa, F.J. Rodriguez, E. Arenas, Persephin-overexpressing neural stem cells regulate the function of nigral dopaminergic neurons and prevent their degeneration in a model of Parkinson's disease, Mol. Cell. Neurosci. 21 (2002) 205–222.

[35] J. Takahashi, T.D. Palmer, F.H. Gage, Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures, J. Neurobiol. 38 (1999) 65–81.
[36] J.C. Conover, G.D. Yancopoulos, Neurotrophin regulation of the developing nervous system: analyses of knockout mice, Rev. Neurosci. 8 (1997) 13–27.
[37] R. Garcia, J. Aguiar, E. Alberti, K. de la Cuetara, N. Pavon, Bone marrow stromal cells produce nerve growth factor and glial cell line-derived neurotrophic factors, Biochem. Biophys. Res. Commun. 316 (2004) 753–754.
[38] Y.T. Tai, C.N. Svendsen, Stem cells as a potential treatment of neurological disorders, Curr. Opin. Pharmacol. 4 (2004) 98–104.
[39] J. Ourednik, V. Ourednik, W.P. Lynch, M. Schachner, E.Y. Snyder, Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons, Nat. Biotechnol. 20 (2002) 1103–1110.
[40] T. Ostenfeld, Y.T. Tai, P. Martin, N. Deglon, P. Aebischer, C.N. Svendsen, Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons, J. Neurosci. Res. 69 (2002) 955–965.
[41] G. Viglietto, S. Dolci, P. Bruni, G. Baldassarre, L. Chiariotti, R.M. Melillo, G. Salvatore, G. Chiappetta, F. Sferratore, A. Fusco, M. Santoro, Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia, Int. J. Oncol. 16 (2000) 689–694.
[42] R.O. Heuckeroth, P.A. Lampe, E.M. Johnson, J. Milbrandt, Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro, Dev. Biol. 200 (1998) 116–129.
[43] C.J. Thiele, C.P. Reynolds, M.A. Israel, Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma, Nature 313 (1985) 404–406.
[44] A. Oliva, A. Borriello, S. Zeppetelli, A. Di Feo, P. Cortellazzi, V. Ventriglia, M. Criscuolo, V. Zappia, F. Della Ragione, Retinoic acid inhibits the growth of bone marrow mesenchymal stem cells and induces p27Kip1 and p16INK4A up-regulation, Mol. Cell. Biochem. 247 (2003) 55–60.
[45] P.P. Roux, P.A. Barker, Neurotrophin signaling through the p75 neurotrophin receptor, Prog. Neurobiol. 67 (2002) 203–233.
[46] P.C. Holm, P. Akerud, J. Wagner, E. Arenas, Neurturin is a neuritogenic but not a survival factor for developing and adult central noradrenergic neurons, J. Neurochem. 81 (2002) 1318–1327.
[47] S.H. Thang, M. Kobayashi, I. Matsuoka, Regulation of glial cell line-derived neurotrophic factor responsiveness in developing rat sympathetic neurons by retinoic acid and bone morphogenetic protein-2, J. Neurosci. 20 (2000) 2917–2925.
[48] C. Marcos, V. Pachnis, The effect of the ret- mutation on the normal development of the central and parasympathetic nervous systems, Int. J. Dev. Biol. Suppl. (1996) S137–S138.