Stem Cells International

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Guest Editors: Oscar Gonzalez-Perez, Jose M. Garcia-Verdugo, Alfredo Quinones-Hinojosa, Sonia Luquin, Graciela Gudino-Cabrera, and Rocio E. Gonzalez-Castaneda

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Editorial Board

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 378356, 2 pages
doi:10.1155/2012/378356

Editorial

Neural Stem Cells in the Adult Brain: From Benchside to Clinic

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Received 19 September 2012; Accepted 19 September 2012

Increasing evidence indicates that neural stem cells (NSCs) play an important role in sustaining cellular homeostasis and brain tissue restoration. The study of all mechanisms that control and modulate the function of NSC is a crucial step for the design of therapies against chronic neurodegenerative processes. In this special issue of the journal, we had the pleasure to edit the topic entitled "*Neural Stem Cells in the Adult Brain: From Benchside to Clinic.*" This special compilation of paper was aimed to provide a global forum for publications of original peer-reviewed manuscripts that reported original research findings in the field of adult neural stem cell, including short communications, full-length research, and review articles. Below, we briefly discuss the papers you may find in this issue.

K. Nakaguchi et al., in their paper entitled "Growth factors released from gelatin hydrogel microspheres increase new neurons in the adult mouse brain", suggest that new neurons born in the subventricular zone (SVZ) may be able to replace neurons lost in degenerative disease or injury and improve or repair neurological deficits. In this excellent paper, they tested whether delivering growth factors via gelatin hydrogel microspheres can support neurogenesis in the SVZ. Their findings indicated that hepatocyte growth factor-containing microspheres increased the number of new neurons migrating from the SVZ towards the injured striatum in a stroke model in mouse. Therefore, they propose that gelatin hydrogel microspheres may be a good delivery tool for the sustained release of growth factors to promote neural regeneration of damaged brain tissues.

J. Tu and S. O. Ugoya discuss that traumatic brain injury (TBI) is one of the leading causes of major disability and death worldwide. Neural stem cells (NSCs) have recently been shown to contribute to the cellular remodeling and may represent a possible therapy for TBI. In their work the authors nicely summarized a critical assessment of recent data and developed a view comprising of six points to possible quality translation of NSCs in TBI.

B. P. Carreira et al., in their paper entitled "Regulation of injury-induced neurogenesis by nitric oxide", sustain that nitric oxide (NO), a pleiotropic signaling molecule in the central nervous system, is able to modulate neurogenesis, acting as a pro- or antineurogenic agent. In their interesting review, they discussed the relevance of the NO system for the treatment of neurodegenerative diseases or several pathological conditions that affect the brain.

J. Namiki et al. analyzed the phosphorylation of nestin, an intermediate filament protein commonly used as a neural stem/progenitor cell marker. Nestin is required for the survival and self-renewal of neural stem cells. In this study, the authors nicely reported CNS-specific phosphorylation sites in nestin that allow distinguishing vascular expression of nestin from other intermediate filament protein subtypes.

Hormonal signals from the pancreatic islets influence the energy homeostasis of the brain and *vice versa*. In an excellent review, M. Machida and coworkers explain the correlation between the insulin-mediated regulatory system of the CNS and the pancreatic endocrine system. Remarkably, adult neurogenesis from undifferentiated neural stem cells is

greatly decreased in diabetic patients, and as a result their learning and memory functions decline. In their paper, the authors summarized latest research regarding this endocrinal and neurological relationship.

L. Calatrava-Ferreras et al. explained that cerebellar ataxias, a heterogeneous group of diseases characterized by motor incoordination, may be treated using cell transplantations. Specifically they propose the use of human umbilical cord blood mononuclear cells as a promising approach for restoration of cerebellar function

S. Martinez-Herrero et al. discuss that adrenomedullin (AM) acts as a growth and cell fate regulatory factor for adult neural stem cells. AM regulates the proliferation rate and the differentiation of stem/progenitor cells into neurons, astrocytes, and oligodendrocytes, probably through the PI3K/Akt pathway. AM gene is also able to regulate the cytoskeleton rearrangements, which is important for cellular morphogenesis. In addition, AM appears to contribute to neural stem cell growth regulation by allowing cells to pass through mitosis. Consequently, AM may contribute to program stem cells for future clinical uses.

Finally, R. Ramos-Zuniga et al. discussed the ethical implications on the use of stem cells. They explain that every clinical project should take that into account, along with potential clinical applications, the principle of "*primum non nocere*" (first, do no harm). The authors also indicate the importance of keeping a close clinical surveillance to establish the possible risks in the use of stem-cell-based therapies.

With this compendium of cutting-edge review articles and original articles written for experts in the stem-cell field, we hope these in the field of adult neural stem cells will help be helpful and educational for readers.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 135187, 14 pages
doi:10.1155/2012/135187

Research Article

Effects of Intravenous Administration of Human Umbilical Cord Blood Stem Cells in 3-Acetylpyridine-Lesioned Rats

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Received 25 June 2012; Accepted 1 August 2012

Academic Editor: Oscar Gonzalez-Perez

Cerebellar ataxias include a heterogeneous group of infrequent diseases characterized by lack of motor coordination caused by disturbances in the cerebellum and its associated circuits. Current therapies are based on the use of drugs that correct some of the molecular processes involved in their pathogenesis. Although these treatments yielded promising results, there is not yet an effective therapy for these diseases. Cell replacement strategies using human umbilical cord blood mononuclear cells (HuUCBMCs) have emerged as a promising approach for restoration of function in neurodegenerative diseases. The aim of this work was to investigate the potential therapeutic activity of HuUCBMCs in the 3-acetylpyridine (3-AP) rat model of cerebellar ataxia. Intravenous administered HuUCBMCs reached the cerebellum and brain stem of 3-AP ataxic rats. Grafted cells reduced 3-AP-induced neuronal loss promoted the activation of microglia in the brain stem, and prevented the overexpression of GFAP elicited by 3-AP in the cerebellum. In addition, HuUCBMCs upregulated the expression of proteins that are critical for cell survival, such as phospho-Akt and Bcl-2, in the cerebellum and brain stem of 3-AP ataxic rats. As all these effects were accompanied by a temporal but significant improvement in motor coordination, HuUCBMCs grafts can be considered as an effective cell replacement therapy for cerebellar disorders.

1. Introduction

Cerebellar ataxias (CAs) include a heterogeneous group of infrequent diseases characterized by lack of motor coordination [1]. According to their etiology, they can be divided into sporadic forms and hereditary diseases. All of them have in common cerebellum and associated neuronal circuits dysfunction, in particular spinocerebellar afferents [2–5]. Current therapeutic approaches are based on the use of drugs that correct some of the molecular processes involved in the pathogenesis of this group of diseases [1, 6–8]. Furthermore, other studies have assayed the potential therapeutic activity of intracerebroventricular, peripheral, or intranasal administration of neurotrophic factors such as insulin-like growth factor (IGF-I), or glial-derived growth factor (GDNF), in different experimental models of cerebellar ataxia in rodents [9–13]. Although the above-mentioned treatments (drugs and trophic factors) yielded promising results, there is not yet an effective therapy for these types of diseases to date [1].

Cell replacement strategies using stem cells (SCs) as donor tissue have emerged as a promising approach for restoration of function in neurodegenerative diseases [14–19]. Hematopoietic stem cells from human umbilical cord blood (HuUCBCs) have been proposed as an excellent source of embryonic SCs in regenerative therapies for

the Central Nervous System [20–23]. HuUCBCs are easily accessible they retain certain properties of embryonic SCs such as the expression of transcription factors specific to embryonic antigens [24] and are well tolerated by the host due to their low immunogenicity [25]. Additionally, in vitro manipulation of HuUCBCs has shown their plasticity. Thus, after exposure to different agents, these cells are able to express antigens of diverse cellular lineages, including the neural type [26–31].

HuUCBCs were used successfully for the first time in 1989, as a bone marrow transplant in a patient with Fanconi's anemia [32]. Other studies have shown that systemic administration of HuUCBCs to different experimental models of neurodegenerative diseases improved their neurological symptoms and life expectancy [22, 23]. The beneficial effects of HuUCBCs seemed to be due to their ability to synthesize and release trophic factors involved in cell survival, rather than having a role in neuronal replacement [23, 33–36].

Stem cell-reparative approaches have been proposed for cerebellum-related disorders [37–40]. However, the type of stem cells most appropriate for future human cell therapy is not clearly defined at present [37]. Considering the possibility that HuUCBCs could be used as a therapeutic agent in CA, we analyze their potential neuroregenerative and/or neuroprotective activity in the 3-acetylpyridine (3-AP) experimental model of CA in rats. The rationale for using this CA model was because the neurotoxin 3-AP selectively lesions calbindin expressing neurons in the inferior olive [9], and this nucleus plays a key role in the control of the cerebellar function by sending glutamatergic excitatory signals to Purkinje cells [41, 42].

Here, we report that intravenous administration of HuUCB mononuclear cells (HuUCBMCs) reaches the cerebellum and brain stem of 3-AP-lesioned rats. Grafted cells reduce neuronal loss in the brain stem, prevent glial reactivity in the cerebellum, and improve motor coordination in ataxic rats. In this study, we also show that HuUCBMCs upregulate the expression of proteins that are critical for cell survival, such as phospho-Akt and Bcl-2, in the cerebellum and brain stem of 3-AP-lesioned rats. The role of activated microglia in HuUCBMCs-mediated neuronal protection in the brain stem is also discussed.

2. Materials and Methods

2.1. Experimental Model of Cerebellar Ataxia in Rats. A total of 40 female Sprague Dawley rats weighing 220–250 g were used in accordance with the European Union Council Directive (86/609/EEC). Rats received an intraperitoneal (i.p.) injection of the neurotoxine 3-AP (40 mg/kg) that selectively lesioned calbindin expressing neurons in the inferior olive [9]. This nucleus plays a key role in the control of the cerebellar function by sending glutamatergic excitatory signals to Purkinje cells (PCs) [41, 42]. From a histological point of view, PC and granule neurons of the cerebellar cortex are the most commonly affected population of neurons in CA [3].

2.2. Behavioral Testing. Motor performance was analyzed using the rotarod test. Before 3-AP lesions were produced, rats received 9 independent training sessions in the rotarod (PanLab S.L., Mod. LE 8500, Cornellá, Spain), with 4 1-minute evaluations at 40 rpm (fixed speed), and 4 1-minute evaluations at 4 to 40 rpm (accelerating rod). Those animals that withstood more than 1 minute at 40 rpm and at 4 to 40 rpm were selected for 3-AP lesions. Motor coordination was evaluated at 72 hours after lesion. Those animals resulting in mean latencies to fall on the accelerating rod of approximately 19 ± 3 s ($n = 16$) were selected for HuUCBMCs or vehicle administration. Starting 10 days after 3-AP lesion procedure, animals were monitored once a week until the end of the study period.

2.3. HuUCBMCs Isolation of Blood Cell Concentrate. Assessment, processing, and cryopreservation of HuUCBCs were carried out by the Centro de Transfusiones de la Comunidad de Madrid (Valdebernardo) in accordance with the Spanish Directive for Donors' Selection (Edition 4/May 2009/PO.CO.01). The donated units met the criteria for minimal cellularity and volume showing the following parameters: mononuclear cells (MCs): $337.8 \times 10^6 \pm 32.56$, total nucleated cells (TNCs): $910.8 \times 10^6 \pm 86.77$, and cells positives for CD34: $0.7620 \times 10^6 \pm 0.09140$.

For isolation of HuUCBMCs we followed a methodology previously described [43]. Briefly, HuUCBCs were drawn from the bag and divided into 2 Falcon tubes with half volume of Lymphoprep and centrifuged at 800 g and 20°C for 40 min to create a Ficoll gradient. The band of mononuclear cells located at the interface (2 to 7 mL) was taken and washed 3 times with PBS. An aliquot of 40 μL was used to determine the number of living cells by Trypan Blue using a Neubahuer chamber.

2.4. Cell Transplantation. At 3 days after 3-AP lesion, rats were anesthesized by inhalatory administration of Isoflurane (2%). One group of animals ($n = 14$) received a single injection of 4.5×10^6 HuUCBMNCs in 250 μL of sterile PBS into the lateral vein of the tail (3-AP + cells). Another group of 3-AP-lesioned rats ($n = 10$) received the same volume of sterile PBS (3-AP + vehicle). As controls we used a group of naïve rats that did not receive HuUCBMNCs grafts ($n = 6$). All experimental groups received cyclosporine (5 mg/kg i.p.) once a day to avoid rejection of human cells 24 hours before cell transplantation, and until the end of the study. Animals were sacrificed at 1, 7, 21 and 44 days after transplantation (4, 10, 24, and 48 days after lesion, resp.).

2.5. Tissue Processing. At 4, 10, and 21 days after lesion the animals were perfused intracardially under deep anesthesia with 50 mL of isotonic saline, followed by 250 mL of 4% paraformaldehyde. Brains were postfixed in the same solution for 24 hr at 4°C, cryoprotected and frozen, before sectioning on a cryostat. For the inferior olive 20 μm thick coronal sections were performed at three levels separated by a distance of approximately 400 μm. These levels correspond

to the following coordinates of the stereotaxic atlas of Paxinos and Watson [44]: −13.30 mm from Bregma (Zone 1), −12.80 mm (zone 2) and −11.96 mm (zone 3).

For immunohistochemical analysis of the cerebellum, 20 μm thick coronal sections were obtained in the cryostat, and mounted on positively charged slides (Dako REAL Capillary Gap microscope slides).

2.6. Antibodies and Immunochemicals. The primary antibodies used in this study were rabbit antiproliferating cell nuclear antigen (PCNA, 1 : 75; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit antiglial fibrillary acidic protein (GFAP, 1 : 200; DakoCytomation), mouse antineuronal nuclei (NeuN, 1 : 1000; Chemicon International Inc.), mouse anti-OX6 (1 : 250; AbD Serotec, Oxford, UK), rabbit antilaminin (1 : 25; Sigma Chemical Co., St. Louis, MO, USA), mouse anti-Bcl-2 (1 : 25; Santa Cruz Biotech), rabbit anticalbindin (1 : 500; Millipore, Temecula, CA, USA), mouse anti-human leucocyte antigen ABC (HLA-ABC 1 : 500; AbD Serotec, Oxford, UK), mouse anti-human nuclei protein (HuNu, 1 : 25; Millipore, Temecula, CA, USA), and rabbit anti-Bax (1 : 250; Santa Cruz Biotech). The secondary antibodies and other immunochemicals used were peroxidase-labeled isolectin B4 (IB4, 1 : 20; Sigma Chemical Co, St. Louis, MO, USA), biotinylated goat anti-mouse IgG (Zymed Laboratories; South San Francisco, CA, USA), streptavidin-biotin-peroxidase complex (DakoCytomation), diaminobenzidine (DAB) + substrate-chromogen system (both from DakoCytomation), Alexa Fluor-568 goat anti-mouse IgG, and Alexa Fluor-488 goat anti-rabbit IgG (1 : 400; all from Molecular Probes; Eugene, OR, USA), fluorescein-conjugated goat anti-mouse IgG (1 : 25; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), Cy3-conjugated donkey anti-guinea pig IgG (1 : 500, Jackson ImmunoResearch Laboratories Inc.), and rhodamine-conjugated goat anti-rabbit IgG (1 : 100, Chemicon International Inc.).

2.7. Immunohistochemistry and Morphometric Analysis. Tissue sections were treated with sodium acetate 10 mM, pH 6.0, at 95°C for 4 min, and preincubated with 5% normal goat serum (NGS) in Tris-buffered saline (TBS: 0.15 M NaCl and 0.1 M Tris HCl, pH 7.4)/0.1% Triton X-100 for 30 min. Primary antibodies were applied for 24 hr at 4°C, and most of them were visualized using immunofluorescence procedures. The slides were coverslipped in a medium containing p-phenylenediamine and bisbenzimide (Hoechst 33342; Sigma) for the detection of nuclei. Some series of sections were preincubated with 5% NGS in TBS and then processed for the histochemical detection of IB4, a marker of microglia and macrophages, by incubating for 2 hr with IB4 conjugated to peroxidase. Finally, the reaction product was detected with DAB chromogen.

For quantitative estimation of calbindin immunostaining in the inferior olive, measurements were performed in several coronal sections of the brainstem, at the three levels indicated previously (see tissue processing). The area of the olive was demarcated and measured in each zone, and the number of cells with caldindin staining was assessed using the 20X objective. Immunohistochemical results were expressed as the number of positive cells/section with the aid of the Computer-Assisted Stereology Toolbox (CAST) grid system (Olympus, Ballerup, Denmark). Fluorescence images were acquired and analyzed by confocal microscopy (Nikon C1 plus ECLIPSE Ti-e microscope).

2.8. Western Blotting Protein Analysis. After 44 days of cells transplantation, the brain stem and cerebellum of 3-AP-lesioned rats that received vehicle ($n = 8$) or cells ($n = 9$) were removed and dissected following a previously described methodology [45]. Tissue was homogenized (1 : 8, w/v) with homogenization buffer (20 mM Tris-HCl, pH 7.5: 140 mM potassium chloride; 5 mM magnesium acetate; 1 mM dithiothreitol, 2 mM benzamidine, 1 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, and 10 μg/mL antipain; 20 mM sodium β-glycerophosphate; 20 mM sodium molybdate; 200 mM sodium orthovanadate). Homogenates were centrifuged at 11,000 g for 20 min, and proteins were processed for Western blot analysis to determine the relative levels of several proteins. The procedures were performed at 4°C, and samples were kept at −80°C until use. Aliquots of 30 μg of protein were separated by electrophoresis on 10–15% SDS-polyacrylamide minigels and transferred to nitrocellulose filters. Membranes were soaked in blocking solution (0.1 M PBS and 5% dry skimmed milk, pH 7.4) and incubated with the following primary antibodies diluted in 0.1 M PBS and 1% dry skimmed milk, pH 7.4: mouse anti-Bcl-2 (1 : 400; Santa Cruz Biotechnology Inc., Burlingame, CA, USA), rabbit anti-Bax (1 : 300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antiproliferating cell nuclear antigen (PCNA, 1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antiglial fibrillary acidic protein (GFAP, 1 : 5000; DakoCytomation, Denmark), rabbit anti-Glut5 (1 : 500; Abcam), mouse anti-OX6 (1 : 1000, AbD Serotec, Oxford, UK), rabbit anti-HuNu protein (1 : 200, Millipore, Temecula, CA, USA), rabbit anticalbindin (1 : 5000; Millipore, Temecula, CA, USA), rabbit anti-Akt (Ser473P) (1 : 2000; Cell Signaling Technology, Beverly, MA, USA), rabbit anti-Akt (1 : 2000; Cell Signaling Technology). After extensive washing in 0.05% PBS-Tween, membranes were incubated with the peroxidase-conjugated or alkaline-phosphatase-conjugated secondary antibodies diluted 1 : 2000 in blocking solution. The membranes were developed with enhanced chemiluminescence Western blotting, following the manufacturer's instructions (Amersham, Buckinghamshire, England), and were exposed to hyperfilm. Membranes were also immunolabeled for loading control using mouse anti-β actin (1 : 5000; Sigma Aldrich) and anti-mouse IgG alkaline phosphatase-conjugated (1 : 3000, Sigma Aldrich) and were developed with alkaline phosphatase reagent. The density of stained bands was scanned and quantified with the Image QuantTL software package, and the data were normalized in relation to β actin levels.

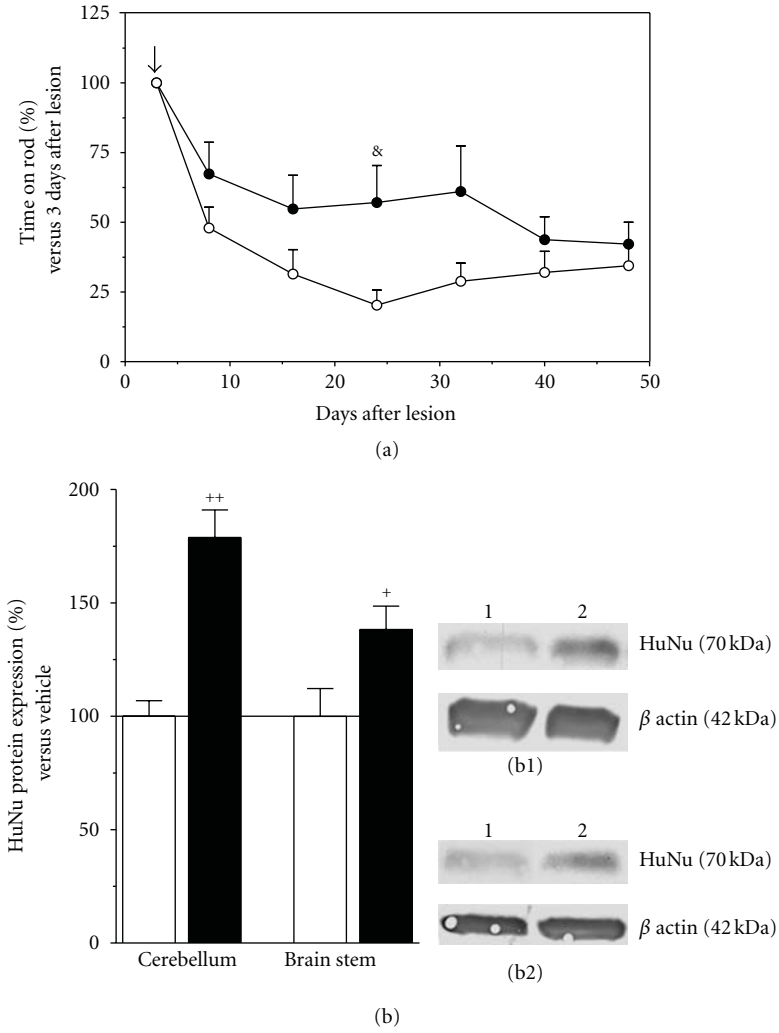

Figure 1: HuUCBMCs grafts improve motor coordination and reach the cerebellum and brain stem of 3-AP ataxic rats. Motor performance, as assessed by the rotarod test, shows a progressive impairment in 3-AP-lesioned rats receiving vehicle ((a) white circles) that reaches a plateau between 15 and 24 days after lesion. Motor coordination in 3-AP-lesioned rats that received HuUCBMCs grafts ((a) black circles) is significantly improved at 24 days after lesion. Time of implantation of HuUCBMCs (↓). (b) shows the detection by western blot of HuNu protein in the cerebellum and brain stem of 3-AP-lesioned rats treated with vehicle (white bars), or with HuUCBMCs (black bars). (b1) and (b2) show representative blots for HuNu protein in the cerebellum (b1) and brain stem (b2). Lane 1: 3-AP-lesioned rats treated with vehicle (3-AP + vehicle); lane 2: 3-AP-lesioned rats treated with HuUCBMCs grafts (3-AP + cells). Results represent the mean ± SEM of 4 (b) to 10 (a) individual animals. $^{\&}P \leq 0.05$ versus 3-AP + vehicle rats at 24 days after lesion, $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ versus 3-AP + vehicle rats at 48 days after lesion.

2.9. Data Analysis. Results are expressed as mean ± SEM of (n) independent animals. Statistical analyses for immunohistochemical and biochemical studies were performed using one-way ANOVA followed by the Newman-Keuls multiple comparison test. For behavioral studies, a two-way ANOVA followed by Student's t-test was used. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. HuUCBMCs Grafts Ameliorate Motor Coordination in 3-AP-Lesioned Rats. To determine whether HuUCBMCs transplantation was functional in vivo, we have analyzed motor coordination using the rotarod test. Motor performance of naïve rats was relatively stable over repeated tests, resulting in mean latencies to fall on the accelerating rod of approximately 52.31 ± 2.7 s ($n = 16$). At 3 days after 3-AP lesion, the latency to fall from rotarod was reduced to 19 ± 3 sec ($n = 16$). As shown in Figure 1(a), 3-AP-lesioned rats showed a progressive impairment that reached a plateau between 15 and 24 days after lesion. By contrast, in the 3-AP + cells group of animals, motor performance was stable between 8 and 32 weeks after lesion (Figure 1(a)). Moreover, 21 days after HuUCBMCs transplantation (24 days after lesion), their motor coordination was significantly

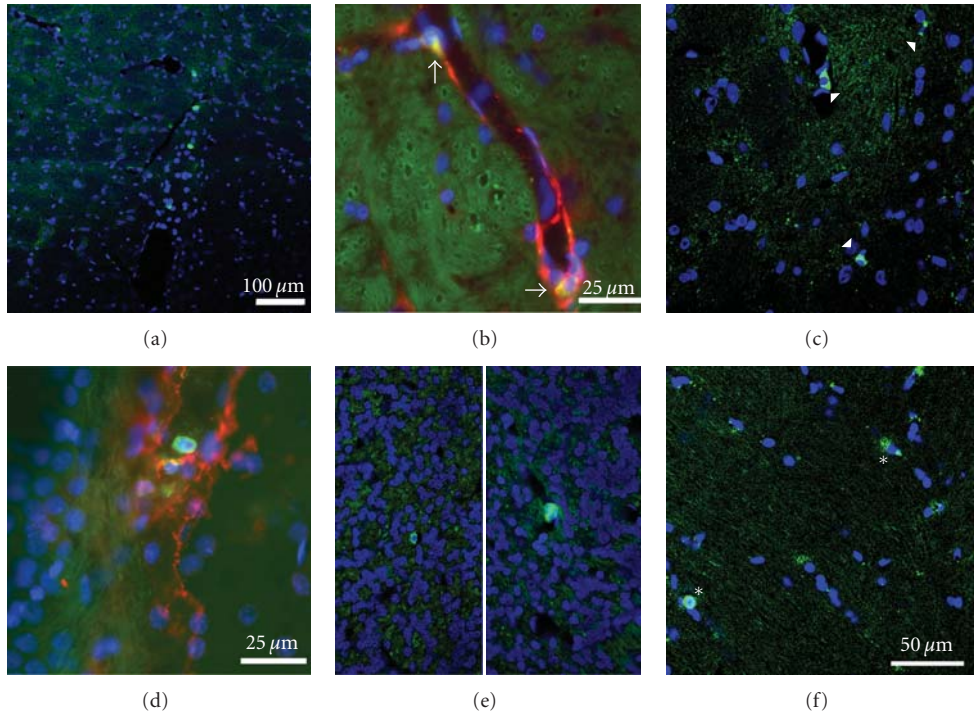

Figure 2: Immunodetection of human leucocyte antigen-ABC in the brain stem and cerebellum of 3-AP ataxic rats. (a) and (c) show HLA-ABC immunostaining (green) in the ventral (a) and dorsal (c) brain stem of 3-AP-lesioned rats receiving HuUCBMCs grafts. (b) Shows HLA-ABC (b, green) and laminin (b, red) immunoreactivity in the ventral brain stem. Note how HLA-ABC-positive cells are associated to laminin-positive blood vessels (b, yellow, white arrows) or integrated in the parenchyma (c, white arrowheads). In the cerebellum HLA-ABC-positive cells (d–f, green) are located near to the vermis in laminin-positive blood vessels (d, red), and in the parenchyma of the granular (e, green) and molecular layers (f, green, white stars) of the cerebellar cortex. Nuclei were counterstained with Hoechst 33342 (blue). Scale bar: 25 μm (b and d), 50 μm (c, e, and f), and 100 μm (a).

improved, to compared with 3-AP-lesioned rats receiving vehicle (Figure 1(a)).

3.2. Detection of HuUCBMCs in Brain Stem and Cerebellum of 3-AP-Lesioned Rats. Immunohistochemical analysis for the human endogenous marker HLA-ABC was performed to determine if intravenous transplanted HuUCBMCs were able to reach the brain stem and cerebellum of 3-AP-lesioned rats. Seven days after HuUCBMCs transplantation, 3-AP-lesioned rats showed HLA-ABC-positive cells in ventral (Figure 2(a)) and dorsal (Figure 2(c)) zones of the brain stem. These cells were associated to laminin-positive blood vessels (Figure 2(b)), or integrated in the parenchyma (Figure 2(c)). Similarly, the cerebellum of 3-AP + cells treated rats showed HLA-ABC immunoreactivity (Figures 2(d)–2(f)). Thus, HLA-ABC-positive cells were observed in the vermis associated to blood vessels (Figure 2(d)), and in the parenchyma of the granular (Figure 2(e)) and molecular (Figure 2(f)) layers of the cerebellar cortex. Under our experimental conditions, HLA-ABC immunoreactivity was not observed at longer periods after transplantation (i.e. 21 days). However, by western blot analysis we found that 45 days after HuUCBMCs grafts were performed (48 days after lesion), the cerebellum and brain stem of 3-AP-lesioned rats showed significant levels of the nuclear antigen expressed by human cells HuNu, compared to 3-AP + vehicle-treated rats, where HuNu protein expression was very low in both structures (Figure 1(b)). We were unable to confirm these results by immunohistochemistry because the antibody used for HuNu detection gave a high background in rat brain slices.

3.3. HuUCBMCs Grafts Partially Prevent Neurotoxin-Induced Neuronal Loss in the Brain Stem. A single injection of 40 mg/kg 3-AP significantly ($P \leq 0.001$, $n = 6$) reduced the number of calbindin-positive neurons in zone 3 (Z3) of the inferior olive from 616 ± 22 to 220 ± 51 calbindin-positive cells/section in naïve and 3-AP-lesioned rats, respectively. A similar effect was observed in zone 1 (Z1) where calbindin-positive cells were reduced by 1.7-fold in 3-AP-lesioned rats ($P \leq 0.01$, $n = 4$). As shown in Figure 3, after 48 days after lesion calbindin immunoreactivity was slightly higher in both zones of the inferior olive of 3-AP-lesioned rats that received HuUCBMCs (Figures 3(b) and 3(d)), compared to 3-AP + vehicle-treated rats (Figures 3(a) and 3(c)). We also analyzed the expression of calbindin and NeuN, a nuclear antigen expressed by neurons, by western blot. In the brain stem of 3-AP + vehicle-treated rats calbindin (Figure 4(a)) and NeuN (Figure 4(b)) protein levels were significantly lower than those found in naïve and 3-AP + cells treated rats. The neurotoxin 3-AP also reduced calbindin and

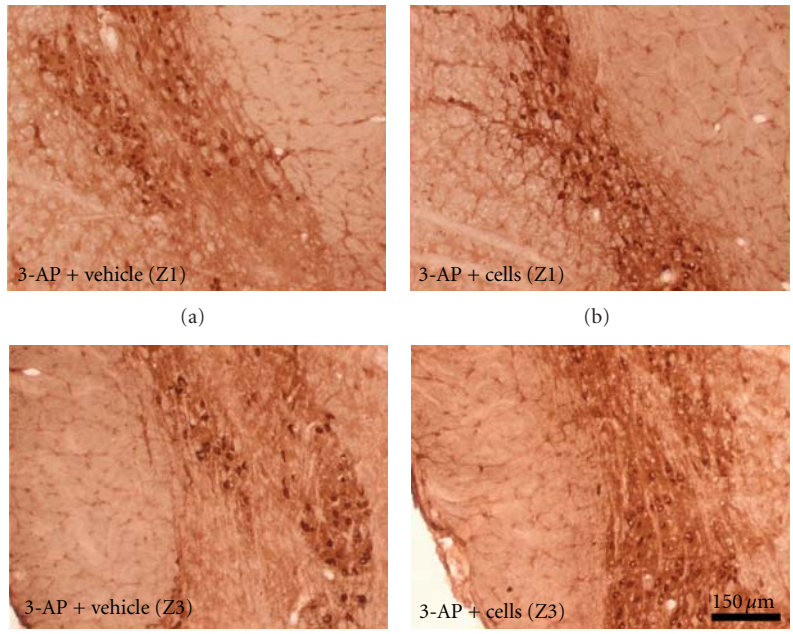

Figure 3: Immunodetection of calbindin in the inferior olive of 3-AP ataxic rats. (a) and (c) show calbindin immunostaining in two different levels of the inferior olive of 3-AP-lesioned rats separated by a distance of approximately 800 μm. Note how HuUCBMCs grafts increase calbindin-positive cells in both zones of the structure (b, c). Scale bar: 150 μm.

NeuN protein expression in the cerebellum, but HuUCBMCs transplantation was unable to recover the levels of both proteins in this structure (Figures 4(a) and 4(b)).

3.4. HuUCBMCs Grafts Modulate Glial Reactivity in 3-AP-Lesioned Rats. Previously, we observed that 3-AP induced a time-dependent invasion of cells expressing the vital marker of microglia IB4 in the inferior olive, that was maintained up to 24 days after lesion [46]. In agreement with those studies, the inferior olive of 3-AP + vehicle-treated rats showed a higher number of IB4-positive cells at 10 days after lesion than 48 hours after 3-AP was injected (Figures 5(a) and 5(b)). Moreover, IB4 labeling was increased in the inferior olive of 3-AP + vehicle-treated rats, as compared with 3-AP lesioned rats that received HuUCBMCs (Figures 5(b) and 5(c)). At 48 days after lesion, western blot analysis for the glucose transporter expressed by microglia GLUT5 gave similar results. Thus, GLUT5 protein expression was significantly raised in the brain stem of 3-AP + vehicle group of animals, compared to naïve and 3-AP + cells-treated rats (Figure 6(a)).

The anti-OX6 antibody recognizes a histocompatiblility Class II antigen expressed by activated microglia. As shown in Figure 6(b), OX6 protein levels were raised by 1.76-fold in the brain stem of 3-AP + cell-treated rats. Similarly, OX6 immunoreactivity was increased in the inferior olive of 3-AP + cells rats (Figure 5(f)), compared to naïve (Figure 5(d)), and 3-AP + vehicle-treated animals (Figure 5(e)). Proliferation is another feature of microglial activation. HuUCBMCs transplantation upregulated PCNA protein expression by 1.6-fold in brain stem ($^{**}P \leq 0.01$ and $^{+}P \leq 0.05$ versus naïve and 3-AP + vehicle rats, resp.). In addition, some of the OX6-positive cells were PCNA positive in the inferior olive of 3-AP + cell-treated rats (Figure 5(g)).

In the cerebellum, neither GLUT5 (Figure 6(a)), or OX6 (Figure 6(b)), nor PCNA protein levels were affected by HuUCBMCs transplantation, compared to naïve and 3-AP + vehicle-treated rats. However, the intravenous injection of HuUCBMCs prevented the increase in GFAP protein expression induced by 3-AP (Figure 6(c)). Besides, the cerebellum of 3-AP + cell-treated rats showed lower GFAP immunoreactivity than the cerebellum of 3-AP + vehicle rats (Figures 5(h)–5(j)).

