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Research report

Embryonic neural stem cells in a 3D bioassay for trophic stimulation studies

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ABSTRACT

Progenitors were discovered in the corpus striatum several years ago, but little is known about their proliferation and differentiation. The aim of this study was to analyze embryonic progenitor cells from the corpus striatum using a bioassay with trophic stimulation. Primary cells obtained from brains of rat embryos at E13–14 were dissected from striatum niches and cultured in stem cell media. These floating dispersed cells clumped together to forming floating bodies like irregular spheres (spheroids), which were placed in type I collagen gel and cultured under basal conditions or with the addition of NGF, NT-3, or NTN. Optimum growth of neurites was obtained, and after 24 and 48 h, they were measured for number and length. The expression of proliferation markers such as PCNA and Ki67, and of neural progenitor markers such as GFAP, nestin, vimentin, O4, A2B5, Pax6, S100, TubIII, and NeuN, was then analyzed. The initial behavior in cell cultures showed distinguishable spheroids that, when placed in 3D gels and with trophic support, generated neurites. A similar effect was observed in glial cell outgrowth from the spheroids. Our assay showed high reproducibility, short culture time, and high resolution for tracing neuron-neurite outgrowth or visualizing glial outgrowth in a few hours.

1. Introduction

The formation of neurons in the adult brain has contributed to knowledge on brain plasticity. In 1962, Joseph Altman reported the incorporation of tritiated thymidine into neural cell DNA (Altman, 1962, 1969; Altman and Das, 1966), which indicated proliferation in the adult brain. Then, decades later, neuron formation in adulthood was confirmed by Eriksson et al. (1998). There are several regions in the mature brain, such as the olfactory bulb and the hippocampus, where the neuron formation process takes place. New neurons seem to derive from neural stem cells that are first transformed into a progenitor which later forms a neuron. This stem cell resides in the subventricular zone of the lateral ventricles (Corotto et al., 1993) and in the subgranular zone of the dentate gyrus (Gould et al., 1992).

Neural stem cells may increase neuroblast populations by rapid proliferation. Later, they generate a group of progenitors that will remain quiescent in these regions until they replace neurons by migration and differentiation, to finally form part of a network. The same finding has been reported for the subventricular zone in the human brain by Sanai et al. (2004) and for the hippocampus by Eriksson et al. (1998).

Knowledge on brain progenitors is growing exponentially, which may be explained by current interest in brain mechanisms by which different populations of neural cells give rise to cells with a multipotent capacity of differentiation and proliferation. These are stem cells that could give rise to progenitors. This is why the subventricular zone of mammalian brain has become a focus for experimental research but there are also true several aspects that still remain incomplete, among which the potential replacement of neurons in diseases, causing cell loss and presents an interesting challenge. A possible way to induce neurogenesis could be learned from manipulating these pluripotent cells that live inside the brain niches.

The formation of new cells from quiescent stem/progenitors must be regulated precisely to avoid an alteration in circuits where neurogenesis is activated through epigenetic signals. This involves

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activation from quiescent cells to controlled amplification and final differentiation of neurons. Culture systems in collagen gel are specific bioassays that have been intensively used as models to evaluate trophic factors in the survival, differentiation and stimulation of nerve outgrowth *in vitro* (Ebendal et al., 1985; Carri et al., 1998; Morel et al., 2010). However, there are few reports concerning their use for stem and progenitor cells (Cruz Gaitan et al., 2012; Carri et al., 2014).

On the other hand, there is abundant experimental evidence for the adult mammal subventricular zone and hippocampus, where a subgroup of quiescent stem cells is able to generate neurons. The corpus striatum could provide important stem cell *reserves*, but little is yet known about their rate of proliferation and the possible paths of differentiation from the embryonic stage. Recent research studies on neural progenitor cells have provided further knowledge on neural regeneration. A number of chemical factors which mediate these effects have been well characterized and have been studied through different development stages of neuronal and glial cells, though little has been done on progenitors, especially by means of 3D assays. The analysis of this response, neuritogenesis, in spheroids explanted to 3D cultures with type I collagen would facilitate their evaluation since this substrate allows the extension of neuronal cells and their neurites.

Several reports suggest that neuron development in the nervous system may be controlled by similar or analogous mechanisms but they have not been applied to stem cells. In order to study this we prepared trophic factors at 20 ng/ml. These factors derive from families of molecules that may influence survival, differentiation, and outgrowth in length and number (density).

In this paper we first characterize the assay necessary for brain progenitors to be cultured under basal conditions and trophic stimulation with the analysis of survival, differentiation, and neurite outgrowth. In this assay, it was possible to evaluate cells by the effects of trophic molecules, particularly those with clear activity on embryonic cells. We were also able to evaluate the action of other epigenetic signals recently discovered for embryonic stem cells in 3D bioassays of type I collagen. It is essential to identify signals that lead the stem to leave quiescence and express a neuronal or glial phenotype under basal conditions and trophic stimulation. This paper also includes the analysis of the immunoreactive profile of stem cells for the identification of neuronal and glial phenotypic epitopes.

2. Materials and methods

2.1. Donor tissue, stem cells assay

Stem cells were obtained from the corpus striatum of E14.5 fetal Sprague Dawley rats, and kept in sterile Hank's medium (CRL:CD SD BR® Charles River Laboratories, USA). Surgery was performed with a stereo-microscope under sterile air flow in a tissue culture room following a previously described protocol (Rojas Mayorquin et al., 2010). Briefly, an oblique cephalic cut above eye level was performed, and skin and cartilage of the head were removed to expose the brain. The corpus striatum is a clear anatomical region in the developing rat brain and has a niche with considerable neural stem cells. This region has a readily recognizable vascular irrigation, which makes it easy to identify it during explantation. The niche was removed from brain hemispheres, and placed in sterile plastic tubes containing Hank's medium, in a humid chamber at 37 °C for 20 min (see Flow chart phase 1). The explanted brain zones were then mechanically dissociated by gently pipetting resulting in a cell suspension that was centrifuged for 5 min (>1000 rpm) for cell concentration. Pellets were resuspended into mitogenic medium defined for stem cells (75% DMEM, 25% HAM F-12, 1% B27 – all from GIBCO, USA) and a mitogenic factor (bFGF at 10 ng/ml – Invitrogen, USA) (see Table 1).

