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Mesenchymal Stem Cells Stimulate Endogenous Neurogenesis in the Subventricular Zone of Adult Mice

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Abstract Mammalian neurogenesis has been demonstrated in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. However, the low rate and the restricted long term survival of newborn cells limit the restorative ability of this process. Adult bone marrow derived mesenchymal stem cells (MSCs) have been extensively studied due to their wide therapeutic potential. The aim of this study was to determine if MSC transplantation to the normally restrictive SVZ of mice housed in an enriched environment stimulates endogenous neurogenesis. In the presented study 30 C57BL/6 female mice were divided into 3 groups: standard environment injected with phosphate buffered saline (PBS) and enriched environment injected with either PBS or MSCs. Bromodeoxyuridine was injected for 6 days, and 3 weeks later the mice were sacrificed and the brain tissue analyzed immunohistochemically. PBS-treated mice housed in enriched cages showed augmented neurogenesis in the SGZ but not the SVZ. MSC transplantation was associated with increased proliferation and neuronal differentiation of neural progenitors within the SVZ and an increase in the proportion of the newborn neurons out of the total proliferating cells. Histological analysis confirmed the survival of a significant amount of the transplanted cells at least 3 weeks after transplantation, and the presence of brain-derived neurotrophic factor expression. To our knowledge, this is the first study to show that MSCs might interfere with the tight regulation of the SVZ, independent of the induced brain lesion.

Keywords Adult stem cell · Neurogenesis · Subventricular zone · Subgranular zone · Neural stem cell · Enriched environment · Cell transplantation · Brain-derived neurotrophic factor

Abbreviations

BMP	bone morphogenic protein
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
GFAP	glial fibrillary acidic protein
MSCs	mesenchymal stem cells
NeuN	neuronal nuclei
PBS	phosphate buffered saline
SGZ	subgranular zone
SVZ	subventricular zone

Introduction

Studies conducted in the last decade have challenged the traditional view that the mammalian central nervous

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system is a nonrenewable tissue. Findings demonstrate that neural stem cells, also referred to as neural progenitor cells, exist in both the developing and adult nervous systems of all mammalian organisms, including humans. Adult neurogenesis was detected in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus.

The adult SVZ contains three main types of progenitor cells: glial fibrillary acidic protein (GFAP) positive, slowly dividing cells (type B); actively dividing transient amplifying cells (type C); and migrating neuroblasts (type A) [1–4]. Accordingly, SGZ contains: GFAP-expressing cells with radial processes (type 1), GFAP-negative cells with short processes (type 2) and doublecortin (DCX) expressing neuroblasts [2, 3]. Researchers hypothesize that the radial slowly dividing cells (type B in the SVZ and type 1 in the SGZ) have the potential to generate dividing neural stem cells (type C in the SVZ zone and type 2 in the SGZ) which in turn give rise to DCX^+ neuroblasts and thereby repopulate neurogenic niches [2, 3, 5–8]. Evidence of a direct relationship among the different residents of these niches is still lacking. According to neurogenesis studies, endogenous neural stem cells may contribute to the repair and integrity of lesioned circuits. In adults, however, neurogenesis occurs at a very low rate, and the long-term survival of newborn cells is limited [9]. Therefore, novel strategies are needed to stimulate the process and increase the restorative ability of newborn cells [10].

Environmental enrichment has been suggested as a potential method to stimulate hippocampal endogenous neurogenesis and the integration of newborn cells into existing neural circuits [11–17]. Several processes may mediate this effect, including increased levels of neurotrophins and synaptic proteins, local expression of vascular endothelial growth factor, recruitment of T cells, and activation of microglia [17]. The theory of enriched environment has led to the hypothesis that the standard cages used to house laboratory animals constitute a non-natural state that limit neurogenesis [4].

Mesenchymal stem cells (MSCs) are known for their ability to adhere to cell culture plastic surfaces and to proliferate extensively [18]. Adult bone-marrow-derived MSCs, under specific conditions, can differentiate into various mesenchymal phenotypes, such as bone, cartilage, and fat [18–20]. Therapeutic strategies based on autologous transplantation of MSCs circumvent the problem of immune rejection and the risk of teratoma formation while raising very few political concerns [21]. These advantages have made MSCs an appealing candidate for the treatment of a broad range of diseases [22–28]. The mechanisms suggested to underlie their beneficial effect include replacement of damaged cells, delivery of trophic factors, and immune modulation [29, 30].