3.5. HuUCBMCs Grafts Stimulate Bcl-2 Protein Expression and Phosphorylation of Akt. Several studies have proposed a neuroprotective role for HuUCBMCs [23]. Using western blot analysis, we studied the effects of HuUCBMCs grafts in the expression of proteins involved in cell survival. The Bcl-2 family comprise proteins that have either antiapoptotic (such as Bcl-2), or proapoptotic (such as Bax) effects [47–49]. In the cerebellum of 3-AP + cell-treated rats, the ratio Bcl-2/Bax was significantly raised compared to naïve rats and 3-AP + vehicle-treated animals (Figure 7(a)). This effect was due to the increase by 1.6-fold observed in Bcl-2 protein levels ($^{*}P \leq 0.05$ and $^{+}P \leq 0.05$ versus naïve and 3-AP + vehicle rats, resp.), while Bax levels remained unchanged in all experimental conditions studied (Figure 7(a1)). Neither 3-AP + vehicle rats, nor 3-AP + cell-treated animals showed significant changes in Bcl-2 and Bax protein expression in the brain stem (Figure 7(a)).

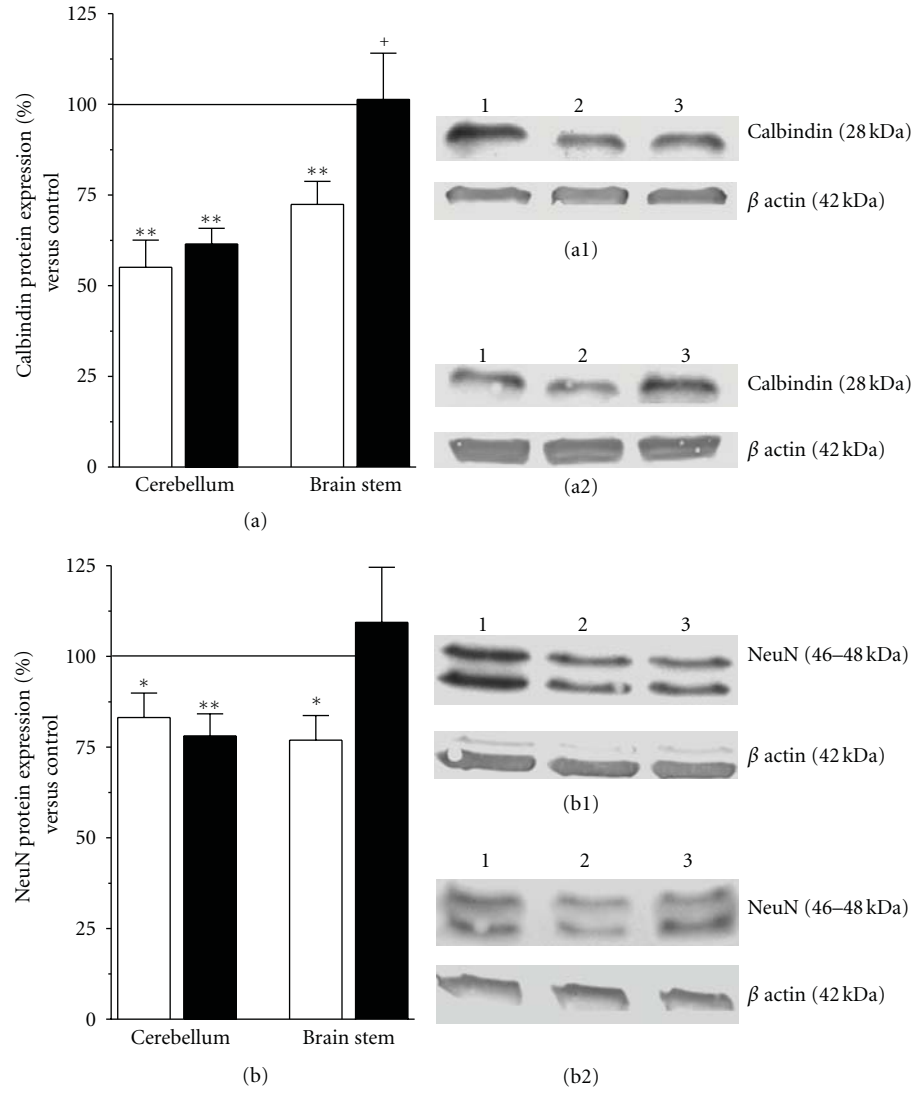

Figure 4: HuUCBMCs grafts partially prevent 3-AP-induced neuronal loss in the brain stem. (a) and (b) show the detection by western blot of calbindin (a) and neuronal nuclei (b, NeuN) in the cerebellum and brain stem of 3-AP-lesioned rats receiving vehicle (3-AP + vehicle rats, white bars) or HuUCBMCs grafts (3-AP + cells rats, black bars). (a1) and (a2) Show representative blots for calbindin, and (b1) and (b2) for NeuN in the cerebellum (a1, b1) and brain stem (a2, b2). Lane 1: naïve rats (control); lane 2: 3-AP + vehicle; lane 3: 3-AP + cells. Results represent the mean ± SEM of 6 to 9 individual animals. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus naïve rats, $^{+}P \leq 0.05$ versus 3-AP + vehicle rats.

The protein Akt is a key downstream effector of the PI3K/Akt-signaling pathway which phosphorylation plays a critical role in the regulation of neuronal survival [24, 50–54]. As shown in Figure 7(b), the ratio phospho-Akt/Akt was significantly increased in the cerebellum and brain stem of 3-AP + cells rats. HuUCBMCs grafts did not modify total Akt protein expression in both structures, but upregulated phospho-Akt levels in the cerebellum and brain stem of 3-AP-lesioned rats by 2- and 1.4-fold, respectively ($^{*}P < 0.05$ versus naïve cerebellum and $^{+}P < 0.05$ versus 3-AP + vehicle brain stem).

4. Discussion

In the present study we show that intravenous administered HuUCBMCs were able to reach the cerebellum and brain stem of 3-AP-lesioned rats. Implanted cells partially blocked the loss of neurons induced by the neurotoxin in the brain stem, prevented the overexpression of GFAP in cerebellum, and stimulated the expression of proteins involved in cell survival in both structures. All these effects were accompanied by a temporal but significant improvement in motor coordination, suggesting the potentiality of HuUCBMCs grafts as a cell replacement therapy for cerebellar disorders.

HuUCBMCs are considered an excellent source of stem cells that can be used for cell replacement therapies in neurodegeneration [20, 22, 23]. However, to our knowledge there are only two studies using HuUCBCs for the treatment of CA [39, 40]. By using an anti-HLA-ABC antibody, we found that intravenous administered HuUCBMCs reached the cerebellum and brain stem of 3-AP-lesioned rats. Under our experimental conditions, HLA-ABC immunostaining

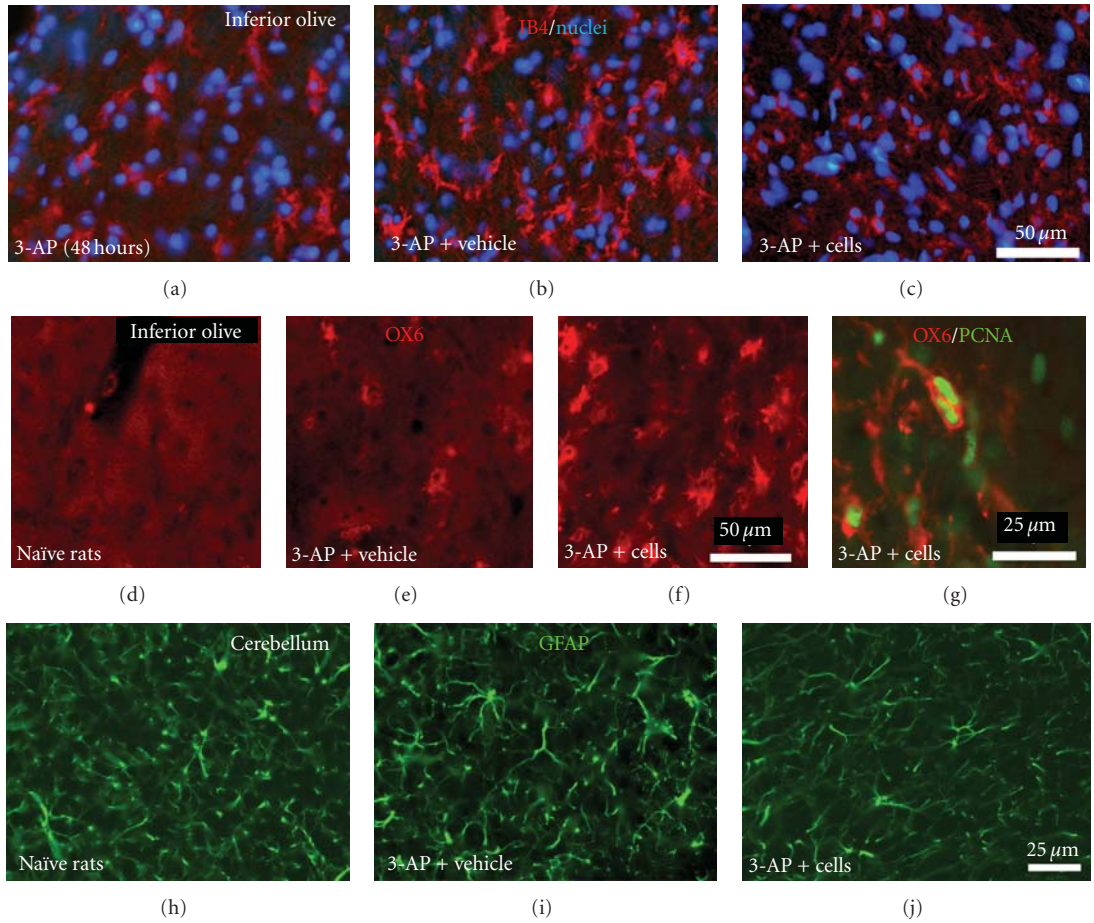

FIGURE 5: Immunodetection of glial cells in the inferior olive and cerebellum of 3-AP ataxic rats. (a) and (b) show the histochemical detection of isolectin B4 (IB4, red) in the inferior olive of 3-AP-lesioned rats 48 hours (a) and 10 days (b, c) after lesion. Note how 3-AP lesioned rats that received HuUCBMCs grafts (c, red) show lower IB4 staining at 10 days after lesion than ataxic rats treated with vehicle (b, red). (d) to (g) show OX6 immunostaining in the inferior olive of naïve rats (d, red) and 3-AP-lesioned rats treated with vehicle (e, red) or HuUCBMCs (f, red). Cell transplantation increases OX6 immunoreactivity (f, red). (g) shows double immunolabeling for OX6 (red) and PCNA (green) in the inferior olive of 3-AP + cells-treated rats. (h) to (j) show GFAP immunoreactivity in the cerebellum. Note how HuUCBMCs grafts reduce GFAP immunolabeling in this structure (j, green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar: 25 μm (g, h, I, and j), 50 μm (a, b, c, d, e, and f).

was only observed during the first three weeks of transplantation. However, western blot analysis showed HuNu protein expression in the cerebellum and brain stem of 3-AP + cell-treated rats two months after the administration of HuUCBMCs. These apparently contradictory results could be explained by a potential reduction in the expression of HLA-ABC at two months of transplantation.

In vitro and in vivo studies have demonstrated that HuUCBMCs are able to differentiate into neurons [31, 34, 43, 55, 56]. In ataxic rats HuUCBMCs implantation slightly recovered calbindin-positive neurons from 3-AP neurotoxicity in the inferior olive and restored calbindin and NeuN levels in brain stem. Although we found HLA-ABC-positive cells in the brain stem of 3-AP + cells-treated rats, none of these cells were located in the inferior olive. For this reason, we may infer that HuUCBMCs did not differentiate in calbindin-positive neurons in this structure. However, from our studies we cannot exclude the possibility that HuUCBMCs are able to differentiate in neurons in the brain stem as we were unable to detect HLA-ABC-positive cells after one month of transplantation.

Increasing evidence strengthens the hypothesis that the beneficial role of transplanted HuUCBMCs is associated with the production of neuroprotective factors [14, 33, 36, 40, 57]. We did not analyze the expression of neurotrophins or cytokines and chemokines with anti-inflammatory properties, but we found that HuUCBMCs grafts potentiated the activation of microglia in the inferior olive and the brain stem of 3-AP-lesioned rats, as analyzed by OX6 and PCNA immunohistochemistry and immunoblot. Although activated microglia have been associated with the pathogenesis of several neurodegenerative diseases [58, 59], these cells could play a key role in neuroprotection through the production and release of neurotrophic factors [60, 61]. A recent study associated the therapeutic benefits of HuUCBMCs transplantation in a rat model of neonatal

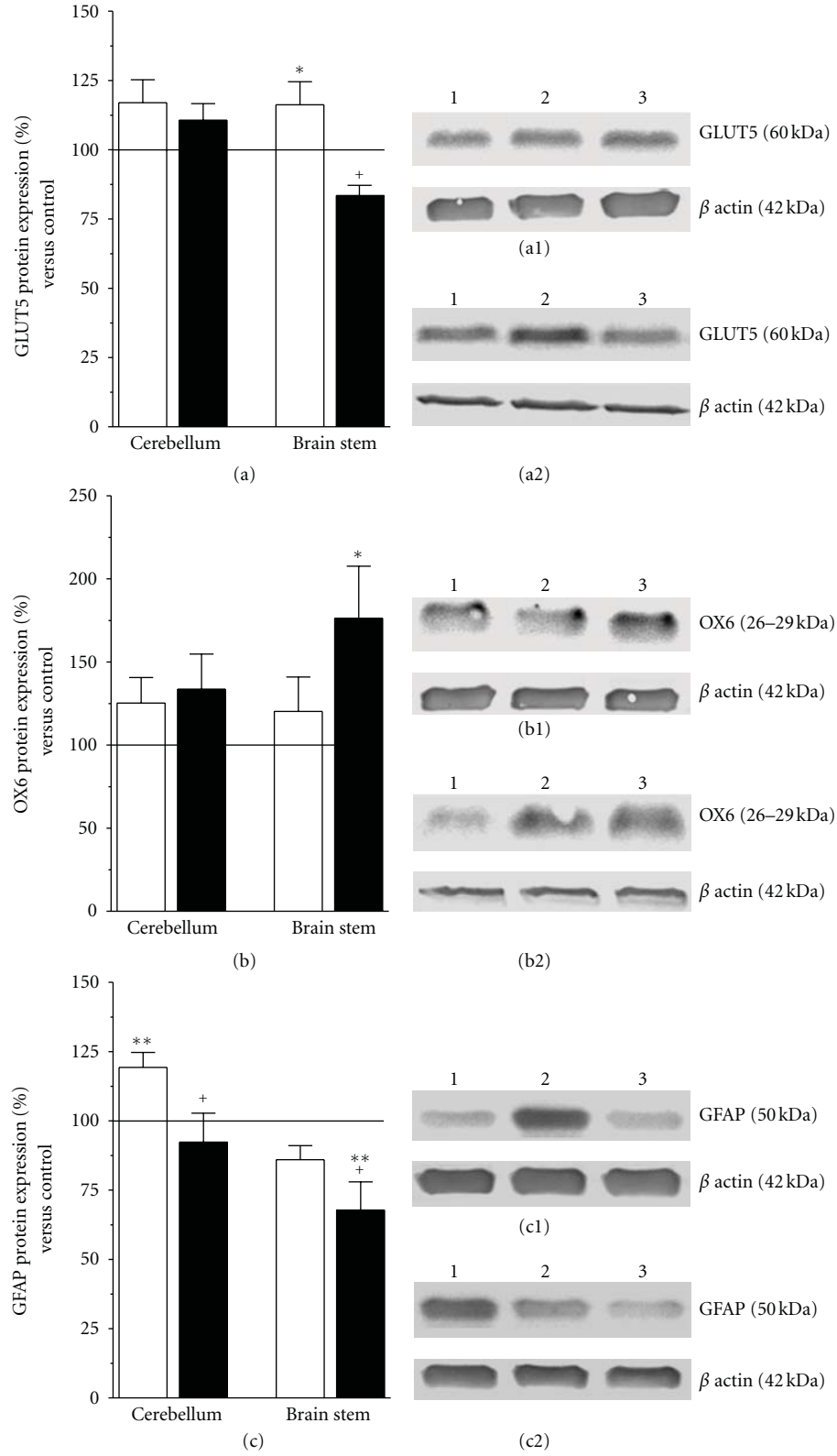

Figure 6: HuUCBMCs grafts modulate glial reactivity in 3-AP ataxic rats. (a), (b), and (c) show GLUT5 (a), OX6 (b), and GFAP (c) protein levels in the cerebellum and brain stem of 3-AP-lesioned rats receiving vehicle (3-AP + vehicle rats, white bars) or HuUCBMCs grafts (3-AP + cells rats, black bars). Note how HuUCBMCs transplantation upregulates OX6 protein expression in the brain stem and reduces GFAP protein levels in the cerebellum of 3-AP-lesioned rats. (a1) and (a2) show representative blots for GLUT5, (b1) and (b2) for OX6, and (c1) and (c2) for GFAP in the cerebellum (a1, b1, and c1) and brain stem (a2, b2, and c2). Lane 1: naïve rats (control); lane 2: 3-AP + vehicle; lane 3: 3-AP + cells. Results represent the mean ± SEM of 9 to 15 individual animals. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus naïve rats, $^{+}P \leq 0.05$ versus 3-AP + vehicle rats.

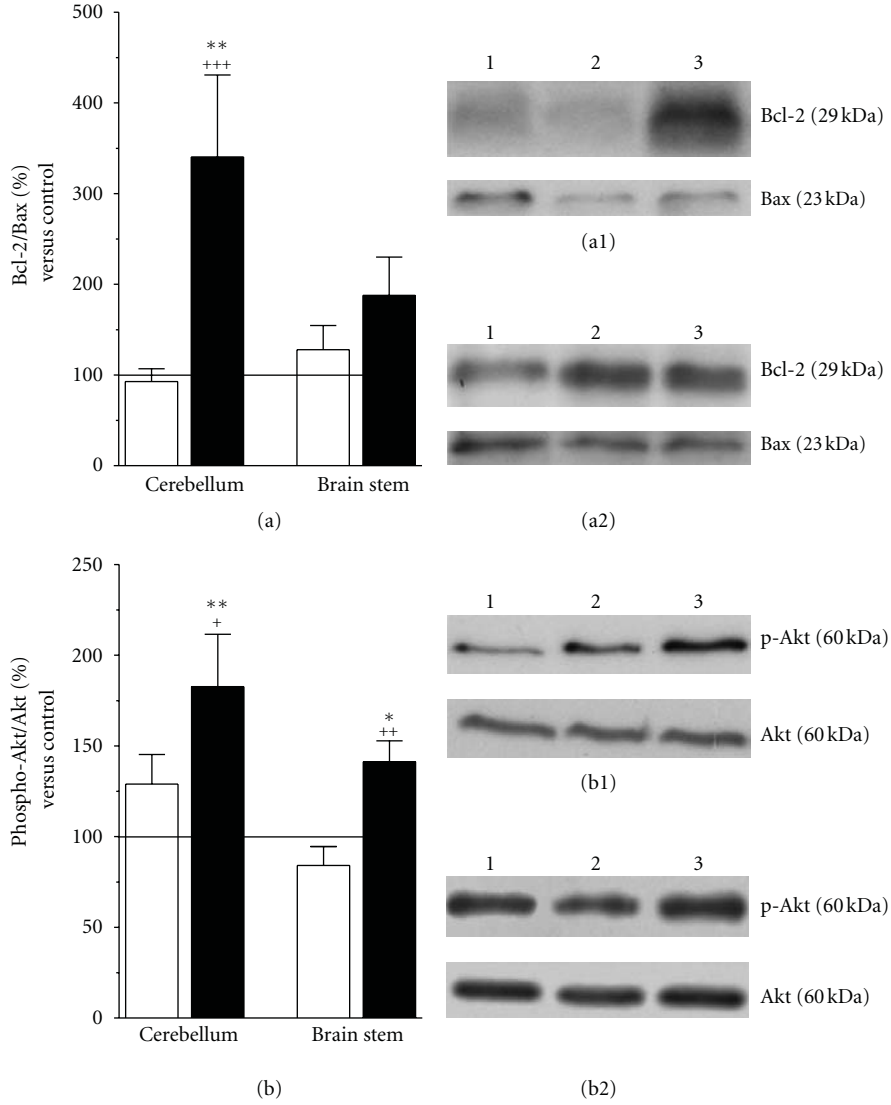

Figure 7: HuUCBMCs grafts upregulate Bcl-2 and phospho-Akt protein levels in the cerebellum and brain stem of 3-AP ataxic rats. HuUCBMCs transplantation raises the ratio Bcl2/Bax in the cerebellum of 3-AP-lesioned rats (a, black bars). Note how this effect is due to an increase in the expression of the antiapoptotic protein Bcl-2 (a1). HuUCBMCs also upregulate the ratio phospho-Akt/Akt in the cerebellum and brain stem of 3-AP ataxic rats (b, black bars). In both structures cell transplantation significantly enhances phospho-Akt levels (b1, b2), which is a protein involved in neuronal survival. (a1) and (a2) show representative blots for Bcl-2 and Bax, and (b1) and (b2) for phospho-Akt and Akt in the cerebellum (a1, b1) and brain stem (a2, b2). Lane 1: naïve rats (control); lane 2: 3-AP-lesioned rats treated with vehicle (3-AP + vehicle); lane 3: 3-AP-lesioned rats treated with HuUCBMCs grafts (3-AP + cells). Results represent the mean ± SEM of 9 to 15 individual animals. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ versus naïve rats, $^{+}P \leq 0.05$, $^{++}P \leq 0.01$, $^{+++}P \leq 0.001$ versus 3-AP + vehicle rats.

hypoxia with a transient up-regulation of microglial activity [62]. By contrast, the blockage of microglia activation enhanced neuroprotection and functional recovery induced by HuUCBMCs grafts in cortical ischemia [63]. Whether HuUCBMCs-driven activated microglia mediates the up-regulation of neuronal markers in the brain stem of 3-AP-lesioned rats or not, will be analyzed in the near future by blocking microglia activation with agents such as minocycline.

Up-regulation of GFAP is a feature of reactive astrocytes that was reported in the cerebellum of ataxic rats [64, 65], and patients suffering from progressive ataxia [66]. In agreement with those studies, we found increased GFAP protein levels in the cerebellum of 3-AP ataxic rats. This overexpression of GFAP could be the consequence of glial activation induced by a loss of neurons, as has been reported in several diseases and neuropathologies [67–69]. Although we did not analyze the number of neurons in the cerebellum, we found that 3-AP significantly decreased the expression of the neuronal markers calbindin and NeuN.

Rat umbilical cord stem cells grafts prevented reactive astrogliosis and rendered neuronal protection in the hyppocampus of ischemic rats [70]. In our study, HuUCBMCs transplantation reduced GFAP protein levels

and immunoreactivity, but was unable to prevent the fall in calbindin and NeuN levels due to 3-AP neurotoxicity in the cerebellum. These results suggest that grafted cells could be involved in the modulation of astrogliosis, but they are not enough efficient to prevent neuronal damage in the cerebellum of 3-AP-ataxic rats.

An interesting finding was that HuUCBMCs transplantation modulated the expression of proteins involved in cell survival. As shown here, intravenous administration of HuUCBMCs significantly raised the phospho-Akt/Akt ratio in the cerebellum and brain stem of 3-AP + cell-treated rats. This effect was due to an increase in phospho-Akt levels, while total Akt remained unchanged in both structures. As other studies have shown that phospho-Akt plays a critical role in the regulation of neuronal survival induced by HuUCBMCs [71, 72], we may consider that this protein could mediate the neuroprotective activity of HuUCBMCs grafts observed in the brain stem. Phospho-Akt also contributes to glial cells survival [72–74]. However, there are no reports showing its possible role in preventing astrogliosis to our knowledge. The PI3K/Akt-signaling pathway regulates the expression of the antiapoptotic factor Bcl-2 [69, 75–77]. Here we found that HuUCBMCs grafts upregulated Bcl-2 protein expression in the cerebellum of 3-AP-lesioned rats. Bcl-2 overexpression enhanced the survival of different types of neurons [69, 77, 78], including those of the granular layer of the cerebellum [79]. Additionally, Bcl-2 may contribute to the maintenance of grafted cells in the cerebellum of 3-AP ataxic rats. In fact, Bcl-2 overexpression mediated the survival of human hematopoietic precursors during fetal life [80] and prolonged the survival of myoblasts transplantation in acute myocardial infarction [81] and chronic heart failure [82].

Finally, our results show that HuUCBMCs implantation ameliorates motor coordination in 3-AP-lesioned rat. This beneficial effect was probably due to neuronal protection elicited by the graft in the brain stem. In addition, HuUCBMCs could modulate neuronal activity in ataxic rats. In this respect, our preliminary studies have detected increased levels of glutamate and GABA in the brain stem of 3-AP-lesioned rats that were significantly reduced to basal levels in those animals receiving HuUCBMCs grafts (our unpublished observations). Under our experimental conditions, functional improvement was not maintained up to one month after transplantation. Recent studies have reported that repeated injections of HuUCBCs improved motor skills in ataxic mice and functional symptoms in patients with hereditary ataxia for longer periods of time [39, 40]. We administered a single intravenous injection of 4.5×10^6 cells/rat, so further experiments are needed to determine the effectiveness of the repeated application of HuUCBMCs in the 3-AP-experimental model of cerebellar ataxia.

5. Conclusions

In summary, our results show that intravenous administered HuUCBMCs reach the cerebellum and brain stem of 3-AP-lesioned rats. Implanted cells stimulate the expression of proteins involved in cell survival in both structures. These proteins could mediate the survival of grafted cells, and the neuroprotective effect observed in the brain stem of ataxic rats. As HuUCBMCs grafts also ameliorate motor coordination in 3-AP-lesioned rats, they can be considered as a potential source of cells useful for cerebellar disorders treatment.

Author Contributions

L. Calatrava-Ferreras, R. Gonzalo-Gobernado, A. S. Herranz, and D. Reimers made the experimental design. L. Calatrava-Ferreras, R. Gonzalo-Gobernado, A. S. Herranz, D. Reimers, T. Montero Vega, L. A. R. López, and E. Bazán contributed in experimental realization. L. Calatrava-Ferreras, R. Gonzalo-Gobernado, A. S. Herranz, D. Reimers, and E. Bazán carried out data analysis. L. Calatrava-Ferreras, R. Gonzalo-Gobernado, A. S. Herranz, D. Reimers, A. Jiménez-Escrig, and E. Bazán wrote the paper.

Acknowledgments

This work was funded by Agencia Pedro Laín Entralgo (NDG7/09) and Fundación Ataxias en Movimiento. L. Calatrava-Ferreras and R. Gonzalo-Gobernado were the recipients of Agencia Pedro Laín Entralgo fellowship and Contrato de Personal de Apoyo a la Investigación (Fondo de Investigaciones Sanitarias), respectively. We are grateful to Maria José Asensio and Judit Muñoz for technical help and Kerry Davis for her paper.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 915160, 7 pages
doi:10.1155/2012/915160

Research Article

Growth Factors Released from Gelatin Hydrogel Microspheres Increase New Neurons in the Adult Mouse Brain

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Received 23 June 2012; Accepted 9 August 2012

Academic Editor: Oscar Gonzalez-Perez

Recent studies have shown that new neurons are continuously generated by endogenous neural stem cells in the subventricular zone (SVZ) of the adult mammalian brain. Some of these new neurons migrate to injured brain tissues and differentiate into mature neurons, suggesting that such new neurons may be able to replace neurons lost to degenerative disease or injury and improve or repair neurological deficits. Here, we tested whether delivering growth factors via gelatin hydrogel microspheres would support neurogenesis in the SVZ. Insulin-like growth factor-1 (IGF-1)-containing microspheres increased the number of new neurons in the SVZ. Hepatocyte growth factor (HGF)-containing microspheres increased the number of new neurons migrating from the SVZ towards the injured striatum in a stroke model in mouse. These results suggest that the strategy of using gelatin hydrogel microspheres to achieve the sustained release of growth factors holds promise for the clinical regeneration of damaged brain tissues from endogenous neural stem cells in the adult SVZ.

1. Introduction

Neural stem cells (NSCs) reside in the brain of adult animals, including humans [1–6]. NSCs residing in the subventricular zone (SVZ) at the lateral walls of the lateral ventricle and in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus produce new neurons and glial cells throughout adult life in mammals [7–11]. The NSCs in the SVZ have the potential to regenerate lost neurons and glia in response to various pathological conditions [12–17]. Neuroregenerative therapy using endogenous NSCs in the SVZ is a highly anticipated emerging strategy for treating human brain diseases because it avoids the risk of immunological incompatibility and the ethical problems inherent in harvesting human cells, and it may reduce the risk of tumorigenesis—which are all problems associated with transplanted stem cells [18, 19]. However, the spontaneous regeneration that takes place in the injured brain is insufficient for its structural or functional restoration [12, 17]. For future clinical applications, the ability to regulate each step in neuronal regeneration, including the generation, migration, differentiation, survival, and functional maturation of new neurons to promote efficient regeneration will be crucial for developing novel and reliable neuronal self-repair strategies [9, 20–23].

Various proteins, including neurotrophic factors and paracrine signaling molecules, are reported to enhance neurogenesis in the SVZ [24]. However, one limitation of using these factors in the treatment of brain diseases is the lack of appropriate delivery systems. It is difficult to engineer systemically administered proteins to cross the blood-brain barrier, and such proteins can cause systemic toxicity at high concentrations [25, 26]. On the other hand, a single local injection of liquid drugs into the brain parenchyma

may not enhance neuronal regeneration effectively, given the limited volume and persistence of substances administered in this way. Therefore, for clinical applications, safe and effective methods for the sustained delivery of neurogenesis-enhancing factors to the SVZ or injured neural tissues must be developed.

Here, we report that growth factors released from biodegradable gelatin hydrogel microspheres increase new neurons in the adult mouse brain. Gelatin hydrogels consist of gelatin polymers, which can be electrically complexed with growth factors [27, 28]. They have been used clinically to deliver growth factors in the treatment of patients with diseases including sudden sensorineural hearing loss, Bell's palsy, and peripheral artery diseases [29–32]. In this paper, we tested the effects of insulin-like growth factor (IGF) [33, 34] and hepatocyte growth factor (HGF) [35–37] delivered by gelatin hydrogel microspheres on neurogenesis in the SVZ in the normal brain, and then on the recruitment of SVZ-derived new neurons to the injured brain after stroke in a mouse model.

2. Materials and Methods

2.1. Preparation of Gelatin Hydrogel. Gelatin hydrogel microspheres (MedGel P15; MedGel, Osaka, Japan) with a diameter within 30 μm were prepared as described previously [27, 28]. The microspheres were incubated with phosphate buffered saline (PBS) (control) or PBS containing recombinant human IGF-1 (PeproTech, Rocky Hill, USA; 0.25 μg) or recombinant human HGF (PeproTech, Rocky Hill, USA; 0.5 μg) for 1 hour at room temperature.

2.2. Animals. Adult (8 weeks old) male ICR mice were purchased from SLC (Shizuoka, Japan) and maintained on a 12-hour light/dark cycle with unlimited access to food and water. All animal-related procedures were approved by the Laboratory Animal Care and Use Committee of Nagoya City University.

2.3. Injection of Gelatin Hydrogel. In intact mice, 3 μL of the gelatin hydrogel suspension or 2 μL of the solutions without gelatin hydrogel were stereotaxically injected using a capillary micropipette (Drummond Scientific Company, Broomall, PA, USA) into the striatum of anaesthetized 8-week-old ICR mice at the following position relative to bregma: 1.0 mm anterior, 1.5 mm lateral, and 3.2 mm deep ($n = 5$ and 3 animals for groups treated with and without gelatin hydrogel, resp.). In the MCAO model, 11 days after MCAO, 5 μL of gelatin hydrogel suspension or 2 μL of the solutions without gelatin hydrogel were stereotaxically injected using a capillary micropipette into the striatum of anaesthetized 8-week-old ICR mice at the following position relative to bregma: 1.0 mm anterior, 2.0 mm lateral, and 3.2 mm deep ($n = 6$ and 4 animals for groups treated with and without gelatin hydrogel, resp.). After surgery, the animals were left on a heat mat and constantly monitored until recovery. After 7 days, the mice were killed and their brains were prepared for immunohistochemistry.

2.4. Middle Cerebral Artery Occlusion. Middle cerebral artery occlusion (MCAO) was accomplished using the previously described intraluminal filament technique [17, 38, 39]. A laser-Doppler flowmeter probe (model ALF21; Advance, Tokyo, Japan) was attached to the surface of the skull to monitor the regional cerebral blood flow. A silicone-coated 8-0 filament was inserted into the internal carotid artery through an incision in the external carotid artery and then advanced to occlude the middle cerebral artery (MCA). The occlusion of the MCA was confirmed by observing a reduction in the value of laser-Doppler flowmetry of about 30%. The filament was withdrawn 50–60 minutes later, and reperfusion was confirmed by laser-Doppler flowmetry, and the incision was then closed. Gelatin hydrogel microspheres were injected 11 days after MCAO. Seven days after the injection, the mice were killed and their brains were prepared for immunohistochemistry.

2.5. Immunohistochemistry and Quantification. Brains were perfusion-fixed with 4% paraformaldehyde, postfixed in the same fixative overnight, and 50 μm sections were cut on a Vibratome sectioning system (VT1200S; Leica, Heidelberg, Germany) as described previously [17, 38, 39]. After three rinses in PBS, the sections were incubated for 40 min in blocking solution (PBS containing 10% donkey serum and 0.2% Triton X-100) and then overnight at 4°C with primary antibodies, which were diluted in the same solution. The next day, the sections were incubated for 2 hours at room temperature with biotinylated secondary antibodies (1 : 500) (Jackson, West Grove, PA, USA) or Alexa Fluor-conjugated secondary antibodies (1 : 500) (Invitrogen, Carlsbad, CA, USA), unless otherwise noted. Biotinylated antibodies were visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and DAB (diaminobenzidine tetrahydrochloride).

The primary antibodies (final dilution and source) used in this study were goat anti-doublecortin (anti-DCX) (1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Ki67 (1 : 200; Novocastra, Newcastle, UK), and mouse anti-NeuN (1 : 100; Merck Millipore, Billerica, MA, USA).