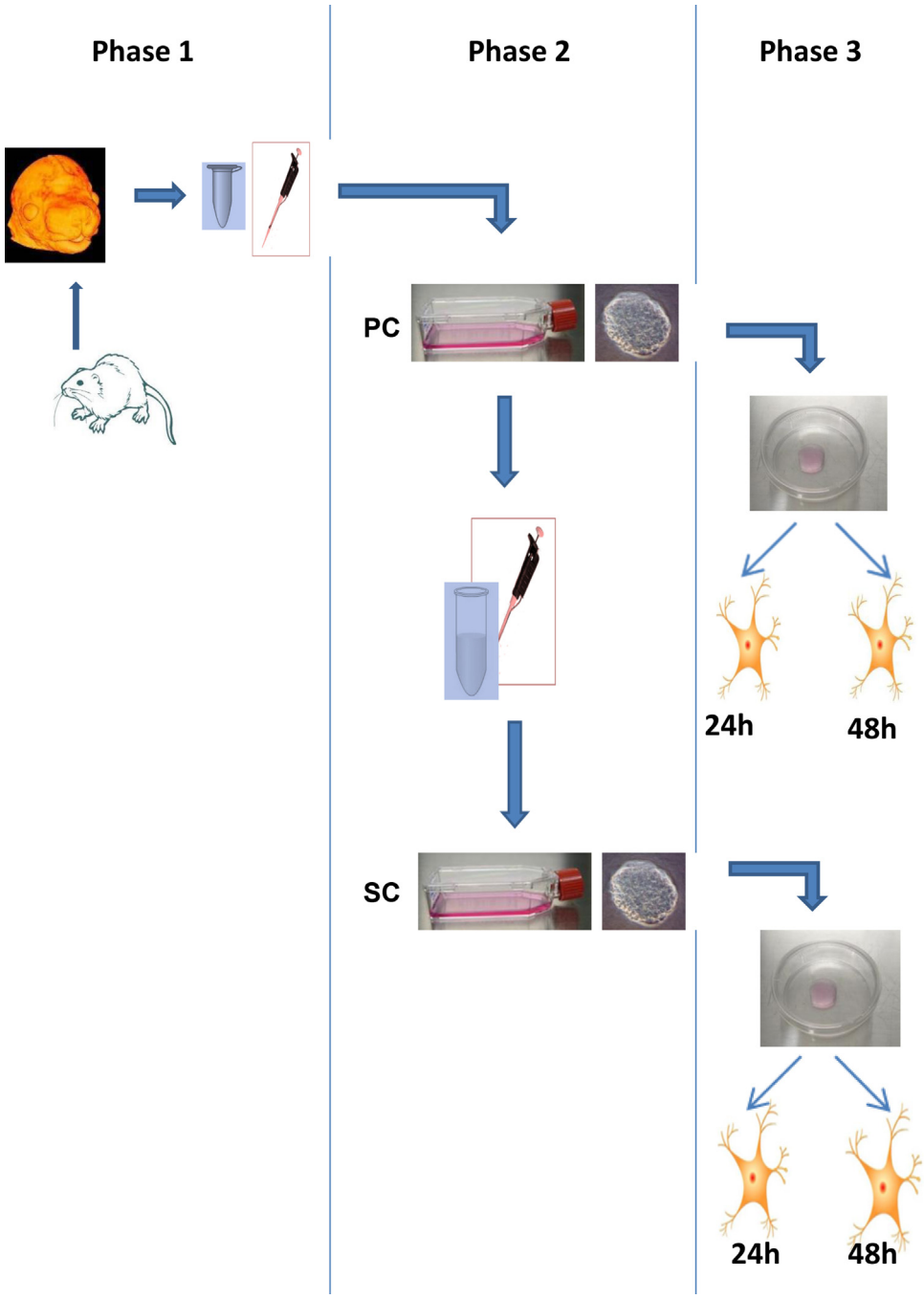
The first step of cell culture was resuspension, seeding in culture bottles (Corning, USA) and incubation at 37 °C in a 5% CO_2 in a humid atmosphere. After the first 48–72 h of incubation, spheroids of primary cultures were obtained and collected by centrifugation for 5 min. The supernatant was then removed, and the spheroids were mechanically dissociated for resuspension in 1 ml medium defined for stem cells. After this procedure we obtained the spheroids of secondary cultures. The cell suspension was resuspended in a double volume and incubated under the same conditions as those of the primary culture. At 48 h from beginning of the culture floating spheroids contained in an aliquot of 8 μl medium were placed in the center of a 35 mm plastic dish for inclusion in collagen gel for the 3D bioassay. The action of trophic factors on the spheroids of secondary culture was then analyzed using the same protocol as that used to evaluate the primary culture.

2.2. The 3D bioassay and treatments

Our 3D bioassay is based on cell clumps inside collagen gel produced in our lab according to the original procedure described by Elsdale and Bard (1972), Ebendal's protocol (1985) and our group (Carri et al., 1998; Reynaldo et al., 2007; Cruz Gaitan et al., 2012; Carri et al., 2014). Briefly, spheroids were pipetted out from the flask, placed in the center of the 35 mm plastic dish inside collagen gels. The trophic factors like neurturin (NTN), neurotrophin-3 (NT-3) and nerve growth factor (NGF) from Peprotech® were added over the surface of the gel at a final concentration of 20 ng/ml. We also performed cultures without trophic factors, covering them with DMEN to prevent dihydratation (see Table 1). The bioassay was cultured for 48 h and neurite outgrowth was then measured at 24 and 48 h (see Flow chart) with an inverted microscope IM35 from Carl Zeiss with long focal distance lens of 10× or 20× and phase contrast illumination.

