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Prevention of seizures and reorganization of hippocampal functions by transplantation of bone marrow cells in the acute phase of experimental epilepsy

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ABSTRACT

In this study, we investigated the therapeutic potential of bone marrow mononuclear cells (BMCs) in a model of epilepsy induced by pilocarpine in rats. BMCs obtained from green fluorescent protein (GFP) transgenic mice or rats were transplanted intravenously after induction of status epilepticus (SE). Spontaneous recurrent seizures (SRS) were monitored using Racine's seizure severity scale. All of the rats in the saline-treated epileptic control group developed SRS, whereas none of the BMC-treated epileptic animals had seizures in the short term (15 days after transplantation), regardless of the BMC source. Over the long-term chronic phase (120 days after transplantation), only 25% of BMC-treated epileptic animals had seizures, but with a lower frequency and duration compared to the epileptic control group. The density of hippocampal neurons in the brains of animals treated with BMCs was markedly preserved. At hippocampal Schaeffer collateral-CA1 synapses, long-term potentiation was preserved in BMC-transplanted rats compared to epileptic controls. The donor-derived GFP^+ cells were rarely found in the brains of transplanted epileptic rats. In conclusion, treatment with BMCs can prevent the development of chronic seizures, reduce neuronal loss, and influence the reorganization of the hippocampal neuronal network.

1. Introduction

Temporal lobe epilepsy (TLE) is the most prevalent form of refractory symptomatic epilepsy.[1] TLE is generally associated with an initial brain insult followed by a variable latent period, which is when epileptogenic processes take place. Subsequently, this process eventually leads to spontaneous recurrent seizures (SRS), i.e., the chronic phase.[2]

At the structural level, temporal lobe epilepsy in humans and in animal models is associated with a loss of neurons in CA1-CA3 areas of the hippocampus, astrogliosis and formation of recurrent connections between mossy fibers.[3,4] Surgical treatment is usually indicated for patients with medically refractory mesial temporal lobe epilepsy, but is limited to those with unilateral hippocampal sclerosis and adequate functional reserves contralateral to the proposed surgical site (see Paglioli).[5,6] Given the high incidence and the limited treatments available for this disease, the development of new therapeutic strategies to decrease or prevent the neuronal alterations associated with epilepsy is of great interest.

Transplantation of stem cells is a potential strategy for the treatment of neurological diseases such as epilepsy. Transplanted cells may have therapeutic effects either by preventing or by suppressing SRS.[7,8] Several reports have also indicated that adult stem cells are pluripotent and generate cell types characteristic of host organs after in vivo transplantation. A number of studies have demonstrated that bone marrow stem cells can differentiate into other cell types, including osteoblasts, adipocytes, chondrocytes, tenocytes, skeletal myocytes,[9] cardiomyocytes,[10] hepatocytes[11] and neural cells.[12–15]

Some studies have examined the therapeutic potential of bone marrow mononuclear cells (BMC) and mesenchymal stromal cells (MSC) in CNS disorders using different experimental models. Recently, in a rodent brain ischemia model, rat BMCs[16–21] and/or MSCs[22–24] were shown to be highly efficient in inducing functional

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neurological recovery. Functional amelioration and remyelinization were also observed in rat models of spinal cord injury and brain trauma.[25,26]

Moreover, neuroprotection could only be observed at low levels and these therapeutic effects seemed to be explained by paracrine actions of the transplanted cells. This therapeutic mechanism has been suggested by studies showing that BMCs stimulate endogenous glial cells or neural stem cells, reduce neuronal apoptosis and neurodegeneration, modulate inflammatory responses and promote self-repair mechanisms in the brain.[16,19,21,27,28] In addition, these cells are able to release many trophic factors and cytokines involved in tissue repair and regeneration.[22,25,29–31]

In this report, we investigated the effects of transplantated BMCs on the epileptogenic process. We examined these effects during early epileptogenesis and during the chronic phase in a model of epilepsy induced by pilocarpine in rats.[32,33]

2. Methods

2.1. Animals

Male Wistar rats (40–45 days of age, 110–130 g) were used to induce epilepsy and also as normal controls. Food and drinking water were available *ad libitum*. EGFP transgenic adult male C57BL/6 mice and male Wistar rats were used as BMC donors. EGFP transgenic mice were bred at the Animal Facility of Gonçalo Moniz Research Center, FIOCRUZ, BA, Brazil. All animal procedures were approved by the Animal Care and Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil. All experiments were conducted in a blinded manner relative to the treatment condition of the animals. Every effort was made to minimize the animal's suffering and to reduce the number of animals used. The "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1996) were strictly followed.

2.2. Induction of SE and epilepsy

For the induction of SE and the subsequent epileptic state, all rats had seizures received lithium chloride (127 mg/kg, i.p.; Sigma–Aldrich, St. Louis, MO). On the following day, methylscopolamine bromide (1 mg/kg, i.p. in 0.9% NaCl) was administered to limit the peripheral effects of the convulsant. SE was induced by injecting pilocarpine (40 mg/kg, i.p.; adapted from Clifford)[33] 30 min later. The severity of convulsions was rated according to Racine's scale,[34] and only those animals that displayed class V behavioral seizures were used in the study. Seizure activity was terminated by diazepam injection (10 mg/kg, i.p.) at 90 min post-onset of SE. The lithium (Li) and pilocarpine (pilo) treated rats with SE are referred to as the epilepsy group, $n = 135$. The normal control group was treated only with saline solution and are referred to as non-epileptic controls, $n = 28$.