For laser scanning microscopy, we used an LSM700 Microscope (Carl Zeiss, Oberkochen, Germany). For the quantification of immunolabeled cells, the images of stained cells were acquired using a fluorescence microscope, BX51 (Olympus, Tokyo, Japan), and a CCD camera, DP71 (Olympus). The actual number of DCX+ cells was counted in three sections: the one containing the injection site and two sections taken 600 μm and 1200 μm, respectively, anterior to the injection site. For analyses of the brain after MCAO, the section including the injection site and an additional 5 (DCX) and 2 (Ki67) anterior sections spaced 300 μm apart were used. The total cell number was estimated by multiplying the sum of the counted cells by 12 and 6, respectively.

2.6. Infarct Volume Evaluation. Brain sections prepared after MCAO (thickness, 50 μm) were immunostained for NeuN

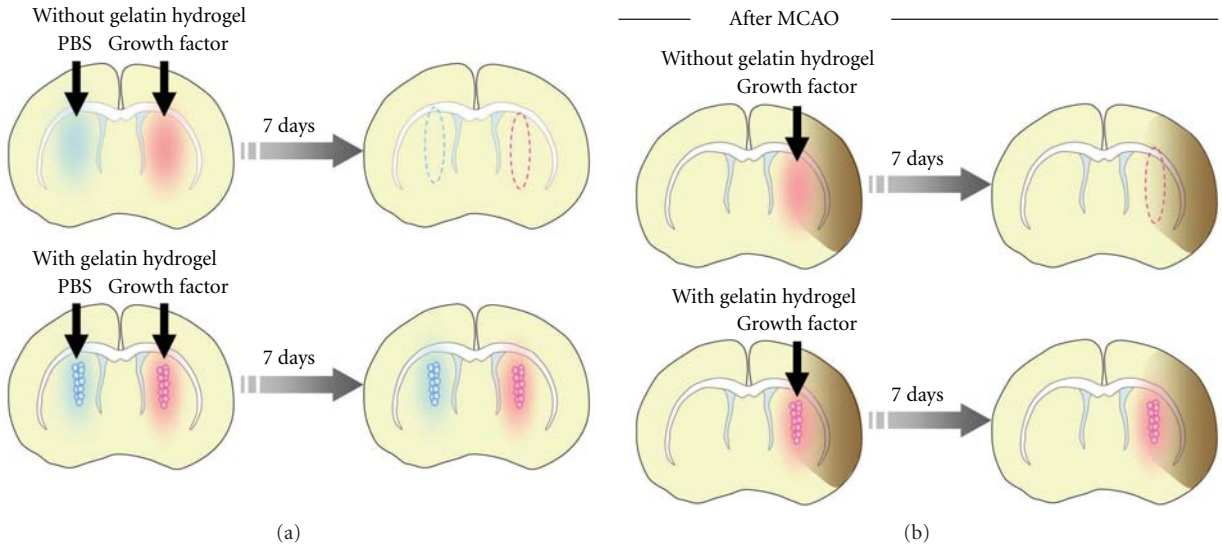

Figure 1: Study design. Schematic illustration of experiments performed to test the effects of growth factors released from gelatin hydrogel on neurogenesis in the SVZ. Effects of growth factors injected into normal (a) and injured brains 11 days after MCAO (b) with or without gelatin hydrogel on the number of new neurons in the SVZ and injured striatum, respectively, were compared 7 days after the injections. Blue and pink circles represent gelatin hydrogel microspheres.

and visualized with DAB using a standard procedure. The images of the sections including the injection site and two additional anterior sections spaced 600 μm apart were captured using a fluorescence microscope, BX51 (Olympus, Tokyo, Japan), and a CCD camera, DP71 (Olympus).

For this analysis, we used only mice in which the striatal infarction (NeuN negative area) was observed within 1 mm of the SVZ in at least one section. The areas that lacked NeuN immunoreactivity (infarct area) and those of the ipsilateral hemisphere in the three sections were measured using the Photoshop CS 8.0.1 (Adobe Systems, San Jose, CA, USA) image software. The percentage of the infarct area was determined by dividing the infarct area by that of the ipsilateral hemisphere for each mouse and used for the analysis of infarct volume.

2.7. Statistics. All data are presented as the mean ± SEM. Comparisons between experimental groups were analyzed by two-tailed Student's t-test, and differences were regarded as statistically significant when $P < 0.05$.

3. Results and Discussion

3.1. Study Design. In this study, we focused on two growth factors, IGF-1 and HGF, incorporated into gelatin hydrogel, which can slowly release proteins after implantation into the body [27, 28]; both IGF-1 and HGF have been approved for clinical applications in the treatment of various diseases [29–32, 40–42]. The gelatin hydrogel microspheres were injected into the striatum of normal brain or close to the injured tissue of a stroke model (Figure 1). Seven days after the injection, the brains were fixed and number of DCX+ new neurons was compared.

3.2. IGF. IGF-1 promotes the proliferation of neural stem or progenitor cells as well as neuronal differentiation and survival [43–46]. It has a neuroprotective effect in animal models of stroke [47]. IGF-1-containing gelatin hydrogel microspheres have been used in a clinical trial for the treatment of patients with glucocorticoid-resistant sudden sensorineural hearing loss [29]. However, it is unknown whether the controlled release of IGF-1 using gelatin hydrogel is useful to stimulate neurogenesis in the SVZ.

We tested the effects of gelatin hydrogel containing IGF-1 on the number of new neurons in the intact adult mouse SVZ. First, we injected PBS containing IGF-1 (0.25 μg) without hydrogel into the striatum close to the SVZ (Figure 2(a)). Seven days later, there was no significant difference in the numbers of DCX+ new neurons in the SVZ between the IGF-1-injected and PBS-injected control brains (Figure 2(c), PBS: 1621 ± 50 cells, IGF-1: 1633 ± 205 cells, $n = 3$ animals for each group), indicating that the IGF-1 injection without gelatin hydrogel was not effective in stimulating neurogenesis under these experimental conditions. We then injected a gelatin-hydrogel suspension in PBS, with or without the same amount of IGF-1, into the striatum (Figure 2(b)). Seven days later, significantly increased numbers of DCX+ new neurons were observed in the SVZ of brains that received the IGF-1-containing gelatin hydrogel compared with the control group (Figures 2(b) and 2(c), PBS: 1469 ± 99 cells, IGF-1: 1916 ± 143 cells, $n = 5$ animals for each group). These results indicate that gelatin hydrogel is suitable as a vehicle for the delivery of IGF-1 to the SVZ. In addition, these results are consistent with the sustained release of IGF-1 from gelatin hydrogel efficiently promoting IGF-1's actions on the proliferation and differentiation of neural stem or progenitor cells [43–46], resulting in increased numbers of new neurons in the SVZ.

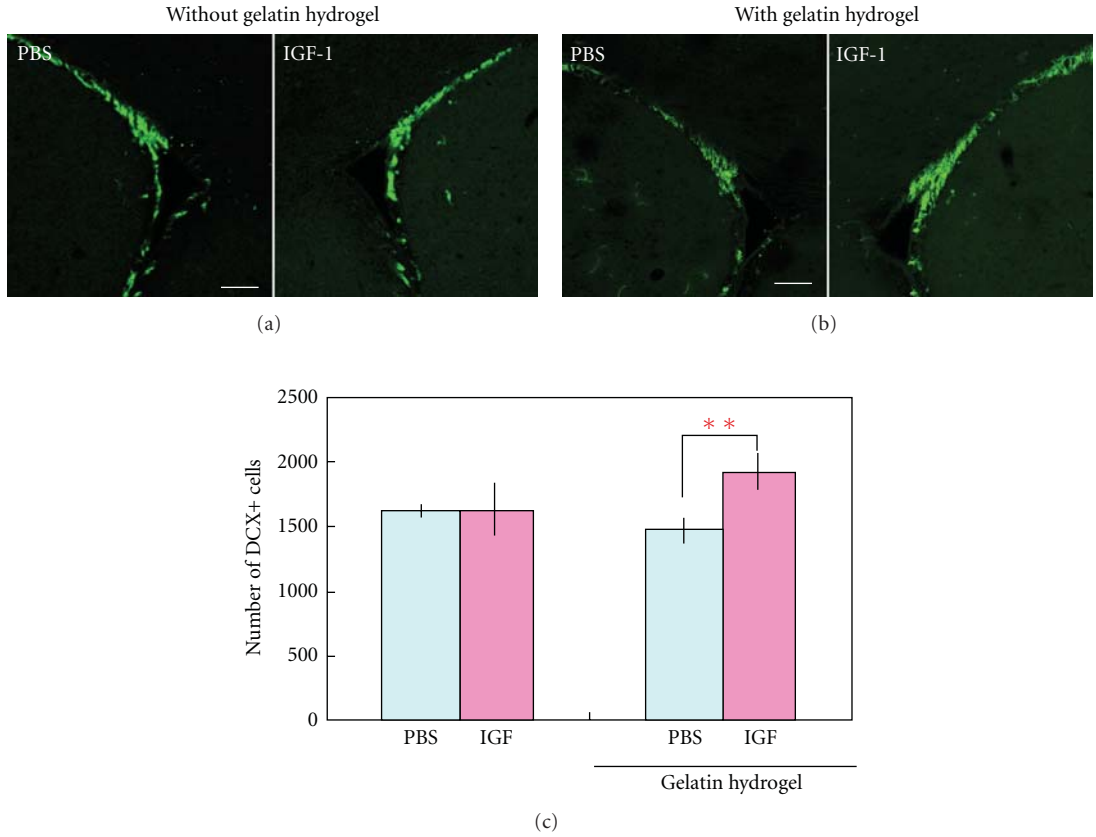

Figure 2: Effects of IGF-1-containing gelatin hydrogel on neurogenesis in the SVZ. (a) Coronal sections of brains that received an IGF-1 or PBS injection without gelatin hydrogel 7 days before, showing DCX+ new neurons in the SVZ (green) ($n = 3$ animals for each group). (b) Coronal sections of brains that received an injection of microspheres containing IGF-1 or microspheres plus PBS, showing DCX+ new neurons in the SVZ (green) ($n = 5$ animals for each group). (c) Quantification of DCX+ cells in the SVZ. Scale bars: 100 μm (a and b). $^{**}P < 0.01$.

3.3. HGF. Previous studies indicated that HGF stimulates the proliferation of neural stem or progenitor cells in the adult SVZ, as well as the migration and differentiation of new neurons [48–52]. Acute injection of HGF into the adult striatum has a neuroprotective effect and promotes neurological recovery in a mouse model of stroke [53]. Topical application of HGF-containing gels on the surface of the cerebral cortex increases the number of new migrating neurons in the striatum of the MCAO model [54]. HGF-containing gelatin hydrogel has been used for the treatment of animal models of several diseases including noise-induced hearing loss [55] and collagen-induced arthritis [56]. These studies strongly suggest that HGF-containing gelatin hydrogel is likely to be useful to enhance neuronal regeneration after stroke.

We first tested the effects of HGF in PBS, with or without gelatin hydrogel, injected into the striatum, on the number of new neurons in the SVZ of the normal adult brain. Seven days after the injection, there was no significant difference in the number of DCX+ cells between the HGF and PBS-control groups regardless of the use of gelatin hydrogel: with gelatin hydrogel, animals treated with PBS showed 1790 ± 102 new cells, and those given HGF generated 1750 ± 106 cells ($n = 3$ animals for each group); without gelatin hydrogel, animals treated with PBS showed 2111 ± 82 cells; those treated with HGF generated 1992 ± 78 cells ($n = 5$ animals for each group, t-test).

Next, we tested the effects of injecting the same substances in a mouse model of stroke induced by MCAO. In this model, SVZ-derived new neurons can be found migrating toward the injured striatum 2-3 weeks after the induction of ischemia [17]. Therefore, we injected HGF in PBS, with or without gelatin hydrogel, into the striatum 11 days after MCAO (Figures 3(a) and 3(b)). Eighteen days after MCAO, we quantified the infarct volume and number of new neurons in the striatum. The HGF administration did not affect the infarct volume, regardless of the use of gelatin hydrogel microspheres: with gelatin hydrogel, PBS yielded an infarct volume of 26.3 ± 3.4%, and those treated with HGF had an infarct volume of 25.3 ± 2.0% ($n = 6$ animals for each group); without gelatin hydrogel, animals treated with PBS showed an infarct volume of 26.6 ± 1.6% and those treated with HGF had an infarct volume of 29.6 ± 3.6% ($n = 4$ animals, t-test).

The administration of HGF without gelatin hydrogel also did not affect the number of DCX+ cells in the striatum (Figures 3(c) and 3(d)). However, the HGF-containing gelatin hydrogel significantly increased the number of DCX+ cells migrating in the striatum after MCAO (Figures 3(c) and 3(d)). Interestingly, the majority of the DCX+ cells extended

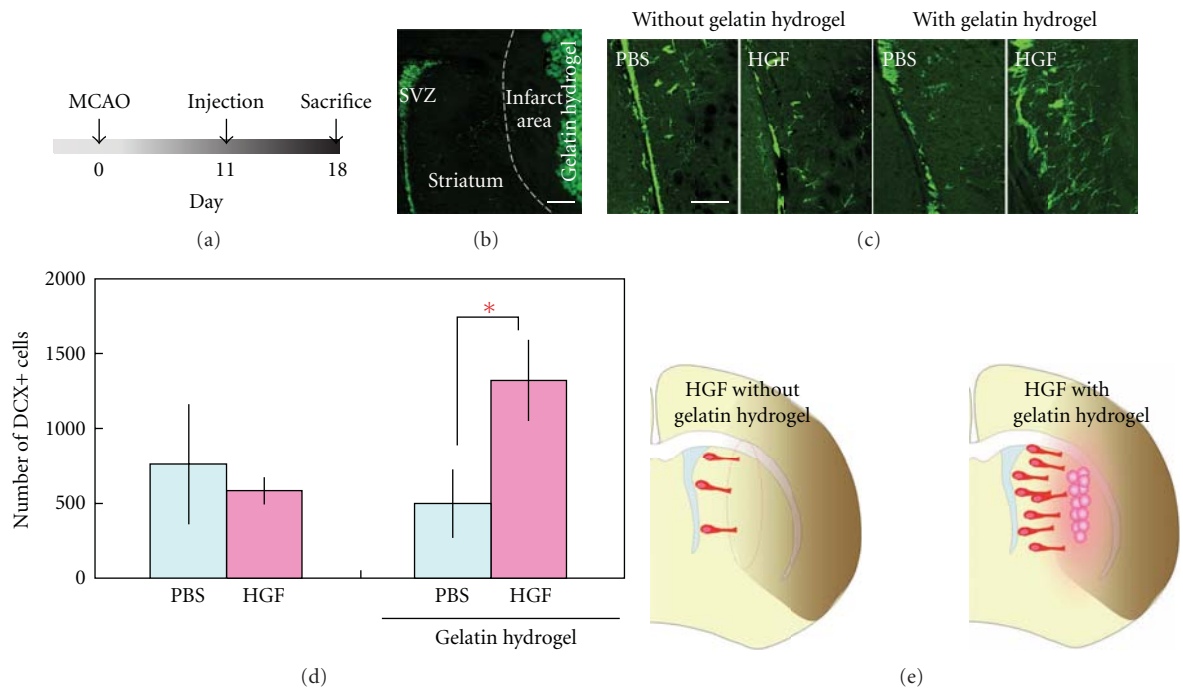

Figure 3: Effects of HGF-containing gelatin hydrogel on the number of new neurons migrating in the ischemic striatum after middle cerebral artery occlusion. (a) Experimental design. (b) A coronal brain section showing DCX+ new neurons in the SVZ (green) and gelatin hydrogel that was injected into the striatum. (c) Coronal sections stained for DCX (green) from brains that received a PBS or HGF injection with ($n = 6$ animals for each group) or without ($n = 4$ animals for each group) gelatin hydrogel. (d) Quantification of the number of DCX+ cells separated by at least 50 μm from the SVZ in the ipsilateral striatum. (e) Summary of the results. HGF administered as HGF-containing gelatin hydrogel microspheres significantly increased the number of new neurons. Scale bars: 200 μm (b and c). $^*P < 0.05$.

a long leading process toward the injected gelatin hydrogel, suggesting that these cells were migrating laterally. Because HGF has been reported to stimulate the proliferation of SVZ progenitors [48], we also examined the effects of HGF on cell proliferation in the SVZ after MCAO. There was no significant difference in the number of Ki67+ proliferating cells between the HGF and PBS-control groups, regardless of the use of gelatin hydrogel (with gelatin hydrogel, PBS: 796.5 ± 87.65 cells, HGF: 798.8 ± 96.12 cells, $n = 6$ and 5 animals, respectively; without gelatin hydrogel, PBS: 837.5 ± 78.77 cells, HGF: 926.0 ± 109.4 cells, $n = 4$ animals for each group, t-test). These results suggest that the increased number of new neurons in the injured striatum induced by treatment with HGF with gelatin hydrogel was due to an increased efficiency of the new neurons' migration but not to their production in the SVZ (Figure 3(e)).

Since the receptor for HGF, c-Met, is expressed in adult DCX+ migrating neurons [49] as well as GFAP+ astrocytes [50], it is possible that DCX+ cells are attracted by the HGF-releasing gelatin hydrogel. It is also possible that the HGF-induced increase in the number of DCX+ cells in the striatum was caused by angiogenic activity [54], since new neurons use blood vessels for their migration [17, 38].

4. Conclusions

The benefits reported here of using gelatin hydrogel microspheres to deliver growth factors to simulate neurogenesis in the SVZ demonstrate that gelatin hydrogel is a promising vehicle for the local and sustained release of drugs in the brain. Previous studies demonstrated that the epicortical delivery of EPO [57] and HGF [54] enhance neurogenesis in the SVZ in mouse models of stroke. However, considering the longer distance between the brain surface and the SVZ in the human brain, direct injection inside the brain parenchyma may be needed to efficiently stimulate neurogenesis from neural stem cells in the SVZ. The local delivery of drugs using hydrogels should result in a lower total dose of medications and thus in fewer side effects. In addition, this technology should be applicable to any charged protein that can enhance neural regeneration [58]. Further studies are needed to improve the injectability of the gelatin hydrogel, which will result in a less invasive procedure. Finally, how the newly generated neurons produced by this treatment contribute to neurological improvement needs to be elucidated.

Authors' Contribution

K. Nakaguchi and H. Jinnou contributed equally to this work.

Acknowledgments

The authors are grateful to Hiroshi Funakoshi (Asahikawa Medical University) and members of the Sawamoto

Laboratory for discussions. M. Sawada is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported by the Project for Realization of Regenerative Medicine (Ministry of Education, Culture, Sports, Science and Technology, Japan) and the Funding Program for Next Generation World-Leading Researchers (Japan Society for the Promotion of Science).

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 804717, 18 pages
doi:10.1155/2012/804717

Review Article

Adrenomedullin as a Growth and Cell Fate Regulatory Factor for Adult Neural Stem Cells

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Received 25 May 2012; Revised 16 August 2012; Accepted 21 August 2012

Academic Editor: Graciela Gudino-Cabrera

The use of stem cells as a strategy for tissue repair and regeneration is one of the biomedical research areas that has attracted more interest in the past few years. Despite the classic belief that the central nervous system (CNS) was immutable, now it is well known that cell turnover occurs in the mature CNS. Postnatal neurogenesis is subjected to tight regulation by many growth factors, cell signals, and transcription factors. An emerging molecule involved in this process is adrenomedullin (AM). AM, a 52-amino acid peptide which exerts a plethora of physiological functions, acts as a growth and cell fate regulatory factor for adult neural stem and progenitor cells. AM regulates the proliferation rate and the differentiation into neurons, astrocytes, and oligodendrocytes of stem/progenitor cells, probably through the PI3K/Akt pathway. The active peptides derived from the AM gene are able to regulate the cytoskeleton dynamics, which is extremely important for mature neural cell morphogenesis. In addition, a defective cytoskeleton may impair cell cycle and migration, so AM may contribute to neural stem cell growth regulation by allowing cells to pass through mitosis. Regulation of AM levels may contribute to program stem cells for their use in medical therapies.

1. Introduction

A continuously increasing number of research articles reporting new experimental data on stem cells confirm the trend that began in 1999. Due to the prospects for the translation of stem cell biology advancements to treatment of many severe conditions such as Parkinson disease [1, 2], Alzheimer disease [3], Duchenne muscular dystrophy [4], amyotrophic lateral sclerosis [3], diabetes [5], stroke [6], myocardial regeneration [7], cartilage repair [8], or acute fail liver [9], stem cells are common in the popular press and great hopes are stirring in the public about their therapeutic potential.

The dogmatic view of an ever-immutable neural tissue in mammals is now been replaced by the notion that cell turn over does occur in the mature central nervous system thanks to the persistence of precursor cells that possess the functional characteristics of neural stem cells [10]. In modern society, where neurodegenerative diseases are becoming a major public health problem, neural stem cells have become one of the main attention points of the scientific community. Their capacity to self-renew and to produce all cell types of the mature central nervous system leads to hypothesize about their potential use in transplantation therapies for severe neurodegenerative diseases such as Parkinson or Alzheimer disease [4].

All the processes of self-renewal, proliferation, progressive maturation, and differentiation, which are needed for stem cell physiology, are orchestrated by a set of transcription factors, cell-to-cell interactions, niche-to-cell interactions, and many soluble diffusible signals [11]. One of these signals is adrenomedullin (AM), a 52-aminoacid peptide with structural homology to calcitonin gene-related peptide [12, 13]. AM displays a large variety of physiological functions including cell growth and differentiation regulation [14]. In addition, recent studies point to specific roles of this regulatory peptide in the behaviour of several stem cells, including neural stem and progenitor cells. This paper tries to sum up the current knowledge about this topic.

2. Adrenomedullin

This regulatory peptide was isolated from human pheochromocytoma by Kitamura et al. in 1993 [15]. This peptide was able to stimulate cAMP production in human platelets and exerted potent and long-lasting hypotensive activity in rats. AM is synthesized by both tumor cells and normal adrenal medulla as well as by many other tissues. AM is a circulating hormone, although it functions also as a local paracrine and autocrine mediator with multiple biological activities such as vasodilatation, cell growth, regulation of hormone secretion, natriuresis, and antimicrobial effects [16].

2.1. Structure of Adrenomedullin. Human AM consists of 52 amino acids and it belongs to the amylin/calcitonin gene-related peptide (CGRP) family. Intermedin, also named adrenomedullin 2, has also been identified as a novel member of this family [12, 13]. AM contains a 6-amino acid ring formed by a disulfide bond between residues 16 and 21. The C-terminal tyrosine residue is amidated (-$CONH_2$). Both structural features are essential for its biological activity [17].

The three-dimensional structure of AM comprises a central α-helical region, covering approximately one third of its total length, flanked by two disordered segments. The presence of the α-helix at the centre of AM seems to be a general feature of the calcitonin peptide family, which is important for the physiology of these peptides and the recognition of their specific receptors [18].

2.2. Adrenomedullin Gene Expression and Release. AM is encoded by a gene contained in human chromosome 11 and consisting of 4 exons and 3 introns, with TATA, CAAT, and GC boxes in the 5'-flanking region [16]. In mice, the AM gene is localized in chromosome 7.

The mature AM peptide is derived from preproadrenomedullin, which contains 185 amino acids in humans. After cleaving of 21-residue N-terminal signaling peptide, preproadrenomedullin is converted to proadrenomedullin, which is a precursor of mature AM (amino acids 95-146 of preproadrenomedullin) as well as of another active peptide, proadrenomedullin N-terminal 20-peptide or PAMP (amino acids 22-41 of preproadrenomedullin) [17].

AM production is mostly regulated by oxidative stress and inflammation-related substances such as lipopolysaccharide and inflammatory cytokines like TNF-α and IL-1, which increase AM secretion rate. There are several binding sites for activator protein-2 (AP-2) and c-AMP-regulated enhancer element. It has also been discovered that there are nuclear factor-Kβ sites on the promoter of the AM gene [14]. Hypoxia is also a potent inducer of AM expression. This overexpression is mediated by transactivation of the AM promoter by hypoxia inducible factor 1 (HIF-1) transcription factor, as well as by posttranscriptional mRNA stabilization. Hypoxia response elements (HREs) have been found in the promoter of the human AM gene [19].

2.3. Metabolism of Adrenomedullin. AM is a circulating peptide and it can be found in plasma at a concentration of 2–10 pM in humans. AM is also present in other biological fluids such as urine, saliva, sweat, milk, amniotic fluid, and cerebrospinal liquid [16]. In plasma, AM is specifically bound to adrenomedullin binding protein-1 (AMBP-1), which was later identified as complement factor H [20]. AM bound to complement factor H cannot be detected in plasma, so it is thought that total plasma AM could be higher than reported in most studies. Circulating AM is rapidly degraded with a halflife of 16–20 minutes. Matrix metalloprotease 2 seems to be responsible for the initial degradation of AM, which is followed by an aminopeptidase [21, 22].

2.4. Adrenomedullin Receptor Distribution. Specific binding sites for AM are located in many cell types and tissues like the heart, lungs, spleen, liver, vas deferens, kidney glomerulus, skeletal muscle, hypothalamus, spinal cord, and so on [16]. The wide distribution of binding sites for AM has to be related with its great variety of biological functions. In addition, recent studies reveal that AM is able to bind to many areas of the brain, providing the anatomical basis for the involvement of AM in the physiology of the central nervous system (CNS) [23].

The same studies also provide evidence for the possible existence of heterogeneous populations of AM binding sites in the brain and peripheral tissues [23] with different affinities for AM.

The AM receptor belongs to the 7-transmembrane domain G-protein-coupled receptor superfamily and is named calcitonin receptor-like receptor (CLR). However, CLR needs the presence of modulating proteins with a single transmembrane domain known as receptor activity modifying proteins (RAMP) [24]. Three RAMP isoforms have been identified in the human genome: RAMP1, RAMP2 and RAMP3. RAMPs bind to the CLR in the endoplasmic reticulum promoting the plasma membrane localization [25]. RAMP1 transports and present the CLR at the membrane surface as a mature glycoprotein, and this heterodimer functions as a CGRP receptor. The CLR molecules transported by RAMP2 and RAMP3 are core-glycosylated and function as AM receptors (AMR); CLR/RAMP2 is known as AMR_1, whereas CLR/RAMP3 is AMR_2 [26].

Qi et al. [27] studied the sequence differences between RAMP1, RAMP2, and RAMP3 to identify individual residues that could be able to alter their pharmacology. They hypothesized that residues present in RAMP2 and RAMP3 but not in RAMP1 are responsible for making CLR/RAMP2 and CLR/RAMP3 AM receptors. It was reported that position 74 in RAMP2 and RAMP3 is critical to establish their affinity for AM while Phe93 in RAMP1 contributes in a very important way to αCGRP affinity for CGRP receptors [27].

The expression of RAMP isoforms in a particular cell may change between physiological and pathological conditions, determining the degree of response to AM and CGRP. In physiological conditions the more abundant isoform is RAMP2, suggesting that most CLR molecules bind to RAMP2 to form a functional receptor for AM. Despite this observation, dynamic changes in the expression pattern of RAMP genes may take place during disease and in some

physiological conditions like pregnancy. The most robust changes in RAMP expression levels coincide with those situations in which plasma AM is most elevated, as in pregnancy or diseases like sepsis or heart failure. In those situations in which AM levels are higher, there is an elevation in RAMP3 expression apparently in order to decrease AM responsiveness [28].

2.5. Signal Transduction Mechanisms. The signal transduction pathways activated by AM vary between species, organs, tissues, and cells. However, there are three main signaling pathways whereby AM exerts its actions: cAMP, Akt, and mitogen-activated protein kinase (MAPK) extracellular signal-regulated protein kinase (ERK).

The main signal transduction pathway activated by AM seems to be the adenylyl cyclase/cAMP system. In many cell types, AM and CGRP receptors are coupled to G_s proteins that activate adenylate cyclase and increase intracellular levels of cAMP [17]. In bovine aortic endothelial cells and vascular smooth muscle cells (VSMC), the accumulation of cAMP causes the activation of protein kinase A (PKA) which in turn increases calcium (Ca^{2+}) efflux leading to relaxation of the vascular cells [29].

Moreover, AM can induce Ca^{2+} mobilization independently from cAMP levels. This fact suggests that other signalling mechanisms may be responsible for AM-induced positive ionotropic action. This theory was confirmed by Szokodi et al. [30]. AM activated phospholipase C through its specific receptor and accelerated Inositol-1,4,5-P_3 formation to stimulate Ca^{2+} release from the endoplasmic reticulum intracellular stores [29, 30]. In addition, the activation of phospholipase C is also involved in ion channel opening. However, other studies have shown that AM administration does not have any effects in intracellular Ca^{2+} concentration and even decreases Ca^{2+} content in cultured HUVECs [31] or in porcine coronary arteries [32]. These results suggest that the regulation of Ca^{2+} mobilization by AM may depend on the cell type and context.

It has been shown that intracellular Ca^{2+} increases, in response to AM, caused activation of nitric oxide (NO) synthase and NO release leading to relaxation of cardiac myocytes [33]. AM activation of NO pathway has a very important role in the regulation of the cardiovascular system by regulating blood flow [34]. The NO pathway and the reactive oxidative species produce an elevation of collateral flow in ischemic tissues having a cytoprotective action against ischaemia/reperfusion injury and against myocardial ischaemia-induced arrythmias in rats [35]. Furthermore, Sata et al. demonstrated that AM inhibited endothelial cell apoptosis through a NO-dependent pathway [36]. Their results suggest that the antiapoptotic effect of AM mediated by NO is independent of cGMP, whereas the cGMP/cGMP-dependent kinase pathway mediates many biological functions of NO-like vasodilatation [36]. Some authors postulate that NO prevents apoptosis by S-nitrosylating caspases [37–39].

AM has been shown to activate the PI3K/Akt pathway in vascular endothelial cells where it regulates many steps like vasodilation, cell survival, proliferation, migration, and vascular cord-like structure formation [40]. The specific role of AM in the multistep process of angiogenesis is regulated via a mechanism that requires the activation of the CLR/RAMP2 and CLR/RAMP3 receptors [41].

Other findings suggest that AM also acts directly on myocardium by the presence of CLR in myocytes and induces cardioprotective and antiapoptotic effects through the activation of the PI3K/Akt pathway after ischemia/reperfusion and enhances neovascularization in ischemic tissues [42].

The role of AM in growth and mitogenesis has led to investigate the regulation of MAPK by AM. AM signalling directly promotes endothelial cell growth and survival through activation of MAPK/ERK downstream signalling pathways [43]. Interestingly, in glomerular mesangial cells AM causes an opposite effect by increasing apoptosis during serum deprivation [44]. AM activates apoptosis in mesangial cells but protects against it in endothelial cells and tumors; these results suggest a cell type-dependent effect of AM on apoptosis [14]. Activation of MAPK and other kinases such as cAMP-PKA, JNK, and protein phosphatase 2A (PP2A) has been proposed to mediate the proapoptotic effect of AM in mesangial cells. On the other hand, AM protects malignant cells from hypoxia-induced cell death by upregulation of Bcl-2 in an autocrine/paracrine manner [45].

AM appears to either stimulate or inhibit cell proliferation depending on the particular cell type. Cell proliferative response induced by AM is thus mediated via activation of protein tyrosine kinase-MAPK. However, in cells lacking MAPK activation signalling pathways, stimulation of AM receptors results in activation of cAMP, leading to growth inhibition [46].

On vascular smooth muscle cells (VSMCs) there are conflicting results. Initially it was suggested that AM had an antiproliferative effect [47]. But more recent studies suggest that AM exerts a potent mitogenic effect in serum-deprived VSMCs [48, 49]. Under serum deprivation, AM promotes DNA synthesis and cell proliferation in VSMCs. These responses are mediated by p42/p44 MAPK activation. AM stimulates proline-rich tyrosine kinase 2 (PYK2) which, in turn, activates c-Src and induces recruitment of adaptor proteins (Shc/Grb2), thereby leading to activation of the Ras-dependent MAPK cascade in VSMCs.

All signal mechanisms in which AM is involved are the basis of its extensive repertoire of biological functions as vasodilation, cellular proliferation, apoptosis modulation, or inflammatory regulation.

The main role played by AM in mammalian development has become apparent following the generation of different knockout (KO) models. In AM gene KO mice, in which the expression of AM and PAMP are suppressed, the null phenotype is embryonically lethal due to the absence of placental vascularisation, malformation of the basement membrane in the aorta and cervical arteries, detachment of the endothelial cells from the basement structure, and the presence of edema [50]. In addition, other groups have generated a KO mouse model in which only AM expression is affected [51]. $AM^{-/-}$ mice are also embryonic lethal and both genotypes $AM/PAMP^{-/-}$ and $AM^{-/-}$ caused embryonic

lethality at the same embryonic age, between embryonic day 14 (E14.5) and embryonic day 15 (E15.5).

To demonstrate the *in vivo* importance of the CLR, a gene-targeted KO model of its gene (Calcrl) was generated. Mice heterozygous for the targeted Calcrl allele appear normal, survive to adulthood, and reproduce normally. However, $Calcrl^{-/-}$ pups are not viable, the embryos die between E13.5 and E14.5 of gestation and they exhibit a very similar phenotype to $AM^{-/-}$ and $AM/PAMP^{-/-}$ mice [52]. This result demonstrates that Calcrl is essential for embryo survival.

In models of mice lacking RAMP2 the results are similar to the ones shown above. $RAMP2^{-/-}$ embryos die in utero at midgestation due to severely deformation, vascular fragility, severe edema, and hemorrhage. In contrast, $RAMP2^{+/-}$ mice are viable, they reach adulthood but they exhibit a great variety of phenotypes due to vascular hyperpermeability and impaired neovascularization [53]. These data show that RAMP2 is a key determinant of the effects of AM on the vasculature and is essential for angiogenesis and vasculature integrity.

Surprisingly, a complete absence of RAMP3 has no effect on survival. RAMP3-null mice appear normal until old age (9-10 months), at which point they have less weight than their wild-type littermates [54]. These results provide support to the hypothesis that RAMP2 and RAMP3 have distinct physiological functions in embryogenesis, adulthood, and old age.

To continue with the study of the lack of AM in adult tissues and organisms, tissue-specific conditional KO models have been generated using Cre/loxP technology [55]. For example, loss of AM receptor resulted in abnormal jugular lymphatic vessels in association with reduction in lymphatic endothelial cell proliferation [43]. Another report, by Fernández et al., showed that lack of AM in the mouse nervous system results in behavioural changes, anxiety, and lower survival under stress conditions [56]. In addition, AM suppression causes significant infarct size increase following cerebral artery occlusion [57].