2.3. Expression markers in cultured cells.

Cultures grown inside gels were fixed *in toto* with 4% paraformaldehyde at room temperature, and then washed three times each in PBS for 5 min. Permeabilization was performed with 0.02% Triton X-100 for 15 min at room temperature followed by overnight incubation at 4 °C with normally diluted (1:100 in PBS) primary antibody, then washed again three times for 5 min each in PBS, and finally incubated with the secondary antibody diluted in 1:200 PBS for 1 h. DNA nuclei were stained with DAPI (Sigma, USA) at 1 μg/ml concentration for 15 min. After washing three times for 5 min each with PBS, the culture was preserved with Fluoromount-G® (Southern Biotechnology Association, USA) antifading mounting medium. Several primary antibodies were used for detecting antigens, especially two antibodies which show expression of proliferation related identifier proteins (anti-PCNA – from Santa Cruz Biotech SC56 – and anti-Ki67 – from AbCam, USA). Several other antibodies showed neural lineage (anti-GFAP – from SIGMA 63893; anti-nestin from Developmental Studies Hybridoma Bank at the University of Iowa DSHB-RAT401; anti-vimentin – from SIGMA B6630; anti-O4 – anti-oligodendrocyte marker O4 MAB345; anti-A2B5 – prepared by us with DSHB-Clone 05; anti-Pax6 from DSHB-PAX6-Kawakami; anti-S100 from SIGMA, USA; anti-Tub III – from SIGMA USA; anti-NeuN from Chemicon International MAB377B). These primary antibodies were detected by a secondary antibody with red Alexa 555 or green Alexa 488 from Molecular PROBES, USA. Then cultures were washed and mounted with Fluoromount-G. This method provided excellent detection of cellular antigens for visualization of the markers and for morphometric analysis. The immunoprofile analysis was carried out



Flow chart. The chart illustrates different phases in the bioassay to evaluate trophic factors on stem cells. At the first step, in phase 1, tissue is taken from the embryonic brain, disaggregated and in phase 2 cultured as primary cells in a PC flask for the next 48 h to produce floating spheroids. In phase 3, the spheroids are placed in a 3D assay to evaluate the action of trophic factors, as indicated in the figures below the arrow at 24 and 48 h. Spheroids continue in culture in the same flask for a total of 72 h and are then taken, disaggregated and resown/recultured in a new SC flask for 48 h continuing phase 2. The newly formed spheroids from passaged cells are placed in phase 3 in a 3D assay to evaluate the action of trophic factors as indicated in the chart at 24 and 48 h.

Table 1
Spheroid trophic treatments.

Treatments	Stem cell media	Differentiation media
1 Control	75% DMEM + 25% F12 + 1 ml B27 + bFGF at 10 ng/ml	Collagen gel type I + DMEM
2 NTN		Collagen gel type I + DMEM + NTN at 20 ng/ml
3 NT-3		Collagen gel type I + DMEM + NT-3 at 20 ng/ml
4 NGF		Collagen gel type I + DMEM + NGF at 20 ng/ml

The 3D bioassay of spheroids in collagen gel with trophic factors over first and second passages.

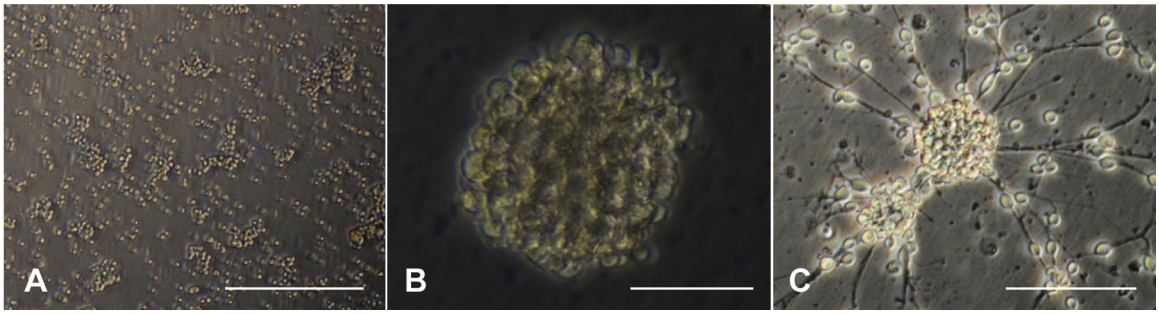


Fig. 1. Images of brain stem cells, floating spheroids and 3D bioassay. (A) Brain disaggregated cells. The explanted brain contains good viable cells that can be seen here in culture before forming the typical clumps as real spheroids. Magnification bar: 250 μm. (B) Floating spheroids in stem cell media before implanting them in 3D assay. Magnification bar: 50 μm. (C) Spheroids with neurites after trophic treatment in 3D collagen substratum. Magnification bar: 100 μm.

on spheroids cultured under basal as well as experimental conditions. The specimens were observed and photographed with a Nikon Eclipse microscope, attached to a D100 digital camera.

2.4. Cell-image processing for measurement

After 24 and 48 h culture, spheroids in 3D gels were clipped in a universal mechanical stage with *x*–*y* translation movement controls. Then the spheroids were field-selected at random and drawn using a clear double glass prism to keep records of the experiment. The cultures drawn were digitalized and measured, outlining the spheroids with a semi-automatic quantitative software (Image-Pro Plus, Media Cybernetic, USA). We measured the number and length of neurites emitted. Neurites are considered as the maximum exponent of trophic factor action on neural tissues producing differentiation. A total of 720 explants were randomly drawn and analyzed as a group (5 per treatment dish for 24 and 48 h, with as much initial culture as the spheroid of secondary culture). Experiments were repeated nine times under the same conditions.

2.5. Numerical data and statistics

Numerical data from experimental cultures measured by the software were transferred to Excel spreadsheets, and assigned to individual groups. We measured two variables, number and length of emitted neurites, classified in three groups: (i) Treatments (NTN, NT-3, and control), (ii) Types of culture (primary and secondary), and (iii) Culture times (24 and 48 h). To obtain comparable data we analyze each spheroid, quantified number and length of neurites in each of them, and calculated the mean. We used the statistical package STATA 7.0 and the NESTED ANOVA TEST to evaluate differences among the Treatments, Types of culture inside Treatments, and Culture times inside Types of culture. The analysis was performed with the original data and those previously submitted to logarithmic transformation to homogenize variance (significance level $p < 0.05$).

3. Results

3.1. Developing stem cells

Explants from the striatum provide tissue with a great amount of proliferative cells, which become detectable when the tissue is disaggregated and cultured in appropriate culture media. These disaggregated cells are randomly distributed in the culture dish (Fig. 1A), reaching a final density that allows good proliferation, and forming floating bodies like irregular spheres (spheroids) after 4 h incubation. They reach a uniform size of 70 μm after 48 h culture. Small pieces of disaggregated tissues produce hundreds of spheroids like the one shown in Fig. 1B. The cell structure of the spheroid is similar to an organoid of classical organotypic explants (Fig. 1C).