The aim of the present study was to determine if MSC transplantation to the normally restrictive SVZ of mice placed in an enriched environment stimulates endogenous neurogenesis.

Materials and Methods

Isolation and Culture of Human MSCs

This study was approved by the Helsinki Committee of the Israel Ministry of Health and Tel Aviv University. Fresh bone marrow aspirates harvested from the iliac crests of adult donors (age 19–76) after informed consent were diluted 1:1 with Hanks' balanced salt solution (Biological Industries, Beit Haemek, Israel). Mononuclear cells were recovered from the fractionated suspension following centrifugation in Unisep-Maxi tubes (Novamed, Jerusalem, Israel) at 1,000 g for 20 min at room temperature. The cells were plated in polystyrene plastic 75 cm^2 tissue culture flasks (Corning, Corning, NY), and nonadherent cells were removed after 24 h by medium replacement. The cells were grown in growth medium containing Dulbecco's modified Eagle's medium (Biological Industries) supplemented with 15% fetal calf serum (Biological Industries), 2 mM L-glutamine (Biological Industries), 100 μg/ml streptomycin, 100 U/ml penicillin, and 12.5 U/ml nystatin (Biological Industries). Adherent cells were cultured to 70–90% confluence and reseeded at a density of 5,000–10,000 cells/cm^2. The cells were maintained at 37°C in a humidified 5% CO^2 incubator.

Animals

All animal studies were approved by the Animal Care and Use Committee of Tel Aviv University. Every effort was made to reduce the number of animals used and to minimize their suffering. Only female mice were used because male mice show territorial behavior with stressful consequences that may affect neurogenesis.

Thirty C57BL/6 female mice weighing 20 g (Harlan, Jerusalem, Israel) were randomly assigned to 3 experimental groups: (1) 10 mice placed in two standard cages and injected with phosphate buffered saline (PBS); (2) 10 mice placed in enriched environment and injected with PBS; (3) 10 mice placed in enriched environment and injected with MSCs. The enriched environment consisted of a large specially designed cage measuring 75×48×36 cm and equipped with a rearrangeable set of plastic tubes, a running wheel, and toys. All mice were housed at a constant

temperature of 23±2°C and lights on 06.00–18.00 h. Food and water were provided *ad libitum*.

Cell Transplantation

Using a stereotactic frame (Stoelting, Wood Dale, IL) under chloral hydrate anesthesia, 100,000 cells in 1 μl PBS or 1 μl PBS was injected into the right and the left SVZ according to the mouse brain atlas [31] at the following coordinates (relative to the bregma and dura): +0.5 mm anterior-posterior; −1.1/1.1 mm medial-lateral; −2.5 dorsal-ventral.

BrdU Administration

To examine the fate of the constitutively proliferating population, the mice were injected intraperitoneally with 50 mg/kg body weight of bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO), 1 injection per day for 6 consecutive days after the PBS/MSCs injection.

Tissue Preparation

Three weeks after the BrdU administation, the animals were anesthetized with chloral hydrate and transcardially perfused with cold PBS, followed by paraformaldehyde (PFA) 4% in phosphate buffer. The brains were then immersed in 4% PFA for 24 h at 4°C followed by cryoprotection in 30% sucrose for an additional 48 h. The brains were frozen in chilled 2-methylbutane, stored at −70°C, and subsequently sectioned with a sliding microtome at −20°C. Coronal cryosections measuring 10 μm were directly mounted onto Superfrost Plus slides (Thermo Scientific, Waltham, MA) for analysis.

Immunohistochemical Analysis

Sections were initially treated with 2N HCl for 30 min at 37°C, washed with PBS, and blocked/permeabilized for 1 h with PBS containing 20% normal horse serum and 0.5% Triton X-100 [32]. Specified combinations of the following primary antibodies diluted in the blocking solution were applied for 24 h at 4°C: rat anti-BrdU (1:200; AbD Serotec, Raleigh, NC), mouse anti-neuronal nuclei (NeuN) (1:200; Chemicon/Millipore, Billerica, MA), mouse anti GFAP (1:200, Chemicom), mouse anti-brain-derived neutrophic factor (BDNF, 1:200; Chemicom) and mouse anti-human nuclei (hNu; 1:100; Chemicon). Secondary antibodies diluted in PBS, highly absorbed goat anti-rat Alexa 488 (1:500, Molecular Probes/Invitrogen, Carlsbad, California) and goat anti-mouse Alexa 568 (1:500, Molecular Probes), were applied for 1 h at room temperature. Sections were mounted with fluorescent mounting solution (Dako, Glostrup, Denmark), covered with a cover slide, and sealed with nail polish. Digital images were obtained with a fluorescence Olympus BX52TF microscope.