2.6. Physiological Activity of Adrenomedullin. In the adult organism, AM has been located in many cell types and in most tissues throughout the body [58]. This distribution suggests that AM has diverse physiological activities and that it needs a tight regulatory system. The locations of AM expression include the nervous system and related structures, cardiovascular system, endocrine organs, digestive tube, excretory system, respiratory system, reproductive tract, and integument, among others.

AM has a variety of biological actions which are of potential importance for cardiovascular homeostasis, growth and development of cardiovascular tissues, and regulation of body fluid. Systemic AM administration has demonstrated that this peptide reduces arterial pressure, decreases peripheral vascular resistance, and increases heart rate and cardiac output [59]. *In vitro,* AM dilates blood vessels of different vascular beds from different animal species. Regarding the mechanism of the vasodilatory effect of AM, most data indicate that AM may induce endothelium-independent relaxation by acting on $CGRP_1$ receptors and elevating cAMP level in vascular smooth muscle cells [17]. In addition, AM binds to specific receptors in endothelial cells and elicits endothelium-dependent vasorelaxation mediated by nitric oxide [60], endothelium-derived hyperpolarizing factor [61], and/or vasodilatory prostanoids [62]. On the other hand, accumulating evidence supports a compensatory role for AM in heart failure. It has been established that plasma AM levels increase in patients with heart failure in proportion to the severity of the disease. Furthermore, recent studies suggest that plasma AM level is an independent prognostic indicator of heart failure. This peptide may regulate myocardial hypertrophy and remodelling in arterial hypertension or heart failure in a paracrine/autocrine way [63].

AM exerts a tight control on renal function and body fluid volume. First, AM exerts direct control over the kidney, affecting diuresis and natriuresis. Furthermore, AM may regulate the hypothalamic-pituitary-adrenal axis at all levels. It has been demonstrated that intracerebral infusions of AM inhibit salt intake and thirst. In the pituitary, AM is able to inhibit the secretion of vasopressin and ACTH, whereas in the adrenal gland it regulates the secretion of aldosterone [64].

AM regulates hormone secretion in many tissues and organs. Levels of this peptide have effects in the hypothalamic-pituitary-adrenal axis as shown above. In addition, AM is synthesized in pancreatic polypeptide-producing F cells of the pancreatic islets and AM receptors are expressed in insulin-producing β-cells. Incubation with a monoclonal antibody against AM raised five times the basal insulin secretion by islets [65]. This indicates that endogenous AM tonically inhibits insulin secretion.

In the reproductive tract, AM is synthesized by the granulosa cells of the ovarian follicles. Plasma AM level increases during the follicular phase and decreases during the luteal phase of the menstrual cycle [66]. Both AM and its receptors are expressed in the uterus and their expression is higher during pregnancy [67]. AM can also be detected in the placenta and in many fetal tissues suggesting that it may be involved in growth and embryogenesis [68].

In the digestive system AM immunoreactivity is widely distributed in the mucosal and glandular epithelia of the stomach, esophagus, intestine, gallbladder, bile duct, and acini of the pancreas and salivary glands [69]. AM is a potent inhibitor of basal gastrin-stimulated HCl secretion [70].

Finally, AM has been found in all epithelial surfaces which separate the external and internal environment and in all body secretions. This wide distribution suggests the possibility that AM has an immunity-related function. It has been proven that both AM and PAMP display potent antimicrobial action against Gram-positive and Gram-negative bacteria [71]. In septic shock patients, a marked elevation of AM blood levels have been reported, probably as a defensive action [72]. However, excessive AM release during septic shock may provoke adverse effects such as hypotension which may threaten the patient's life.

3. Adrenomedullin in the Central Nervous System

AM and its receptors are abundantly expressed in the CNS and its cellular components. By a combination of techniques it has been proven that AM appears from day 10 of embryonic development in maturing cells of the ventral horn of the spinal cord and by day 14 mRNA for AM and its receptors could be observed in specific neuron groups [73]. This was followed by the demonstration of the presence of human AM mRNA in the following regions: frontal, temporal and occipital cortex, pons, thalamus, hypothalamus, cerebellum, and in the pituitary gland [74]. With respect to the rat cerebral cortex, AM immunoreactivity was shown to be widely distributed in all areas under basal conditions. In particular, high levels of immunoreactivity were found in the thalamus, hypothalamus, adenohypophysis, and neurohypophysis [75].

Given the wide distribution of AM immunoreactivity in the adult CNS, it has to be admitted that the function of AM at most of these sites is still largely unknown. It is apparent that AM can act as a neurotransmitter, neuromodulator or neurohormone; but in sight of its ample distribution, it could have other functions apart from those. The prominent localization of AM in dendritic structures and the cytoskeleton also argues against an exclusive role as a neurotransmitter [75].

The AM system may be especially important in cerebral circulation. The concentration of AM is about 50% higher in cerebral blood vessels than in other regional circulations due to an astrocyte-induced elevation of AM production by cerebral endothelial cells [76]. However, the biological actions of AM in cerebral blood vessels have only been partially defined.

AM, as an endothelium-derived autocrine/paracrine hormone, plays an important role in the regulation of specific blood-brain barrier properties [77]. AM may be one of the physiological links between astrocyte-derived factors, cAMP and the induction and maintenance of the blood-brain barrier. Moreover, the role of AM in the differentiation and proliferation of peripheral endothelial cells and in angiogenesis suggests a more complex function for AM in the cerebral circulation and in the development of blood-brain barrier [78].

The hypotensive effect of AM in the periphery is not paralleled in the brain. Both, AM and PAMP, given by intracerebroventricular (icv) infusions, exerts significant hypertensive actions in conscious animals by a central action which stimulates α-adrenergic, sympathetic function. In addition to increasing blood pressure after icv administration, both peptides stimulated significant increases in heart rate in these conscious, unrestrained animals [79]. Direct effects of AM centrally administered on sympathetic nerve activity in conscious rats were observed and these data suggest that icv AM induced an increase in preganglionic sympathetic discharge [80]. The temporal aspects of CNS-induced hypertension by AM are similar to those effects seen after the administration of angiotensin II [79, 80]. This suggests a possible common mechanism for the hypertensive action of both peptides in the brain.

Regarding cerebral physiopathology, using the rat focal stroke model of middle cerebral artery occlusion (MCAO), Wang et al. [81] showed increases in AM mRNA expression in the ischemic cortex. Immunohistochemical studies localized AM to ischemic neuronal processes. However, this study reports that AM exacerbates focal brain ischemic damage, in contrast with all other posterior studies. Two years later, Dogan et al. [82] reported in a MCAO model that pre- and postinfusions of AM decreased the volume of ischemic brain injury, partly by increasing regional cerebral blood flow in a dose-dependent manner in rats. More recent studies have definitely proven the protective role of AM in ischemic brain. In transgenic mice that overproduce AM, the infarct area and gliosis after MCAO were reduced, in association with suppression of leukocyte infiltration, oxidative stress and apoptosis in the ischemic core. In addition, vascular regeneration and subsequent neurogenesis were enhanced, preceded by increase in mobilization of CD34+ mononuclear cells (which are endothelial cell precursors). Brain edema was also significantly reduced via suppression of vascular permeability [83]. On the other hand, Hurtado et al. [57] studied the effect of AM and its binding protein, complement factor H, in the physiopathology of stroke using a brain-specific conditional KO for AM and a complete KO for factor H. They found that animals lacking AM had got a significant infarct size increase compared with their WT littermates following middle cerebral artery occlusion. In contrast, lack of complement factor H did not affect infarct volume. They suggest that the neuroprotective action of AM in the brain may be mediated by regulation of iNOS, matrix metalloproteases, and inflammatory mediators [57].

Fos protein and NO-producing neurons of the rat brain, which are involved in cardiovascular regulation, are also induced by central administration of AM. Following icv administration of AM, Fos-like immunoreactive neurons were markedly increased in several brain areas of the rat, including the forebrain, the hypothalamus, and the brainstem [84, 85]. In the same conditions, the number of double-labeled neurons for Fos and NO synthase was increased in the paraventricular and supraoptic nuclei [86].

AM is hypothesized to be a physiologically relevant regulator of fluid and electrolyte homeostasis. Centrally administered AM inhibits water drinking [87] and salt appetite [88]. In addition, inactivation of endogenous AM increases water and sodium intake, suggesting that AM tonically inhibits thirst and salt intake [89]. AM may therefore participate in the central control of fluid balance by a variety of mechanisms. This peptide has direct actions in the hypothalamus to decrease vasopressin secretion and in the pituitary gland to inhibit ACTH release [90]. AM is also able to inhibit water intake after a hypovolemic challenge or a hyperosmotic challenge [87] and could diminish angiotensin II-stimulated water intake in a dose-dependent manner [90]. Another potential target for AM seems to be the area postrema which is involved in many vegetative functions, such as cardiovascular control and eating and drinking behaviour [75].

Icv injection of AM also causes a dose-dependent reduction in feeding in rats [91]. This effect is attenuated

by administration of $CGRP_{8-37}$ showing that AM action is mediated by CGRP receptors. In the same way, Martínez et al. [92] reported that AM inhibits gastric emptying in conscious rats in a dose-dependent manner by acting through adrenal-dependent, β-adrenergic pathways independently of central CRF receptors activation.

Recently, AM has been reported to exert actions at each level of the hypothalamus-pituitary-adrenal (HPA) axis [93]. These data suggest that the peptide plays a role in the organization of the neuroendocrine responses to stress. AM acts within the hypothalamus of unrestrained male rats to increase HPA axis activity. AM given icv stimulates the release of prolactin but does not alter the secretion of GH, thus demonstrating the specificity of AM within the hypothalamus [94]. The role of AM in the modulation of HPA axis activity appears to be quite complex. Icv administration of AM elevated plasma corticosterone levels, which suggests that AM acts within the hypothalamus to stimulate CRH release into the hypophyseal portal vessels, thus increasing activity of the HPA axis [94]. In addition, AM has been shown to induce Fos expression in CRH-positive hypothalamic paraventricular neurons [95] and AM directly caused depolarization of parvocellular paraventricular neurons in brain slices [96]. It can be concluded that AM produced in the brain may be an important neuromodulator of the hormonal stress response.

These results are also supported by the experiments of Fernández et al. [56]. They built a conditional KO mouse model using the Cre/loxP approach, obtaining animals with no AM nor PAMP expression in the CNS but normal levels in other organs. These genetically modified animals appear to have normal lives and do not present any gross morphological defect. Despite the normal appearance, behavioral analysis show that mice with no AM in their brain have impaired motor coordination and are hyperactive when compared to their wild-type (WT) littermates. Interestingly, heterozygous mice behave exactly as WT animals, suggesting that even a partial presence of AM during brain development is enough to prevent damage. Another characteristic of the mice lacking AM in the brain is their excessive anxiety levels. As seen before, icv administration of AM release stress hormones like corticosterone [94]. This clearly suggests that brain AM limits the magnitude of the stress response. Furthermore, KO mice had lower survival rate under hypobaric hypoxia conditions, demonstrating once again the neuroprotective function of AM in the CNS [56].

AM mRNA and protein have been shown to be expressed in dorsal root ganglia which contains nociceptors [97]. This pattern of expression has led researchers to hypothesize that AM could play a role in nociception. Ma et al. [98] demonstrated the presence of AM-like immunoreactivity in both CGRP-containing and lectin IB4-binding nociceptors in dorsal root ganglion and axon terminals in the superficial dorsal horn of the rat spinal cord. Likewise, immunoreactivity for receptor markers such as CLR and the three RAMPs is localized in the superficial dorsal horn. These results supply anatomical evidence that AM may be a pain-related neuropeptide. In addition, this group also provides functional proof that AM works as a pain neuropeptide. Moreover, intrathecal injection of AM induces a long-lasting heat hyperalgesia in rats. AM-induced pain response is mediated by a direct activation of AM receptors located on nociceptive neurons in the dorsal horn and through the activation of the PI3K/Akt/GSK3β signalling pathway. They also suggested that AM potentially induces the release of other pain-stimulating substances such as substance P or glutamate.

Following this line of thought, Fernández et al. [99] explored pain sensitivity in a mouse conditional KO model for AM in neurons of the CNS, including the spinal cord and dorsal root ganglia. Elimination of the AM gene in the CNS of the mouse results in expression changes for several sensory neurotransmitters, including CGRP, substance P, and enkephalin, in the dorsal root ganglia and the spinal cord. Furthermore, lack of AM expression has behavioral consequences when pain sensitivity was tested with the tail-flick and the hotplate latency paradigms. The results of the test suggest the possibility that AM acts as a nociceptive modulator in spinal reflexes, whereas it may have an analgesic function at higher cognitive levels [99]. This study confirms the important role of AM in pain sensitivity processing, but presents a more complex function of AM than previously described.

As seen above, AM is involved in many functions in the CNS, and it also exerts some peripheral actions through CNS mechanisms. However, there is still much work to do in the area to fully understand all the roles that AM play in the nervous system.

4. Adrenomedullin and Stem Cells

4.1. General Aspects about Stem and Progenitor Cells. Stem cells are defined by their ability to self-renew and to differentiate into many functional cell types in response to specific signals [100]. In general, stem cells produce one or more intermediate cell types before reaching the fully differentiated state which is characteristic of adult tissues. These intermediate differentiation steps are known as progenitor cells.

During the last years, stem cells have been found in many organs and tissues, such as bone marrow, peripheral blood, umbilical cord, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, gut epithelium, epidermis, cornea, retina, liver, pancreas, adipose tissue, hair follicle, mammary gland, ovary, prostate, and testis [101]. Among the main functions of these cells, the most relevant is the replacement of tissue cells which die naturally as part of tissue's homeostasis or because of an injury or illness. Stem cells are therefore indispensable for the integrity of complex and long-living organisms.

In mammals, several stem cell niches can be found. The niche microenvironment plays a critical role in cell maintenance and differentiation since it is composed of a specialized group of cells that regulate stem cell survival, self-renewal, and differentiation [102, 103]. Strong evidence suggests now that the niche is indispensable for stem cells regulation [104]. Regulation from the niche includes growth factor signalling, cell-to-cell contact, and cell-to-extracellular-matrix

interactions for homeostatic cell turnover and increased cell production in response to stimulation [105].

Many molecules have been described to regulate stem cell behavior. The main signals are Shh, Wnts, bone morphogenic proteins, transforming growth factor (TGF) beta, angiopoietin-1/Tie2, fibroblast growth factors, SCF/c-kit, Jagged/Notch, insulin/insulin-like growth factors, and leukemia inhibitory factor through the JAK-Stat pathway [11, 101, 106–109].

4.2. AM as a Growth and Development Factor. From the beginning, it was thought that AM was an important tumor growth factor [110]. Nowadays it is well known that AM stimulates proliferation of fibroblasts, keratinocytes, endothelial cells, osteoblasts, and many tumor-derived cells [14].

In Swiss 3T3 cells, AM increases DNA synthesis in a dose-dependent manner by increasing cAMP/PKA [111]. AM also stimulates DNA and cAMP synthesis in human keratinocytes [112]. These data provide evidence for a growth-promoting effect of AM, possibly mediated through cAMP.

However, in some cell types such as VSMC, mesangial cells, glial cells, and glial tumor cells an inhibitory effect was observed for AM on proliferation and growth [14].

Therefore, the role of AM as a growth factor, depends on the cell type.

In addition, AM has also been proposed as an important factor in embryogenesis and differentiation [73, 113] and as an apoptosis survival factor [114]. In conclusion, there is no doubt about the role of AM in cell and tumor growth, and this might be related with a possible role of AM in the growth and differentiation of stem cells.

4.3. Adrenomedullin and Endothelial Progenitor Cells (EPCs). EPCs are precursor cells which are found in circulating blood and/or in the bone marrow. EPCs are mobilized from the bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrating to injured endothelium sites and differentiating into mature endothelial cells *in situ*.

Because of the main role of AM in endothelial cell biology, the number of investigations studying the role of AM in the physiology of EPCs are increasing.

The first discoveries were that AM increases the number of early EPCs and also suppresses their apoptosis [115, 116]. AM could exert its actions on EPCs because these cells express CLR on their surface [117].

Nagaya et al. [116] studied the effects of AM gene-modified EPCs on the treatment of pulmonary hypertension in rats. Intravenous administration of AM-expressing EPCs significantly decreased pulmonary vascular resistance compared with EPCs alone and the AM-expressing cells ameliorated pulmonary endothelium regeneration. In addition, rats with pulmonary hypertension transplanted with AM-expressing EPCs had a significantly higher survival rate. All these results taken together suggest that AM gene transfer into EPCs inhibits cell apoptosis and induces proliferation and migration so that AM gene transfer strengthens the therapeutic potential of EPCs.

Given that AM inhibits vascular endothelial apoptosis and induces angiogenesis, some studies investigated whether AM enhances bone marrow cell-induced angiogenesis after transplantation in a rat model of hindlimb ischemia [115]. Bone-marrow-derived mononuclear cells (MNCs) also express CLR. *In vivo*, rats which received AM infusion plus MNC transplantation showed a significantly higher laser Doppler perfusion index than the control groups and had an important improvement in blood perfusion. Also, capillary density was highest in the AM plus MNC transplantation group. The combination of AM infusion and MNC transplantation enhances MNC-induced angiogenesis. Furthermore, AM increased the number of MNC-derived von Willebrand factor-positive cells and generated α-SMA-positive vascular structures. The same group studied the action of AM in cultured MNCs [115]. *In vitro*, AM inhibits serum starvation-induced MNC apoptosis. The increase in MNC survival achieved by AM depends on the PI3K/Akt pathway. AM also increases MNC adhesiveness to endothelial cells via activation of adhesion molecules such as ICAM-1 and VCAM-1. MNC adhesiveness to endothelial cells is indispensable for MNC differentiation into the endothelial lineage. In addition, AM may accelerate MNC differentiation into endothelial cells since it increases the number of MNC-derived EPCs that express VE cadherin, KDR and CD31.

The same results were obtained after transplantation of mesenchymal stem cells (MSCs) for improving neurological deficits after stroke in rats [6]. The group of animals which received AM plus MSC transplantation had a significant improvement in their health status. They also showed a marked induction of angiogenesis, and AM infusion significantly inhibited apoptosis of transplanted MSCs.

Taken together, these data suggest that AM enhances the therapeutic potency of MNCs and MSCs through inhibition of apoptosis, induction of angiogenesis, and adhesion and differentiation improvement [6, 115].

Direct transplantation of EPCs seems to be a useful strategy for therapeutic neovascularization in ischemic tissue [117]. EPCs have been successfully used in the treatment of renal ischemia after acute kidney injury [118], myocardial regeneration [7, 119–122], and in therapeutic angiogenesis in diabetes [123]. The coadministration of AM enhances the angiogenic effects of EPCs, improves their mobilization, and helps in the development of collateral vessels [115, 116, 119, 124, 125]. In a model of hindlimb ischemia induced by resecting the right femoral artery [126], AM overexpression significantly enhanced the recovery of blood flow and increased capillary density in the ischemic leg. The results of Abe et al. [126] suggest that AM-induced angiogenesis may be associated with mobilization of bone marrow-derived cells. Treatment with AM increased bone-marrow-derived cells in the ischemic tissue, which expressed the endothelial-cell-specific protein CD31. This treatment also increased the number of Sca1 and c-kit double positive cells in peripheral blood and bone marrow. There is a possibility that the mobilization of EPCs is modulated by NO. In eNOS-deficient mice, bone marrow transplantation itself is not enough to recover the ischemic tissue [127]. It would be possible that EPC mobilization promoted by AM

may be related to AM-induced NO release via activation of the PI3K/Akt pathway [126].

Tumors, besides recruiting neighboring blood vessels or epithelial cells, also incorporate EPCs into the developing vasculature. This process is dependent on the mobilization of VEGFR2+ and CD133+ cells into circulation. The use of an AM antagonist *in vivo* significantly reduces tumor growth and microvessel density. In tumor endothelial cells isolated from renal cell carcinoma xenografts both proliferation and migration were blocked by treatment with an AM antagonist [128]. The antagonist also reduced the number of circulating CD133+ and VEGFR2+ cells which demonstrates the importance of AM in the mobilization of EPCs [128].

AM is also involved in the vascular differentiation from progenitor cells. Yurugi-Kobayashi et al. [129] studied *in vitro* the acquisition of arterial or venous identity by endothelial cells induced from VEGFR2+ progenitor cells. Whereas VEGF alone mainly induced venous endothelial cells, addition of AM, which elevates cAMP, supported substantial induction of arterial endothelial cells. Stimulation of cAMP pathway induced Notch signal activation in endothelial cells. All these data indicate that coordinated signalling of VEGF, Notch, and cAMP is required to induce arterial endothelial cells from vascular progenitors. However, more research is needed to understand how arterial differentiation is achieved in the living organism.

In the skin, sensory nerves play a main role in the pattern of arterial differentiation [130–132]. Nerves and blood vessels are branched structures. Arteries, but not veins, are specifically aligned with peripheral sensory nerves in embryonic mouse limb skin. Several lines of evidence suggest that this association reflects a requirement for the nerve to induce arterial differentiation. In mutants lacking peripheral sensory axons and/or Schwann cells, arterial differentiation does not occur and remodelling appears abnormal. Furthermore, *in vitro*, purified peripheral sensory neurons or Schwann cells can induce arterial marker expression in cultured endothelial cells [130]. The fact that this induction can be blocked by a specific VEGF antagonist suggests that nerve-promoted arteriogenesis may be mediated by local secretion of VEGF. It has also been proven that VEGF is necessary for arterial differentiation of a primitive capillary plexus *in vivo* [131]. Nerves may promote blood vessel association and arterial differentiation shortly after their arrival in the periphery in order to ensure access to a local source of survival factors during their growth. The idea that AM may be related in this process cannot be excluded, since many perivascular nerves in the rat mesenteric artery show AM-like immunoreactivity [132].

Very recently, it has been confirmed that AM could augment the growth and angiogenic properties of late outgrowth EPCs as potently as VEGF [133]. Hermansen et al. [134] confirmed that cultured EPCs possess an endothelial phenotype and express the AM receptor complex CLR/RAMP2. AM stimulation induced proliferation of EPCs and increased the formation of tubular networks in the EPC/fibroblast coculture and matrigel assays. These effects seem to be mediated through the PI3K/Akt signalling pathway.

Considering all this information, it is possible to assume that AM has a main role on growth, mobilization, and differentiation of EPCs, which leads us to propose that AM may be a relevant therapeutic target for the treatment of vascular diseases.

4.4. Adrenomedullin and Mesenchymal Stem Cells (MSCs). Mesenchymal stem cells (MSCs) are adult stem cell with capacity of self-renewal and differentiation into mesoderm- and nonmesoderm-derived tissues. They also play an important role in endogenous maintenance of stem cells niches.

There is a strong interest in searching for potential therapeutic effects of MSCs. Mobilization of endogenous MSCs represents an alternative treatment for the regeneration of injured growth plate cartilage [135]. Different aspects of MSCs render them an appropriate cell type for clinical use to promote bone regeneration [136]. Since 1995 it has been known that under *in vitro* conditions, MSCs can differentiate into cells exhibiting features of cardiomyocytes. After this seminal work, several preclinical and clinical studies have supported the notion that MSC therapy may be used for cardiac regeneration [128, 137–139]. *In vitro* works suggest that a great variety of stimuli such as insulin or fibronectin, and direct cell-to-cell contacts induce the differentiation of MSCs into cardiomyocytes. The stem cells which acquired a cardiomyocytes-like phenotype were characterized by the expression of myosin heavy chain, beta-actin, and troponin T [137]. Studies in animal models have demonstrated the ability of transplanted or infused MSCs to engraft and differentiate into cardiomyocytes, VSMCs, and endothelial cells [137, 140] and to secrete factors such as VEGF, FGF, MCP-1, HGF, IGF-1, SDF-1, thrombopoietin, and AM, to reduce tissue injury and/or enhance tissue repair [125, 138]. These factors stimulate cell adhesion, neovascularization, and formation of inflammatory infiltrate without tissue necrosis. The molecular pathways that regulate MSC-mediated regeneration are, however, poorly understood. Recently, Alfaro et al. [139] demonstrated in a murine myocardial infarct model that downregulation of the canonical Wnt pathway, characterized by significant upregulation of specific secreted frizzled-related proteins (sFRPs), was necessary for MSC self-renewal. Using a genetically modified mouse model they found that sFRP2, an inhibitor of Wnt signalling, is a key molecule for the biogenesis of a superior regenerative phenotype in MSCs.

Several studies have investigated the role of AM in MSC transplantation. MSCs are able to secrete large amounts of angiogenic and antiapoptotic factors, including AM [125]. It has been proven that AM enhances the therapeutic potency of MSC transplantation in experimental stroke in rats [6]. AM induces angiogenesis in the ischemic tissue and significantly inhibits the apoptosis of transplanted MSCs. PI3K/Akt is an important cell survival pathway in MSCs that seems to mediate cytoprotective effects in these cells [141]. AM is able to activate the PI3K/Akt pathway, so it is reasonable to think that AM may promote MSC survival and help to cardiac repair through this pathway.

Adipose tissue-derived stem cells (ASCs) are also adult stem cells with capacity for self-renewal and differentiation,

like MSCs. Both have similar characteristics, but ASCs are closer to the endodermal and/or early hepatic differentiation stages. As with MSCs, extensive research has been done to find therapeutic roles for ASCs. Undifferentiated ASCs have the ability to improve hepatic function in mice with acute liver injury in a short-time span [142]. After the transplantation of ASCs into mice with acute liver failure, markers of liver injury decreased. In addition, injection of ASCs into the corpus cavernosum seems to be a potential therapy for the treatment of erectile dysfunction in rats with hyperlipidemia [143] and with Streptozotocin-induced diabetes [144]. This functional restoration is associated with improvements in the histology of the cavernous body, and an increased expression of VEC markers such as VE-cadherin and endothelial nitric oxide synthase. In a KO model of AM, the effect of AM-null ASCs on erectile dysfunction was significantly diminished. Furthermore, an overexpression of AM in the same cells significantly improved erectile function in the diabetic rats [144]. These results suggest that AM secreted by ASCs plays a main role in the restoration of erectile function. ASCs have also been used for the regeneration and repair of infarcted heart but its efficiency is under debate. The major problem is the poor survival rate of implanted cells. Overcoming this problem may improve stem cell therapy [145]. As AM is a factor that improves the survival in most cells, one might think of it as a way to improve ASC-induced cardiac regeneration. However, the role of AM in proliferation or apoptosis of ASCs has not been tested yet.

4.5. Adrenomedullin and Hematopoietic Stem and Progenitor Cells. Hematopoietic stem cells (HSCs) possess the unique capacity to undergo self-renewal *in vivo* throughout the life of an individual while also providing the complete repertoire of mature hematopoietic and immune cells.

Previous attempts to identify the soluble factors that regulate HSC self-renewal have been poorly successful. Chute et al. [146] demonstrated that primary human brain endothelial cells (HUBECs) support the *ex vivo* amplification of primitive human bone marrow and cord blood cells and they analyzed the gene expression of HUBEC trying to find factors with hematopoietic activity. Functional analyses demonstrated that AM synergizes with stem cell factor and Flt-3 ligand to induce the proliferation of primitive human CD34+CD38-lin-cells and promotes the expansion of CD34+ progenitors in culture.

Apart from bone marrow, cord blood is an important source of HSCs and an alternative for allogenic transplantation. AM, which is also expressed by cord hematopoietic stem cells, has been used in combination with endothelin-1 to magnify the cord blood hematopoietic cell middle-term expansion. This effect seems to be mediated by the CLR/RAMP system [147]. AM may be used for improving the expansion of the HSC from the cord blood, which is of great importance for tissue engineering and clinical use.

In an elastase-induced emphysema mouse model, AM infusion significantly inhibited the increase in lung volume and static lung compliance. AM also increased the number of mononuclear cells, stem cell antigen-1-positive cells in circulating blood, and the number of bone-marrow-derived cells incorporated into the elastase-treated lung [148]. Addition of AM to cultured alveolar epithelial cells and endothelial cells also attenuates elastase-induced cell death [148].

4.6. Adrenomedullin and Adrenocortical Stem Cells. The theory that adrenocortical homeostasis is maintained by undifferentiated stem or progenitor cells can be traced back nearly a century. It is thought that these rare stem cells remain relatively undifferentiated and quiescent until needed to replenish the organ. Some experiments suggest that the adrenocortical progenitors reside in the outer periphery of the adrenal gland [149]. Early microscopic studies of the adrenal glands of different vertebrates suggest the presence of peripheral undifferentiated cells containing conspicuously large euchromatin-rich nuclei. In addition, the restricted circumferential expression of preadipocyte factor 1 (Pref-1) in the glomerulosa of the rat supports the hypothesis that the glomerulosa layer contains cells on different degrees of differentiation. Moreover, experiments using radioactive thymidine indicate that subcapsular cells of the rat adrenal replenish the neighboring steroidogenic zones, suggesting that this population provides a pool of progenitors that serve to maintain the functional capacity of the cortex [149].

AM gene expression changes during rat adrenal regeneration after enucleation and contralateral adrenalectomy [150]. AM mRNA expression suffers a marked rise between days 1 and 5 of the regeneration. During the early stages of regeneration, local stem cells are exposed to relative hypoxia and enucleation induces an inflammatory response. Both hypoxia, via HIF, and inflammation, through NO pathways, are known to upregulate AM gene expression [62, 151]. The increase in AM gene transcription and translation may be considered one of the early events in the enucleation-induced activation of local adrenocortical stem cells. Furthermore, the two AM receptors complex, CLR/RAMP2 and CLR/RAMP3, are upregulated in enucleated adrenals, perhaps as a response to the increased local production of AM [152]. The concerted increase in AM and its receptors expression might improve the autocrine/paracrine mechanisms by which AM enhances proliferation of zona glomerulosa stem cells during adrenal regeneration.

5. Adrenomedullin and Neural Stem Cells (NSCs)

The dogmatic view of an ever-immutable neural tissue in mammals is now been replaced by the notion that cell turn over, including neurons, does occur in the mature CNS throughout the adulthood, thanks to the persistence of precursor cells that possess the functional characteristics of neural stem cells (NSCs) within restricted brain areas [10]. The generation of a particular class of neuron or glial cell from a multipotent progenitor is a complex process that can be subdivided into a series of sequential steps. First, progenitor cells acquire unique positional identities through a process of spatial patterning. Multipotent progenitors

produce daughter progenitor cells that are restricted to produce only one of the primary neural cell types, neurons, oligodendrocytes, or astrocytes [10]. Committed neuronal progenitors also become specialized to produce neurons of a particular kind. Neural progenitors stop dividing, migrate towards more differentiated areas, and then initiate a programme of terminal differentiation [153].

In the adult mammalian brain, neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles [10, 154]. The SVZ is the adult brain region with the highest neurogenic rate and it represents a remnant of the embryonic germinal neuroepithelium. From the SVZ, newly generated neurons reach their destination in the olfactory bulb (OB) after a long migration through the rostral migratory stream [10]. Neurons born in the adult SGZ migrate into the granule cell layer of the dentate gyrus and become dentate granule cells [154]. Neurogenesis declines with aging in both the SVZ and the SGZ [154].

Postnatal neurogenesis is subject to tight regulation, confined to isolated microenvironments, and is sensitive to neuronal activity, stress and aging. This control may be required to prevent network instability, maintain memory and behavioral patterns, and prevent tumorigenesis. Numerous researches are trying to find the molecular mechanisms that regulate NSC self-renewal, proliferation, progressive maturation, and terminal differentiation. These studies have identified many growth factors, cell signals, and transcription factors, which regulate neurogenesis [11]. After intracerebral administration of growth factors such as EGF, FGF2, or TGF-α, cell proliferation in the SVZ is dramatically increased and the progeny fate can change depending on the type of factor used [10]. Cultured neurospheres (Figure 1) synthesize FGF2/EGF which regulate endogenous cytokines that participate in the growth and differentiation of the neurosphere cells [155]. Some angiogenic proteins with neurotrophic effects, such as VEGF and AM, have been shown to stimulate proliferation and neuroblast production in the SVZ, while IGF-1 promotes proliferation and migration in the same niche [11]. Neurogenesis also responds to other cell signals such as neurotransmitters. While GABA and glutamate may not control the fate commitment of stem cells or progenitor cells, they could control the synthesis and release of other diffusible molecules such as growth factors. In addition, they can control the expression of transcription factors and other intracellular molecules such as microRNAs [11]. Emerging epigenetic mechanisms are critical for orchestrating nearly every aspect of neural development and homeostasis, including brain patterning, neural stem cell maintenance, neurogenesis and gliogenesis, neural subtype specification, and synaptic and neural network connectivity and plasticity [156]. Recent evidence indicates that combinations of transcription factors of the homeodomain (HD) and basic helix-loop-helix (bHLH) families establish molecular codes that determine, both where and when the different kinds of neurons and glial cells generated [157].

NSCs differentiate into three distinct cell types as seen above: neurons, astrocytes, and oligodendrocytes [10].

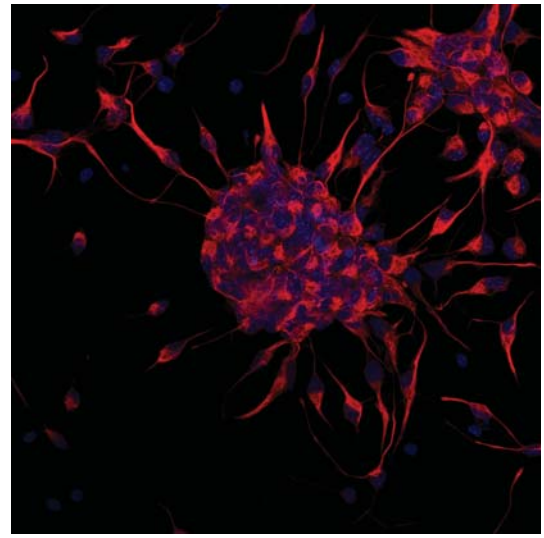

Figure 1: Confocal microscopy image of a neurosphere. Cells were stained with an antibody against nestin (red), a marker of stem cells, and counterstained with DAPI which labels the nuclei in blue.

The proportion of each lineage varies considerably depending on internal and external cues.