3.2. 3D gel culture for trophic evaluation

Inside the collagen matrix, the spheroid is an excellent assay to evaluate the effects of trophic factors on stem cells (see neuritogenesis in Fig. 1C). The 3D gel prepared in our lab proved to be of optimal consistency for proliferation and differentiation, and good mechanical support for survival, neurogenesis and vigorous outgrowth (see spheroids in Fig. 3). Treatments with NT-3, NTN, or NGF were used to evaluate trophic factor action. Untreated cultures were used as controls. After stimulation, they started a rapid process of differentiation, increasing survival and extending a neurite. The number of neurites as well as their length can be noted and the treatments compared with respect to untreated spheroids.

3.3. Analysis of the differentiation process

3.3.1. Neuritogenesis measured as emitted neurites

Treatments were measured as the number of neurites based on two different parameters of the experiments: types of culture and culture times for each treatment and controls. We observed great number of neurites in the spheroid after trophic stimulation, in comparison with cultures under basal conditions (control). Spheroids of secondary cultures inside collagen gel after 48 h treatment with NT-3 presented the highest number of neurites (mean = ±45). These were followed by NTN (mean = 32 neurites), and NGF (25 neurites), evaluated at 24 and 48 h. The control experiment showed few neurites in comparison with treatments, with a mean of seven neurites in secondary cultures (Fig. 2A), and the highest response in the differentiation process compared with neurite number in all treatments (10 between spheroids of primary and secondary cultures for NT-3, 6 for NGF, and 2 for NTN). Culture times (24 and 48 h) did not influence the differentiation process. These results demonstrate that stem cells respond to trophic factor stimulation (Fig. 2B). Highly significant differences ($p < 0.001$) were observed between the number of neurites under the action of trophic stimulation among different molecules and experiments under basal conditions. Differences were also observed between primary and secondary cells per treatment (p value < 0.001), whereas culture times (24 and 48 h) for the same secondary cells showed no differences (significance level = 0.661).

3.3.2. Length of emitted neurites

Neurite length measurement showed longer neurites in spheroids under trophic stimulation than in controls. NT-3-treated spheroids of secondary cultures (48 h) presented the longest neurites (86.9 μm) compared to the other treatments. These were followed by NTN (72.86 μm), and NGF (60.48 μm). Controls did not

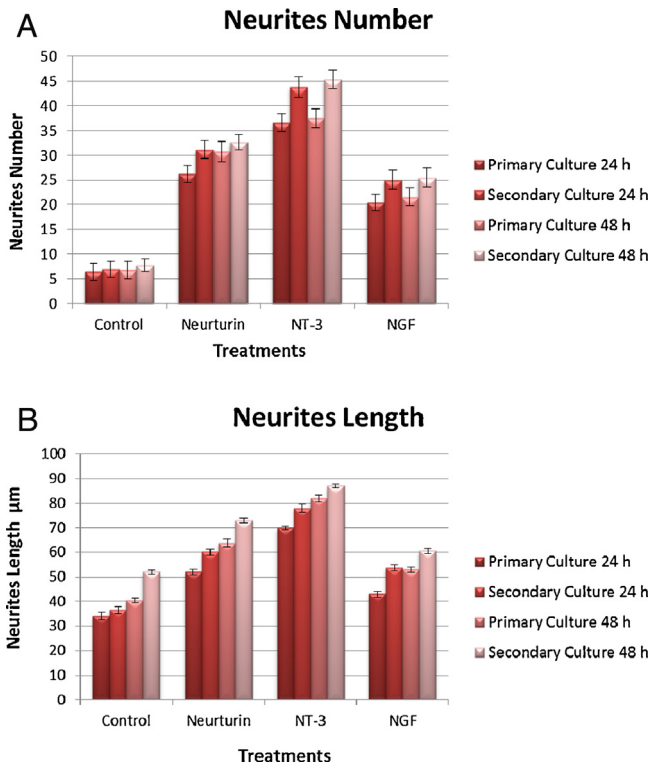


Fig. 2. Histograms of neurite number and neurite length. Neurite outgrowth induced with NTN, NT-3, and NGF on spheroids of primary culture and spheroid of secondary culture evaluated at 24 and 48 h. (A) Neurite number. (B) Neurite length. NT-3 showed the best effect compared to control, followed by NTN, and finally NGF. After 48 h culture, a greater elongation of neurites was observed.

show significant neurite elongation in comparison with all treatments. Spheroids of secondary cultures presented highest response in the differentiation process of neurite length in all treatments. An important influence of culture times (24 and 48 h) on this differentiation process could be observed in terms of neurite elongation. These results were confirmed by the existence of significant differences in neurite length among all trophic factors tested with respect to the control (significance level = 0.045). Differences were also observed between both culture times (24 and 48 h) ($p = 0.015$). Moreover, highly significant differences were found between types of culture ($p < 0.001$).

Culture times also showed significant neurite differences: higher number and length of neurites were observed for NT-3-treated spheroids of secondary 48 h cultures. This demonstrates the excellent action of these factors on the neurite elongation process and the growth cone of neural cells. All cells analyzed in collagen gel showed significant differences among treatments and in spheroids of secondary cultures tested ($p < 0.001$). The highest length and number of neurites were observed for spheroids of secondary cultures where NT-3 exerted a stronger stimulation on these parameters than the other trophic factors assayed, to develop neurogenic processes (Fig. 3C). The same though lower effect was observed for NGF (Fig. 3D) and NTN (Fig. 3B).

3.4. Neural profile of spheroids

Neural markers have been characterized in these cells and the multipotency of the cells has already been shown. It was also possible to trace that cells can be differentiated into at least two types.

In floating cultures (spheroids of primary cultures), the expression was dominated by antigen PCNA and A2B5, while S100, KI67, and Pax6 were notably expressed in the spheroids of secondary cultures. GFAP, nestin, PCNA, O4, and tubulin III were expressed in spheroids of primary and secondary cells of both types of culture (Fig. 4).