2.3. Preparation of bone marrow cells

Bone marrow cells were harvested from EGFP transgenic mice or male Wistar rats. The animals were killed with 200 μl of 8% ketamine hydrochloride (Cristália, Brazil) and 2% chlorpromazine (União Química, Brazil), which was followed by dissection. Fresh bone marrow was extracted from the humerus, femurs and tibiae by flushing with PBS. After centrifugation, the cell pellet was

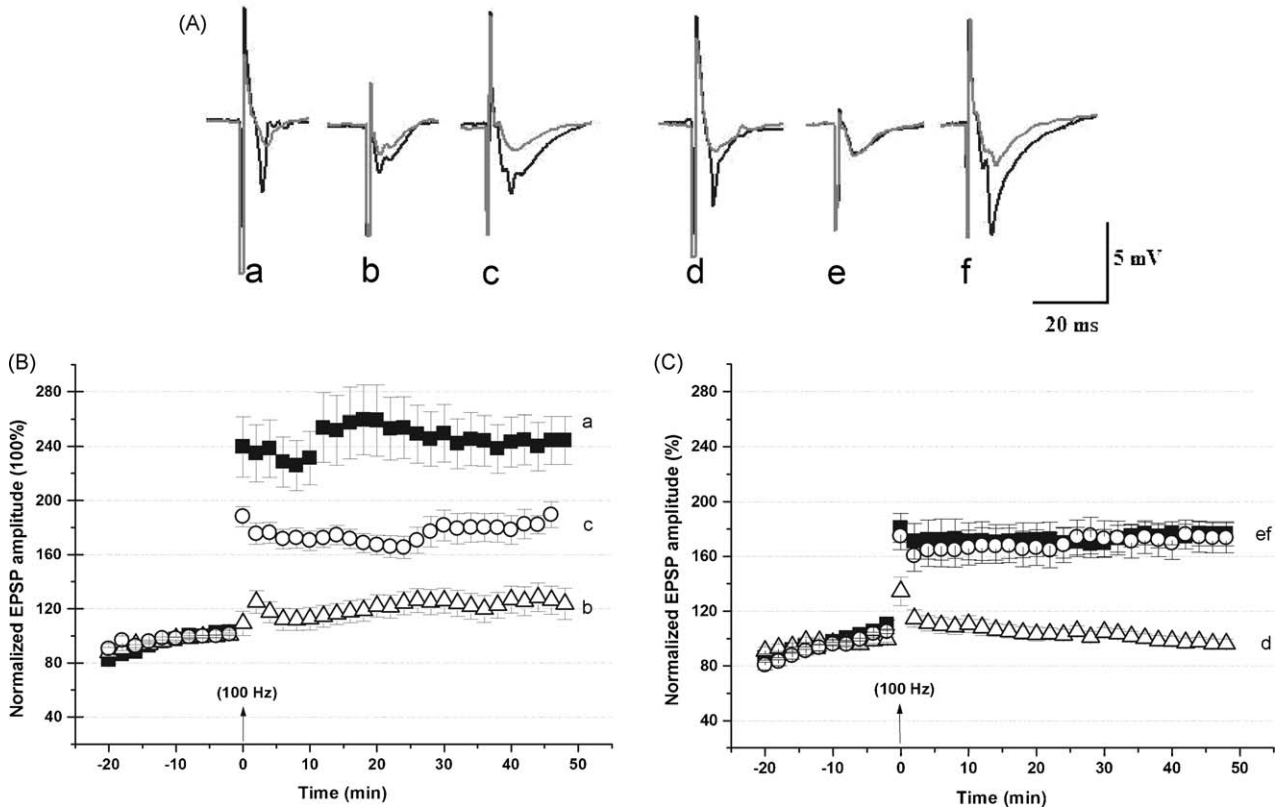

Fig. 1. Electrophysiological properties of brain sections of normal and epileptic rats. Hippocampal slices were prepared from, saline-epileptic, BMC-epileptic and non-epileptic controls at 10 and 120 days after SE. (A) Insets are superimposed representative traces of the averaged fEPSPs before (−20 to −15 min; gray line) and after (44–48 min; dark line) tetanic stimulation in non-epileptic control (a and d), in saline-epileptic (b and e), and BMC-epileptic (c and f) groups. Normalized amplitudes of fEPSPs in non-epileptic control (dark squares), saline-epileptic (open triangles), and BMC-epileptic (open circles) groups killed 10 (B) and 120 (C) days after SE ($n = 9–10$ slices/group). Data are presented as mean ± S.E.M.

resuspended with RPMI medium and fractionated on a density gradient generated by centrifugation over a Ficoll-Hypaque solution (Histopaque 1119 and 1077, 1:1; Sigma, St. Louis, MO) at 400 × *g* for 30 min at room temperature. The mononuclear fraction over the Ficoll-Paque layer was collected and washed twice with PBS. BMCs obtained from rats were incubated with Qtracker (Invitrogen – Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Flow cytometric analysis

For detection of surface antigen, mononuclear cells were washed with saline and incubated at 4 °C for 30 min with conjugated antibodies against murine CD34 PE, CD90 PE, CD11b PE, CD44 PE, CD117 Cy5, CD45 APC and Sca-1 Cy5 (BD Pharmingen, San Diego, CA). Excess antibody was removed by washing. Labeled cells were collected and analyzed using a FACScalibur cytometer (Becton Dickinson, San Diego, CA) with CellQuest software. At least 50,000 data points were collected. The mean ± S.D. for the markers expressed by mononuclear cells were CD34 (4.860 ± 0.43), CD45 (96.36 ± 1.80), CD117 (13.95 ± 0.88), CD11b (60.22 ± 3.30), CD44 (95.61 ± 1.78), CD90 (8.100 ± 1.28), Sca1 (36.06 ± 1.8) and GFP (96.52 ± 0.76).

2.5. Transplantation of BMCs

After injection of diazepam, epileptic animals were randomly assigned to the following groups: BMC-epileptic group receiving transplantation of mouse BMC ($n = 80$) or rat BMC ($n = 5$), saline-epileptic group (was injected with saline solution $n = 50$) and non-epileptic-BMC ($n = 4$, receiving mouse BMC). The BMC suspension was prepared for transplantation in saline at a density of 1×10^7 cells/ml cells in 100 μl of total fluid and was administered by an intravenous route via the tail vein. The same volume of saline was injected into the epileptic control animals.