Quantification

Proliferation was assessed by counting the $BrdU^+$ cells bilaterally in the SGZ of the dentate gyrus (defined as a zone of the hilus, the width of two cell bodies, along the base of the granular layer) and the SVZ of the lateral ventricles. Neurogenesis was assessed by counting the cells that were co-labeled with BrdU and NeuN. Using the mouse brain atlas [31] we quantified the cells in the SGZ by staining 6 coronal sections (360 μm apart) from −1.06 to −3.8 (posterior to bregma) per mouse brain and manually counting $BrdU^+$ and $BrdU^+/NeuN^+$ cells. In the SVZ, 4 coronal sections (150 μm apart) from the appearance of the third ventricle (1.18, anterior to bregma) to the disappearance of the commissural anterior (−0.34, posterior to bregma) per mouse brain were stained, and the positive cells were counted. To estimate the number of cells per dentate gyrus/lateral ventricle wall, the total number of cells counted in the selected coronal sections from each brain was multiplied by the volume index (ratio of the volume of the dendate gyrus/lateral ventricle wall to the total combined volume of the selected section). Four brains were used for quantification. This method of quantification was based on a previously described protocol with minor modifications [32].

Statistical Analysis

Data were analyzed using SPSS software. Comparisons between two groups were performed with the two-tailed *t*-test. The results were considered significant at $p<0.05$.

Results

Enriched Environment Enhances the Number of Newborn Neurons in the SGZ but not in the SVZ

To determine whether an enriched environment affects neurogenesis in the SVZ and SGZ, we assessed the number of cells that incorporated BrdU and stained positive for neuronal marker NeuN in mice placed in regular or specially designed enriched cages 3 weeks after the BrdU injection (Fig. 1a, b). There was a statistically significant difference in the number of $NeuN^+/BrdU^+$ cells in the SGZ of the dentate gyrus between the animals housed in standard conditions (standard cage + PBS) and enriched environment (enriched cage + PBS; 2,108±429 cells vs. 4,439±428 cells, respectively, $p=0.003$; Fig. 1e, f, g). No such difference was noted in the SVZ ($p=0.34$; Fig. 1c, d, g).