The generation of different cell types depends first on the presence of proneural factors, which integrate spatial and temporal cues and transform this information into neuronal subtype-specific differentiation programmes [157]. A number of progenitor proteins have been shown to select the cell types produced by progenitors of different domains in the neural tube. Among the best studied are the HD proteins Pax6 and Nk2 homeobox 2, as well as the bHLH protein oligodendrocyte transcription factor 2 (Olig2) [158]. Pax6 expression in mouse spinal cord progenitors induces the formation of neurons whereas the loss of Pax6 results in reduced neurogenesis and increased oligodendrocyte and astrocyte formation [158]. Loss of Olig2 in the mouse spinal cord results in the absence of both motor neurons and oligodendrocytes [158]. Once a progenitor cell has acquired a particular neural or glial identity, the next step is to arrest cell division and to initiate a programme of terminal differentiation [157]. Transcription factors of the bHLH family have a main role in the differentiation of neural progenitors into neurons [106]. The expression of proneural bHLH proteins, that in the mouse include Mash1, Ngn1-3, and Math1, is both necessary and sufficient to enhance the generation of differentiated neurons. Ngn2 expression in proliferating neuronal progenitors in the hippocampus support the idea that it plays a significantly role in adult neurogenesis [154]. The expression of Mash1 or Ngn2 in forebrain progenitor cells promotes the generation of GABAergic and glutamatergic neurons respectively [159]. The downstream effectors of proneural genes in the telencephalon include members of the Notch and Wnt pathways, adhesion proteins, and transduction factors [160]. Proneural genes also control later aspects of the neurogenesis process, including the arrest of progenitor division, migration of

newborn neurons and terminal differentiation [157]. On the other hand, a key step in the switch of neural progenitors to gliogenesis is the induction of Sox9 (a target gene of Ngn2 [161]) and NFIA, two proteins that promote astroglial and oligodendroglial fates (Figure 2). The choice between astroglial or oligodendroglial fates is controlled by different transcription factors. The progenitor proteins Olig2 and Nkx2.2 promote oligodendrogenesis and inhibit astrogenesis [161]. The proneural proteins Mash1 is also expressed by a subset of oligodendrocyte precursors as soon as they are generated. Using transgenic mice, it has been proven that Mash1 is required for the generation of an early population of oligodendrocyte precursors in the ventral forebrain [162]. The bHLH protein SCL Tal1 has the opposite role of promoting astrogenesis by inducing astrocyte precursor-specific genes and inhibiting oligodendrogenesis via repression of Olig2 [163].

External cues can also modify the proportion of each lineage that is produced from the undifferentiated progenitor cells. One of these external factors is AM. The NSCs located in the OB, known as olfactory bulb stem cells (OBSCs) [164, 165], retain properties of stem cells and they can self-renew and generate neurons, astrocytes, and oligodendrocytes [166, 167]. Lack of AM results in profound changes in the proliferation rate and differentiation in the progeny of OBSCs [168]. The progeny of stem/progenitor cells isolated from the OB of AM-null mice contains a lower proportions of neurons and astrocytes and a higher proportions of oligodendrocytes than cells from WT animals. *In vitro*, this effect can be partially reversed by the addition of synthetic AM to the culture medium. These data indicate the existence of a molecular switch where AM signalling stimulates neural precursors to generate either neurons and astrocytes or oligodendrocytes [168].

Otaegi et al. [169] found that the PI3K/Akt pathway is involved in OBSC differentiation into neurons and astrocytes. As AM signalling involves the activation of Akt [170], it is possible that AM regulates the proportion of OBSC progeny through this mechanism.

Hypoxia, via HIF-1, is known to upregulate AM gene expression [151] and, recently, it has been described that physiologic hypoxic conditions, which stimulates HIF-1 production, could strongly influence the growth of neural stem cells and their differentiation mechanisms both *in vivo* and *in vitro* [171]. Lowered oxygen tension enhances dopaminergic differentiation and survival of NSCs in a human ventral mesencephalic stem cell line. AM gene expression may be influenced by HIF-1 during NSCs growth and differentiation suggesting a possible role for AM in this process.

Furthermore, the KO mice for AM in the CNS shows morphological changes in some areas of the brain with tubulin hyperpolymerization and an increase on Glu-tubulin immunoreactivity [56]. These data can be explained because interactions between AM and several microtubule-associated proteins (MAPs) and between PAMP and tubulin have been found [172]. Downregulation of the gene coding for both, AM and PAMP, through small interfering RNA technology results in morphological changes, microtubule stabilization, increase in posttranslational modifications of tubulin, reduction of cell motility, and partial arrest at the G2 phase of the cell cycle [172]. Cell cycle and cell migration, two cell features that require the intervention of the cytoskeleton, are very important events for all stem cells and, therefore, a defective cytoskeleton may endanger the features and functions of stem cells. Moreover, the cytoskeleton is a main point in the formation and maintenance of mature neural cells morphology and dendritic processes, and its defective function may alter both the morphology and physiology of nerve cells [173]. Although membrane receptors activated by AM and/or PAMP cannot be completely excluded, these data suggest that both peptides may have an intracellular contribution to cell growth regulation by allowing cells to pass through mitosis [172]. The AM gene-null cells show important changes in their cytoskeleton, with tubulin hyperpolymerization and changes in the actin cytoskeleton [168]. Modifications in the tubulin and actin cytoskeleton may lead to profound changes in the morphology of the stem/progenitor cells which show modifications in the cell shape and they display abundant filopodia [168].

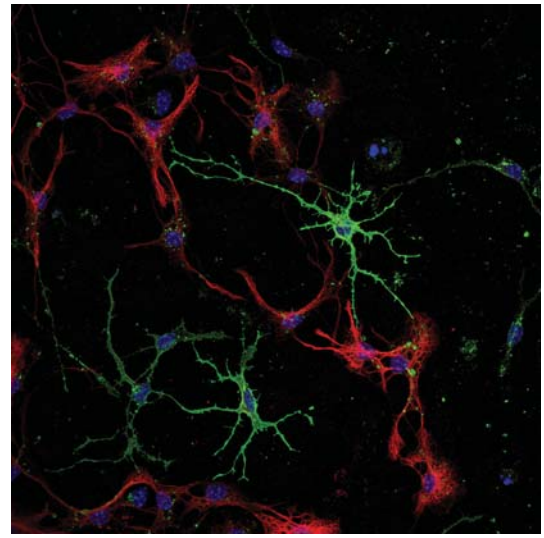

Figure 2: Confocal microscopy image of astrocytes and oligodendrocytes obtained from OBSC. Oligodendrocytes are stained with marker O4 (green) and astrocytes with glial fibrillary acidic protein (GFAP) (red). Cell nuclei are stained with DAPI (blue).

Surprisingly, there are not apparent morphological changes in neurons or astrocytes derived from neurospheres lacking AM and PAMP, but the oligodendrocytes present serious modifications with shorter and less numerous cell processes. Both microtubules and microfilaments are extremely important for oligodendrocyte morphogenesis, and the differences observed in the cytoskeleton of the stem cells may be responsible for the immature phenotype described for oligodendrocytes lacking the AM gene [168]. The morphological and proliferative characteristics of the AM KO cells do not revert when synthetic AM is added to the culture medium, in contrast with what occurs with the percentage of cells of the three lineages produced by the neurospheres [168]. This suggests the existence

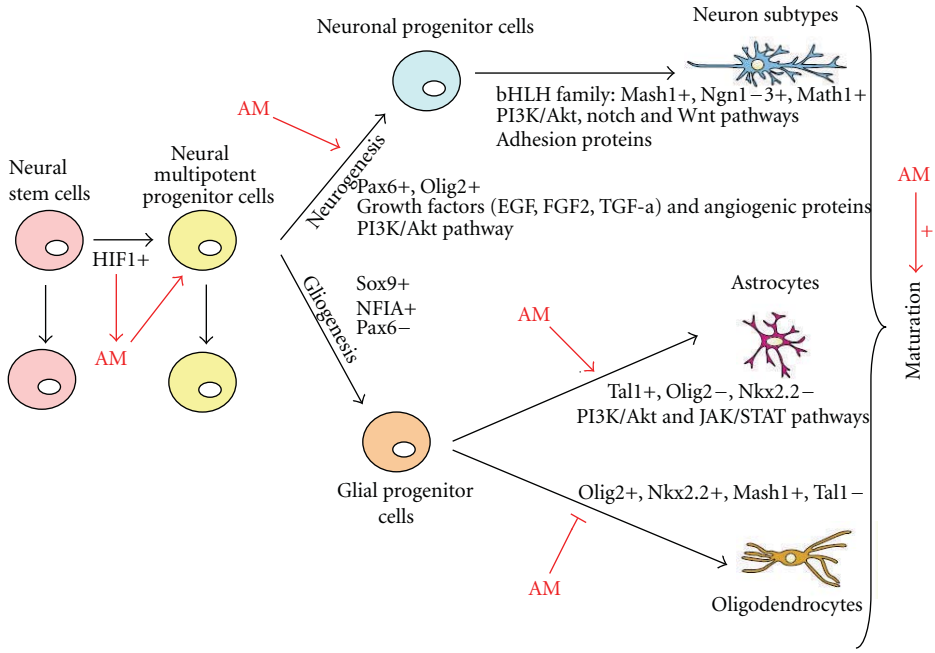

Figure 3: Schematic cartoon of the potential mechanisms of action of AM in NSC. AM increases the proliferation of neural multipotent progenitor cells probably through the activation of the transcription factor HIF-1. In addition, AM regulates the differentiation rate of progenitor cells into neurons, astrocytes, or oligodendrocytes. Some of these actions may be mediated by the interaction of AM and PAMP with the cytoskeleton.

of distinct mechanisms regulating AM-mediated stem cell differentiation and morphology. Cell fate may be mediated through the membrane receptor and the activation of the PI3K/Akt pathway, whereas morphological features and cell cycle might be more dependent on the intracellular pool of these peptides.

Taken together, these data demonstrate that AM is an important factor in regulating the proliferation and differentiation of adult NSCs or adult neural progenitor cells (Figure 3), and that AM might be used to modulate stem cell renewal and fate in an attempt to produce and control neural stem cells for regenerative therapies.

6. Concluding Remarks

AM and its receptors are widely distributed throughout the CNS. AM regulates some properties of the blood-brain barrier, increases preganglionic sympathetic discharge, exerts neuroprotective actions in the brain against stroke damage, and regulates fluid balance and electrolyte homeostasis when centrally administered. Recently, it has been reported that AM can organize the neuroendocrine response to stress and play a role in nociception.

These facts and the idea that AM acts as a growth factor and as cell fate determinant for a number of stem and progenitor cells lead us to hypothesize that AM may regulate NSC growth and differentiation.

In this paper we have shown that AM can modify the proportion of each lineage of neurons, astrocytes, and oligodendrocytes produced from undifferentiated progenitor cells, probably through the membrane receptors for AM and the activation of the PI3K/Akt pathway. Furthermore, both AM and PAMP may be able to influence NSC proliferation, growth, and maturation through interactions with the cytoskeleton.

AM could be used for the regulation of growth and differentiation of neural cells derived from neural progenitors as a step towards their potential therapeutic applications in the treatment of a number of neurodegenerative diseases.

Acknowledgment

This study was supported by a Grant from Spain's Ministry of Science and Innovation, SAF-2009-13240-C02-01.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 141624, 8 pages
doi:10.1155/2012/141624

Review Article

Bench to Bedside of Neural Stem Cell in Traumatic Brain Injury

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Received 1 June 2012; Revised 10 August 2012; Accepted 21 August 2012

Academic Editor: Rocio E. Gonzalez-Castaneda

Traumatic brain injury (TBI) is one of the leading causes of major disability and death worldwide. Neural stem cells (NSCs) have recently been shown to contribute to the cellular remodelling that occurs following TBI and attention has been drawn to the area of neural stem cell as possible therapy for TBI. The NSCs may play an important role in the treatment of TBI by replacing the damaged cells and eventual remyelination. This paper summarized a critical assessment of recent data and developed a view comprising of six points to possible quality translation of NSCs in TBI.

1. Introduction

Traumatic brain injury (TBI) has remained a major cause of mortality, morbidity and leading cause of large-scale disabilities worldwide. TBI results in a large number of deaths and a cause of permanent disabilities with enormous losses to individuals, families, and communities [1]. World Health Organization (WHO), in 2004, has estimated that 25% of road traffic collisions requiring admission to a hospital suffered TBI [1–3].

Moreover, WHO has introduced the new metric tool, the disability-adjusted life year (DALY), which quantifies the burden of diseases, injuries and risk factors. The worldwide leading causes of TBI include road traffic accidents that were estimated being 41.2 million DALYs in 2008, violence being responsible for 21.7 million DALYs, and self-inflicted injuries being 19.6 million DALYs, respectively. All these will leave disability associated with TBI in survivors [2, 3].

However, no effective therapy or program is available for treatment of individuals with TBI; nonetheless, researchers had tried some therapeutic agents like levodopa/carbidopa and some neurotrophic factors in brain injury with persistent vegetative state with the aim of augmenting and slowing the progression from persistent vegetative state into some degree of consciousness. This still needs experimentation to confirm if these dopamine precursors and other neurotrophic factors have any role in TBI. Several other therapeutic agents like cannabinoid dexanabinol, erythropoietin, and gamma-glutamylcysteine ethyl ester have all shown to have neuroprotective effect in human at experimental stage with remarkable improvement in post-TBI outcome [4–8].

Recently, more attention has been drawn to the area of stem cell therapy, largely due to advanced knowledge about stem cells. The stem cells may play an important role in the treatment of TBI by replacing damaged cells, and helping functional recovery. The search for stem cell therapy for TBI is progressing. Since the pathophysiology of TBI is largely unknown, it makes a search for an effective stem cell therapy difficult. This is because multiple cell types like neuronal cells, glial, and endothelial cells are usually involved in TBI. Furthermore, cerebral vasculature, especially the blood brain barrier (BBB), may be affected in TBI; this injury may be focal or diffuse axonal injury (DAI). Taming these burgeoning effects of TBI will require NSCs which can differentiate into neurons and glial cells. It has been reported that progenitor cells differentiated into neurons and glial in adult brain, and an increase in astrocytic progeny is forming reactive astrocytes to primarily limit cyst enlargement in posttraumatic syringomyelia [9–12].

This review is an optional extra to see if we can achieve the translation of basic knowledge of neural stem cells into therapeutic options in persons with TBI by enhancing and integrating these neural progenitor cells (NPCs) unto neurogenesis and directing these cells to the specified targets

or through multipotency where the transplanted cells can differentiate into glial cells, neurons, and endothelial cells, as the injuries are not always selective but diffuse and we may need to induce these transplanted cells into appropriate phenotype. This is a critical review of existing current literature on neural stem cell research and proposing an approach for quality clinical translation in TBI. We will look at the pathophysiology of TBI and proposing the "six-point schematic approach" to achieve standard and quality bench to bedside in neural stem cell of TBI. We also highlighted the need for suitable clinical translation, coordination, and administration of research in the field of neural stem cell therapy of TBI.

2. Pathophysiology of TBI

Pathophysiology of TBI involves two main phases: these are primary injury following the trauma, and the secondary injury which is mediated by inflammatory response to trauma.

2.1. Primary Injury. Pathophysiology of initial injury has been postulated to include acceleration, deceleration, and rotational forces which may or may not be as a result of the trauma. This flow of events leads to initiation of inertia which is both acceleration and rotational head movements. This impact on the cortical and subcortical brain structures causes focal or diffuse axonal injury (DAI) and these inertial forces will disrupt the BBB [13]. The primary events also involve massive ionic influx referred to as traumatic depolarization. The major inflammatory neurotransmitters released are excitatory amino acids. This may explain the pathophysiology of DAI in TBI. This is followed by cerebral edema with associated increase in intracranial pressure, which usually forms the major immediate consequences of TBI. Brain edema may come from astrocyte swelling and disruption of the BBB [14, 15]. The BBB is disrupted in acute phase of severe TBI. The expression of high levels of glucose transporter 1 (GLUT 1) was observed in capillaries from acutely injured brain, which occurs in association with compromised BBB function. Vascular endothelial growth factor also plays a role in neuronal tissue disruption and increases the permeability of the BBB via the synthesis and release of nitric oxide [16]. Figure 1 depicts the pathophysiology of the primary injury.

2.2. Secondary Injury. The secondary events are a complex association of the inflammatory response initiated by the trauma leading to diffuse neuronal degeneration of neurons, glial, axonal tearing, and genetic predisposition (Figure 2). Furthermore, excitatory amino acid release, oxygen radical reactions, and nitric oxide production will lead to activation of N-Methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA), alpha-7 nicotinic receptor ($\alpha 7$), and nicotinic acetylcholine receptor (nACR) [17–19] and subsequent calcium influx. All these cascades of events will cause mitochondrial disruption and free radical release with eventual tissue peroxidation. One theory is that excitatory amino acid release leads to calcium influx into neurons and other brain cells which promote oxygen-free radical reactions. High calcium and the presence of free-radical molecules create an unstable environment in the cell that may lead to increased production and release of nitric oxide and excitatory amino acids (e.g., glutamate). Nitric oxide may participate in oxygen radical reactions and lipid peroxidation in neighboring cells [20]. A summary is shown in Figure 2. The secondary injury plays a major role in the outcome of TBI. Therapeutic interventions should target this phase as it is the major determinant of morbidity and mortality in TBI [16]. Genes implicated to influence the outcome of TBI include *apoe*. *Apoe* multifactorially affects the clinicopathological consequences of TBI [21]. *Apoe* is associated with increased amyloid deposition, amyloid angiopathy, larger intracranial hematomas, and more severe contusional injury. *Comt* and *drd2* are genes which may influence dopamine-dependent cognitive processes, such as executive or frontal lobe functions. The *ace* gene may affect TBI outcome *via* alteration of cerebral blood flow and/or autoregulation and the *cacna1a* gene may exert an influence *via* the calcium channel pathways and its effect on delayed cerebral edema [22]. Increased signal transducers and activator of transcription (STAT) 3 signaling has been reported in a rat model of TBI [23]. Although several potential genes that may influence the outcomes following TBI have been identified, future investigations are needed to validate these genetic studies and identify new genes that might contribute to the outcomes following TBI.

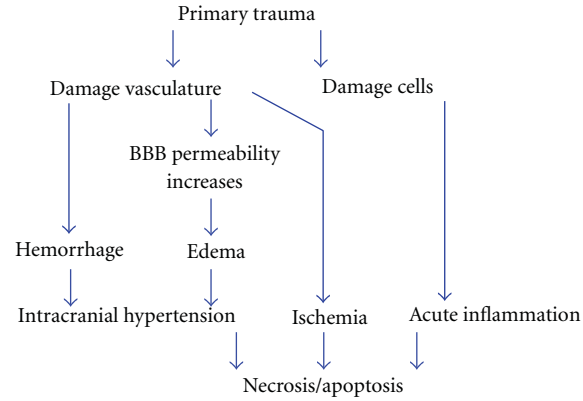

Figure 1: Sequential events of primary injury in TBI. Initial impact is usually by directing trauma to the head either open or closed head injury. This trauma will cause mechanical damage to neurons, axons, glia, and blood vessels by shearing, tearing, or stretching. Blood vessel ruptures cause hemorrhage. Even in unruptured blood vessels, BBB permeability increases resulting in edema. Hemorrhage and edema often lead to intracranial hypertension. Following hemorrhage, ischemia could occur in brain tissue. TBI-caused cell damage induces macrophage and lymphocytes migrant to the injury site releasing inflammatory mediators that triggers a cascade of events towards necrosis and/or apoptosis. Necrosis and/or apoptosis also can be a consequence of hemorrhage and ischemia.

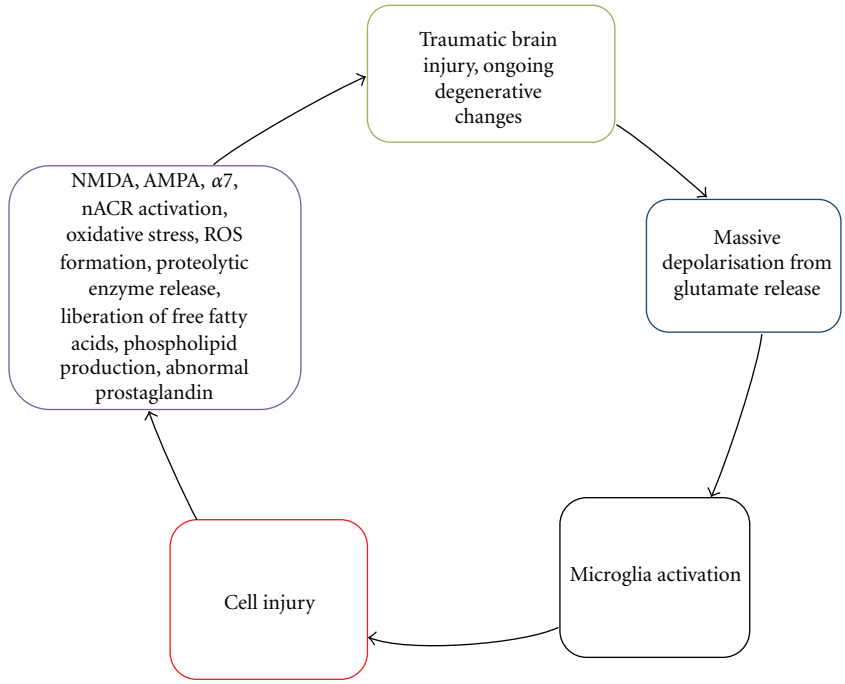

Figure 2: Sequential events of secondary injury in TBI. This includes variety of processes such as depolarization, disruption of ionic homeostasis and release of neurotransmitters, lipid degradation, and oxidative stress. These events are a result of interaction between the excitatory amino acid released with an influx of oxygen-free radicals that ultimately set up NMDA, AMPA, $\alpha 7$, and nACR to sustain the unstable environment for cell injury and degenerative changes.

3. Application of NSCs in TBI

There are at least two possible strategies involving neural stem cells (NSCs) to repair injured brain. They are transplantation of exogenous NSCs and stimulation of endogenous NSCs.

3.1. Transplantation of Exogenous NSCs. There have been attempts to transplant various types of cells, such as neurons and neural stem cells to repair damaged brain. The main objectives of these transplantation experiments are (1) growth facilitation: the transplant fills the lesion site and serves as a cellular bridge; (2) new neurons: the transplant can provide new neurons, which in turn provide new targets and sources of innervations and thus repair the damaged neural circuits; (3) factor secretion: the transplant can produce a variety of substances, such as neurotrophic factors, that may aid in the repair process [24]. Several characteristics of NSCs make them potentially suitable for repair after TBI. Firstly, they can serve as a renewable supply of transplantable cells by clonally expansion in culture. Secondly, they are of CNS origin and the cells generated from the grafts have neural characteristics. Thirdly, NSCs can be manipulated by genetic engineering methods to produce specific proteins, such as neurotrophins, neurotransmitters, and enzymes [25].

It has been reported that autologous-cultured cells harvested at time of emergency surgery from patients with TBI and subsequently engrafted into damaged part of the brain can be detected using MRI [26]. The efficacy of transplantation largely depends on a grafting method that optimizes the survival of the transplanted cells and minimizes the graft-induced lesion. Most transplantation studies involved intraparenchymal injection into the CNS, in which cells were grafted directly into or adjacent to the lesion [27–29]. The optimal time for transplantation may not be immediately after injury. The levels of various inflammatory cytokines (TNFα, IL-1α, IL-1β, and IL-6) in the injured brain peak 6–12 hours after injury remain elevated until the 4th day. Although these inflammatory cytokines are known to have both neurotoxic and neurotrophic actions, they are believed to be neurotoxic within a week after injury, which causes the microenvironment to be unsuitable for survival of the grafted cells [30]. However, if too much time passes after the injury, glial scar forms a barrier around the lesion site and inhibits local blood circulation which is needed for graft survival. Thus, it is considered that those 7 to 14 days after injury are the optimal time for transplantation [31, 32].

3.2. Stimulation of Endogenous NPCs. Since the description of endogenous neurogenesis in adult brain by Luskin in 1997 [33] and Alvarez-Buylla and co-workers in 2000 [34], several publications have confirmed their findings. They demonstrated the presence of NSCs in adult rodent ventricular zone (VZ) that migrated to the olfactory bulb and integrated into the neuronal network called the rostral migratory stream (RMS).

However, the potential success of stimulating endogenous NPCs is hinged on delivery of various growth factors. More so, this seems to be the most common way

to stimulate NPCs. The following growth factors have been reported: EGF, FGF-2 [35–37], bFGF [38], aFGF [39], BDNF [40], NGF, NT-3 [40, 41], VEGF [42], GDNF [43], IGF-1 [42], and SDF-1 alpha [44]. They were administrated by intraventricular [35], intraparenchymal [40, 42, 45] or intrathecal [36–38, 43] injection. They were reported not only to enhance the proliferation, migration, and gliogenesis of NPCs [35–37, 44] but also to protect the spinal cord from further damage [41, 42]. In addition, these growth factors facilitated the regrowth of axons and remyelination [39, 40, 46]. Functional recovery was also reported after they were delivered into injured spinal cord [35–37, 39]. However, the details of functionary recovery are still not clear.

Not only growth factors, other molecules, were shown to stimulate endogenous NPCs. Proliferation of endogenous NPCs was demonstrated when the sodium channel blocker tetrodotoxin and the glycoprotein molecule sonic hedgehog were injected into the parenchyma [47, 48]. Imitola and colleagues reported that cognate chemokine receptor type 4 (CXCR4) expressed by NSCs can regulate their proliferation and direct their migration towards the injury site [44]. In addition, antibodies blocking IL-6 receptors were reported to not only inhibit differentiation of endogenous NSCs into astroglia *in vivo* and *in vitro*, but also to promote functionary recovery [49, 50]. Okano and colleagues assumed that the functionary recovery is probably due to blocking IL-6 and consequently inhibiting the formation of glial scars and promoting axonal regeneration [49, 51]. Notably, studies of ATP-binding cassette (ABC) transporters have emerged as a new field of investigation. ABC transporters (especially ABCA2, ABCA3, ABCB1, and ABCG2) are found to play an important role in proliferation and differentiation of NSCs [45, 52–56].

In contrast to transplantation of exogenous NPCs, stimulation of endogenous NPCs to repair damaged spinal cord has three main advantages: (1) there is no ethical issue of embryonic and foetal cells, (2) it is usually less invasive, and (3) no immunogenicity; it avoids immunorejection that observed in transplantation of exogenous NPCs [57].

Like adult NPCs transplantation studies in SCI, no neurogenesis has been reported from the stimulation of endogenous NPCs. Yamamoto and colleagues reported that lack of neuronal differentiation is related to upregulation of the Notch signal pathways [58]. The increased level of various cytokines within the microenviroment surrounding the area of injury may also cause a lack of trophic support for differentiation into neuronal lineage [59–62].

Recently, more attention has been drawn to CBP/p300-phosphorylated Smad complex. It was found that CBP/p300-phosphorylated Smad complex can be bound in NSCs, which may decide the differentiation of NSCs. If the complex is bound with phosphorylated STAT 3, the NSCs differentiate into astroglia lineage cells. On the other hand, if the complex is bound with proneural-type of the basic helix-loop-helix (bHLH) factor, such as neurogenin 1 and 2, they differentiate into the neuronal lineage [51, 63, 64]. Apart from that, Peveny and Placzek reported that *SOX* gene may also play an important role in neural differentiation [65].

Once NSCs decide to differentiate into neuronal lineage, a cascade of hundreds of genes is regulated over time to lead the immature neuron into its mature phenotype. Many of these neural genes are controlled by RE1-silencing transcription factor (REST). REST acts as a repressor of neural genes in nonneural cells, while regulation of REST activates large networks of genes required for neural differentiation [66–68].

4. Bench to Bedside Translation of Stem Cell Therapy

The main purpose of scientific studies is to put our discoveries into daily clinical practice. The basic science laboratory takes its observations obtained at cellular or molecular levels in a cutting edge condition and implements this into acceptable practice clinically to the benefit of the public. However, this is always met with a lot of challenges, such as ethics, governmental regulations, funding constraints, paucity of adequate collaboration among clinical and basic science, and the challenges of conducting a clinical study.

The authors, nonetheless, propose six-point schema for improving bench to bedside translation of stem cell therapy (Figure 3(a)) involving a rigorous network of six stakeholders: basic researchers, pharmaceutical companies, patient or general public participating in clinical trials, regulatory bodies or agencies for grant approval, collaborative research between basic and clinical scientist with the plan of developing biomarkers for potential drug targets, and creating a concerted network of groups that identifies some of the medical problems relating to TBI. We are still faced with the need to formulate hypothesis both at experimental and clinical epidemiologic levels and implementing these into clinical practice while the translational researcher serves to collaborate and coordinate all these strategies.

Indeed, communication and dissemination (Figure 3(b)) which are patient centeredness will not only impact on the public, but will also help to tame the ethical problems in this field. Communication will involve both patients and other clinicians involved in conducting randomized clinical trials (RCTs). With strong feedback on outcomes, pharmacovigilance, and health promotion, education of the populace in form of scientific advocacy is so paramount as this will impact on improved scientific collaboration, quality public control, and increased transparency among researchers and may improve funding of research work [69].

Research in neural stem cell is still a grey area and much knowledge needs to be gained, to actually close the gaps. There is inadequate understanding of secondary injury process, insufficient preclinical testing in diffuse axonal injury models, species differences, and lack of understanding of the mechanism of drug-receptor interactions. Smith and colleagues had suggested the need to use gyrencephalic models for proper translation of TBI [70]. There is need for increased linkages and networking between academician, researchers, and clinicians for greater reward of what is being generated.

Methodological disparities between experimental models of TBI and clinical studies cannot be overemphasized.

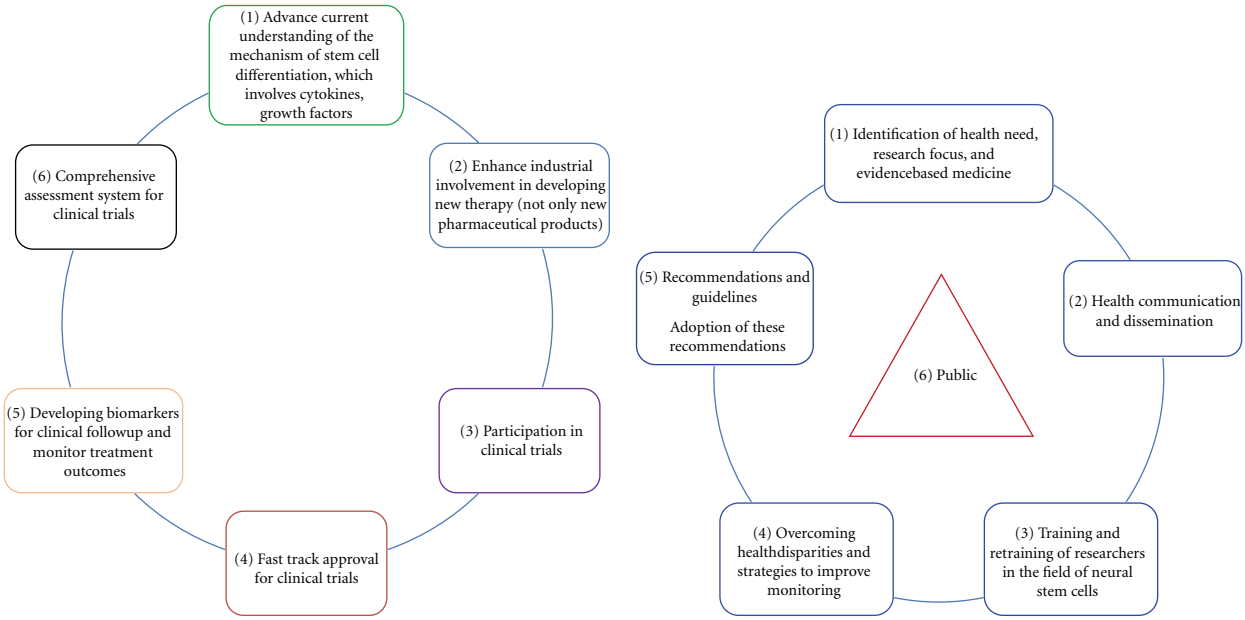

Figure 3: (a) Proposed schema for effective translation involving concerted effort of multilevel strategies of six main stakeholders. (b) Proposed framework for the reinforcement of the multi-level strategies effective bench to bedside translation of NSCs in TBI.

The intent to treat models, differences in statistical analysis as a result of differences in sample size, and different behaviours between human and animals. Injury severities in animals differ from humans; while they are well defined in animals, they could take any direction in human. The need to improve study quality score has recently being called for by stroke therapy academic industry roundtable (STAIR), which was recently updated and this includes the following recommendations: (1) elimination of randomizations and assessment bias, (2) use of a priori definitions of inclusion/exclusion criteria, (3) inclusion of appropriate power and sample size calculations, (4) full disclosure of potential conflict of interest, (5) evaluation of therapies in male and female animals across the spectrum of ages, and with comorbid conditions such as hypertension and/or diabetes. Furthermore, some researchers had also expanded on these proposed recommendations for improved clinical trials in brain injury with special focus on neuroprotective therapies in TBI [70, 71]. Nonadherence was the single most important determinant of trial failure in the past.

Finally, the International Mission on Prognosis and Clinical Trial Design in TBI (IMPACT) proposed ways of overcoming the above disparities and challenges. The recommendations include a robust inclusion criteria and recommendations for general research in TBI [70]. The six-point schema is an overview recommendation with the public, patient, or the society as the core and the fulcrum of all activities of research and if implemented may yield quality research outcome in neural stem cells translation in TBI.