2.6. Monitoring of spontaneous recurrent seizures

Groups of Li-pilo injected rats were video recorded during different periods for the observation of motor seizure occurrence, duration and severity of SE. The occurrence of recurrent class V seizures according to Racine's scale (showing forelimb clonus, rearing and a loss of righting reflex)[34] was quantified. First, all seizures were observed during handling by direct observation of rats in their home cages. Second, SE animals were video recorded (light-sensitive black and white cameras - Sony, Brazil) from the days 15 through 22 and from days 110 through 117 after SE, for 12 h per day (7.00 a.m. to 7.00 p.m.) to determine their epileptic condition. After the last observation, the animals were submitted to electrophysiological studies and histopathological analyses.

2.7. Electrophysiological studies

2.7.1. Slice preparation

Rats were anesthetized with thiopental (40 mg/kg, i.p.) before decapitation. The brains were rapidly removed and cooled in dissection artificial cerebrospinal fluid containing (in mM): 130 NaCl, 3.5 KCl, 1.3 NaH_2PO_4, 5 Mg^{2+}, 0.2 $CaCl_2$, 10 d-glucose and 24 $NaHCO_3$, which was previously gassed with a 95% O_2 and 5% CO_2 mixture to obtain a pH value of 7.3–7.4. Subsequently, brains were sectioned coronally and glued with cyanoacrylate to the stage of a vibratome (Vibroslice 752M, Campden Instruments, Lafayette, IN, USA). Four-hundred μm thick transversal hemislices were prepared

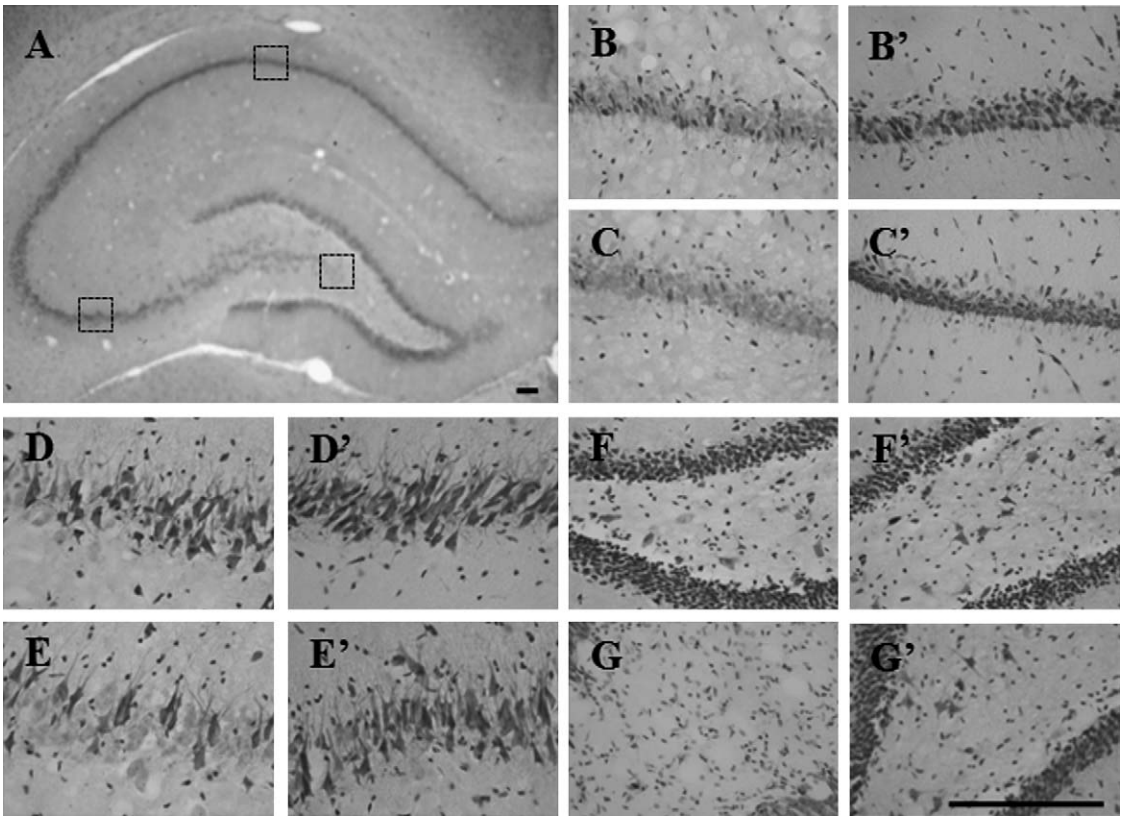

Fig. 2. Effect treatment with bone marrow in the hippocampal cells. Nissl-stained sections showing hippocampal structure [A = in a coronal section of non-epileptic rats, B = CA1; D = CA3; F = dentate gyrus] of epileptic rats and (B′, D′, F′; CA1, CA3 and dentate gyrus, respectively) from BMC-treated rats during epileptogenesis (10 days after transplantation). In the chronic phase (120 days after transplantation) (C = CA1; E = CA3, G = dentate gyrus) of epileptic rats and BMC-treated rats (C′, E′, G′, CA1, CA3 and dentate gyrus, respectively). Scale bar = 100 μm.

from the medial part of the hippocampus and allowed to recover for at least 1 h before being transferred to a submersion-type recording chamber and placed on a nylon net submerged in normal artificial cerebrospinal fluid (in mM: 130 NaCl, 3.5 KCl, 1.3 NaH_2PO_4, 2 Mg^{2+}, 2 $CaCl_2$, 10 d-glucose and 24 $NaHCO_3$, pH 7.4), which was continually gassed with 95% O_2 and 5% CO_2 at room temperature.