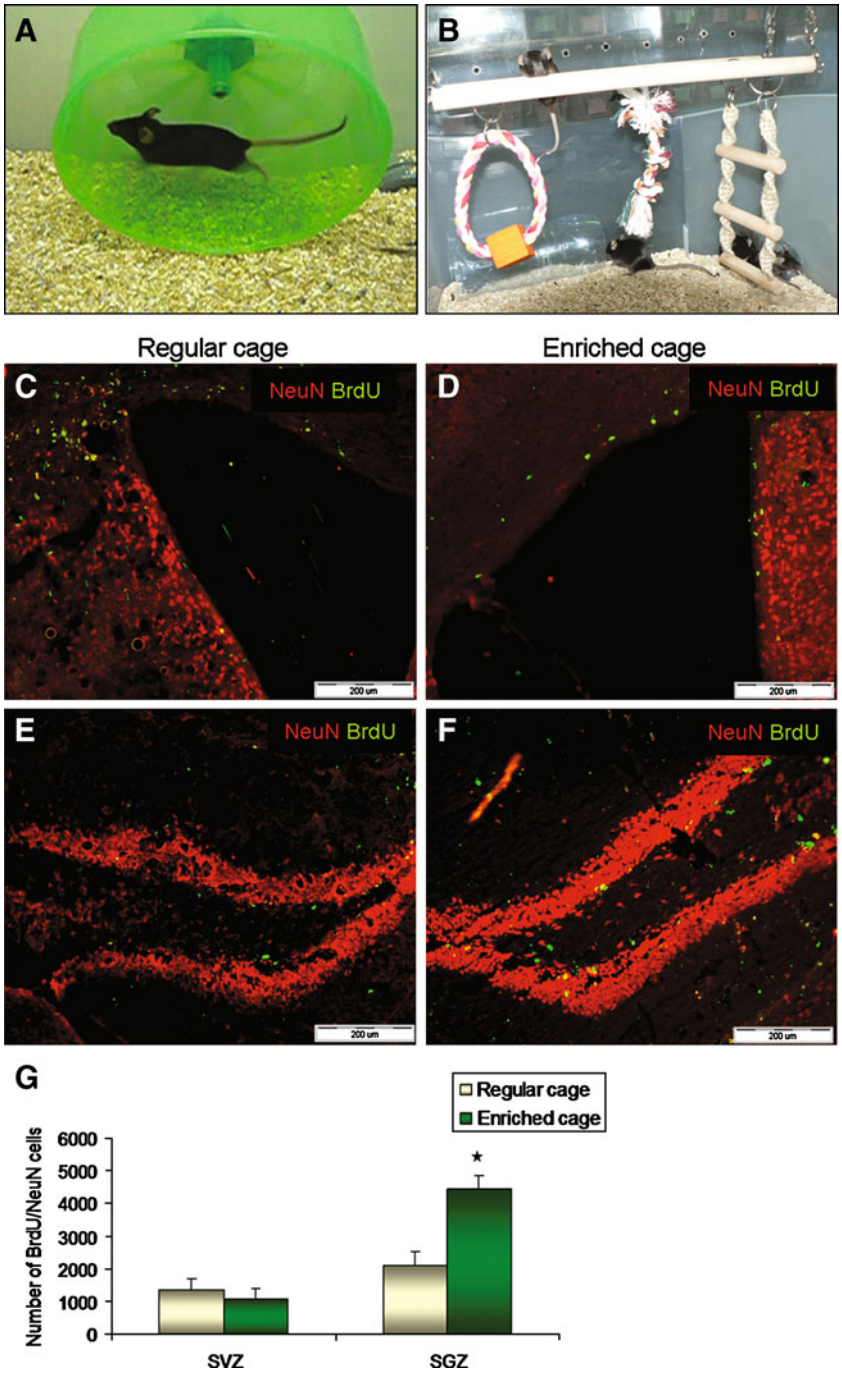

Fig. 1 Effects of enriched environment on neurogenesis in the SVZ and SGZ. **a–b** The enriched environment, consisting of a large, specially designed, cage equipped with a rearrangeable set of plastic tubes, a running wheel, and toys. **c**–**f** NeuN (*red*) and BrdU (*green*) staining of newborn neurons in the SVZ/SGZ of mice injected with PBS and housed in a regular cage (**c**, **e**) or enriched cage (**d**, **f**). **g** Quantification of the $NeuN^+$ and $BrdU^+$ cells in the SVZ and the SGZ of mice injected with PBS and placed in a regular or enriched cage. Values are mean number of cells and standard deviation. *$p<0.05$

Transplantation of MSCs into the SVZ of Mice Housed in an Enriched Environment Stimulates Cell Proliferation and Neuronal Differentiation

On the basis of the hypothesis that animals housed in standard cages are in a non-natural state that may inhibit neurogenesis [4], we used enriched cages as a common platform to evaluate the effects of MSC transplantation into the SVZ. Immunohistochemical analysis revealed a significant increase in the number of the $BrdU^+$ cells in the SVZ of MSC transplanted group compared to PBS injected group (from 2,153±343 to 6,432±665, $p=0.0006$; Fig. 2a, b, e) and in the number of newborn neurons, $BrdU^+$ and $NeuN^+$, (from 1,056±340 to 4,756±1,731, $p=0.02$; Fig. 2a, b, f). Furthermore, the SVZ was characterized by an increase in the proportion of newborn neurons out of all $BrdU^+$ cells, from ~50 to ~75% (Fig. 2g). In the SGZ, MSC transplantation did not induce a further effect beyond the stimulation of hippocampal neurogenesis by the enriched environment ($p=0.36$ for $BrdU^+$ cells; $p=0.18$ for $NeuN^+/BrdU^+$ cells; Fig. 2e, f, g).