5. Conclusions

Mortality and disability from TBI are projected to rise globally. Neural stem cell therapy is a strategy that offers hope in the future for treatment of brain injury. In addition, we are now able to monitor autologous neural stem cells *in vivo*, cell migration and clearly demonstrate that neural stem cells could selectively target injured brain or spinal cord tissue and undergo neurogenesis. Finally, the proposed six-points cyclical schema should be implemented with determined effort of all stakeholders for effective bench to bedside translation of neural stem cell therapy in TBI.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

S. O. Ugoya is a recipient of International Macquarie University Research Excellence Scholarship.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 430138, 5 pages
doi:10.1155/2012/430138

Research Article

Nestin Protein Is Phosphorylated in Adult Neural Stem/Progenitor Cells and Not Endothelial Progenitor Cells

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Received 1 July 2012; Accepted 3 August 2012

Academic Editor: Oscar Gonzalez-Perez

An intermediate filament protein, Nestin, is known as a neural stem/progenitor cell marker. It was shown to be required for the survival and self-renewal of neural stem cells according to the phenotypes of Nestin knockout mice. Nestin expression has also been reported in vascular endothelial cells, and we recently reported Nestin expression in proliferating endothelial progenitor cells, but not in mature endothelial cells. Using quantitative phosphoproteome analysis, we studied differences in phosphorylation levels between CNS Nestin in adult neural stem cells and vascular Nestin in adult bone-marrow-derived endothelial progenitor cells. We detected 495 phosphopeptides in the cell lysates of adult CNS stem/progenitor cells and identified 11 significant phosphorylated amino acid residues in the Nestin protein. In contrast, endothelial progenitor cells showed no significant phosphorylation of Nestin. We also measured neoplastic endothelial cells of the mouse brain and identified 13 phosphorylated amino acid residues in the Nestin protein. Among the 11 phosphorylated amino acids of adult CNS Nestin, five (S565, S570, S819, S883, and S886) were CNS Nestin-specific phosphorylation sites. Detection of the CNS-specific phosphorylation sites in Nestin, for example, by a phospho-specific Nestin antibody, may allow the expression of CNS Nestin to be distinguished from vascular Nestin.

1. Introduction

Nestin is a class VI intermediate filament protein expressed in undifferentiated central nervous system (CNS) cells during development. The protein is known as a neural stem/progenitor cell marker and required for the survival and self-renewal of neural stem cells (NSCs) [1]. Nestin expression is downregulated when CNS stem/progenitor cells differentiate into neurons or glial cells [2, 3], and the expression is kept in adult CNS stem/progenitor cells that reside in the forebrain neurogenic regions [4, 5]. Nestin expression has also been reported in vascular endothelial cells (ECs) from a variety of adult human non-CNS tissues [6, 7]. We recently reported Nestin expression in proliferating endothelial progenitor cells (EPCs), but not in mature ECs [8]. We utilized E/*nestin*:EGFP transgenic mice using its second intronic enhancer element to study neural-specific *nestin* gene expression [9, 10] and demonstrated that vascular *nestin* expression is not activated by the CNS-specific enhancer of the *nestin* gene [8]. This finding indicated that the Nestin expressed in EPCs is cytochemically similar to the protein expressed in CNS stem/progenitor cells, but the regulatory mechanism of gene expression is different.

The reversible phosphorylation of proteins results in a conformational change that alters their function. Many proteins, including cellular receptors, enzymes, and intracellular signaling molecules, are activated/deactivated by phosphorylation/dephosphorylation. Thus, reversible phosphorylation plays a significant role in the regulation of cellular processes. Nestin protein in the cytoplasm of CNS stem/progenitor cells is thought to play a role in distributing Vimentin from copolymerized intermediate filaments to daughter cells during cell division [11]. Elevated phosphorylation of Nestin

has been observed to accompany the mitotic reorganization of Nestin in an immortalized CNS precursor rat cell line [12]. Concerning the developing mouse brain, more than 500 phosphorylation sites on proteins, including Nestin, have been identified by phosphoproteomic analysis [13]. However, Nestin phosphorylation has not been investigated in adult NSCs. Using quantitative phosphoproteome analysis, we demonstrate in the present study that different phosphorylation levels are found among CNS Nestin in adult NSCs and vascular Nestin in adult bone-marrow-derived EPCs.

2. Materials and Methods

2.1. Animals. Adult (8 to 10 weeks old) wild-type C57BL/6J mice were purchased from SLC (Shizuoka, Japan). All animal-related procedures were approved by the Laboratory Animal Care and Use Committee of Keio University and conducted in accordance with the guidelines of the National Institutes of Health, USA.

2.2. Primary Neural Stem/Progenitor Cell Culture. Neurospheres were generated from adult mouse forebrain as described previously [8, 10, 14, 15]. Briefly, the striata from adult mice were dissected, incubated with trypsin solution for 15 min at 37°C, triturated, and then trypsin inhibitor solution added. Dissociated cells (5000 cells/mL) were seeded in neurosphere culture medium composed of DMEM-F12 (1 : 1), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (13.4 mM), HEPES (5 mM), insulin (25 μg/mL), transferrin (100 μg/mL), progesterone (20 nM), sodium selenate (30 nM), and putrescine (60 nM) supplemented with recombinant human epidermal growth factor (EGF, 20 ng/mL) and recombinant human basic fibroblast growth factor (bFGF, 20 ng/mL). Cells were cultured for 7 days *in vitro* (DIV) and formed floating cell clusters of neural stem/progenitor cells (neurospheres). Primary neurospheres were collected and mechanically triturated. Dissociated cells were counted and stored at −20°C.

2.3. EPC Culture. EPCs were cultured from mononuclear cells (MNCs) under previously reported culture conditions [8]. The femurs and tibias of adult mice were crushed, suspended in αMEM (#11900, Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G (10,000 units/mL) streptomycin sulfate (10,000 μg/mL) (PS), and filtered through a 70-μm filter (Cell Strainer #352350, Falcon, Bedford, MA). MNCs were isolated from bone marrow cells by Ficoll density-gradient centrifugation (Ficoll-Paque Plus, 1.077 g/mL, GE Healthcare, Uppsala, Sweden). Cells (1×10^6 cell/mL) were plated on fibronectin-coated 6-well plates (#140675, Nunc, Roskilde, Denmark) in endothelial basal medium supplemented with 5% FBS, vascular endothelial growth factor (VEGF), bFGF, recombinant analog of insulin-like growth factor-1 (R^3-IGF-1), EGF, hydrocortisone, ascorbic acid, and gentamicin/amphotericin-B (EGM-2-MV Bullet Kit CC-3202, Lonza, Walkersville, MD). The medium was changed after 24 hours to remove nonadherent cells and renewed every week. At 21 DIV, cells were lifted by incubation with 0.25% trypsin and 1 mM EDTA and then stored at −20°C.

Although a unique EPC marker has not been identified, EPCs are characterized as cells with a high proliferative potential that display typical endothelial characteristics and differentiate into ECs *in vitro* [16]. EPCs obtained under the above culture conditions were positive for the proliferation marker Ki67, positive for EC lineage marker CD31 and vascular endothelium cadherin or the uptake of 1,1′dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate Ac-LDL (DiI–Ac-LDL), negative for the mature EC marker von Willebrand factor (vWF), and capable of differentiating into mature ECs [8].

2.4. EC Line. To compare phosphorylation between CNS Nestin and vascular endothelial Nestin in proliferative cells further, we prepared neoplastic ECs. Cells from a mouse brain endothelioma cell line (bEnd.3 cells CRL-2299, ATCC, Manassas, VA) were characterized as proliferative endothelial cells expressing vascular Nestin similar to EPCs and positive for mature EC marker vWF [8].

The EC line was cultured according to the manufacturer's instructions. Briefly, cells were maintained in DMEM (#12699, Gibco Invitrogen) supplemented with 10% FBS and 1% PS. The medium was renewed every 3 to 4 days. Cells were harvested by incubation with 0.25% trypsin and 1 mM EDTA and stored at −20°C.

2.5. Quantitative Phosphoproteome Analysis. Cells were processed for phosphoproteome analysis based on mass spectrometry (MS) coupled with miniaturized on-line liquid chromatography (LC). Proteins were extracted from cells (100,000 cells from adult neurospheres; 1,000,000 cells from EPCs and the neoplastic ECs) using 12 mM sodium deoxycholate and 12 mM sodium lauroyl sarcosinate, and digested with Lys-C and trypsin [17]. Phosphopeptides were enriched by aliphatic hydroxy acid-modified metal oxide chromatography with titania [18] and analyzed by nanoLC-MS/MS using an LTQ-Orbitrap instrument (Thermo Fisher Scientific, Bremen, Germany). Peptides and proteins were identified using Mascot version 2.3 (Matrix Science, London) with the SwissProt database. Label-free quantitation was performed based on the peak areas of extracted ion chromatograms for identified phosphopeptides using Mass Navigator (Mitsui Knowledge Industry, Tokyo, Japan).

3. Results

3.1. Protein Phosphorylation of CNS Stem/Progenitor Cells, EPCs, and Neoplastic ECs. CNS stem/progenitor cells were obtained from striata of the lateral wall of the lateral ventricles in the adult mouse brain and grown as neurospheres *in vitro*. Phosphoproteome analysis detected 495 phosphopeptides in the cell lysates (Table 1). Approximately 90% of the peptides detected in neurosphere cells were phosphorylated. A similar percentage of phosphopeptides was measured in neoplastic ECs. However, approximately 60% of peptides in nonneoplastic proliferative endothelial cells,

Table 1: Protein phosphorylation of adult CNS stem/progenitor cells, EPCs, and neoplastic ECs.

	pPeptides (pPeptides/total peptides)	pSites (pSite/total pSites)			Multi-pPeptides (single or multi-pSite peptides/total pPeptides)		
		Serine	Threonine	Tyrosine	1p	2p	>2p
Neurospheres	495 (91.2%)	443 (84.2%)	71 (13.5%)	12 (2.3%)	401 (81.0%)	74 (14.9%)	20 (4.0%)
EPCs	250 ± 5 (59.7%)	194 ± 8 (87.4%)	20 ± 3 (9.0%)	8 ± 0 (3.6%)	228 ± 6 (91.0%)	19 ± 0 (7.4%)	4 ± 0 (1.6%)
Neoplastic ECs	980 (97.8%)	896 (88.1%)	103 (10.1%)	18 (1.8%)	675 (68.9%)	256 (26.1%)	49 (5.0%)

pPeptides, phosphopeptides; pSites, phosphorylation sites; multi-pPeptides, multi-phosphorylated peptides; 1p, single-phosphorylated site; 2p, two phosphorylated sites. Data are mean ± standard deviation.

Nestin phosphorylation		Cell samples		
Phosphorylated sites	Peptide sequence	Neurospheres	EPCs	Neoplastic ECs
S169	RPPAPAHASPIRAPEVEELAR	1.4.E + 05	0	2.4.E+ 05
S565, S570	ENCNSSIEENSGTVKSPEK	1.5.E + 04	0	0
S575	ENCNSSIEENSGTVKSPEK	0	3.1.E + 02	7.5.E + 04
S688	FPRSPEEDQQAFRPLEK	3.7.E + 03	0	1.9.E + 05
S728	ERQESLKSPEEEDQQAFR	1.8.E + 05	0	1.1.E + 06
S728, S731	ERQESLKSPEEEDQQAFR	9.9.E + 04	0	4.4.E + 05
S731	ERQESLKSPEEEDQQAFR	4.1.E + 05	0	6.2.E + 04
S813	ESQESLKSPEEEDQR	0	0	3.9.E + 04
S813, S816	LVEKESQESLKSPEEEDQR	0	0	1.2.E + 05
S819	ESQESLKSPEEEDQR	2.9.E + 04	0	0
S883, S886	VSQVSLESLEK	1.2.E + 05	0	0
S1010	SLEDESQETFGSLEK	1.6.E + 05	0	5.2.E + 05
S1216	SLGEVEWELPGSGSQQR	0	0	4.8.E + 04
S1562, S1565	SPASPKWEQAGEQR	0	0	5.1.E + 04
S1565	SPASPKWEQAGEQR	2.8.E + 05	0	6.1.E + 04
S1837	APLVGSPVHLGPSQPLK	1.1.E + 05	0	3.9.E + 05
S1860, S1861	FTLSGVDGDSWSSGED	0	0	1.5.E + 06

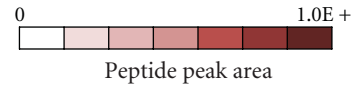

Figure 1: Quantitative phosphoproteome analysis of Nestin. A peak area of more than 1.E+04 indicates significant phosphorylation detected by MS/MS. pSites, phosphorylated sites of amino acid residues.

EPCs, were phosphorylated, indicating that intracellular and cell membrane proteins were activated overall in neurosphere cells compared to EPCs. Generally, phosphorylation occurs most commonly on serine, followed by threonine. More than 80% of phosphorylated sites were serine residues in our samples, and the ratio of phosphorylated amino acids was not different between neurosphere cells, EPCs, and neoplastic ECs (Table 1). We tabulated phosphorylated proteins and their phosphorylated amino acid residues in Figure 1 in Supplementary Material (available online at doi:10.1155/2012/430138).

3.2. Nestin Phosphorylation of CNS Stem/Progenitor Cells, EPCs, and Neoplastic ECs. Quantitative phosphoproteome analysis identified 10 phosphopeptides and 11 significant phosphorylated amino acid residues (a peak area >1.E + 04) in the Nestin protein from adult neurosphere cells (Figure 1). In contrast, EPCs derived from adult mouse bone marrow showed no significant phosphorylation of Nestin ($n = 2$). In neoplastic ECs, 13 significant phosphorylated amino acid residues were identified. Thus, the finding that Nestin is not phosphorylated in EPCs is not likely due to the tissue specificity of ECs. All phosphorylated amino acid residues found in neurosphere cells and neoplastic ECs were serine. Although phosphothreonine was reported in samples of Nestin from an immortalized rat cell line [12] and human HeLa cells (cervical carcinoma) [19, 20], we did not detect the phosphorylation of threonine residues in the Nestin proteins from our samples. Five of the phosphorylated amino acid residues (S565, S570, S819, S883, and S886) were detected in neurosphere cells only, eight (S575, S668, S813, S816, S1216, S1562, S1860, and S1861) were detected

in neoplastic ECs only, and six (S169, S728, S731, S1010, S1565, and S1837) were detected in both neurosphere cells and neoplastic ECs (Figure 1).

In CNS stem/progenitor cells, Nestin protein preferentially forms heterodimers and heterotetramers with a variety of intermediate filament proteins, particularly type III Vimentin and type IV α-Internexin [21, 22]. Phosphoproteome analysis detected phosphorylation of Vimentin but not α-Internexin from the samples of adult neurospheres, EPCs, and neoplastic ECs (Figure 1 in Supplementary Material).

4. Discussion

Recent investigations of *nestin*-knockout mice have reported that Nestin deficiency results in embryonic lethality with the neuroepithelium of the developing neural tube exhibiting low numbers of NSCs and high levels of apoptosis [1]. The downregulation of *nestin* in the embryonic cerebral cortex using small interference RNAs against *nestin* mRNA results in G1 cell-cycle arrest and a severe reduction in the generation of neurons [23]. However, no data have been reported on the *in vivo* function of adult Nestin. Transient transfection of *nestin*-non-expressing cells with an expression vector carrying rat *nestin* cDNA has been shown to promote the disassembly of phosphorylated Vimentin intermediate filaments in the cytoplasm during mitosis [11]. Our phosphoproteome analysis detected phosphorylated Vimentin in the adult neurosphere sample. Thus, Nestin in adult NSCs is likely to mediate the distribution of Vimentin protein to daughter cells during self-renewal and neurogenesis.

In a rat neuronal progenitor cell line, the mitotic reorganization of Nestin was accompanied by the elevated phosphorylation of Nestin, and T316 was identified as a Nestin phosphorylation site [12]. Phosphorylated threonine was not detected in Nestin from adult CNS stem/progenitor cells, EPCs, or neoplastic ECs in the present study. However, we identified 11 significant phosphorylation sites at serine residues in Nestin protein from adult CNS stem/progenitor cells using quantitative phosphoproteome analysis. The difference in phosphorylated amino acids and the number of phosphorylated sites may be due to technological advances in phosphoproteome analysis and/or the difference in cell sources. Phosphorylated serine residues have been reported in Nestin protein from the brain of mouse embryos [13] and mouse skin melanoma [24] and have been assumed by similar data in human HeLa cells [19, 20]. Among the 11 phosphorylated serine residues we identified in Nestin protein, only two (S565 and S1010) were reported previously; the other nine (S169, S570, S728, S731, S819, S883, S886, S1565, and S1837) were newly identified in the present study.

Nestin protein is expressed not only in NSCs, but also in tissue stem/progenitor cells beyond the germ layers, including mesenchymal stem cells [25], vascular endothelium [6–8], muscle [26–28], testes [29], and teeth [30]. Nestin is also abundant in progenitor cells derived from embryonic stem cells that have the potential to develop into neuroectodermal, endodermal, and mesodermal lineages [31]. We recently reported that Nestin is expressed in proliferating ECs and may be useful as a marker of neovascularization [8]. Nestin expression has been reported in the angiogenic endothelium of cancers [32, 33]. Independent cell-type-specific elements of the *nestin* gene are identified in transgenic mice; the first intron directs reporter gene expression to the mesodermal somite, and the second intron contains enhancer that functions in NSCs [28]. Although the regulatory mechanisms underlying *nestin* gene expression in proliferative vascular cells are different from those in NSCs, the protein expression of vascular Nestin is cytochemically similar to CNS Nestin [8]. The phosphorylation of Nestin protein from adult neurospheres can allow it to be distinguished from Nestin in EPCs. Although Nestin phosphorylation was also observed in neoplastic ECs, our phosphoproteome analysis identified CNS-specific phosphorylation sites, suggesting that a phospho-specific Nestin antibody may distinguish between the expression of CNS and vascular Nestin proteins.

5. Conclusions

Quantitative phosphoproteome analysis identified phosphorylated serine residues in Nestin from adult mouse CNS stem/progenitor cells. Phosphorylation was not observed in Nestin from EPCs. Detection of the CNS-specific phosphorylation sites in Nestin, for example, by a phospho-specific Nestin antibody, may allow the expression of CNS Nestin to be distinguished from vascular Nestin.

Acknowledgment

This study was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Science, Sports, and Technology to J.N.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 895659, 15 pages
doi:10.1155/2012/895659

Review Article

Regulation of Injury-Induced Neurogenesis by Nitric Oxide

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Received 1 June 2012; Accepted 19 July 2012

Academic Editor: Oscar Gonzalez-Perez

The finding that neural stem cells (NSCs) are able to divide, migrate, and differentiate into several cellular types in the adult brain raised a new hope for restorative neurology. Nitric oxide (NO), a pleiotropic signaling molecule in the central nervous system (CNS), has been described to be able to modulate neurogenesis, acting as a pro- or antineurogenic agent. Some authors suggest that NO is a physiological inhibitor of neurogenesis, while others described NO to favor neurogenesis, particularly under inflammatory conditions. Thus, targeting the NO system may be a powerful strategy to control the formation of new neurons. However, the exact mechanisms by which NO regulates neural proliferation and differentiation are not yet completely clarified. In this paper we will discuss the potential interest of the modulation of the NO system for the treatment of neurodegenerative diseases or other pathological conditions that may affect the CNS.

1. Introduction

Neurogenesis is not limited to embryonic development as previously thought and occurs throughout the entire adult life of mammals, including humans. New neurons are continuously added to neural circuits and originate at two principal brain regions: the subventricular zone (SVZ) of the lateral ventricles, which generates olfactory bulb (OB) neurons, and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. Both regions harbor neural stem cells (NSCs) that can be isolated and cultured *in vitro* in the presence of growth factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), or both. The absence of growth factors results in the differentiation of cells into neurons, astrocytes, or oligodendrocytes as discussed in [1]. Neurogenesis has been exhaustively studied over the past years, and despite the great progress that has been achieved, the knowledge of the multiple aspects controlling proliferation, differentiation, or survival of NSCs is far from being known or understood. It was shown that neurogenesis decreases with aging and is impaired in several pathological conditions affecting the brain. Whether the insult is acute, such as ischemic brain stroke, traumatic brain injury, or epileptic seizures, or is a slow-progressing disease like Alzheimer's disease, Huntington's disease, or Parkinson's disease, all these conditions are accompanied by an inflammatory response in the brain [2]. Furthermore, the blockade of neuroinflammation restores adult neurogenesis [3, 4]. When an inflammatory response in the brain appears following an injury, activation of the brain immune cells takes place, particularly microglial cells. In inflammatory conditions, microglial cells become "activated", and among a plethora of morphological and immunological alterations, they are able to express the inducible nitric oxide synthase (iNOS), producing high levels of nitric oxide (NO).

NO is a multifaceted gaseous signaling molecule with several distinct functions in the central nervous system (CNS) [5]. This molecule is simultaneously involved in neuroprotection and in neurotoxicity, being also involved in inflammatory mechanisms in the CNS [6, 7]. NO was shown to modulate neurogenesis in the adult CNS as reviewed in [8]. In physiological conditions, NO tonically inhibits neurogenesis in the brain, while in pathophysiological conditions

it exerts a proneurogenic effect on the dividing population of neuronal precursors. Moreover, the physiological effect of NO is mostly mediated by the neuronal nitric oxide synthase (nNOS), which is constitutively expressed, while pathophysiological levels of NO are attained following expression of iNOS [9–12]. Depending on the insult and on its source, NO can act as an antiproliferative agent [9–11] or stimulate neuronal precursor proliferation and differentiation [12]. However, the exact mechanisms by which NO regulates neuronal proliferation and differentiation are not yet clarified, and further investigation on this matter is needed. Since neuroinflammation is detrimental for adult neurogenesis, it would be of great interest to elucidate the role of inflammatory NO on the ongoing neurogenesis in these conditions. Therefore, the main goal of this paper is to elucidate the potential of the NO system modulation for the treatment of neurodegenerative diseases or other pathological conditions that may affect the CNS.

2. Neurogenesis following Brain Injury

Adult neurogenesis is implicated in many forms of plasticity in the CNS. The neurogenic process can be summarized in five main stages: (a) precursor cell proliferation, (b) fate determination, (c) migration, (d) differentiation and integration, and (e) survival.

Various models of injury in the rodent brain have been used to demonstrate that proliferation of stem cells is particularly enhanced in the SVZ and DG after an insult, which has been suggested to be a repair attempt from the lesioned brain, as reviewed in [13]. It has been observed that injury and pathological conditions affect adult neurogenesis, having a particular impact in neurogenic regions, but also in areas that are not normally considered as classical neurogenic regions, as discussed in [14, 15].

Regarding the type of insult to the brain, this may be acute, as ischemic brain stroke, traumatic brain injury or prolonged seizures, or a slow-progressing neurodegenerative disease. Neurogenesis decreases with aging and is impaired in several neurodegenerative disorders, such as Huntington's disease [16, 17] or Alzheimer's disease [18]. All these conditions are accompanied by an inflammatory response in the brain. However, the factors that attract neural progenitors to the lesioned areas are still under investigation. Another matter of hot debate is whether these new neurons are functionally integrated and survive in the existing neuronal circuitry.

3. Injury and Neuroinflammation

Inflammation is, by definition, a complex biological response to certain noxious stimuli such as stress, injury, or infection by external agents [19, 20]. After injury or exposure to pathogens, an inflammatory response takes place, with the involvement of two major groups of immune cells: (a) CNS resident microglial cells and astrocytes and (b) infiltrating lymphocytes, monocytes, and macrophages from the hematopoietic system [21, 22]. Therefore, the neuroinflammatory response attempts to protect the affected organism by removing harmful stimuli or removing dead and damaged cells, thereby initiating the healing process and return the tissue to homeostasis. When activated, immune cells release different regulating substances, such as complement molecules, cytokines-like interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, IL-18 and IL-6, chemokines such as stromal-derived factor (SDF)-1alpha and monocytes chemoattractant protein-1 (MCP-1), glutamate, reactive oxygen species (ROSs), and reactive nitrogen species (RNSs) like NO, as extensively reviewed in [23]. These inflammatory mediators are responsible for the recruitment of resident microglia, stimulation of astrogliosis, but also for the disruption of the blood-brain barrier (BBB) and further recruitment of monocytes and lymphocytes from the hematopoietic system to the site of inflammation [24–26].

Although inflammation in the CNS should be considered as a process that seeks to protect, we also must take into account its harmful properties as reported in [27]. The activation of recruited cells to the site of inflammation leads to the release of inflammatory factors that contribute to create a positive feedback loop of inflammatory activation, resulting ultimately in neuronal loss and/or neuronal damage. Thus, the inflammatory response may have a dual effect on the cellular environment, beneficial and/or detrimental. The severity of neuroinflammation can range from mild acute to uncontrolled chronic inflammation, resulting in different activation states of inflammatory cells and distinct biological outcomes [28]. It is believed that neuroinflammation may be involved in the mechanisms that lead to various CNS diseases, also affecting the process by which new neurons are generated in the brain [29].

3.1. Neurodegeneration. Neurodegeneration is characterized by the slow progressive dysfunction and loss of neurons in the CNS. Immune activation within the CNS is a classical event following infections, ischemia, trauma, and neurodegenerative diseases. The inflammatory response often contributes to collateral CNS injury, which is characterized essentially by neuronal loss and atrophy in different brain regions. Neuronal susceptibility to cell death [30, 31] and concomitant failure in self-repair mechanisms [32], combined with inhibition of axonal growth and limited repopulation by neuronal precursor cells are singled out as the main causes for neurodegenerative events that follow brain inflammation [33, 34]. However, not all immune response in the CNS should be considered harmful, and in many cases they actually are an important aid for cell repair and regeneration. Particularly, microglial cells seem to play an important role in facilitating the reorganization of neuronal circuits and in triggering repair [35]. Thus, like inflammation, microglial activation also appears to play a dual role in neurodegeneration, acting either as detrimental or beneficial, as reported in [36].

The relationship between neuroinflammation and neurodegeneration is being studied in numerous models of CNS disorders such as Alzheimer's and Parkinson's disease, suggesting neuroinflammation as a critical process, if not the primary cause, for CNS lesions seen in these diseases, as

Table 1: Regulation of adult neurogenesis by inflammatory mediators.

Inflammatory factor	Proliferation of NSC	Differentiation of NCS	Survival of NSC	References
IL-1	↑ or ↓	—	↓	[51]
IL-6	↓	↓ neuronal	↓	[4, 46, 52]
IFN-gamma	↓	↑ neuronal	↓	[42, 53–56]
	↑	—	=	[57, 58]
	—	↓ neuronal	↓	[40]
	—	—	↓	[43, 54]
TNF-alpha	↑	↓ neuronal (TNF-R1)	=	[59]
	↑	↑ neuronal (TNF-R2)	↑	[60]
	↑	↑ neuronal (TNF-R1) ↑ astrocytic	↑	[61]
	↑ or ↓	↑ neuronal (TNF-R2) ↓ neuronal (TNF-R1)	↑ or ↓	[36, 44]
SDF-1alpha	↑ or ↓	↑ neuronal	↑	[62, 63]

The effects listed here may not be direct. ↑: increase; ↓: decrease; =: no change; —: no report.

extensively reviewed in [23, 37]. However, these studies also revealed complex neuroimmune interactions, both at cellular and molecular levels, thus demonstrating that immune cells secrete both neurotoxic and neuroprotective molecules [2]. Although different triggering events could occur, a common feature for the neurodegenerative event seems to be the chronic activation of microglial cells.

3.2. Neuroinflammation and Production of New Neurons. As mentioned in previous sections, neuroinflammation is a complex process with different outcomes in neurogenesis, which can be enhanced or suppressed [38]. Besides differences between mild acute and uncontrolled chronic inflammation, the shift from pro- to antineurogenic inflammatory status appears to be dependent on (a) the mechanism by which microglia, macrophages, and/or astrocytes are activated, (b) the type of inflammatory mediators released, and (c) for how long inflammatory cells, particularly microglia, are activated [36].

3.2.1. Impaired Formation of New Neurons. Inflammation and microglia activation were initially thought to inhibit adult neurogenesis [3, 4], while recent studies indicate that microglia can also support neurogenic events, as described in [39]. It was shown that lipopolysaccharide-(LPS-) induced activation of microglia impairs neurogenesis in rats [4], apparently through the increased production of TNF-alpha [40]. Additional evidences corroborating the detrimental effect of LPS-activated microglia was provided by another study, which showed that acute activation of microglia with LPS reduces NSC survival and neuronal differentiation [41]. Furthermore, suppression of microglial activation with an antibiotic, such as minocycline, was also used to demonstrate increased neurogenesis in the hippocampus, thus indicating that the severity of impaired neurogenesis correlates with the number of activated microglial cells [4]. Several other authors reported that the mechanism by which microglia exert these effects involves the release of proinflammatory mediators, such as IL-1, IL-6, IFN-gamma, and TNF-alpha, which seem to play an essential role in suppressing neurogenesis [42–45] (Table 1). It has also been suggested that ROS and RNS, particularly NO, can inhibit adult neurogenesis in inflammatory conditions [3, 46, 47]. In addition, several studies demonstrated that neurogenesis could be restored following treatment with anti-inflammatory drugs [3, 4, 48, 49]. Neurogenesis was restored after treatment with indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), after irradiation-induced inflammation [3] or focal cerebral ischemia [49]. Other studies also reported an increased survival of newly generated neuroblasts in the striatum after stroke [49], or in the DG after middle cerebral artery occlusion (MCAO) [50] when the activation of microglia is inhibited by indomethacin or minocycline, respectively.

3.2.2. Enhancement of Neurogenesis. Contrary to what was initially thought that neuroinflammation is detrimental to adult neurogenesis, recent evidence indicates that under certain circumstances inflammation can also benefit the neurogenic process (Table 1). Apparently, neural stem cells become "activated" following brain injury and migrate into the lesioned areas, thus suggesting the inflammatory microenvironment as an important trigger for the migration of newborn cells [64, 65]. Microglia was reported to play a dual role on neurogenesis, suggesting neurogenesis inhibition to be caused by microglial activation under inflammatory conditions [53]. Other studies showed a persistent production of neurons from adult NSC, even after the inhibition of acute microglial activation, during recovery after stroke [66, 67]. Moreover, it was demonstrated that long-term survival of newborn neurons after *status epilepticus* (SE), with concomitant chronic activation of microglia [68]. *In vitro* studies have also showed an important role for microglia in directing the replacement of damaged or lost cells [52, 69–71]. LPS-activated microglia and inflammation increase the integration of newly generated neurons into the adult rat hippocampus [72]. More recently, long-term accumulation of activated microglia, although with

a downregulated inflammatory profile, was shown to be concomitant with persistent neurogenesis in the adult SVZ after stroke [73]. Other inflammatory mediators have also been implicated in the improvement of migration and proliferation of new neurons following brain damage, such as SDF-1alpha and its receptor CXCR4 [62, 66] or trophic factors such as GDNF and BDNF, who are involved in the removal of damaged synapses [73]. In summary, all these studies suggest a neuroprotective role of microglia for newborns cells. Although microglia may have a detrimental action in early stages of the inflammatory response that follows acute insults, it could be converted into a protective state during chronic activation.

3.2.3. Dual Role of Inflammation in Neurogenesis. It is now widely accepted that microglia have a dual role in neurogenesis by favoring it or, alternatively, hindering neurogenesis. Apparently, microglial cells and the inflammatory factors they release, like NO (to be discussed below), seem to have opposite roles in neurogenesis under inflammatory conditions [38, 74]. However, it is important to reinforce the idea that inflammation, essentially characterized by activation of microglia, has distinct roles in various stages of neurogenesis, this effect being dependent on the degree of activation of immune cells, type of inflammatory mediator released, and duration of the inflammatory response [38]. Nevertheless, there are lines of evidence for some of the most important inflammatory mediators in the regulation of neurogenesis and/or neuroprotection [23, 75, 76].

As noted, further studies should be conducted to assess the interaction between neuroinflammation and neurogenesis, particularly how neuroinflammation modulates self-renewal, proliferation, migration, differentiation, integration in the neuronal network, and, more importantly, survival of newborn cells. As different authors have reported that chronic inflammation can stimulate one or more stages of neurogenesis, such as migration, proliferation, or differentiation, the problem remains in the reduced long-term survival of newborn neurons [23]. Moreover, since different microglial phenotypes and morphologies can be identified during inflammation, an extensive genetic and proteomic characterization will be of great interest to understand more accurately this complex crosstalk.

4. Nitric Oxide

Nitric oxide, a short-lived gaseous-free radical, is synthesized by the nitric oxide synthase (NOS) family of enzymes present in most of the cells of the body. NO is implicated in a wide range of physiological processes within the cardiovascular, immune, and nervous system, where it can act as a non-canonical neurotransmitter [77], but it can also be an important player in pathophysiological events. Different members of the NOS family control different functions of NO. The discovery of NO in the CNS was a breakthrough in the concept of neuronal communication. NO was characterized in the CNS for the first time as an intracellular messenger to increase cyclic guanosine 3′,5′-monophosphate (cGMP) levels, after the activation of glutamate receptors [78]. Later, the same authors also described NO as a neuromodulator, particularly due to its diffusible properties [79], thus acting not only in cells that release NO, but also in neighboring cells where it can therefore trigger its autocrine and/or paracrine functions. Unlike other neurotransmitters, NO is synthesized on demand, diffusing from nerve terminals since it is not stored in vesicles nor released by exocytosis [5]. In the CNS, NO is also associated with cognitive function, having an important role in synaptic plasticity, and controls biological functions, including body temperature, sleep-wake cycle, appetite, and modulation of hormone release, as reviewed in [7]. Another distinctive feature from classical neurotransmitters is that, unlike them, NO ends its action after reacting with a substrate and not by enzymatic degradation or reuptake. In addition, the key mechanism to regulate the activity of NO is the control of its synthesis.

Physiologically, NO interacts with several intracellular targets activating different signaling pathways with a stimulatory or inhibitory response. However, NO can also be toxic to cells, in a mechanism dependent on the formation of RNS [80, 81]. Oxidative stress and nitrosative stress, a consequence of high levels of NO and RNS, have been implicated in the pathogenesis of several neurodegenerative disorders [80, 82, 83], which will be explored in Section 4.2.

4.1. NO as an Inflammatory Mediator. The NOS family of enzymes is responsible for the synthesis of NO. Three different enzyme isoforms have been identified in mammalian cells: (a) neuronal NOS (nNOS, type I), which is constitutively expressed in brain neurons and is activated by calcium/calmodulin, particularly following stimulation of NMDA-type glutamate receptors; (b) endothelial NOS (eNOS, type III), constitutively expressed in endothelial cells and astrocytes and is regulated by calcium/calmodulin and phosphorylation/dephosphorylation; (c) inducible NOS (iNOS, type II) which is calcium-independent and its regulation depends on de novo synthesis [80, 84, 85]. iNOS is not normally expressed in the "healthy" brain but is induced in glial and endothelial cells by proinflammatory stimuli such as cytokines, bacterial/viral agents, and/or hypoxia [80]. iNOS is mainly expressed in macrophages, astrocytes, and microglial cells, upon neurotoxic, traumatic, and inflammatory damage [7, 84, 86, 87], but it could also be found in neurons [88, 89]. Once expressed, iNOS continuously produces high amounts of NO, even for several days [31, 87, 90–92]. The massive production of NO by iNOS is toxic, since it inactivates the mitochondrial respiratory chain enzymes that ultimately induce apoptosis in target cells. Moreover, NO has been described as an important activator of cyclooxygenase-II (COX-2) in glial cells, also regulating leukocyte adhesion in vessels [80]. The concentration achieved by NO seems to be a determining factor for the effects observed locally in the brain. Thus, in physiological concentrations, which are believed to range from 0.1 to 100 nM, NO is relatively nonreactive, and its actions are mainly mediated by binding to the heme group

of soluble guanylate cyclase (sGC), leading to its activation and subsequent production of cGMP [93].