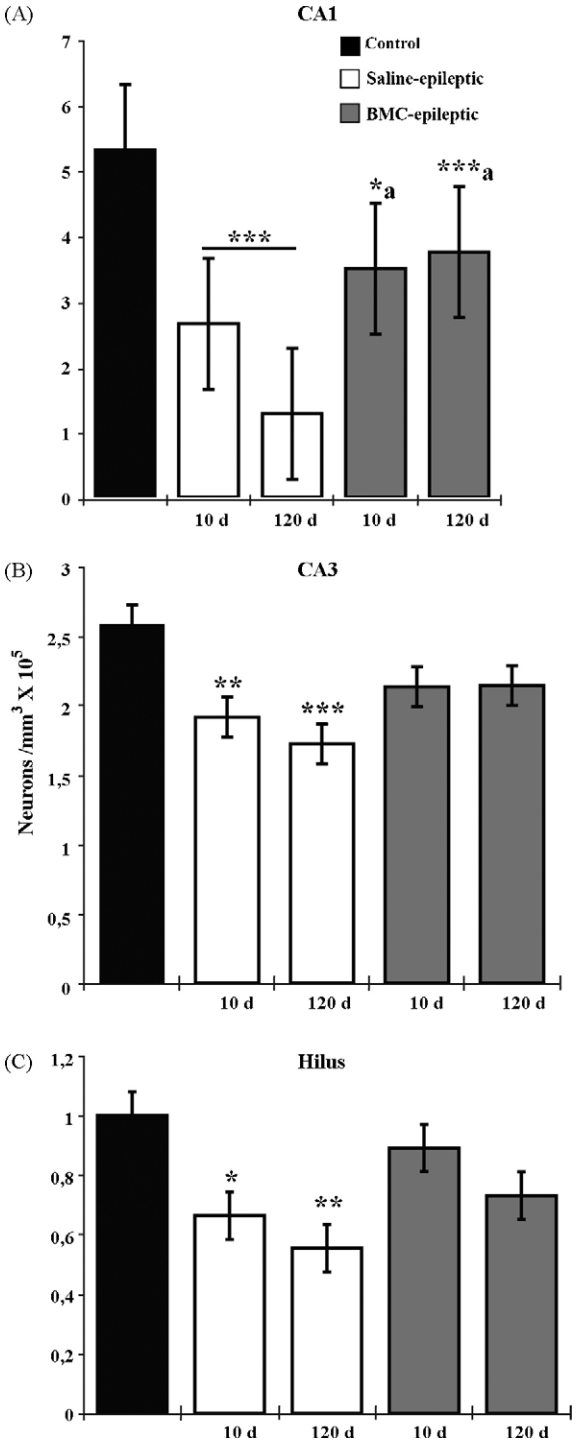

Fig. 3. (A–C) Effect of Li-pilo-induced epilepsy and treatment with bone marrow on cells on neuronal density. Comparisons of neuronal density per cubic millimeter volume of tissue among different hippocampal sections were performed during epileptogenesis (10 days after Li-Pilo) and/or during the chronic phase (120 days after Li-Pilo) in BMC-epileptic, saline-epileptic and non-epileptic controls (2 hemispheres of 5 rats, per group). The groups were compared by one-way ANOVA followed by Tukey *post hoc* test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ indicate significant differences compared to baseline; symbol (a) indicates a significant difference between the BMC-epilepsy and saline-epilepsy groups. AOI = area of interest (800 μm^2). Data are reported as mean ± S.E.M.

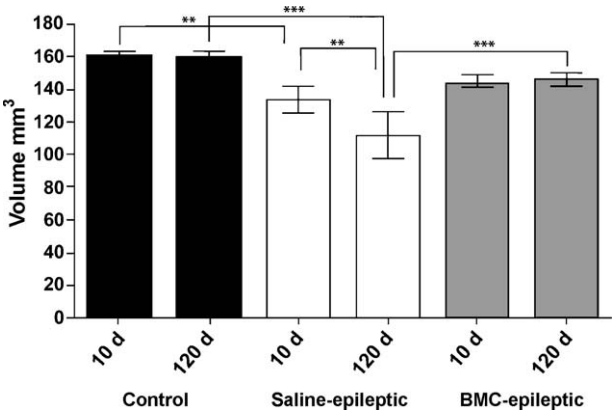

Fig. 4. Effects of BMC treatment on hippocampal volume in Li-pilo-induced epilepsy. The hippocampal volume analyzed (24.000 μm^3) was calculated from 5 rats in each group (non-epileptic control, saline-epileptic, and BMC-epileptic) killed 10 and 120 days after SE. The groups were compared by one-way ANOVA followed by Tukey *post hoc* test. $^{**}p < 0.01$; $^{***}p < 0.001$. Results are expressed as mean ± S.D.

2.7.2. Long-term potentiation (LTP) induction

Field potentials were used in this study to avoid biasing the data based on the activity of a single cell. The Schaffer collateral afferent pathway of the hippocampal slice was stimulated (0.2 ms constant-current pulses every 20 s) using a differential alternating current (Isoflex M.P.I., Israel). The stimulation electrode consisted of a twisted bipolar pair of 75 μm platinum–iridium wires (A-M Systems, Carlsborg, WA, USA). Borosilicate glass capillary electrodes were filled with 0.9% NaCl solution (electrode impedance 2–20 MΩ) and placed in the dendritic region of the CA1 neurons (stratum radiatum). Excitatory postsynaptic potentials (fEPSPs) were recorded using an Axoclamp 1-D amplifier (Axon Instruments, Foster City, CA, USA) connected to a computer. At the beginning of each recording, an input–output (I/O) curve for the fEPSP amplitude relative to the stimulus intensity (increased in 50 μA stepwise manner ranging from 50 to 250 μA) was recorded until fEPSP amplitudes were saturated. This current intensity was adjusted to evoke a baseline fEPSP amplitude that was 50–60% of the maximal fEPSP amplitude obtained in the I/O curve.[35] Baseline responses to 0.05 Hz paired-pulse stimuli (0.2 ms) were recorded for 20 min before long-term potentiation induction. After a stable baseline-evoked response was observed, the high-frequency stimulation protocol was applied (four trains of 1 s duration at 100 Hz, pulse duration of 0.2 ms, with an intertrain interval of 20 s). Field potentials were monitored for at least 60 min after the tetanic stimulus. Evoked fEPSPs were amplified and low-pass filtered at 600 Hz (Cyber Amp 320, Axon Instruments), digitized (Digidata, Axon Instruments) and recorded (Axoscope 9.2, Axon Instruments). The amplitude of the evoked synaptic response was measured by

Table 1
Effects of bone marrow cells on spontaneous seizures of Li-pilo-treated rats.