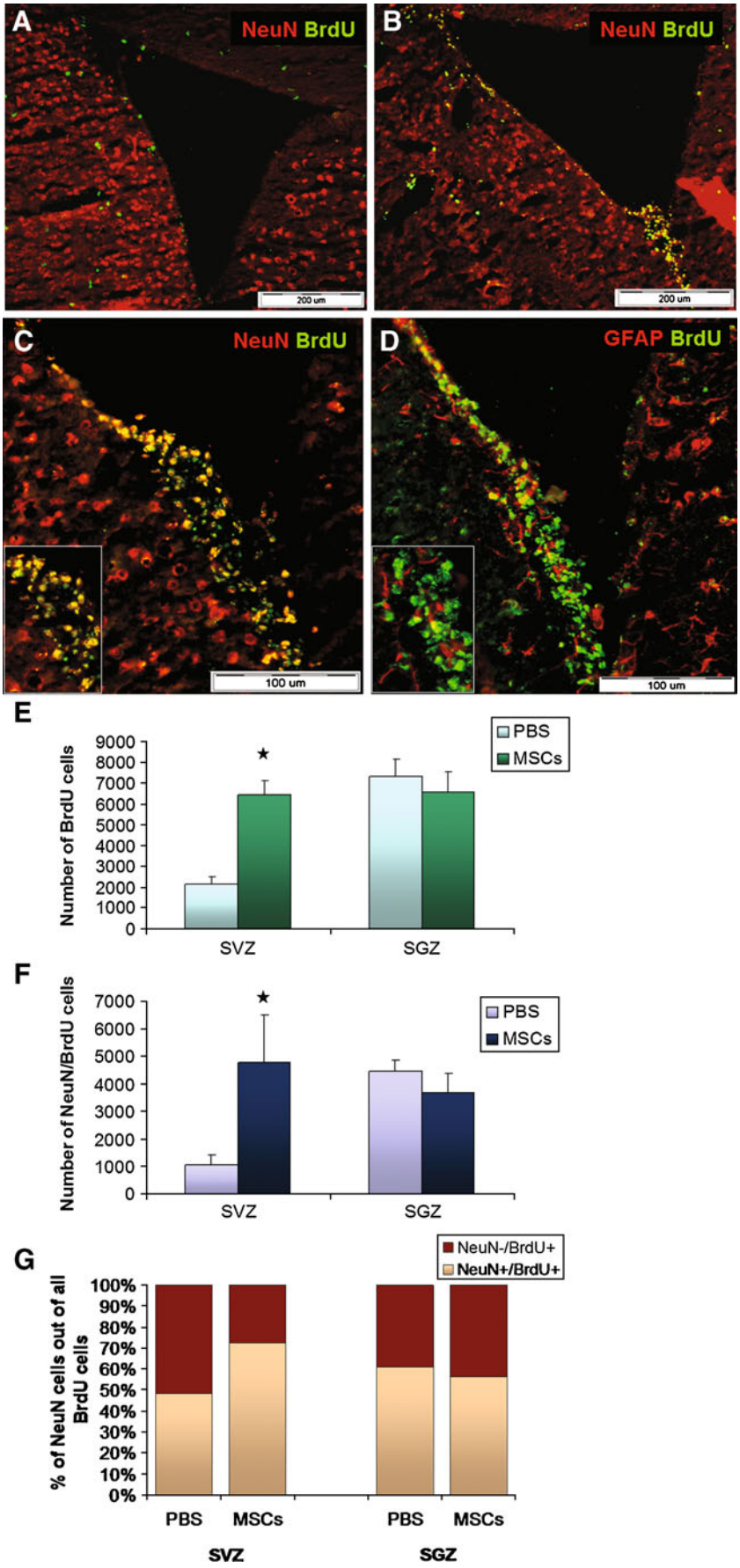

Fig. 2 Neurogenic effect of MSC transplantation into the SVZ of mice housed in enriched cages. a–b NeuN (*red*) and BrdU (*green*) staining of the newborn neurons in the SVZ of mice injected with PBS (**a**) or MSCs (**b**). **c–d** Detection of $NeuN^+/BrdU^+$ cells (**c**) and $GFAP^+/BrdU^+$ cells (**d**) in the sequential sections in the SVZ region of mice transplanted with MSCs. **e–f** Number of proliferating progenitors, $BrdU^+$ cells (**e**) or newborn neurons, $NeuN^+/BrdU^+$ cells (**f**) in the SVZ and SGZ of mice injected with PBS or MSCs. Values are mean number of cells and standard deviation. *$p<0.05$. **g** Percentage of $NeuN^+$ cells out of total $BrdU^+$ cells in the SVZ and SGZ of mice injected with PBS or MSCs