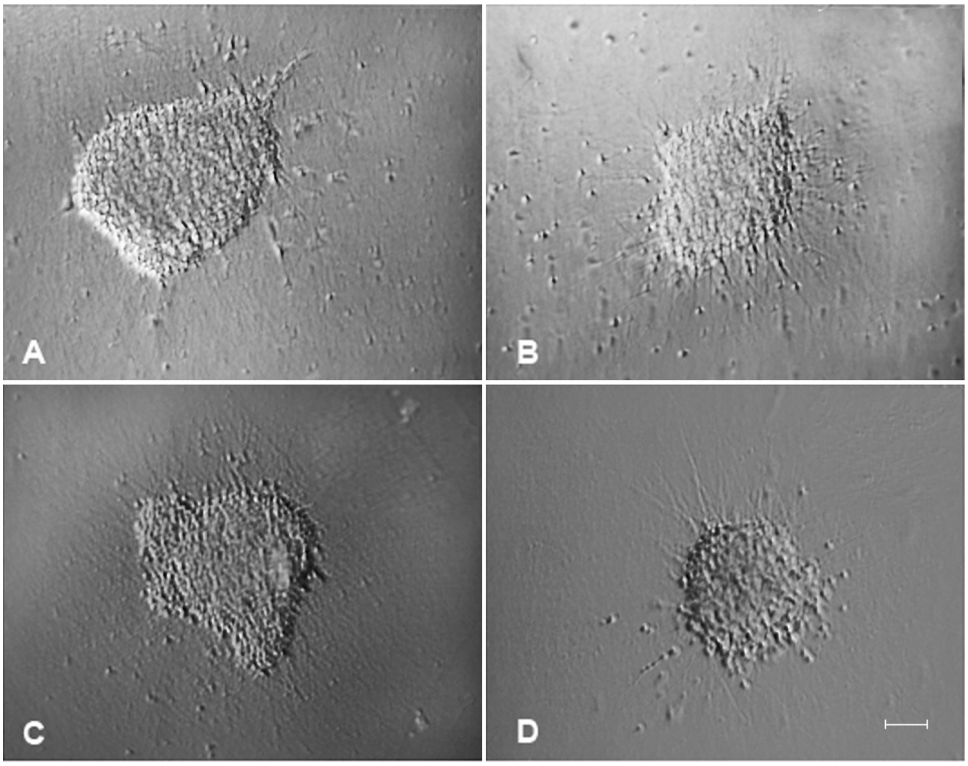


Fig. 3. (A–D) Images of spheroids in 3D bioassay. Images illustrate microscopic photos of cultured spheroids inside collagen gel with trophic stimulation. Note the differences between the control and the stimulated spheroids: (A) untreated (control); (B) NTN; (C) NT-3; (D) NGF. Magnification bar: 150 µm.

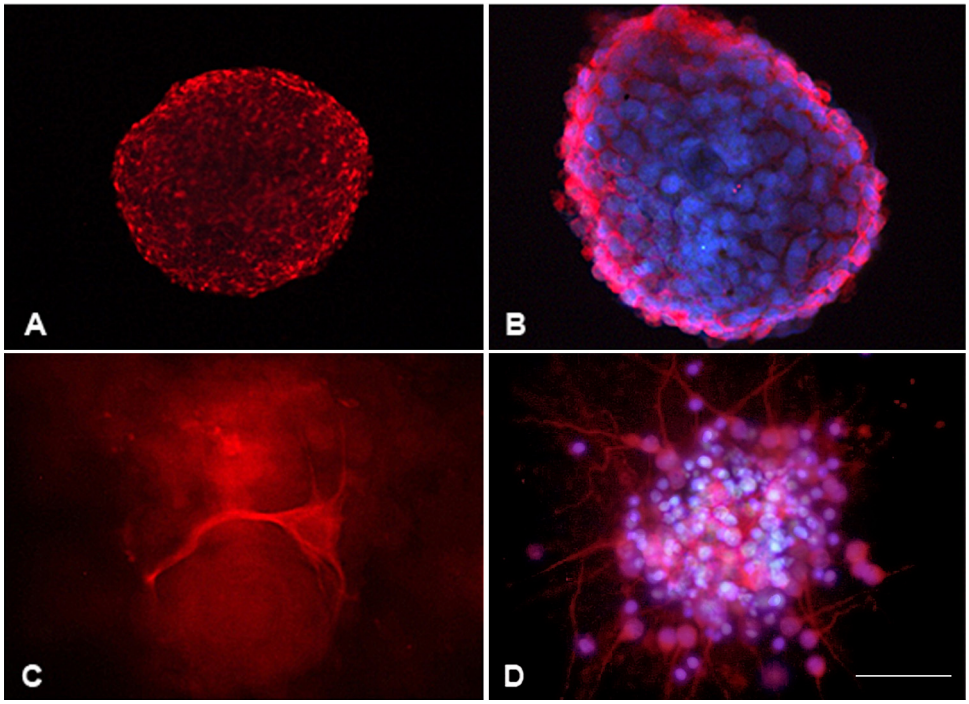


Fig. 4. (A–D) Spheroids with immunolabelling. Spheroid of primary culture was stained with anti-vimentin antibody (A), expression of anti-β III tubulin antibody in spheroid treated with NTN (B), anti-A2B5 antibody (C). Note the excellent cell staining of glial cells lineage. Anti-β III tubulin antibody in NT-3 treated spheroids (D). Nuclei are DAPI counterstained. Magnification bar: 50 μm.

3.5. Expression markers under trophic induction

Trophic stimulation with NT-3, NTN, and NGF is sufficiently effective to activate the expression of specific neuronal markers like tubulin III (Fig. 4D). Spheroids of primary cells also expressed tubulin III. Spheroids of secondary cells only expressed glial marker O4 with NTN, and neuronal marker NeuN with NT-3. Moreover, spheroids of primary cells and untreated spheroids showed positive marking for neuronal NSE, typical of neurons. Images of positive findings are shown in Fig. 4.

4. Discussion

The neural system arises from multipotent cells capable of providing neurons and glia (Reynolds et al., 1992; Doestch, 2003a, 2003b; Weinandy et al., 2011; Quadrato et al., 2014). After the initial proliferation, the stem/progenitor is restricted to a small area (Miller and Gauthier-Fisher, 2009; Weinandy et al., 2011; Gould and Tanapat, 1999; Magavi et al., 2000; Sabelström et al., 2013), which demonstrates that proliferating cells in the brain generate granule neurons and those in the SVZ give rise to both telencephalic neurons and glia.