Treatment	Period after SE (days)	Seizure frequency	Duration of seizure (s)
Saline-epileptic group ($n = 20$)	15–21	1.069 ± 0.65[a]	42.6 ± 6.93
	110–117	1.712 ± 1.20[a]	40.0 ± 7.22
BMC-epileptic group ($n = 20$)	15–21	0	N/A
	110–117	0.096 ± 0.20[a,b]	20.5 ± 5.28

Data are expressed as mean ± S.D.
[a] Spontaneous seizures of class V according to Racine's scale, per day.
[b] $p < 0.0001$ when compared to saline-epilepsy group.

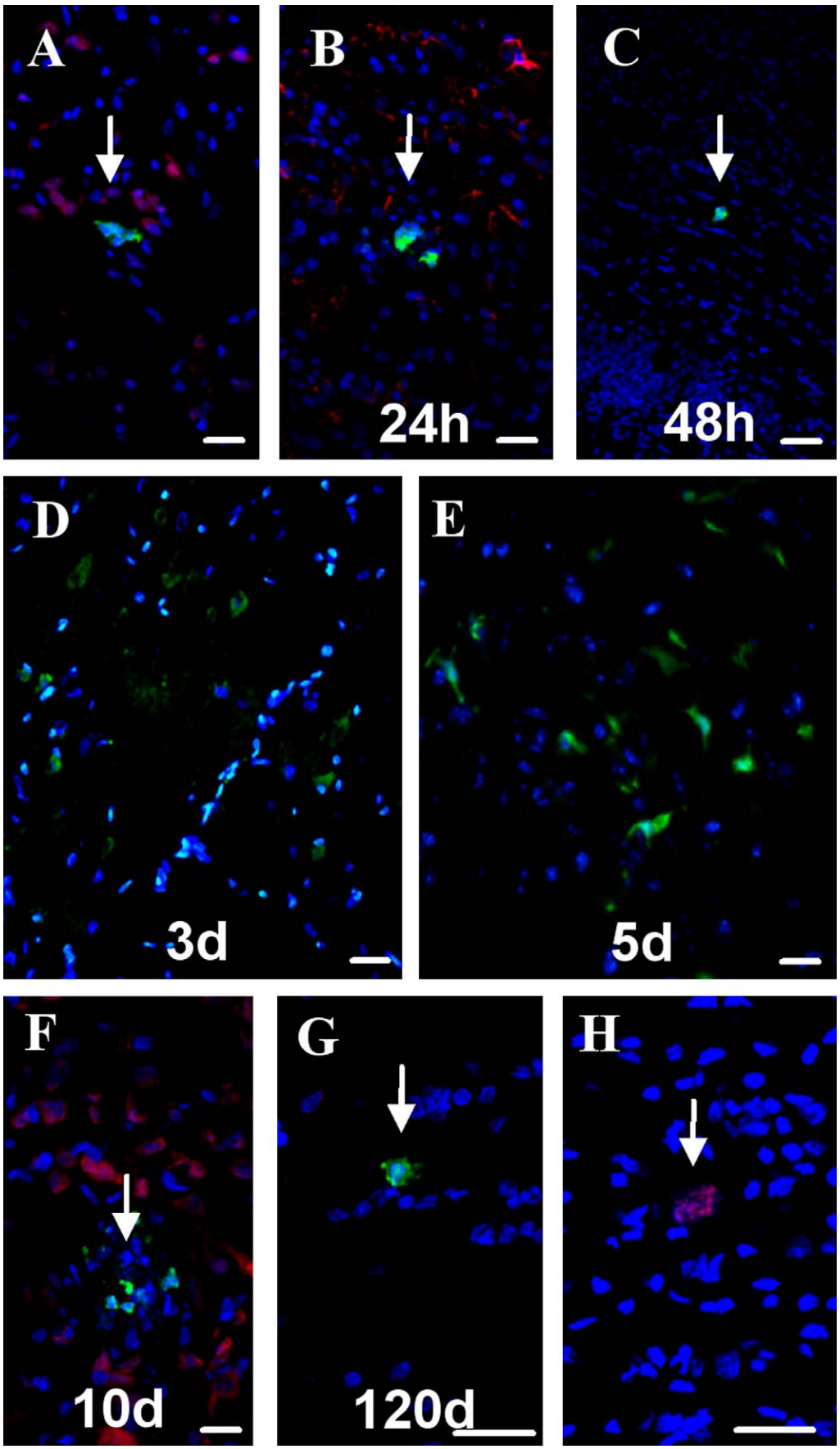

Fig. 5. Presence of donor-derived cells in brains of epileptic rats. Photomicrographs of coronal sections obtained 24 h after transplantation, stained for visualization of $NeuN^+$ (red) and GFP^+ (green) cells in ectorhinal cortex (A), and hilus of GD (B). Section of the stratum radiatum of the hippocampus (C) obtained 48 h after transplantation showing

Clampfit, Axon Instruments.[35,36] Values reported in Fig. 1 were normalized on a per recording basis and then plotted as the mean of 2 min (6 fEPSPS) ± S.E.M. of one to three slices per rat, with 5 rats per group per time point (i.e., 10–15 and 120–127 days after SE).

2.8. Neuronal density estimation

The animals were deeply anesthetized with thiopental (40 mg/kg, i.p.) and transcardially perfused with saline solution followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed from the skulls, post-fixed in the same solution at room temperature for 24 h and cryoprotected by immersion in 30% sucrose solution in phosphate buffer at 4 °C until they sank. The brains were then quickly frozen in isopentane that was previously cooled in liquid nitrogen (−70 °C).