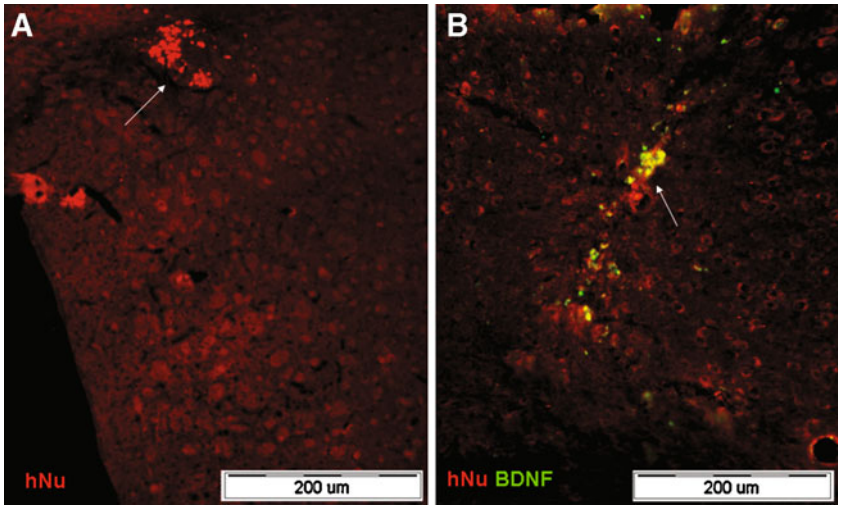

Fig. 3 Detection and characterization of the transplanted MSCs three weeks after transplantation into the SVZ of mice housed in enriched cages. a hNu staining of the transplanted cells, detected in proximity to the transplantation site. **b** hNu (*red*) and BDNF (*green*) staining of the transplanted cells

To further characterize the newborn cells in the SVZ of the mice transplanted with MSCs, sequential slides were stained for neuronal marker (NeuN and BrdU) and astrocytic marker (GFAP and BrdU). As seen in Fig. 2, most of the $BrdU^+$ cells co-expressed NeuN, whereas only a few cells were co-labeled with GFAP (Fig. 2c, d).

Mesenchymal Stem Cells Survive Three Weeks after Transplantation and Produce BDNF

We next sought to determine if the newborn neurons in the SVZ of MSC transplanted brains may derive from the introduced cells. As seen in Fig. 3a, the transplanted MSCs, detected by immunostaining for human antigen hNu, were detected in the transplantation site, in proximity to the SVZ, for at least 3 weeks after transplantation. No cells positive for hNu^+ could be detected in the lateral wall of the lateral ventricle, ruling out the possibility that the newborn neurons were derived from the transplanted cells. Co-staining with hNu and BDNF showed that the transplanted cells expressed BDNF 3 weeks after transplantation (Fig. 3b).

Discussion

This study showed that female mice placed in specially designed enriched cages show an increase in neurogenesis in the SGZ of the dentate gyrus but not in the SVZ. Under these conditions, transplantation of human MSCs into the SVZ stimulates the proliferation and maturation of endogenous progenitors towards the neuronal phenotype. The newborn cells express the neuronal marker NeuN but not the astrocytic marker GFAP. The detected newborn neurons are not the transplanted cells as indicated by the finding that the transplanted cells remain in close proximity to the site of transplantation and were never detected along the lateral walls of the lateral ventricles. In addition, the transplanted cells were found to express a neurotrophin, BDNF, which may provide a feasible explanation for the reported data.

The present study is part of the search for novel therapeutic strategies in brain injury and disease that are based on the concept of adult neuronal regeneration. Our findings are in agreement with previous reports on the stimulatory effect of enriched environment on hippocampal neurogenesis [11–17]. We showed that housing mice in large cages equipped with plastic tubes, a running wheel, and toys, while better mimicking the natural environment than standard cages [4], stimulates neuronal cell proliferation and differentiation in the SGZ.