However, the existence of corpus striatum stem cells in the embryonic rat brain is controversial. Evidence of a mechanism producing these cells in this area is disputed (Fentress et al., 1981). But several groups disagree and demonstrate from brain embryos of Sprague Dawley rats that the corpus striatum is a well-established neurogenic region. Palmer et al. (1995) have shown the existence of neuronal and glial progenitors in the corpus striatum similar to the progenitors identified in the subventricular zone and the hippocampal dentate gyrus. These progenitors will differentiate to neurons or glial cells *in vitro* only if they receive the appropriate trophic treatment. In fact, Doestch (2003a, 2003b) has proposed re-analyzing the function as well as the progeny of cells, especially when they are located in the niche under the influence of trophic factors. In this paper, we have analyzed the action of some trophic factors on striatum stem/progenitor brain cells using our collagen gel 3D assay (Reynaldo et al., 2007; Cruz Gaitan et al., 2012; Carri et al., 2014).

4.1. The clump of cells as spheroids in collagen gels as a model

The bioassay used in these experiments is simple, easy to perform, with repeatable good results. Tissue explanted is easy to dissociate by simple mechanical procedures and the results are reliable and show a good number of proliferating stem cells. These proliferative cells seem to be the same as those reported by Dayer et al. (2005). Bédard et al. (2006) proposed that new neurons at the striatum come from subventricular progenitors migrating through different pathways toward the olfactory bulb. There are some remaining progenitors from the same striatum (Tropepe et al., 1997). In contrast, Luzzati et al. (2007) demonstrated that the striatum and migrating neuroblast are different progenitors that never mix. There are some clusters of proliferative cells in the caudate from which neuroblasts start migrating (Luzzati et al., 2007). These are the corpus striatum proliferative cells that were used for our culture assays. These cells, like hippocampal neurons, express DCX. In the adult brain, this neurogenic striatum is in intimate contact with another two neurogenic regions (Luzzati et al., 2007; Jiao and Chen, 2008).

The explantation zone is easy to identify during surgery. Access is rapid and precise. Explanted tissue contains good viable cells that form spheroids in culture (Fig. 1B) (Rojas Mayorquin et al., 2010). Mitosis of the peripheral cells into spheroids has been attributed to stimulation of bFGF within the stem cell medium (Reynolds et al., 1992); this mitosis can be seen in Fig. 1A.

Progenitors and stem cells grown together as spheroids and then placed in a 3D culture are an excellent assay for trophic factors. The response could be differentiation of progenitors as neurons that can produce a neurite. As soon as the cells are stimulated with trophic factors, they start to increase survival and rapidly differentiate, extending a neurite. Fig. 3 illustrates spheroids placed in collagen and under the effect of NT-3, NTN and NGF. NT-3 exerts a greater

stimulation than other trophic factors in both length and number. NTN and NGF have the same effect but with small differences.

4.2. Action of trophic factors on proliferating cells

Trophic factors have multiple actions on cells throughout embryo development. Neurotrophins and their receptors have been shown *in vitro* and *in vivo* to be localized in numerous nerve structures, influencing proliferation, neurite outgrowth and cell survival (Von Bartheld, 1998; Wang et al., 2010; Delgado et al., 2014; Gransee et al., 2015). Various effects of trophic factors have also been observed on embryonic stem cells. In our bioassay, bFGF promotes progenitor proliferation. Cattaneo and McKay reported that NGF increases proliferation of explanted nestin-positive precursors from E13.5–E14.5 embryos when they were previously treated with bFGF, but in our bioassay bFGF seemed to promote only proliferation of nestin-positive stem cells and NGF acted as an inductor of neuritogenesis (see Fig. 4D).bFGF induces proliferation of precursors, which migrate without complete differentiation of neurons from the subventricular zone through the striatum or s. nigra (Jin et al., 2005). It also directs progenitor differentiation to glial oligodendrocytes (Palmer et al., 1995) and increases survival and differentiation of dopaminergic neurons *in vitro* (Reuss and Unsicker, 2000). We analyzed oligodendrocyte markers and found similar results of 04 and A2B5expression when stem cells were in our medium with bFGF, demonstrating induction of oligodendrocyte differentiation.

NT-3 has important effects on the developing nervous system and during regeneration, such as the regulation of the survival and proliferation of glia in the visual system (Von Bartheld, 1998). Thoracic dorsal root ganglia dissected from rats at embryonic day 13 show neurite outgrowth, especially when they are under NT-3 and NGF in the collagen matrix (Hari et al., 2004). Our results demonstrate that NT-3 induces neurogenesis and neuritogenenesis in embryonic stem cells from the corpus striatum when they are in a 3D collagen matrix.

More recently, GDNF was found to be expressed at the developing striatum (Choi-Lundberg and Bohn, 1995) but reduced during the postnatal days as NTN increased (Arekud et al., 1999). GDNF and NTN may protect and restore neural function in injured animals (Gasmi et al., 2007) and these factors seem to work together in neural development. It is clear that the action of GDNF and NTN is to specifically promote cell morphology and stimulate neurite outgrowth (Zihlmann et al., 2005) and our results clearly confirm that NTN is neuritogenic for striatal precursors.

4.3. The trophic factors direct neural stem cells to become neurons

Neural stem cells that are stimulated by a trophic factor will change shape and morphology following a process to generate a neuron that will finally produce an extension outgrowth that will be a neurite. This process will show varicosities, branching along the elongation axis on the neurite body, and a final growth cone. These varicosities and growth cones are easily recognizable in phase contrast illumination during evaluation in the assay (Fig. 1C). This process is known as neuritogenesis; it has been very well regarded for assessing the action of trophic molecules on nerve cells (Ebendal et al., 1985), and may be attributed to their action on specific cell receptors (Huang and Reichardt, 2001).

In cultures, the elongation of neurites from the spheroid offers a good parameter for measurement. The outgrowth is measured as two magnitudes: (i) number of neurites, and (ii) length of neurites (see Fig. 2A and B). These magnitudes represent the number of cells transformed to neurons and the elongation of neurons in response to trophic action. In our experiments we found very good outgrowth in both number and length of neurites. There are significant differences with untreated control cultures. Spheroids of secondary cultures treated with NT-3 at 48 h gave a spectacular 86.9 μm extension of neurites. It was clear that NT-3 acts on both aspects of neurite magnitude: number and length. The results found with NT-3 are manifestly different from those produced by NGF and NTN (Fig. 2A and B). No other treatment gave the same result. Spheroids of secondary cultures with NTN gave 72.8 μm at 48 h and classical NGF gave a length of 60.4 μm at 48 h. The control elongated only 51.9 μm. Spheroids of secondary cultures have a greater response in length in all treatments with minimal difference between primary and secondary. Elongation was very good at 48 h culture.