Thirty-μm serial coronal sections obtained using a cryostat (Leica, Germany) were mounted on poly-L-lysine coated slides, stained with a solution of 0.01% cresyl violet and glacial acetic acid 0.01%, washed in distilled water, cleared in a graded series of ethanol and xylene, and coverslipped for observation by light microscopy [objects (20×), Olympus AX 70 photomicroscope coupled to a digital Leica DC 300F camera and Image-Pro Plus Software 6.1 (Media Cybernetics, San Diego, CA, USA)].

Coronal sections from the hippocampus (stratum pyramidale of the CA1 and CA3 subfields) and hilus of the dentate gyrus were identified according to Paxinos and Watson,[37] and neuronal counts were made between the coordinates of Bregma (1.46–3.64 mm). Neurons were identified morphologically by their large, pale nuclei surrounded by dark cytoplasm containing Nissl bodies. Glial cells were identified and excluded from the counts by their relative size and lack of stained cytoplasm. The nucleolus of the neuron was used as the counting marker. Neuronal density (number of neurons/mm^3) was estimated using an adaptation of the optical dissector method.[38] Equidistant coronal sections of the brain were analyzed in the rostral-caudal direction.

In a counting frame or area of interest (AOI), the nucleoli were counted at different focal planes obtained during the course of focusing through the tissue slice (in our case, Z axis = 30 μm). Nucleoli found overlaying the left and upper borders of the counting frame were counted together with the nucleoli located within the analyzed square. In contrast, nucleoli overlaying the right and lower border of the counting frame were not counted.

Neuronal density was estimated using the following formula: $Nv = (1/a{\cdot}h){\cdot}(\sum Q/\sum P)$, where Nv = estimated neuronal density; a = area of counting frame (800.00 μm^2); h = slice height (30 μm); $\sum Q$ = sum of nucleoli counted; $\sum P$ = sum of analyzed counting frames. At least 10 counting frames were analyzed per region in each hippocampus with 5 rats per group per time point.

2.9. Volume estimation

The Cavalieri method[39,40] was used to estimate the volume of the hippocampus. Cross sectional areas of the hippocampus were captured (with 150 μm intervals) using the image analysis system previously described. The hippocampal formation was outlined and measured. The boundaries of these regions were defined in accordance with criteria of Amaral and Witter,[41] whereby the hippocampal formation "hippocampus" was composed of three-layered structures, including the dentate gyrus, the hippocampus proper (CA1, CA2 and CA3 subfields) and the subiculum. The hippocampal volume was calculated by summation of the areas analyzed and multiplication of the distance between the sections (150 μm). At least 10 areas were measured in each analyzed brain.

2.10. Immunofluorescence analysis

Following euthanasia, rat brains were fixed by transcardial perfusion with saline, which was followed by perfusion and immersion in 4% paraformaldehyde. After post-fixation overnight, brains were cryoprotected and frozen in methylbuthane at −80 °C. A series of 20 μm thick sections were obtained, with 9 sections per animal at various levels (100 μm interval) from the BMC-mouse-epileptic group: n = 12, BMC-rat-epileptic group: n = 5, and BMC-control group: n = 3. To detect the distribution of transplanted BMCs in other organs (liver, lung, spleen, and heart), three sections (20 μm thick, 100 μm interval) from each organ were obtained. Sections were incubated with biotinylated anti-neuronal nuclei antibody (NeuN, 1:100, Chemicon International, Temecula, CA, USA), rabbit anti-GFAP (1:400; Dako North America Inc., CA, USA), and chicken anti-GFP (1:400, Aves Labs Inc., Oregon, USA). After incubation overnight, the slices were washing twice for 5 min in PBS Tween 0.05% and in PBS. Sections were incubated with streptavidin-Alexa Fluor 555-conjugated or goat anti-rabbit IgG Alexa Fluor 568-conjugated and goat anti-chicken IgG Alexa Fluor 488-conjugated antibodies (all secondary antibodies were used at a dilution of 1:200, Invitrogen-Molecular Probes, Carlsbad, CA, USA) for 1 h at room temperature. Sections were washed twice in PBS Tween 0.05% and in PBS, dried and coverslipped using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, California, USA).

Brains of the rats transplanted with Qtracker-stained BMC were frozen at −20 °C and 30 μm thick slices of the dorsal hippocampus were fixed in 4% paraformaldehyde and dried and coverslipped using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, California, USA). Negative control sections from each animal received identical treatment, except than the primary antibodies were omitted. The presence of fluorescent cells was determined by observation with a BX61 microscope (Olympus) using appropriate filters or using a confocal microscope (FV 1000 Olympus Inc., Japan). Images were captured using a color digital video SPOT flex camera (15.2, 64 Mp, Shifting Pixel, Diagnostic Instruments Inc., USA).

2.11. Statistical analysis

Data are presented as mean ± S.D. or mean ± S.E.M. as indicated in the figure legends. A two-sided, nonpaired t-test was used to analyze the flow cytometry, as well as differences in SRS end duration. We used one-way analysis of variance (ANOVA), followed by the Tukey Kramer multiple comparisons test to evaluate neuronal density, volume estimation and electrophysiological data. The analyses were done using Origin version 5.0 (Microcal Software Inc., Northampton, MA, USA) and Prism version 4.0 (GraphPad Software, San Diego, CA) software. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Transplantation of BMCs prevents spontaneous seizures in pilocarpine-treated rats

The epileptic rats developed status epilepticus (class V of Racine's scale) with a mean latency of 31.57 ± 10.41 s. The duration

GFP$^+$ cells (green). GFP$^+$ cells (green) shown in sections of the CA1 region (E) and hilus (F) 3 and 5 days after transplantation, respectively. Section of the plexus 10 days after transplantation showing cells expressing microglia markers (F) and the (G) amygdalostriatal transition area showed GFAP$^+$ (red) and GFP$^+$ (green) cells. (H) Section of perirhinal cortex 120 days after transplantation showing GFP$^+$ cells (green). DAPI-stained nuclei are shown in blue. Images (A)–(C) and (G) and (H) were obtained by confocal microscopy, and the micrographs (D–F) by conventional florescence microscopy. Arrows indicate GFP$^+$ cells. Bar = 100 μm.

of SE was monitored for 90 min. Mortality during the 5 days after SE was 30% (24/80) for the BMC-epileptic group; 20% (1/5) for the BMC-rat-epileptic group and 40% (14/50) for the saline-epileptic group. No mortality was observed after this period. A relationship between the latent period duration (i.e., the interval between Li-pilo-induced SE) and the first SRS was not observed. However, 80% of the animals had seizures in the first day of video recording and all animals had SRS during the second day of observation (from days 15 to 21 after SE). All rats in the saline-epileptic group developed SRS, whereas none of the BMC-epileptic group had seizures (Table 1). For evaluation of the chronic phase (120 days post-transplantation), all animals in the saline-epileptic group developed SRS, whereas only 25% of BMC-epilepsy animals did. In addition, this group exhibited a lower seizure frequency and duration compared to the saline-epileptic group ($p < 0.0001$; Table 1).