MSCs produce and secrete a variety of factors that regulate angiogenesis, immune response, and neural function [33–36]. We hypothesized that they may stimulate endogenous neurogenesis upon transplantation into the SVZ. Indeed, in the reported study transplantation of MSCs into the SVZ stimulated the proliferation and the neuronal differentiation of endogenous progenitors within the SVZ niche. At 3 weeks after stem cell transplantation, the newborn neurons expressed neuronal marker NeuN and did not express astrocyte marker GFAP. Our assumption that the $NeuN^+$/$BrdU^+$ cells were indeed newborn neurons was based on the finding of Mullen et al. [36] that NeuN is expressed in the majority of neuronal cell types upon withdrawal of the neuron from the cell cycle and the beginning of terminal neuronal differentiation [37]. The finding of an increase in the proportion of the newborn NeuN-expressing neurons out of the total $BrdU^+$ cells within the SVZ may indicate selective stimulation of neuronal differentiation. Further analysis confirmed that the newborn neurons derive from endogenous progenitors and are not the transplanted cells, which remained near the transplantation site.

The neurogenic effects of transplanted MSCs have been previously described in animal models of brain insults, such as stroke and Parkinson's disease [38, 39]. However, in these studies, the neurogenic effect could not be detected in naive brains, suggesting a synergistic action of the transplanted cells and the induced lesion on endogenous neurogenesis. Additional studies showing that MSCs promote endogenous neurogenesis in the hippocampus [40, 41] suggested that the effect of MSCs in the nervous system is mediated mainly by their interaction with local progenitors and not by their differentiation into neural cells.

During adult SVZ neurogenesis, neural stem cells proliferate within the SVZ, form chains that migrate to the olfactory bulb ("chain migration"), and differentiate into inter-neurons [1–4]. According to the developmental timeline of Zhao et al. [2], newborn neurons start to express NeuN only 14–28 days after birth [2]. In our study, $NeuN^{+}/BrdU^{+}$ cells were detected within the SVZ 3 weeks after the BrdU administration. Therefore, we hypothesize that the transplanted MSCs interfered with neurogenesis regulation in the SVZ, making neuronal differentiation possible within the niche. The tight regulation of endogenous progenitors in the SVZ is controlled by several factors. Dutton and Bartlett [41] showed that increasing the cell density of cultured SVZ progenitors reduced the frequency of neuronal differentiation. Others noted that bone morphogenic protein (BMP) 2 and 4 signaling in the SVZ inhibited neurogenesis and directed glial differentiation [42]. They proposed that a wide expression of BMP proteins restricts neurogenesis to regions expressing BMP antagonists and that Noggin, expressed by the ependymal cells in the SVZ, antagonizes BMPs, allowing neurogenesis. Additional molecules that regulate adult neurogenesis include vascular endothelial growth factor, pigment epithelium-derived factor, secreted neurotransmitters, and other growth factors, such as BDNF [3]. Although the role of BDNF in SVZ neurogenesis was confirmed in several studies [43–46], a recent report indicated that BDNF signaling is not necessary for endogenous SVZ neurogenesis [47]. In our study, the transplanted MSCs expressed the neurotrophic factor BDNF 3 weeks after transplantation. We hypothesize that secretion of trophic factors may interfere with the regulation of endogenous neurogenesis in the SVZ, which may explain the presented results.

Cell survival following transplantation is an important issue in cell based therapeutic strategies. In our study human bone marrow derived MSCs, transplanted into the SVZ of mice, stimulated endogenous neurogenesis. Furthermore, we showed that the cells survived 3 weeks after transplantation. We hypothesize that the innate immunosuppressive characteristic of the MSCs may elucidate their survival and function. Several studies have reported the in vitro and in vivo immunosuppressive properties of bone marrow-derived MSCs [48–52]. It is reasoned that the transplanted MSCs face a foreign, inflammatory environment and may induce immunomodulating processes that limit local inflammation in order to enhance their survival. The underling mechanisms of action may include the secretion of soluble factors that create an immunosuppressive milieu [53] and the reduction in infiltration of blood-borne inflammatory cells [54]. Recent study present an additional evidence for the hypoimmunogenic property of MSCs implanted into the rat striatum [55].

To conclude, the present study showed that the transplantation of human bone marrow derived MSCs into the SVZ of adult mice stimulates the proliferation and neuronal differentiation of endogenous progenitors within the otherwise restrictive and tightly regulated neurogenic niche. It is possible that soluble factors secreted by the transplanted cells interfere with the regulation of SVZ neurogenesis, thereby allowing for the proliferation and neuronal differentiation of the SVZ residents. However, additional mechanisms, such as stimulation of astrocytes and/or endothelial cells within the SVZ may also be involved. Further studies are needed to evaluate the significance of the enhanced SVZ neurogenesis in brain injuries and diseases.

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Conflict of Interest The authors declare no potential conflicts of interest.

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