The NT-3 effect could be explained through the expression on the cell membrane of the corresponding receptor TrkC and TrkB (Tessarollo et al., 1994; Klein et al., 1994). The effect of NTN fundamentally supports survival more than differentiation in neural cells (Zihlmann et al., 2005) possibly acting through the Ret receptor (Durbec et al., 1996). NGF also promotes survival, with proliferation and strong differentiation made possible through the high affinity NGF-receptor (Loeb et al., 1991; Bothwell, 1995; Kaplan and Miller, 1997).

In the experiments, neural stem cells which are treated with trophic factors differentiate to neurons and produce neurite outgrowth (Quadrato et al., 2014). This outgrowth, which we named neuritogenesis, has been measured based on three different circumstances of the experiments: (i) the trophic factors, (ii) the type of assay, whether primary or secondary cultures, (iii) evaluation at 24 and 48 h. Under these conditions, the stem cells show vigorous neuritogenesis with a considerable number of neurites (Fig. 3B–D). Experiments with NT-3 and spheroid of secondary cultures evaluated at 48 h show a mean number of 45 neurites per sphere. Treatments with NTN show a mean number of 32 neurites and with NGF, a mean number of 25 neurites at both 24 and 48 h. Controls compared with those treated do not show neurites in the same number and length. There are significant differences ($p < 0.001$) between the number of neurites under the action of trophic stimulation and also in comparison to the experiment under basal conditions. There are also significant differences between types of culture in each treatment, but no differences between evaluations at 24 and 48 h.

4.4. Markers of neural stem cells in cultures

The presence of antigens corresponding to cell proliferation in cellular spheroids indicated that there are progenitors of neural phenotypes in early phases of cultures. Positivity with Ki67 and PCNA has been detected, and this corresponds to proliferation and characteristic self-renewal of stem cells. The expression of the markers cited above also shows in early phases that there is induction of differentiation in the assay. The immunolabeling was performed within the collagen after trophic stimulation. In these spheroids, the expression of nestin, vimentin and GFAP are markers of stem cells and progenitors. Floating spheroids show characteristics of stem cells, with expression of PCNA and A2B5 in spheroids of primary cultures, while S100, KI67 and Pax6 are markedly expressed in spheroids of secondary cultures. GFAP, nestin, PCNA, O4 and tubulin III are expressed in cells of both primary and secondary cultures. Their expression demonstrates the capacity to generate differentiation toward other cell types. Photos of stained anti-vimentin spheroids can be seen in Fig. 4A, while those treated with NTN and stained with anti-β III tubulin can be seen in Fig. 4B. The spheroids stained with anti-A2B5 antibody are shown in Fig. 4C and those treated with NT-3 and stained with anti-tubulin III in Fig. 4D. Spheroids of secondary cultures treated with NTN express O4 according to glial cells, while those of primary cultures treated with all the trophic factors, NT-3, NTN and NGF,

express tubulin III (Fig. 4D). Spheroids of primary cultures treated with NT-3 express tubulin III and Neu-N characteristic of neurons, and those treated with NGF express tubulin III, again typical of the neuronal phenotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2015.04.006