3.2. Electrophysiological studies and LTP induction

Next, we examined the electrophysiological properties of rat brain sections from the different experimental groups. Stimulation of Schaffer collaterals elicited field excitatory post-synaptic potentials (fEPSPs) in slices prepared from non-epileptic control rats (Fig. 1A-a), whereas this response was attenuated in saline-epileptic animals (Fig. 1A-b) 10 days after SE. An incremental increase in fEPSPs amplitude from BMC-epileptic animals (Fig. 1A-c) was observed compared to saline-epileptic animals. In contrast, 120 days after SE, this response was markedly attenuated in saline-epileptic rats (Fig. 1A-e) and similar fEPSP amplitudes were observed in BMC-epilepsy rats (Fig. 1A-f) compared to non-epileptic control rats (Fig. 1A-d).

We further examined changes in fEPSP amplitude following tetanic stimulation to induce long-term potentiation in slices that showed typical postsynaptic responses (10 days after SE, Fig. 1B). The normalized amplitude obtained from brain slices of saline-epileptic animals (104.1 ± 12.49 mV) was significantly lower compared to non-epileptic controls (213.1 ± 19.61 mV; $p < 0.001$). In contrast, the amplitude was significantly increased in BMC-epileptic rats (180.3 ± 14.25 mV), which was not statistically different when compared to non-epileptic control animals ($p > 0.05$).

In the chronic phase (120 days after SE), the amplitude was significantly reduced in slices obtained from brains of saline-epileptic rats (97.33 ± 5.6 mV) compared to non-epileptic controls (175.2 ± 12.85 mV; $p < 0.001$). In contrast, a significant potentiation could also be detected in the BMC-epilepsy rats (174.4 ± 15.75 mV) compared to non-epileptic controls ($p > 0.05$; Fig. 1C).

3.3. Epilepsy-induced changes in hippocampal volume and cell loss are attenuated by transplantation of BMC

Qualitative analysis of Nissl-stained neurons showed no histological lesions in normal control groups (Fig. 2A). In contrast, there was substantial neuronal loss accompanied by gliosis in the stratum pyramidale of the hippocampal subfields. Some of the remaining neurons appeared shrunken, with pycnotic nuclei in the epileptic rats (Fig. 2B–G). In epileptic rats treated with BMC, neuronal lesions were less extended than in epileptic rats (Fig. 2B′–G′).

As demonstrated by the quantitative analysis 10 days after SE, brain sections from animals of the saline-epileptic group had a severe reduction in the neuronal density of the CA1 subfield of the hippocampus (50.1% when compared to non-epileptic control animals; $p < 0.001$). Although a reduction of 34.3% was also observed in the BMC-epileptic animals compared to non-epileptic rats ($p < 0.00$), the number of neurons in BMC-epilepsy animals was significantly higher than saline-epileptic animals ($p < 0.05$). After 120 days of SE, the most severe damage was seen in saline-epileptic rats, which had a 76.9% decrease in the number of neurons compared to non-epileptic controls ($p < 0.001$), thereby showing a progression of pathologies compared to sections obtained 10 days after SE. In contrast, BMC-epileptic animals had moderate neuronal loss (32.0%) compared to controls, and about 3-fold more neurons than saline-epileptic animals ($p < 0.001$, Fig. 3A).

In the CA3 subfield of the hippocampus, a reduction in neuronal density was observed in the saline-epileptic group when compared with non-epileptic controls 10 days (26.3%; $p < 0.01$) and 120 days (34.1%; $p < 0.001$) after SE. In this region, the differences in neuronal density of BMC-epileptic animals were not statistically different than control animals during both time points analyzed ($p > 0.05$; Fig. 3B). Similarly, a decrease in neuronal density was seen 10 and 120 days after SE in the saline-epileptic group in the hilus of the dentate gyrus (34%, $p < 0.05$ and 45%, $p < 0.01$; respectively). In contrast, no significant differences were found in BMC-epileptic rats compared to control animals for both time points analyzed (Fig. 3C).

Measurements of hippocampal volume revealed decreases in this parameter in saline-epileptic rats 10 and 120 days after SE when compared with non-epileptic controls ($p < 0.01$ and $p < 0.001$, respectively). In addition, a significant decrease was observed in saline-epileptic animals 120 days after SE compared to this group 10 days after SE ($p < 0.01$). When BMC-epileptic rats were compared to non-epileptic controls, no significant differences were found for both time points analyzed ($p > 0.05$). Moreover, a significant difference ($p < 0.001$) was found when BMC-epileptic rats were compared to saline-epileptic rats at 120 days, but not 10 days after SE ($p > 0.05$; Fig. 4).