NO can also be converted into more reactive species commonly refereed as RNS. In high concentrations, NO reacts directly with oxygen (O_2) to produce nitrogen dioxide (NO_2), which in turn further reacts with NO originating dinitrogen trioxide (N_2O_3). In addition, NO_2 may oxidize or nitrate, by adding a nitro (NO_2^+) group to a great variety of molecules, being a classic example the nitration of tyrosine to 3-nitrotyrosine [94]. Moreover, NO reacts with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$), an extremely reactive molecule which can oxidize or nitrate other molecules or, instead, decay forming other damaging species, such as NO_2 and/or the hydroxyl radical ($OH^\bullet$). On the other hand, N_2O_3 can add a nitrosonium ion (NO^+) to thiols or amines, an event also designated as nitrosation/nitrosylation, being a good example cysteine than can be nitrosated to S-nitrosocysteine [94]. Both S-nitrosylation and nitration typically lead to alterations in protein function [94].

4.2. Neuronal Death. According to the literature, the role of NO in the brain could be summed up in two radically different outcomes: (a) as an intracellular signaling messenger, regulating a wide variety of physiological events, such as synaptic plasticity, blood flow, and neuronal development [95] and (b) as a cytotoxic agent killing indiscriminately both pathogenic and "healthy" host cells in disease [96, 97]. Strong evidence has been reported in the literature supporting a role of NO in the pathogenesis of neurodegenerative disorders, including autoimmune and chronic neurodegenerative diseases. As stated in previous sections, the role of NO seems to be dependent on the concentration attained locally in tissues. When produced in excess, NO shifts from a physiological to a neurotoxic effector. NO overproduction may be due to nNOS activation following persistent glutamate excitatory input and/or iNOS expression, upon an inflammatory response. Activated inflammatory cells generate increased levels of ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. Moreover, NO can also induce the production of superoxide by mitochondria [7]. NO and superoxide readily react to form $ONOO^-$, an extremely reactive molecule [81].

Likewise, the excessive release of both glutamate and NO, coupled to oxidative stress and mitochondrial dysfunction, appears to be involved in the majority of neurodegenerative diseases. NO from inflammatory origin has been reported as an important contributing factor to the vulnerability of neurons, causing neuronal death both *in vivo* and *in vitro* in rodents [98, 99]. Some authors have suggested this neurotoxic effect as a consequence of enzymatic inhibition of the respiratory chain, resulting in hypoxia, excitotoxicity, and elevated levels of $ONOO^-$, as reviewed in [81]. Furthermore, the excessive NO release by glial cells leads to the formation of $ONOO^-$, which appears to be involved in the mechanisms of neuronal death, some of them linked to protein dysfunction due to nitration or s-nitrosylation [100]. Protein nitration is an irreversible chemical modification affecting tyrosine phosphorylation or dephosphorylation, which seriously affects several signaling pathways involved in the control of cell survival, proliferation, or programmed cell death, as reviewed in [101].

Table 2: Regulation of adult neurogenesis by NO under physiological or inflammatory conditions.

Condition	Proliferation of NSC	Differentiation of NCS	Survival of NSC	References
Physiological	↓	=	=	[8, 9, 106, 107]
	↓	=	↓	[8, 108, 109]
	↓	↑	=	[8, 10, 11, 110–112]
	↓	↓	↓	[8, 113, 114]
Inflammation	↑	=	=	[23, 115]
	↑	↑	=	[12, 23, 74, 116, 117]
	↑	↑	↓	[23, 118]

The effects listed here may not be direct. ↑: increase; ↓, decrease; =, no change or no report.

Although it has been implicated in acute injury events, particularly due to a massive release during an inflammatory response, NO has also been associated to slow progressive disorders that can be genetically inherited or sporadic. Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis are all neurodegenerative disorders in which NO has been suggested to be involved, since all of them show evidence of oxidative and nitrosative stress [80, 102]. ROS and RNS are important factors in neuroinflammation-mediated neurotoxicity [103]. Furthermore, the presence of 3-nitrotyrosine has been reported in several neurodegenerative diseases linked to oxidative stress such as Alzheimer's [104] or Parkinson's disease [105]. Thus, understanding the involvement of NO in the etiology of these disorders may highlight an eventual beneficial potential role of selective NOS inhibitors.

4.3. Nitric Oxide and Neurogenesis. The role of NO as a modulator of neurogenesis is a matter of strong debate. Depending on the source, NO has a dual influence in the neurogenic process both by inhibiting or stimulating neurogenesis (Table 2).

The role of NO in neurogenesis has not been identified until recently [9, 10, 110]. The authors of these contributions had also described a cytostatic function of NO in the CNS, demonstrating that nNOS-derived NO is involved in the regulation of neurogenesis, particularly neural stem cell function [9, 10, 110]. Since blood vessels are part of the SVZ and dentate gyrus SGZ niches, which are also surrounded by differentiated neurons expressing nNOS, NO is produced in close proximity to NSCs. Several authors have described another function for NO in the rostral migratory stream (RMS), where SVZ-derived progenitor cells migrate into the olfactory bulb and differentiate into neurons [119]. These authors demonstrated that nitrergic neurons are in close

Table 3: NO-dependent signal pathways in neurogenesis.

NO source	Effect	Signaling pathway	References
nNOS	↓ proliferation (SVZ)	Nitrosylation of EGF receptor	[127]
		(PI3-K)/Akt pathway	[107, 127]
	↓ neurogenesis (DG)	PSA-NCAM and CREB	[134]
		cAMP phosphorylation	[113]
eNOS	↑ neurogenesis (DG and SVZ)	↑ BDNF and VEGF	[135]
	↑ neurogenesis (DG)	↑ VEGF	[128]
iNOS	↑ proliferation (SVZ)	ERK 1/2 pathway	[74]
		cGMP/PKG pathway	[115]
	↑ migration (NT2 cell line)	cGMP/PKG pathway	[136]
	↑ neurogenesis (DG)	NMDA receptor	[130, 137]
	↑ neurogenesis (DG and SVZ)	L-VGCC	[138]
	↑ astrogliogenesis	JAK/STAT-1 pathway	[112]

↑: Increase; ↓: decrease; Brain-derived neurotrophic factor, BDNF; Vascular endothelial growth factor, VEGF; L-type voltage-gated Ca^{2+} channel, L-VGCC.

vicinity to the RMS and that the NO generated regulates the migration and proliferation of progenitors that could also express nNOS [119]. Other groups have demonstrated NO production to be induced by neurotrophic factors, which in turn act in target cells inducing cell cycle arrest and/or exit favoring differentiation [111, 120, 121].

It should be noted here that the majority of the studies on the effect of NO in adult neurogenesis are focused mainly on the modulation of proliferation. In this context, the evaluation of survival rates of newly formed neurons is also important, since NO is known to be a regulator of apoptosis [118]. Several studies have shown that NO inhibits apoptosis by preventing increases in caspase-3 activity [122], which has been described to increase short-term survival of progenitor-cell progeny in the adult rat DG following SE [123].

Production of NO via nNOS has been demonstrated to have an important antiproliferative effect both *in vitro* and *in vivo*, but also as being involved in neuronal differentiation, survival, and synaptic plasticity [9, 10, 107, 113, 124]. It was shown that chronic nNOS inhibition enhances neurogenesis. Indeed, the selective inhibition of nNOS with 7-nitroindazole (7-NI) greatly increased cell proliferation in the SVZ, RMS, and OB, but not in the DG, in adult mice [10]. This antiproliferative effect of NO has been confirmed by others, that have shown that when NO production is inhibited either by using an intraventricular infusion of an NOS inhibitor in the rat brain or by using an nNOS-knockout mouse model, proliferation is greatly increased in the olfactory subependymal zone and in the DG [9, 108, 113, 125]. Moreover, the inhibitory role of nNOS-derived NO on SVZ and DG neurogenesis has also been demonstrated in the context of cerebral ischemia [126]. Other authors suggested NO to be a negative regulator of SVZ neurogenesis by modulating the activity of the EGF receptor [107], via nitrosylation of specific cysteine residues [127] (Table 3). Accordingly to these studies, the antiproliferative effect can be partially explained by the inhibition of the EGF receptor and the phosphoinositide-3-kinase (PI3-K)/Akt signaling pathway [107, 127]. Moreover, these authors described the antimitotic effect of NO to correlate with the nuclear presence of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ [127].

On the contrary, by using pharmacological or genetic approaches, an opposite role has been found for NO synthesized by eNOS in the SVZ and iNOS in the DG following focal ischemia, which seems to stimulate neurogenesis [12, 128]. Moreover, increased immunoreactivity against iNOS following transient ischemia was shown to correlate with a decrease of nNOS in the hippocampus, which is concomitant with an increased neurogenesis [116, 129]. Numerous works showed that ischemia-induced neurogenesis in DG involves the activation of NMDA receptors [130], which is simultaneous to increased iNOS expression [131, 132] (Table 3). However, in a study regarding the effects of NO in cell proliferation, both nNOS- and iNOS-derived NO increases neurogenesis following seizures in the DG of adult rats [133]. Other authors reported that NO released under inflammatory conditions is involved in NSC differentiation into astrocytes by a mechanism dependent on the activation of the JAK/STAT-1 signal transduction pathway [112]. Recently we showed that supraphysiological levels of NO induce the proliferation of SVZ-derived neural stem cells through the activation of two signaling pathways, in a biphasic manner. Thus, the mitotic effect of NO is initially mediated by the direct activation of signaling pathway downstream of the EGF receptor, but bypassing the EGF receptor [74]. Downstream of the EGF receptor, there is an increased activation of the mitogen-activated protein (MAP) kinase ERK pathway following exposure to NO, which activates several downstream targets, namely p90RSK, and further decreases nuclear levels of $p27^{Kip1}$, thus allowing cell cycle progression [74]. Furthermore, the proliferative effect of supraphysiological levels of NO, following longer periods of exposure (24 h), is mediated by increased signaling through the cGMP/cGMP-dependent kinase (PKG) pathway [115]. In addition, we also showed that NO from iNOS origin promotes proliferation of NSC in the hippocampus of adult mice following SE [74].

Altogether these findings illustrate that NO is a modulator of neurogenesis in diverse ways, and the different NO

synthases are important players in this effect on neurogenesis [11, 139, 140]. NO effects on neurogenesis are dependent on the developmental period and source of NO (Table 3). Furthermore, NO can have concentration-dependent effects, depending on the local concentration and surrounding molecular environment. Apparently, under physiological conditions NO acts as a negative regulator of neurogenesis [9, 10, 110], while in inflammatory conditions a decrease in nNOS and increase in iNOS may act as a mechanism to enhance neurogenesis [12, 74, 107, 141, 142]. However, the exact molecular mechanisms underlying this dual effect of NO on neurogenesis, are not fully clarified and more studies need to be conducted.

5. Potential Neurogenic Targets in Nitrergic Pathways

Repair of damaged tissues and organs is essential for the survival of organisms. Although the CNS has pools of neural stem cells, these have a limited ability for repair and endogenous cell replacement. Some strategies have been studied over the past years to promote brain repair, particularly: (a) neural precursor or stem cell transplantation or (b) stimulation of endogenous neurogenesis. Moreover, excessive proliferation of NSCs associated with tumor formation is a major concern in the clinical application of both these strategies. Since most brain disorders that could benefit from enhanced neurogenesis are normally accompanied by neuroinflammation, understanding how the inflammatory response affects the neurogenic process is of major importance for the design of safe and efficient therapeutic strategies.

As discussed previously, NO was described to have a dual role on the regulation of adult neurogenesis. NO synthesized from nNOS appears to decrease neurogenesis or to act as an antiproliferative agent [9, 10, 107, 108, 110, 113, 127], whereas NO from iNOS and eNOS origin seems to stimulate neurogenesis [12, 74, 128, 142]. Taking this evidence into account, the modulation of the NO system may be a good target for the development of strategies to improve brain repair. Next, some of the most relevant therapeutic strategies for brain repair using the modulation of the nitrergic system will be discussed.

5.1. Nitric Oxide-Releasing Drugs. Nitric oxide-releasing drugs are pharmacologically active substances that release NO *in vivo* or *in vitro*. Two large groups of NO-releasing drugs can be found today: (a) NO donors and (b) NO-releasing nonsteroidal anti-inflammatory drugs. Although the clinical application of these drugs to improve brain repair seems remote, their potential application in the treatment of CNS disorders is a matter of great interest. Several studies have been carried out in order to understand how these drugs control neurogenesis. In fact, there seem to exist good reasons to believe that the use of these drugs may be advantageous in the treatment of brain disorders.

5.1.1. Nitric Oxide Donors. Nitric oxide-releasing compounds are clinically used for the treatment of patients with coronary heart disease [143]. Different types of NO-releasing agents have been developed and are commercially available, such as sodium nitroprusside (SNP), firstly described as a vasodilator, which is used to manage acute hypertensive crisis; or molsidomine, used in the therapy of angina pectoris and heart failure. SIN-1, another NO donor, is known as both NO and $ONOO^-$ donor mainly because during NO release from SIN-1 superoxide is also generated [144, 145]. A wide range of NO-releasing drug classes have been developed recently. Among them are diazeniumdiolates, also known as NONOates (such as DEA/NO, SPER/NO, or DETA/NO) that release NO spontaneously under physiological conditions. Preclinical studies have shown a potential application for NONOate in cardiovascular disease, but further studies need to be conducted for their use in the clinic [145]. Chemically distinct NO donors differ in their half-life time and amounts of NO released *in vitro*. Moreover, depending on pH value, temperature, presence of cofactors, and light, the amount of NO released could be altered [144–146].

These compounds have also been useful to study physiological processes and molecular mechanisms in which NO is involved. NO was described to act as an antiproliferative agent in the CNS under physiological conditions, thus affecting neurogenesis [127]. Interestingly, in this work the authors described NO to be antiproliferative through the inhibition of the EGF receptor by S-nitrosylation [127]. Moreover, other authors have also described NO physiological levels to be antiproliferative in the brain [11, 107].

Numerous studies have used NO donors to investigate the effect of high concentrations of NO on neurogenesis, thus mimicking NO concentrations that can be achieved locally in the brain following an inflammatory response. Several groups reported nitric oxide-releasing drugs to enhance recovery after brain injury, partly by increasing neurogenesis in the DG and SVZ [147–150], following ischemic stroke [147, 151] and traumatic brain injury [148]. One study found that exogenous administration of NO using DETA/NO increases cell proliferation and survival in mice hippocampus [117]. We have shown that high concentrations of NO, which could be attained locally in the brain following an inflammatory response, have a dual effect on the proliferation of SVZ-derived NSCs [74, 115]. In fact, the effect of NO on the proliferation appears to be dependent on the period of exposure and concentration of NO achieved. Thus, a slight elevation on NO levels above the physiological range has a proliferative effect in an initial stage (1 day). On the contrary, continuous release of NO overtime (for 2 days) had an antiproliferative effect in SVZ-derived NSCs [74, 115]. This evidence is important to realize that controlling neuroinflammation, thus controlling NO production, will improve the outcome from the neurogenic process following brain injury. Other groups have shown that high concentrations of NO could also modulate other neurogenic stages, such as migration [136, 152] or differentiation [112].

In fact, the studies published in the literature about the effect of high levels of NO in neurogenesis, using NO donors, seem to bring contradictory evidence. However, it should be noted that in most of these works the NO donors

used are chemically distinct and/or have distinct kinetics on NO release. Therefore, the evidence should be carefully interpreted to prevent misleading conclusions. Nevertheless, all these studies appear to be consensual on the following: NO is an important modulator of neurogenesis.

5.1.2. Nitric Oxide-Releasing Nonsteroidal Anti-Inflammatory Drugs. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NO-NSAIDs) are a group of compounds with potential therapeutic applications in several clinical conditions. These drugs are synthesized by grafting a NO-donating moiety to classical NSAID, such as aspirin (NO-aspirin), flurbiprofen (NO-flurbiprofen), naproxen (NO-naproxen), diclofenac (NO-diclofenac), and ibuprofen (NO-ibuprofen) [143, 153, 154]. At present, NSAIDs are used for the treatment of a variety of inflammatory conditions. However, NSAIDs have a limited therapeutic application in chronic conditions, mainly due to their significant side effects in the gastrointestinal (GI) tract and kidneys. In the last decades, a great effort has been done to improve NSAID safety. Therefore, NO-NSAID may be considered as an important therapeutic attempt to overcome the side effects by NSAID. The release of NO from these drugs mimics the physiological production of NO by constitutive NOS, which appears to reduce the toxicity when compared to the parent NSAID [153, 155]. Moreover, this modification strongly reduces the side effects of NSAID, without affecting the anti-inflammatory effectiveness [153].

Since NSAIDs are primarily used as anti-inflammatory drugs, many of the studies with NO-NSAID have been essentially about its anti-inflammatory effects. Numerous studies in the literature have reported the anti-inflammatory effect of NO-NSAID in animal models of acute or chronic inflammation. More recently, there has been increasing concern about the potential application of these drugs in CNS disorders, particularly in neurodegenerative diseases, such as Alzheimer's disease. Numerous reports suggested NO-NSAID to be a suitable approach for the treatment of Alzheimer's disease, since they are less toxic to the GI tract than NSAID following chronic ingestion. Moreover, NO-NSAID also inhibit caspase activity thus protecting neurons against cytokine-induced apoptosis during Alzheimer's disease [153]. As reported by Hauss-Wegrzyniak and coworkers, chronic ingestion of NO-flurbiprofen reduced the activity state of microglial cells in a rat model of Alzheimer's disease, when comparing to animals treated with aspirin [156]. Other authors also described NO-flurbiprofen to reduce brain beta-amyloid in a mice model of Alzheimer's disease, which was associated with activation of microglial cells, the presumed responsible for clearing beta-amyloid deposits [157]. Interestingly, these authors reported NO-aspirin to be more efficacious than ibuprofen or celecoxib, a selective COX-2 inhibitor [157]. The neuroprotective effect of different NO-NSAID has additionally been described in other animal models of brain damage. Treatment with NO-aspirin was shown to be more neuroprotective than aspirin, following MCAO [158]. In fact, the results from these experiments are of great interest since they strongly suggest that NO release is determinant for the protective action of NO-aspirin in this animal model. Although the mechanism underlying this effect is still unclear, NO improved blood flow to the ischemic region, thereby reducing the lesioned area. Moreover, the ability of NO-NSAID to inhibit caspase activity is also important for this effect [159].

Given the ability of NSAID in crossing the BBB [160], the use of NO-NSAID in the treatment of CNS disorders can be a very useful tool, in particular for the control of neuroinflammation that, as noted above, may affect neurogenesis [155]. Therefore, it is important to conduct more studies to understand the mechanisms and levels within which NO released by NO-NSAID may promote neurogenesis.

5.2. PDE Inhibitors. The main cellular signaling pathway stimulated by NO is the activation of sGC, subsequent production of cGMP, and further activation of protein kinases that regulate various physiological events [161]. Neurons synthesize cGMP in response to NO by activation of sGC, a heterodimeric heme-containing enzyme. NO reacts with the heme group of the sGC, which undergoes a conformational change, converting GTP into the second messenger cGMP [93, 162]. Some studies suggest that NO can also downregulate sGC activity, particularly in neuroinflammatory conditions [163]. cGMP-dependent kinases, which are serine/threonine kinases, are activated by cGMP and are involved in several physiological phenomena including long-term potentiation in the hippocampus and long-term depression in the cerebellum [93, 162]. In physiological conditions, intracellular cGMP levels are controlled by cyclic nucleotide phosphodiesterases (PDEs) [94]. PDEs are enzymes that hydrolyze the 3′-phosphodiester bound of cyclic adenosine monophosphate (cAMP) or cGMP, originating their corresponding monophosphates, 5′-AMP or 5′-GMP, respectively. cGMP-related physiological functions can be regulated by controlling the levels of PDE type 5 (PDE5) enzymes, which specifically hydrolyze cGMP. Moreover, cGMP also modulates the activity of PDE [164].

The use of selective PDE inhibitors has been proven to be useful in the clinic, particularly PDE5 inhibitors, which are drugs used to treat erectile dysfunction and pulmonary arterial hypertension [164–166]. Sildenafil, commercially available as Viagra, is classically considered as a PDE5 inhibitor; however, it also inhibits PDE1 and PDE6 [166–168]. Similarly to sildenafil, two other inhibitors with higher selectivity for PDE5 were developed for the treatment of erectile dysfunction: tadalafil (Cialis) and vardenafil (Levitra). More recently, a new compound was developed, T0156, which potently inhibits PDE5 [169]. In fact, T0156 inhibits PDE5 with higher potency than sildenafil also presenting higher selectivity for PDE5 in comparison to PDE6 [169]. In erectile dysfunction, PDE5 inhibition enhances relaxation of the cavernosal smooth muscle by NO and cGMP, thus allowing blood flow and stimulating penile erection [170, 171]. In the lung, PDE5 inhibitors act as vasodilators, increasing blood supply, antagonizing the vasoconstriction of smooth muscle, and decreasing pulmonary arterial resistance, thus treating pulmonary hypertension (for comprehensive review see [172–174]).

In the CNS, neurogenesis generally declines with aging and is correlated with the emergence of neurodegenerative diseases. Moreover, the levels of NO gradually decrease in aging, which is concomitant with a decrease in cGMP levels. As demonstrated in aged rats, cGMP levels are decreased as a consequence of the increasing phosphodiesterase activity when compared to young adult rats [175]. Several authors described NO and cGMP to be important effectors in the regulation of different events related with the neurogenic process, particularly proliferation, migration, differentiation, growth, axon guidance, and cell survival [115, 136, 152, 176, 177]. Furthermore, brain PDE5 was reported to have a role in learning and memory, physiological events that are closely dependent on neurogenesis. Therefore, targeting PDE5 activity as a strategy to reverse the deleterious effects on neurogenesis, and thus enhancing it, seems to be a promising strategy to be applied in clinic. However it should be noted that the use of PDE5 inhibitors as an effective therapy for neurodegenerative diseases is dependent on their permeability to the BBB. For instance, sildenafil is known to cross the BBB and can be easily administered.

The administration of PDE5 inhibitors as a possible therapy for Alzheimer's disease has been studied, due to their ability to reverse deficits in long-term memory caused by pharmacological agents or aging. Different authors have described that the administration of sildenafil enhances memory and restores learning ability in animal models [178–184]. Beyond this important role, PDE5 inhibitors appear to stimulate neuronal plasticity, particularly through the enhancement of endogenous neurogenesis in the adult brain. In addition, the administration of PDE5 inhibitors, such as sildenafil, but also tadalafil, positively affected neurogenesis in the OB, SVZ, and the DG of rats by a mechanism involving the intracellular increase of cGMP levels [185–187]. Moreover, the administration of PDE5 inhibitors has also been associated to neuronal function recovery in rats following a stroke [188] or after ischemic injury either in young adult rats as in aged rats [187, 189]. Furthermore, PDE5 inhibition by sildenafil stimulated cell proliferation in rat SVZ cultures [190]. In a recent report, it was shown that sildenafil has a neuroprotective role, improving the clinical symptoms and neuropathology in a mouse model of multiple sclerosis, thus suggesting PDE5 as an important target for the therapy of this disease [151].

In summary, this evidence supports the idea that the use of PDE5 inhibitors merits further investigation in order to clarify their involvement on neurogenesis, but also to understand the mechanisms underlying these effects.

6. Future Prospects

Stimulation of endogenous adult neurogenesis and modulation of injury-induced neurogenesis is presently being considered as a potential therapeutic approach for neuronal repair in neurodegenerative disorders, as opposed to the more invasive approach of transplantation of exogenous stem cells. Understanding how the inflammatory response affects neurogenesis is fundamental to better design therapeutic strategies for safe and efficient regulation of endogenous neurogenesis. Therefore, the knowledge of the inflammatory agents that modulate proliferation and/or differentiation of NSCs is of great usefulness if its action could be correctly targeted and controlled, for instance, with selective drugs for the agent of interest.

Nitric oxide, which acts as a nonspecific cytotoxic mediator and a biological messenger, in immunological response, has been attracting increasing importance from pharmaceutical companies. Indeed, several nonsteroidal anti-inflammatory NO-releasing drugs (NO-NSAID) are currently under investigation and were shown to be beneficial in models of several neurodegenerative conditions accompanied by inflammation [153, 191]. As an alternative to conventional NSAIDs with significant side effects, pharmacologically improved and therapeutically enhanced NO releasing nonsteroidal anti-inflammatory drugs with less side effects are being developed as reviewed in [192]. Moreover, besides the clinical applications of PDE5 inhibitors, they appear to be a good strategy for the treatment of certain CNS disorders and further improve neurogenesis. These drugs have already been shown to be important modulators of the nitrergic system, preventing neurodegeneration and favoring neurogenesis.

In light of these facts, the modulation of the NO system seems to be a good target for the development of strategies to improve brain repair. However, despite all good evidence that drugs that modulate the NO system have given, further studies are necessary. In fact, a full understanding of how inflammation affects neurogenesis is essential to the development of therapeutic strategies that can induce neurogenesis from endogenous neural precursor cells, and further investigation needs to be conducted to better understand the mechanisms underlying the effect of neuroinflammation in cellular regeneration in the diseased brain.

Acknowledgments

This work was supported by the Foundation for Science and Technology, (FCT, Portugal), COMPETE and FEDER (project PTDC/SAU-NEU/102612/2008). B. P. Carreira is supported by FCT (fellowship SFRH/BPD/78901/2011).

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 470949, 7 pages
doi:10.1155/2012/470949

Review Article

Ethical Implications in the Use of Embryonic and Adult Neural Stem Cells

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Received 1 July 2012; Accepted 7 August 2012

Academic Editor: Sonia Luquin

The advent and growth of technological advances have led to new routes of knowledge. Thereby, we currently face new challenges. We have just started to get a glimpse of the structural and functional role of neural stem cells in differentiation and migration processes, the origin of synaptic networks, and subsequent readjustments in specific circuits. A whole range of treatment possibilities originates from this knowledge that potentially can be used for different neurological diseases in humans. Although this is an encouraging scenario, it implies that the human brain is the object of such study, as well as its potential manipulation and transplantation. It is, therefore, pertinent that ethical principles should be followed in such research to have proper balance between what can be done and what should be done, according to every specific context. Hence, it is wise to consider ethical implications in every research project, along with potential clinical applications, under the principle of causing no harm, following risk and benefit rules in decision making and with respect of the human condition as a priority.

1. Stem Cells

Biotechnology and gene manipulation, as a result of elucidating the human genome, have set new challenges in view of the enormous amount of information in neurosciences area. On one hand, this new knowledge from the last ten years is huge in terms of molecular and genetic information that led to the identification of stem cells. This happened after Thomson [1, 2] proposed the scientific analysis of embryos not used in protocols for *in vitro* fertilization (IVF). Several cell lines were isolated, compatible with all the different cell phenotypes of the adult. At the time, three features were identified for embryonic stem cells. (a) They derive from embryos in the preimplantation stage. (b) They are preserved as undifferentiated cells for indefinite time in special media. (c) They maintain their pluripotential capacity to generate any line of cells from all embryonic germinal layers [3].

The knowledge around stem cells and their differentiation and maturation processes to correctly make structured tissues is the result of many years of phylogenetic evolution and significant knowledge of the human ontogeny. Currently, we are familiar with many of the variants of pluripotential cells in different types of tissue, as well as advances and different applications of such cells, many of them still under investigation. The greater understanding of the human genome and the normal phenotype evolution gave way to the identification of abnormal phenotype expression in early stages of neurodevelopment disturbances, and also programmed or late stages such as in the case of many degenerative diseases [4–6].

The identification of neural stem cells had a breakthrough associated with studies of animal embryos and subsequently in human beings, and the anatomic and functional confirmation of neuro-ontogeny. When this information was learned, the first debate came about: where do we get tissue from? What is the origin and destination of such tissue?

In a first phase, investigators resorted to embryo studies from miscarriages, but the changes in IVF strategies for the benefit of couples with fertility problems led to the availability of embryos in association with the gestational process.

The question about the origin of such tissue was still unanswered and new questions arose about the care and management of embryos and the final destination of those not selected or successfully implanted, like the ones preserved in the laboratory. This gave way to begin having certain availability of these embryos for scientific research, with variable legal and ethical implications. There are countries that forbid this practice altogether, under the premise that you cannot make decisions about life to propose the cure of certain diseases. In this scenario, the debate about the beginning of life comes up again, as well as the limitations to use embryos in a totally limitless way or the opposite position in countries where embryos are allowed to be created *ex profeso* for research purposes along with the procurement of stem cells [7].

So far, regulations have been issued as legal standards trying to approach the way in which these processes can follow the same line of respect for human integrity, without limiting the potentialities of science and therapy in embryo studies [8].

Recently, the American Association of Cancer Research (AACR) has promoted new agreements endorsed by the U.S. government to validate the continuity of studies in embryonic stem cells, as a key element to find treatment for nearly 200 diseases linked to the concept of "cancer." The rationale is that the origin of cancer follows genetic and epigenetic factors which must be studied in order to find a cure for this disease. Consequently, knowing the differences between normal stem cells and cancer stem cells will allow to provide greater therapeutic options in the future. The agreement postulates the prevalence of respect for the human embryo, abiding to the international standard of making use of embryos up to day 14 of the blastocyst stage, but once again emphasizing the pertinence of continuous research of scientific information that cannot be documented otherwise (AACR, 2010) [9].

Aside from the knowledge of embryonic stem cells, other scientific contributions began changing the paradigm in the information and exploration of other exciting scientific territories. The theoretical concept that the adult central nervous system never regenerates [10] changed when scientists finding the neurogenic capacity of the adult brain. Even in human, neural stem cells were found primarily in two region, the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus [11–14]. In the case of rodents, newly generated cells are able to migrate, differentiate, mature, and integrate into preexisting circuits, where supply functions as cognition and olfaction [15].

2. Adult Stem Cells and Transplants

It is relevant to analyze the role of neural stem cells because of their potential clinical application in regenerative and repair therapy. It is clear that it is important to have discussions with the purpose of avoiding ethical dilemmas in science and in society, as well as their consequences concerning the procurement of these cells so that investigators can approach the field of applied science, in this case in reference to transplants.

Even though at the present time organ and tissue transplants are a real fact in our everyday life, this was accomplished thanks to a series of collaborations and willingness that led to experimental surgical refinements, the development of microsurgery, as well as the training and skill to perform vascular anastomosis and control rejection responses, just to mention some of the most relevant events

Nevertheless, in the case of neural stem cell transplants experience is barely beginning in the sense of systematization of processes, standardization of sources and origin of stem cells, application methods, controlled directionality of effects, clinical impact, and inherent risks and complications of the surgical procedures [16–20].

Part of this promising expectation was originally based on the concept that the brain was a site with a peculiar structure and functionality which made it "privileged," thus an organ with certain good features for receiving transplants. All of this was the result of having structures such as the blood-brain barrier and unique conditions in the behavior and tolerance of immune mechanisms [21].

However, these concepts have changed because we now have more information about the blood-brain barrier not being so airtight, and that it allows the passage of certain type of mediator cells of the immune response, and that it changes when there is an intrinsic inflammatory process [22]. The immune response concept itself underwent a change from learning that the microglia are capable of presenting antigens and activating phagocytic features and inducing *in situ* cytokine activities [23]. Moreover, the brain is currently no longer considered as a "privileged" site but an organ with different immune mechanisms and very peculiar immune response processes. In addition, it has been observed that many pathological and inflammatory conditions significantly affect neurogenic niches. Even more, increasing evidences indicate that chemokines and cytokines play an important role in regulating proliferation, cell fate choices, migration, and survival of neural stem cells under physiological conditions [24].

Even so, the brain is still a site with more favorable conditions for transplantation, compared to other peripheral organs, according to the tolerance experience found in fetal tissues with moderate immune suppression therapy, unlike xenografs, which are rejected. This opened perspectives for transplantation of neural stem cells, presumed to have greater potential in terms of structural and functional regeneration, with reduced risk of immune rejection reaction [25–27].

According to the concepts above, it has been considered that the potential action of these cells, when transplanted to the human nervous system, would have three relevant effects.

(1) Cell Regeneration. Previous studies showed that neural stem cell can differentiate and migrate after transplantation to integrate into the host tissue in models of spinal lesions and stroke [28–32]. This supports and gives hope to the proposed repair therapy. Additionally, active electric connection

mechanisms in the brain cortex have been identified [33], but the functional relevance of this response is still to be established, as well as the reason for the persistence of certain in situ cells as undifferentiated [34].

(II) Neuroprotection. An additional function of neural stem cells is the neuroprotective properties. Transplanted neural stem cells can release specific factors, which promote the survival and prevent cell death in sites where they have been implanted. Some of these released factors that increase their bioavailability are neurotrophic factors [35]. This proposal has led to expectations in case of inflammatory disorder [36], neonatal brain injury [37], and degenerative diseases [38–40]. One of the mechanisms proposed is that the transplanted neurospheres-derived cells have a two way molecular exchange that makes them more sensitive for releasing neurotrophic factors when found in the neuro-glial microenvironment.

(III) Immunomodulation. There is growing evidence about the immune modulating capacity of neural stem cells from in vitro and in vivo studies, in terms of regulating the deleterious inflammatory response and fostering immune conditions for tissue regeneration [41]. This has been shown as a reduced inflammatory response in experimental autoimmune encephalomyelitis, and a reduced proliferation of T-cell derivatives in response to concanavalin A in the oligodendrocytes [42]. Likewise, a reduced inflammatory response has been identified in experimental spinal lesions [43, 44], which would open the way to a new modality in the mechanism of action of transplanted neural stem cells.

3. Potential Applications of Adult Neural Stem Cells

At one point, it was considered that embryonic stem cells were the key for the creation of different potential cell lines with therapeutic purposes, when it became evident that they were available in greater number, easy to identify, and grow in culture. Also, embryonic stem cells grew faster and more easily, compared to adult stem cells, and ultimately they could be more plastic and manageable.