References

Altman, J., 1962. Are new neurons formed in the brains of adult mammals? Science 35, 1127–1128.
Altman, J., Das, G.D., 1966. Autoradiographic and histological studies of postnatal neurogenesis I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions. J. Comp. Neurol. 126, 337–389.
Altman, J., 1969. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J. Comp. Neurol. 137, 433–457.
Arekud, P., Alberch, J., Eketjall, S., Wagner, J., Arenas, E., 1999. Differential effects of glial cell-line derived neurotrophic factor and neurturin on developing and adult substantia nigra dopaminergic neurons. J. Neurochem. 73, 70–78.
Bédard, A., Gravel, C., Parent, A., 2006. Chemical characterization of newly generated neurons in the striatum of adult primates. Exp. Brain Res. 170, 501–512.
Bothwell, M., 1995. Functional interactions of neurotrophins and neurotrophin receptors. Annu. Rev. Neurosci. 18, 223–253.
Carri, N.G., Bengtsson, H., Charette, M., Ebendal, T., 1998. BMP receptor II expression coincides with op-1 effects on developing chick retinal explants. Neuroreport 9, 1097–1101.
Carri, N.G., Bermudez, S.N., Di Napoli, J., Fiore, L., Scicolone, G., 2014. Anaglyph of retinal stem cells and developing axons: selective volume enhancement in microscopy images. Anat. Rec. 297, 770–780.
Corotto, F.S., Henegar, J.A., Maruniak, J.A., 1993. Neurogenesis persists in the subependymal layer of the adult mouse brain. Neurosci. Lett. 149, 111–114.
Choi-Lundberg, D.L., Bohn, M.C., 1995. Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. Brain Res. Dev. Brain Res. 85, 80–88.
Cruz Gaitan, A.M., Reynaldo, M.B., Carri, N.G., I Congreso FALAN-IBRO-LAR, 2012 2012. Activation of neural stem cells to strong differentiation and vigorous outgrowth by trophic factors tandem inside collagen gel (W:162)., pp. 216.
Dayer, A., Cleaver, K., Abouantoun, T., Cameron, H., 2005. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J. Cell Biol. 168, 415–427.
Delgado, A.C., Ferrón, S.R., Vicente, D., Porlan, E., Perez-Villalba, A., Trujillo, C.M., D Ocón, P., Fariñas, I., 2014. Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction. Neuron 83, 572–585.
Doestch, F., 2003a. A niche for adult neural stem cells. Curr. Opin. Genet. Dev. 13, 543–550.
Doestch, F., 2003b. The glial identity of neural stem cells. Nat. Neurosci. 6, 1127–1134.
Durbec, P., Marcos-Gutierrez, C., Kilkenny, C., Grigoriou, M., Suvanto, P., Wartiovaara, K., Smith, D., Ponder, B., Costantini, F., Saarma, M., Sariola, H., Pachnis, V., 1996. Glial cell line-derived neurotrophic factor signalling through the Ret receptor tyrosine kinase. Nature 381, 789–792.
Ebendal, T., Norrgren, G., Carri, N.G., Belew, M., 1985. Nerve growth factors in peripheral tissue and CNS. In: Eccles, J., Dimitrijevich, M.R. (Eds.), Recent Achievements in Restorative Neurology, 1: Upper Motor Neuron Functions and Dysfunctions. Karger, Basel, Basilea, pp. 311–320.
Elsdale, T., Bard, J., 1972. Collagen substrata for studies on cell behavior. J. Cell Biol. 54, 626–637.
Eriksson, P.S., Perfilieva, E., Bjork Eriksson, T., Alborn, A.M., et al., 1998. Neurogenesis in the adult human hippocampus. Nat. Med. 4, 1313–1317.
Fentress, J.C., Stanfield, B.B., Cowan, W.M., 1981. Observation on the development of the striatum in mice and rats. Anat. Embryol. 163, 275–298.
Gasmi, M., Herzog, C., Brandon, E., Cunnungham, G., Ramirez, A., Ketchum, E., Bartus, R., 2007. Striatal delivery of neurturin by CERE-120, an AAV2 vector for the treatment of dopaminergic neuron degeneration in Parkinson's disease. Gen. Ther. 15, 62–68.
Gransee, H., Zhan, W.Z., Sieck, G.C., Mantilla, C., 2015. Localized delivery of BDNF-expressing mesenchymal stem cells enhances functional recovery following cervical spinal cord injury. J. Neurotrauma 32, 185–193.
Gould, E., Cameron, H.A., Daniels, D.C., Woolley, C.S., Mcewen, B.S., 1992. Adrenal hormones suppress cell division in the adult rat dentate gyrus. J. Neurosci. 12, 3642–3650.
Gould, E., Tanapat, P., 1999. Stress and hippocampal neurogenesis. Biol. Psychiatry 46, 1472–1479.
Hari, A., Djohar, B., Skutella, T., Montazeri, S., 2004. Neurotrophins and extracellular matrix molecules modulate sensory axon outgrowth. Int. J. Dev. Neurosci. 22, 113–117.
Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.
Jiao, J., Chen, D.F., 2008. Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells 26, 1221–1230.
Jin, K., Lafevre-Bernt, M., Sun, Y., Chen, S., Gafni, J., Crippen, D., Logvinova, A., Ross, C.A., Greenberg, D.A., Ellerby, L.M., 2005. FGF-2 promotes neurogenesis and neuroprotection and prolongs survival in a transgenic mouse model of Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. 102, 18189–18194.
Kaplan, D., Miller, F., 1997. Signal transduction by the neurotrophin receptors. Curr. Opin. Cell Biol. 9, 213–221.
Klein, R., Silos-Santiago, I., Smeyne, R., Lira, S., Brambilla, R., Bryant, S., Zhang, L., Snider, W., Barbacid, M., 1994. Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature 368, 249–251.
Loeb, D., Maragos, J., Martín-Zanca, D., Chao, M., Parada, L., Greene, L., 1991. The trk proto-oncogen rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 66, 961–966.
Luzzati, F., De Marchis, S., Fasolo, A., Peretto, P., 2007. Adult neurogenesis and local neuronal progenitors in the striatum. Neurodegener. Dis. 4, 322–327.
Magavi, S.S., Leavitt, B.R., Macklis, J.D., 2000. Induction of neurogenesis in the neocortex of adult mice. Nature 405, 951–955.
Miller, F.D., Gauthier-Fisher, A., 2009. Home at last neural stem cell niches defined. Cell Stem Cell. 4, 507–510.
Morel, G., Sosa, Y., Bellini, M., Carri, N.G., Rodriguez, S., Bohn, M., Goya, R.G., 2010. Glial cell line-derived neurotrophic factor gene therapy ameliorates chronic hyperprolactinemia in senile rats. Neuroscience 167, 946–953.
Palmer, T.D., Ray, J., Gage, F.H., 1995. FGF-2 responsive neuronal progenitors reside in proliferative and quiescent regions of the adult brain. Mol. Cell. Neurosci. 6, 474–486.
Quadrato, G., Elnaggar, M., DiGiovanni, S., 2014. Adult neurogenesis in brain repair: cellular plasticity vs cellular replacement. Front. Neurosci. 8, 1–5, Opinion Article.
Reynaldo, M., Torres-Ruiz, N.M., Carri, N.G., 2007. In: Eynard, A.R., Valentich, M.A., Rovasio, R.A. (Eds.), Histologia y Embriologia del ser humano. Bases celulares y Moleculares. Editorial Panamericana, Buenos Aires, pp. 308–309.
Reynolds, B., Tetzlaff, W., Weiss, S., 1992. AmultipotentEGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. 12, 4565–4574.
Rojas Mayorquin, A.E., Torres Ruiz, N.M., Gudiño Cabrera, G., Ortuño-Sahagún, D., 2010. Substrative hybridization identified gen differentially expressed by olfactory ensheating cells and neural stem cells. Int. J. Dev. Neurosci. 28, 75–82.
Reuss, B., Unsicker, K., 2000. Survival and differentiation of dopaminergic mesencephalic neurons are promoted by dopamine-mediated induction of FGF-2 in striatal astroglial cells. Mol. Cell. Neurosci. 16, 781–792.
Sabelström, H., Stenudd, M., Frisén, J., 2013. Neural stem cells in the adult spinal cord. Exp. Neurol. 260, 44–49.
Sanai, N., Tramontin, A.D., Quinones-Hinojosa, A., Barbaro, N.M., 2004. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427, 740–744.
Tessarollo, L., Vogel, K., Palko, M., Reid, S., Parada, L., 1994. Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. Proc. Natl. Acad. Sci. U. S. A. 91, 11844–11848.
Tropepe, V., Craig, C.G., Morshead, C.M., van der Kooy, D., 1997. Transforming growth factor-α null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. J. Neurosci. 17, 7850–7859.
Von Bartheld, C.S., 1998. Neurotrophins in the developing and regenerating visual system. Histol. Histopathol. 13, 437–459.
Wang, J., Yang, J., Gu, P., Klassen, H., 2010. Effects of glial cell line-derived neurotrophic factor on cultured murine retinal progenitor cells. Mol. Vis. 16, 2850–2866.
Weinandy, F., Ninkovic, J., Götz, M., 2011. Restrictions in time and space – new insights into generation of specific neuronal subtypes in the adult mammalian brain. Eur. J. Neurosci. 33, 1045–1054.
Zihlmann, K., Ducray, A., Schaller, B., Huber, A., Krebs, S., Andres, R., Seiler, R., Meyer, M., Widmer, H., 2005. The GDNF family members neurturin, artemin and persephin promote the morphological differentiation of cultured ventral mesencephalic dopaminergic neurons. Brain Res. Bull. 68, 42–53.