Even so, the use of embryonic stem cells presents legal and ethical limitations, such as the obvious destruction of live embryos to obtain stem cells. Additionally, other technical limitations not previously seen were identified: (a) rejection of embryonic stem cells requiring immunosuppressive treatment, (b) the possibility to induce cells of tumor lineage. All these limitations lead investigators to look for other alternatives.

The search for adult stem cells has become all the more important, getting greater availability to their source of origin and limiting ethical conflicts with the use of embryos [45].

Adult stem cells are found in the brain, pancreas, liver, bone marrow, blood, muscle, skin, and other body tissue. They can be crucial to continue forming and generating tissue which is structurally linked to cell lineages from where they have been phenotypically collected.

Currently, a few scenarios are found in which adult stem cells can have potential application leading to the identification and characterization of adult tissue with germinal properties such as the case of the hematopoietic tissue and the skin. Olfactory tissue stem cells have also been proposed as an alternative to be studied and transplanted to repair vascular brain lesions or traumatic spinal cord lesions [30]. A unique feature of adult neural stem cells is that they have been well identified and characterized in the adult brain, particularly in the subventricular zone and the dentate gyrus of the hippocampus [15], where neural stem cells show a potential differentiation to glial and neuronal cells aside from being compatible with radial migration [46, 47].

This sort of application has the potential advantage of implanting predifferentiated cells to certain glial or neuronal cell lineage, as a step forward in the therapy to repair pathologic processes, for example, ischemia, multiple sclerosis, spinal cord injury, or degenerative processes such as Alzheimer, Huntington, or Parkinson's disease [48–51].

4. Risks with Ethical Implications

Risks involved in the clinical application of adult neural stem cells have not been totally evaluated, nor have long-term followup, so it is still necessary to be cautious and alert. Not only because of the ethical implications that can be anticipated in terms to what is morally and socially accepted in each community, but also because of the technical implications and risks to the patient's health.

These risks are basically found within the following possibilities:

(A) Risk of Tumors. This possibility has been considered a real one, according to reports of teratomas in the striate cortex in experimental Parkinson models. A previous report mentioned 20% of new onset tumors in an experimental sample, when undifferentiated stem cells were used [52]. The possibility of using viral vectors or genetic manipulations from regulator genes, trying to guide differentiation and efficacy in dopaminergic neurons, also involves the risk of viral transmission out of control and out of target, plus the risk of mutagenesis. The stem cells themselves pose an additional unknown risk. The longer cells are grown in culture, the more likely they are to acquire genetic and epigenetic changes, in agreement with the previous experience with embryonic stem cells [53–55].

(B) Inadequate Migration. The risk of migration defects gives rise to heterotopias in the white matter, sub-ependymal region, and the cortical gray substance if there is no control in the migration process toward a lesion in a specific area. There might be an out of target aberrant migration, giving rise to potential heterotopias with the ensuing clinical complications such as difficult to control epilepsy (refractory) or other neuropathological conditions. Being able to get greater

differentiation in adult neural stem cells and the necessary refinement in target migration is still a challenge [27, 56].

(C) Transplant Rejection. Immune rejection conditions will always be found in adult neural stem cell transplants. Although there is now greater experience with embryonic mesencephalic stem cells and management of the need to give constant immunosuppression (cyclosporine), not only to avoid rejection, but also to maintain clinical response in adult neural stem cells the experience is not the same. Theoretically, since cells are more differentiated in adult tissues and more antigenic they might require greater use of immunosuppressive drugs with the inherent additional risks such as liver and renal toxicity, hypertension and immunodeficiency [57].

(D) Surgical Risks. In spite that most of the brain cell tissue transplants are done with stereotactic method, with specific mapping and accurate coordinates, the procedure is not devoid of risk. An average of 3% surgical risk has been reported associated to bleedings or infection. Even though the risk involved is less compared to deep brain stimulation procedures, where a foreign body is placed, this condition must be carefully looked into for risk-benefit analysis [34].

(E) Infections. This is a constant risk in every cell transplant process in which pathogens may be transmitted from the donor to the recipient, such as hepatitis B or C, lymphotropic virus, HIV/Aids, cytomegalovirus, and herpes simplex virus. In addition, there is also the risk of infection in the culture media and in handling the samples, either from bacteria (Staphylococcus, Streptococci, *E. coli*), yeasts, spores, and prion diseases [58].

5. Neuroethics and Neural Stem Cells

In view of the response from society to these unforeseen topics resulting from scientific and technological advances applied to medical science, it was necessary to have ethical support from certain deontological criteria and universal concepts (not specific to medical science), such as the UN Declaration of Universal Human Rights [59].

Subsequently, a more specific form is described with the advent of bioethics [60], applied in greater association with life science and survival. The latter school of thought is prevalent mainly in western culture and postulates respect of basic principles of human behavior in interaction with other individuals. This is the reason why every human action, even nonmedical, may be subjected to these precepts.

In more recent times, neuroethics has come to respond to the great demand of topics that neurosciences have put forth in dealing with traditional ethics. It has been necessary to establish more specific study lines, as a result of a great amount of information, research, and potential treatment applications with ethical implications, but more specific and dealt by experts who have deep knowledge of dilemmas prevailing in basic and clinical neuroscience.

Table 1: Ethical considerations in the use of neural stem cells. Ethical considerations related to the origin and source of neural stem cells and the potential clinical applications.

Ethical considerations	
Origin	Embryos from miscarriages and abortions, IVF embryos, *ex profeso* embryos, cultures, cadavers, tissues and somatic cells. Care and preservation of progenitor cells. Patents
Applications	Basic research, characterization, biological behavior, differentiation, migration, neurodevelopment. Transplants, diseases of neurodevelopment, trauma, stroke, neurodegenerative diseases. Regenerative therapy. Risks, long term results. Public health impact. Patents and accessibility

Neuroethics is not proposed with the reductionist view of a single organ, but rather redefining the important role of neuroscience as the object of study in every variant, and the unique condition that it is the human brain itself that ponders, discusses and decides about its own object of study.

Today, there are two points of interest in the field of stem cells proposed by neuroethics: on the one hand, the origin of stem cells and the way in which they are obtained, studied, protected, and preserved. On the other hand, everything around the application of neural stem cells, from feasibility to viability, risk and benefit, the transplant process itself, complications, outcome, public health impact, and also potential deviations [61–63].

At this point, I will particularly refer to the second segment, since there is a great deal of literature about the management and regulation of the origin of embryonic stem cells particularly referring to regulations of human embryos for research, where the dilemma of the difference between zygote, embryo, fetus, and the moment when life starts is still debated. However, in terms of ethical dilemmas from the application of stem cells to neurologic diseases, we do not have the same amount of information (Table 1).

At the present time, it is relevant to promote and motivate the use of adult neural stem cells for three main reasons: (a) to reorient the strategy to a more successful outcome considering cell lineage in the most differentiated possible way, in order to have better certainty about the functional implications of mature cells in the recipient tissue; (b) when the same tissue origin is considered, there would be greater likelihood to reach the therapeutic target, with less risk of variables involved in migration and functional errors, once the rejection reaction is under control; (c) adult neural stem cells involve less ethical objections, as compared to embryonic stem cells [64, 65].

For these specific potential cases for research and transplants, and the challenges and dilemmas they involve, it is wise to once again resort to the pragmatic ethics in bioethical research, that rules its actions within a framework of respect for the subjects of research, particularly groups of sick and vulnerable subjects.

Thus, it is considered healthy that proposals of fully justified new research that produces scientific knowledge, improves public health as well as the quality of medical care, must, above everything else, protect the patients and avoid creating damage. Also, such research must be totally compatible not only with legal regulations but also with precepts of moral and behavior values and virtues that identify a defined social context [66, 67].

Following these concepts, in the case of adult neural stem cell transplants, the same criteria used for transplants in general are applied in which a bioethical triangle is formed.

On one hand, we find the donor line in which there is total freedom, knowledge, informed consent to give, by means of an altruistic action, the tissue, and cells for a transplant. No extraordinary risks for the life or health of the donor are involved, without conflicts of interest or submission to scientific postures. In every case, a reasonable risk-benefit position must prevail, taking into account potential help for control or cure of a disease and the value of contributions to scientific knowledge.

On the other hand, we find the recipient line which would be assumed to be the main potential beneficiary, with the same rights, with informed consent and ethically validated in terms of selection and assignment criteria and with clear information about the expectations of the procedure, without any conflict of interest from secondary compensations. The donor makes the decision according to the established riskbenefit, considering that what is technically viable must also be ethically acceptable.

Finally, at the base of the triangle, we find the line of the human and professional team, participating in the scientific research, the harvesting of cells, cultures, transplant procurement, the transplantation itself, and subsequent followup and control. As any human activity, it is not free of risks and byas. That is the reason why everyone must do their work with self-respect, and respect for the team and obviously everyone involved in the transplantation process; following the principles of professional behavior of technical training, ethically acknowledged and supported.

This rational balance in the bioethical triangle must be totally compatible with principles of truthfulness, justice, equality, autonomy, welfare, and confidentiality, postulated by bioethics and that now neuroethics adopts as fundamental premises to deal with these new dilemmas that science has brought about neural stem cells [68].

This is where neuroethics attempts to create awareness among neuroscientists, geneticists, neurosurgeons, and all the professionals involved in this process about the responsibility of anticipating to the debate due to the use and abuse of these procedures and research. The neuroethicists have a social responsibility to see that the advent of technological advances which have increased our ability and power to carry out new experiments be maintained within the risk-benefit regulatory criteria, with respect for life and within morally and socially accepted strategies [69] (Figure 1).

One of the current and future challenges that has also been settled with specific actions in this scenario has to do with justice and access to this type of therapy as a standard treatment for given diseases in the future. One of the ways

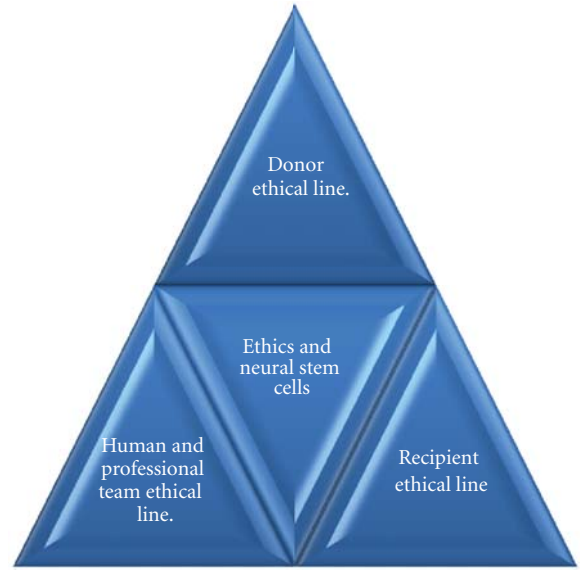

Figure 1: Bioethical triangle in the research and use of neural stem cells.

to deal with this matter has been to discourage development and approval of restrictive patents in this area, in order for this knowledge to prevail as heritage of humanity, and that viability and feasibility can be guaranteed everywhere in the world following the simplest reproducibility strategies with an ethical point of view [70].

6. Conclusions

Extensive and constant discussion is required about the role of biotechnology and its ethical implications in the scenario of neural stem cells. If we can have a balanced position, we shall be able to do scientific work with a tool that has great potential to solve health problems in different stages of life, such as in the case of neural stem cells, with no limitations. But it should certainly prevail in a position of respect for human beings and society that are the main beneficiaries of this promising proposal.

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Hindawi Publishing Corporation
Stem Cells International
Volume 2012, Article ID 959737, 8 pages
doi:10.1155/2012/959737

Review Article

The Insulin Regulatory Network in Adult Hippocampus and Pancreatic Endocrine System

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Received 13 June 2012; Revised 6 August 2012; Accepted 9 August 2012

Academic Editor: Sonia Luquin

There is a very strong correlation between the insulin-mediated regulatory system of the central nervous system and the pancreatic endocrine system. There are many examples of the same transcriptional factors being expressed in both regions in their embryonic development stages. Hormonal signals from the pancreatic islets influence the regulation of energy homeostasis by the brain, and the brain in turn influences the secretions of the islets. Diabetes induces neuronal death in different regions of the brain especially hippocampus, causes alterations on the neuronal circuits and therefore impairs learning and memory, for which the hippocampus is responsible. The hippocampus is a region of the brain where steady neurogenesis continues throughout life. Adult neurogenesis from undifferentiated neural stem cells is greatly decreased in diabetic patients, and as a result their learning and memory functions decline. Might it be possible to reactivate stem cells whose functions have deteriorated and that are present in the tissues in which the lesions occur in diabetes, a lifestyle disease, which plagues modern humans and develops as a result of the behavior of insulin-related factor? In this paper we summarize research in regard to these matters based on examples in recent years.

1. Introduction

Insulin is a hormone that helps cells store sugars and fats as energy sources, and a variety of problems develop in the circulatory organs including heart when its synthesis and secretion become impaired [1–4]. The fact that insulin is also essential to maintaining brain function is important [5–8], and in addition to the retinopathy and peripheral neuropathy that are known complications of diabetes, changes in insulin signals in the brain of diabetic patients have a pronounced impact on neuropathy in the central nervous system (CNS), especially in the hippocampus [9]. Actually, not only are diabetic patients at increased risk of contracting neurodegenerative diseases and psychiatric disorders such as Alzheimer's disease, Parkinson's disease, depression, and Huntington's disease, but diabetes has also been experimentally shown to have a great effect on the functions of the neural circuits in the hippocampal region [9–13].

The hormone insulin is secreted by the β cells that compose the pancreatic endocrine tissue in response to the environment and conditions in which the individual and the organs and tissues find themselves, and its metabolic mechanisms are strictly regulated [1–6]. Insulin also plays a major role in the stage that controls differentiation, especially by tissue stem cells, into almost all of the cells that compose the organ or the body. Under conditions in which the amount of insulin levels is reduced, the proliferative and maintenance functions of undifferentiated stem cells themselves (self-renewal capacity) are often consistently suppressed, even in different lineages. On the other hand, based on research reports in recent years it is now clear that insulin-activated signal transduction mechanisms play a major role in regulating their differentiation pathways at the stem cell differentiation induction stage in the various tissues [5–9].

2. Neural Stem Cells and Insulin

Stem cells retain pluripotency, which is the source of their ability to differentiate into various cell types that compose tissues and to fulfill their functions. When organs are formed during embryonic stage, organogenesis is achieved by

vigorous renewal by embryonic stem cells, which have very high proliferative capacity, and by orderly differentiation of tissue cell groups for the patterning. Stem cells can renew themselves almost indefinitely and differentiate into the cells that compose tissues. Asymmetric cell division allows stem cells to self-renew and produce another cell that undergoes differentiation. Stem cells are broadly classified into two types: stem cells in the embryo stage and adult stem cells, which are present in the tissues of adults. Neural stem cells, which are responsible for neurogenesis, are present not only during embryonic and perinatal stages but also in the adulthood stage.

In recent years, it has been shown that neural stem cells are present in adult mammalian brain. They self-renew by continually dividing, as well as differentiate into functional neurons and glial cells (astrocytes, oligodendrocytes) (Figure 1). The property of neural stem cells, such as self-renewal and the multipotency, is finely modulated by both cell-intrinsic and cell-extrinsic factors. It is well known that dietary restriction and exercise (mild running, etc.) enhance adult neurogenesis. On the other hand, severe stresses, brain inflammation, and the aging impair the rate of neurogenesis significantly. High-fat diets and obesity pose serious health problems as well as the decline of adult neurogenesis. To control the coordinate energy intake and expenditure, insulin signaling is utilized. Insulin is an important neuromodulator, contributing to neurobiological processes, in particular energy homeostasis and the cognitive function. Adult neural stem cells sense and respond to changes in energy homeostasis occurring locally in the brain and systemically in the mammalian organism. The regulatory system from an undifferentiated neural stem cell to a differentiated neuron, astrocyte, or oligodendrocyte is associated with various transcriptional changes, including numerous genes associated with metabolism and energy sensing such as insulin signal transduction pathways and insulin receptors (Figure 1). Understanding the hormonal aspects of how adult neurogenesis is regulated may lead new strategies for treating neurodegenerative disorders.

3. Diabetes and Hippocampal Neurons

As a result of high-calorie diets and sedentary lifestyles, diabetes is rapidly becoming more prevalent and appears to negatively impact the brain, increasing the risk of depression and dementia. The diabetes patients of type-1 (caused by insulin deficiency) or type-2 (mediated by insulin resistance) exhibit impaired cognitive function compared to age [14]. The incidence of Alzheimer's disease is almost twice that in a nondiabetic population [10]. Diabetes-related cognitive dysfunction has been controversial as to its underlying cause, whether it is the result of both microvascular and macrovascular cerebral diseases, hypoglycemic episodes with subsequent neuronal loss, the direct neuronal damage caused by chronically elevated intracellular glucose concentration in hyperglycemia, or the decline of neurogenesis. Recent studies suggest that diabetes produces a variety of neurochemical, neuroanatomical and behavioral changes that are indicative of accelerated brain aging in the hippocampus, including morphological changes [15], accumulation of oxidative stress markers [16], electrophysiological changes [17], neuroendocrine changes [18], and cellular molecular changes [19]. Diabetes rats exhibit dendritic atrophy of hippocampal pyramidal neurons, decreases in spine density, synaptic reorganization in the dentate gyrus (DG), increased neuronal vulnerability and reductions in cell proliferation and neurogenesis [20–22]. Since insulin replacement inhibits or reverses these neurological deficits in diabetes animals [23, 24], these results support the emerging and expanding hypotheses regarding the important role of insulin in the CNS, especially for hippocampal neurons.

4. *NeuroD1* Gene for the Insulin Expression in Both Pancreas and Hippocampus

As an example of control in the hippocampal nervous system in the brain, insulin increases the tolerance of mature neurons to toxicity and has a protective function that keeps the network functions of the neurons in an active state [25, 26] (Figure 1). Insulin also exercises control of the fate-determining mechanism (cell fate choice) of adult neural stem cells, that is, it newly promotes induction of undifferentiated neural stem cells to differentiate into oligodendrocytes [27], which have the function of protecting neurons. Moreover, insulin promotes the function of fibroblast growth factor 2 (FGF2), which has an important role in maintaining neural stem cells in the undifferentiated state, and it also plays a major role in the stem cell self-renewal stage, that is, it strongly activates stem cell proliferation [28–32].

The hippocampus is one of the parts of the brain in which severe damage occurs in both diabetic patients and patients with neurological diseases, including Alzheimer's disease [9–13]. The hippocampus governs human memory and learning functions, and all throughout life new neurons are generated daily by the neural stem cells located in the DG region, which is the region of the hippocampus where adult neurogenesis occurs [33–39]. The newly generated neurons construct new neural circuits with existing neurons, and a variety of extracellular factors, intracerebral neurohormones, and neurotransmitters whose function is to transmit information between neurons are synthesized and secreted, and they support the functions of the neural circuits. Not only a decrease in the neural activity of information transmission in these neurons but also a decrease in the phenomenon of neurogenesis by neural stem cells occurs due to the insulin deficiency in diabetic patients, and as a result there is a large decline in learning and memory capacity, which is governed by the hippocampus [5–13].

Research on the molecular mechanisms that control neurogenesis by the adult neural stem cells in the hippocampus has been vigorously pursued [36, 40, 41]. The paracrine factor Wnt3 is synthesized by astrocytes [42–44], which are a neurogenesis niche environment in the hippocampus. Neuronal differentiation is initiated when the *NeuroD1* gene is activated by the Wnt3 factor produced by the glia [42–44].

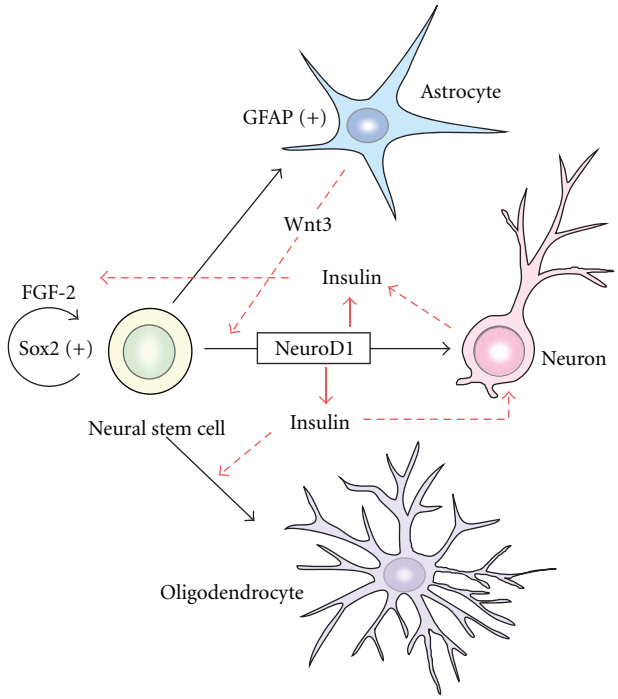

Figure 1: Schematic representation of the signals and transcription factors regulating adult neurogenesis. Undifferentiated adult neural stem cells express Sox2 transcription factor for the self-renewal function. FGF-2 promotes the proliferation of neural stem cells and insulin and IGF-1, and IGF-2 support the process. Astrocyte-secreted Wnt3 promotes the neuronal differentiation from neural stem cells by the activation of NeuroD1 transcription factor in the neuronal progenitor cell. The NeuroD1 transcription factor triggers the expression of insulin gene. Insulin, IGF-1, and IGF-2 promote the oligodendrocyte differentiation from neural stem cells. They also promote neuronal survival and possess the protection ability of mature neurons by preventing their natural cell death.

NeuroD1 is also a transcriptional factor that directly activates the insulin gene [45–47]. *NeuroD1*-gene-deficient mice lack the DG region, which is the site of neurogenesis in the hippocampus, and that causes a fatal functional impairment of the nervous system [47, 48]. NeuroD1-deficiency in mice causes severe diabetes and perinatal lethality because NeuroD1 is required for insulin gene expression [45, 46]. Rubio-Cabezas et al. investigated human subjects and sequenced the *NeuroD1* gene in 44 unrelated patients with permanent neonatal diabetes, result in that homozygous mutations in *NeuroD1* were identified in diabetes patients [49]. The study shows that diabetes, neurological abnormalities including cerebellar hypoplasia, learning difficulties, sensorineural deafness and visual impairment result from the loss of function mutations in *NeuroD1*. These reports indicate the critical role of NeuroD1 in both the endocrine pancreas and the CNS not only in animal models (rodents) but also in humans.

5. Insulin Expressions in CNS

Neuronal synthesis of insulin has been debated for long time. *De novo* insulin synthesis in mammalian brain has been supported by the detection of preproinsulin I and II mRNA in rat fetal brain and cultured neurons and also by insulin immunoreactivity in neuronal endoplasmic reticulum, axon, dendrites, and synapses [50–56]. Singh et al. reported that insulin is expressed in cultured rat hippocampal neurons but is not expressed in peripheral sympathetic neuronal cells [57]. Devaskar et al. reported that rabbit hippocampus expressed insulin mRNA apparently by using RNase protection assays and in situ hybridization analysis [58]. High performance liquid chromatography, radioimmunoassay, and [35S]cysteine metabolic labeling of cultured neuronal and glial cells indicated extracellular secretion of immunoprecipitable insulin by neurons only [58]. Further studies provided clear evidence that insulin synthesis occurs in mammalian brain CNS, where it may reach high levels [57, 59–61]. Consistent with these studies, we also reported that *de novo* insulin synthesis was performed in adult neural stem cell culture *in vitro* and in newborn neurons in the rat and mouse hippocampus *in vivo* [62].

Importantly, the mentioned NeuroD1 transcriptional factor is essential for expression of target insulin gene in CNS. Wnt3 stimulates their activation as stem cell niches-secreting paracrine factor in hippocampal DG [42–44]. The localization of insulin-expressing neurons involves olfactory bulb and higher order association which are rich in dopaminergic norepinephrinergic innervation [57, 62]. These same areas express insulin receptors [63], suggesting autocrine or paracrine action of the insulin in adult hippocampus. Since hippocampal neurogenesis is coupled with expression of NeuroD1 [43, 46–48, 64], the expression (and the secretion) of insulin in newborn neurons via the NeuroD1 transcription factor may be upregulated in a treatment that promotes adult neurogenesis in diabetes patients.

6. Regenerative Therapy for Diabetes Using Adult Neural Stem Cells

Transplantation of pancreatic islets (islets of Langerhans) from the pancreas of a different donor is an effective treatment method for type-1 diabetes. However, the problem of shortage of human donors for pancreatic islet transplantation is extremely serious. As described previously, adult neurons existing in our brain neurogenic area, such as hippocampus, have the intrinsic capacity to produce insulin, implying that adult neural stem cells have a potential as a cell source for the stem cell-based transplantation therapy of diabetes (Figure 2). Adult neural stem cells can be established and cultured by collection from the olfactory bulb [65–68], which is easier location to collect multipotent neural stem cells than the hippocampus. It has been confirmed that when adult neural stem cells are collected from diabetic rats, established as cultures, and transplanted into the pancreases of diabetic rats once a state of ready insulin production has been achieved, a decreased blood glucose level can be maintained [62]. In addition, when the transplanted neural stem cells were removed from the alleviated diabetic rats, the blood glucose level again increased [62]. Since the strategy is based on autologous transplantation of cells (adult stem cells from patient's olfactory bulb: endoscopic collection of neural stem cells from the olfactory bulb is preferable to collection of intracerebral neural stem cells by means of difficult surgery), there are no donor issues and no concerns about the adverse effects of immunosuppressive agents. The insulin-producing cells are continuously replenished by adult neural stem cells and therapeutic efficacy is thus maintained. Another advantage is that the treatment has a low carcinogenesis risk and is considerably safe because the procedure involves no gene transfer.

However, to make it closer to reality of the treatment using adult neural stem cells for diabetes, there are several challenges and issues to be overcome. It is important to evaluate the method using adult neural stem cells derived from large model animals more close to human, such as monkeys and pigs. During the time-period that adult neural stem cells are extracted from the CNS, such as olfactory bulb, and expanded to transplant to the pancreas of the patients, the improvement of the technology and methodology to activate the neural stem cells with high quality to express the insulin is also required. Increasing the insulin expression capability by *ex vivo* culture with recombinant Wnt3 protein (activator of NeuroD1 expression) and antibody against IGFBP-4 (inhibitor of Wnt signaling) is essential for the scheme that was examined in rodent study [62] (Figure 2). This step can be further improved by the various types of drug screening and/or also for order-made cell screening system for the personalized therapy. The screening system usines adult neural stem cells that assume drug discovery for diabetes patients with neurological diseases is considered to be available, in conjunction with a pharmaceutical company with chemical compound libraries. If declined ability of the CNS and the neural stem cell function could be improved by the medication, the synergistic effect of diabetes treatment with combined transplantation therapy would be expected.

In terms of stem cell-based regenerative medicine, it is more elegant to activate the adult stem cells present in the patient's body safely, and in a way that is closer to that which occurs naturally without stressful surgeries in future. Monitoring of patient's insulin production ability on the olfactory bulb-derived adult neural stem cells would be also useful in both diabetes treatments and neurological diseases caused by low-insulin levels.

7. Similarity between Adult CNS and Pancreatic Endocrine System

A basic mechanism of expression of a certain absolutely essential biological factor, insulin, that has correlative similarities has been demonstrated in very different tissues that are far apart from each other in the body: the pancreas and the hippocampus. Because the nervous system is the source of insulin production in invertebrates, such as the fly [69, 70], it is very interesting that in maintaining the function of both the CNS and the pancreatic endocrine system the insulin regulatory system plays a major role in the mechanism that controls stem cell differentiation in both tissues and has been preserved during evolution. Comparisons and analyses of the functions of neural circuits in the CNS and hormone metabolic system in the pancreatic endocrine system are expected to provide new clues to understanding and analyses based on new approaches in the field of research on the control mechanisms of neuronal circuits. For example, is not there a possibility that the insulin control network, that is, proliferation and differentiation of stem cells and protection of neurons in the CNS (Figure 1) functions similarly in the cells that build up pancreatic endocrine tissue (α cells, β cells, δ cells, and γ cells)?

Actually, niche environment correlations are observed. An astrocyte marker in CNS, GFAP, is expressed in a similar manner in the α cells of the pancreas together with glucagon [61, 71, 72]. Another astrocytic gene, S100beta, is also expressed in pancreatic GFAP-positive cells [73]. Pancreatic δ cells may be regulated under gene control in the similar way in part as interneurons at hilus region in the hippocampus, since they share same expression somatostatin as typical marker of the cell population [74]. Somatostatin is a multifunctional peptide with variety of functions, and it has been associated with disease progression, such as neurological disorders including Alzheimer's disease and multiple endocrine neoplasia [75, 76]. The understanding of the regulatory system of differentiation of each lineage from adult neural stem cells may contribute to develop new tools of the therapy of diseases affecting both pancreatic endocrine system and CNS.

8. Sensor Cell Populations That Control the Cell Fate Choice of the Stem Cells-Wnt3 and Insulin

Astrocytes function as the neural stem cell niche in the hippocampus, and they not only have important functions that maintain neuron functions and support neural activity,

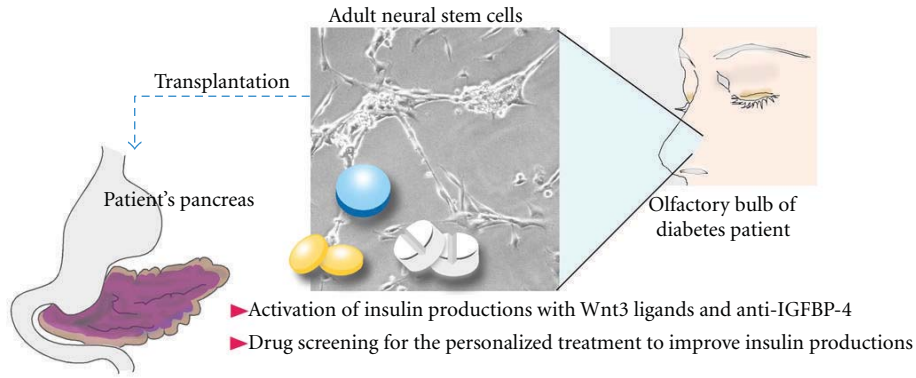

Figure 2: Concept of regenerative therapy for diabetes using autologous neural stem cells from the olfactory bulb. Adult neural stem cells are extracted form the olfactory bulb surgically using an endoscope. Since neural stem cells in diabetic animals had been found to contain higher IGFBP-4 (the Wnt inhibitor) and lower levels of Wnt3 (activators for insulin production via the NeuroD activation) than wild-type animals, treating the cultured neural stem cells with Wnt3 ligands and anti-IGFBP-4 (neutralizing antibody against the IGFBP-4 protein) rescues insulin expressions during *ex vivo* culture on the collagen sheets. This step would be improved by the various types of drug screening and/or also for order-made cell screening system for the personalized therapy.

but also support neurogenesis by undifferentiated adult neural stem cells. One of its molecular mechanisms consists of secretion of Wnt3/Wnt3a protein, which is important for triggering neurogenesis (activation of *NeuroD1* gene) and controlling stem cell behavior. Glycogen synthase kinase 3 (GSK3) is a ubiquitously expressed serine/threonine kinase that is central to insulin and Wnt signaling [77]. In insulin-signaling, GSK3 constitutively inhibits glycogen synthesis by the phosphorylation. In the canonical Wnt signaling that is activated by Wnt3/Wnt3a, beta-catenin is the substrate for the GSK3. Wnt and insulin both negatively regulate GSK3, albeit through two disparate signaling cascades [78]. Insulin target genes contain insulin response elements, which bind a variety of transcription factors [79]. Insulin receptors are abundant in neurons in cell bodies and synapses and less abundant in glia [80–84]. Wnts can signal through several different types of receptors, but the most widely recognized Wnt receptors are Frizzled proteins (Fzd). Fzd receptor subtypes and related signaling molecules are expressed in the adult hippocampus, where they play roles in the survival, function, and plasticity of neurons. In mice lacking Wnt3a, the hippocampal formation is disrupted, suggesting Wnt3a-medated signaling is crucial for the normal growth of the hippocampus [85].

Astrocytes have been found to retain sensor function that transmits the environmental signals to the stem cells, by producing the paracrine factor Wnt3 in response to the circumstances [42–44]. The frequency of neurogenesis declines with age, and it varies with the environment in which individuals find themselves, such as stress or disease, suggesting that neurogenesis in the hippocampus is regulated by molecular mechanisms that are capable of readily changing in response to external stimuli [44, 86]. Some "external stimuli," for example, exercise and an enriched environment, increase the formation of the network of new neurons in the hippocampus, while others, for example, stress, disease, and aging, reduce it, and the patterns of expression of large numbers of gene groups change in a variety of ways.

9. Developing New Strategy to Treat Diabetes and Neurological Diseases

When we observe the homology between the pancreas and hippocampus by comparing them against each other, very interesting questions arose as to whether there might also be a cell population that has a sensor function in the pancreatic endocrine system. Hardly anything has yet been learned about the behavior according to the stage of disease progression or biological information, such as age, of the α cells in pancreatic endocrine tissue, which correspond to the Wnt3 factor producing ability of the hippocampal astrocytes, and future research is being awaited. If it were possible to control the Wnt3 producing ability of hippocampal astrocytes and pancreatic α cell niches, it might lead to improvement of the tissue stem cells whose function is reduced in the diabetic state as well as to progress in new research and development that will be useful in medical care and drug discovery.

In this paper, we described the insulin control network of both the neural stem cells in the brain and the endocrine cells in the pancreas based on their involvement with diabetes patients, and the accumulating academic information linked to overcoming the diminished functions of stem cells whose functions have deteriorated will be very useful. In the future, it appears that it might also be possible to contribute to elucidating the pathology not only of diabetes but also of neuropsychiatric diseases whose risk increases as the pathology of diabetes progresses or to the search for new therapeutic reagents for the treatment of human neuropsychiatric diseases and to the development of treatment techniques.

Acknowledgments

The original work described in the present paper was supported, in part, by the National Institute of Advanced Industrial Science and Technology (to M. Machida, S. Fujimaki, R. Hidaka, M. Asashima and T. Kuwabara),

a Grant-in-Aid for Scientific Research on Innovative Areas (to T. Kuwabara), JSPS Grant-in-Aid for Scientific Research C (no. 23500773 to M. Machida) and a Mochida Memorial Foundation for Medical and Pharmaceutical Research (to T. Kuwabara).

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