3.4. Migration of donor-derived BMC to the epileptic brain

To investigate the presence of transplanted BMC in the brains of BMC-epileptic rats, animals were killed 1–3, 5, 10 and 120 days after SE. BMCs survived and were distributed throughout the damaged brain of recipient rats. These BMCs were localized to the cortex and/or hippocampus after transplantation for 24 h (Fig. 5A and B), 48 h (Fig. 5C), 3 and 5 days (Fig. 5D and E, respectively). After 10 and 120 days of transplantation, GFP^+ cells were also found in the perirhinal cortex and basomedial amygdale (Fig. 5F and G, respectively). We did not observe GFP^+ cells co-stained with NeuN or GFAP at any time point analyzed. Bone marrow cells obtained from rats and incubated with a Q-dot tracer were distributed throughout the damaged brain at all time points analyzed. Fig. 5H shows the expressed Q-dot tracer (red) in the 24 h post-transplantated brain. Although donor cells were not found in brains of normal animals transplanted with GFP^+ cells (BMC-control group, $n = 4$), they were found in other organs such as spleen, lung, kidney and heart (data not show). In addition, we did not find any tissue damage or tumor formation in animals transplanted with BMCs, and no systemic complications or increased morbidity were associated with intravenous injections of BMCs in the epileptic animals.

4. Discussion

The present study was designed to test the hypothesis that transplantation of bone marrow cells in the acute phase of epileptogenesis prevents the development of epilepsy in the pilocarpine model. In our study, the pathologies induced by pilocarpine injection indicated that our model is in accordance with other data published.[42] Most importantly, a significant improvement in the behavioral manifestations of animals treated was found in animals submitted to BMC therapy. In the short-term analysis, we observed that animals treated with BMCs did not

develop SRS. Nevertheless, in the long-term analysis, 25% of the animals treated with BMC developed SRS, although with a lower frequency and duration when compared to the epileptic rats (Table 1). These results demonstrate that BMCs administered by an intravenous route after Li-pilo induced SE can suppress the manifestation of early SRS, and significantly reduce epileptogenesis, since only 25% of animals showed less severe seizures 4 months post-SE and after transplantation of BMCs.

The timing of neuronal death induced by pilocarpine SE may be critical. Some studies have reported that 24 h after SE, edema was present in the amygdala, piriform cortex and entorhinal cortex, which was accompanied by extensive neuronal loss, whereas the process was delayed in CA1 and CA3 neurons.[43] These results suggest that the injury of the piriform and entorhinal cortices during SE is predictive of further epilepsy.[44] In this study, we found that BMCs probably exert a neuroprotective effect by preventing the neuronal damage caused by pilocarpine in the hippocampal formation. This effect may be related to the fact that transplantation took place immediately after termination of SE.

Our data show that intravenously injected bone marrow cells from rats or GFP transgenic mice can gain access to the brain of epileptic rats. Also, we have found that this migration occurs as early as one day after intravenous injection. However, the number of cells that enter organ tissue is relatively small in both rats submitted to allogenic or to xenogenic transplantation. Between 1 and 120 days after grafting, we observed that the donor cells were found to be distributed throughout the whole brain. We did not find donor-derived cells expressing the neuronal (NeuN) or astrocyte (GFAP) cell markers.

The mechanisms by which transplanted BMCs induce functional benefits after epileptic induction are not clear. Acute brain insults (e.g., stroke and SE) have an inflammatory component,[45,46] which contributes to the progression of neuropathological events.[47] In addition, we have found in a model of chronic chagasic cardiomyopathy that i.v. transplantation of BMCs decreased inflammation in the heart.[48] Moreover, the intravenous administration of BMCs and the migration of these cells into the injured tissue may provide trophic factors such as the hematopoietic cytokine, which is also a growth factor in the central nervous system.[49] Herein, we also found that some GFP^+ cells were seen in every analyzed brain section from transplanted mice. These data indicate that, despite their minimal retention in the brain, BMCs can cause functional improvement. In support of our work, others have shown functional recovery using a similar therapeutic modality of BMC treatment after brain ischemia,[16–21] or demyelinating injuries of the spinal cord.[50,51]

Integration of newly formed neurons into the excitatory neural circuitry is a pre-requisite for the reconstitution of brain function. The axonal wiring in the mammalian central nervous system is organized in a precise 'point-to-point' manner.[52,53] The entorhinal–hippocampal excitatory projection is the first connection of the trisynaptic circuit, which is suggested to be involved in the formation of learning and memory.[54,55] Previous reports have indicated that LTP is composed of two independent components. These include synaptic and EPSP-to-spike coupling components, as evidenced by an increase in the fEPSP slope and in the population spike amplitude, respectively.[56] In the present study, we found that treatment with BMCs exerted protective effects on LTP since treated epileptic rats had a higher amplitude fEPSP compared to epileptic animals during early epileptogenesis (10 days after SE) and/or during chronic epilepsy (120 days after SE). Interestingly, at this latter time point, no significant differences were noted in the fEPSP slope at the induction of LTP compared to non-epileptic control rats. This suggests an increase in the efficiency of synaptic transmission and/or in the excitability of the postsynaptic neuron after BMC therapy. The successful LTP induction in hippocampal slices supports the idea that the transplanted BMCs participate in the preservation or regeneration of the synaptic circuitry involved with LTP. These results clearly demonstrate that transplantation of BMCs significantly ameliorate the function of synapses in the hippocampus of BMC-epileptic rats.

In conclusion, our results are the first proof of principle for bone marrow cell therapy in epileptic rats. BMCs have several advantages for transplantation. First, autologous BMCs can be used, thus circumventing the problems of host immunity. Second, intravenous transplantation of BMCs may be an efficient therapeutic approach. Third, the use of freshly isolated cells does not require culture expansion. In addition, since epileptogenesis is a dynamic process with severe consequences and cognitive deterioration[57,58]; the time course of disease should be considered for therapeutic cell transplantation in order to prevent additional epileptic dysfunction. Further studies will be essential to elucidate the mechanism through which intravenous transplantation of BMCs promotes functional recovery after status epilepticus.

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References

1. Engel J. Mesial temporal lobe epilepsy: what have we learned? Neuroscientist 2001;7:340–52.
2. Leite P, Garcia-Cairasco N, Cavalheiro EA. New insights from the use of pilocarpine and kainate models. Epilepsy Res 2002;50:93–103.
3. McNamara JO. Cellular and molecular basis of epilepsy. J Neurosci 1994;14:3413–25.
4. Parent JM, Lowenstein DH. Mossy fiber reorganization in the epileptic hippocampus. Curr Opin Neurol 1997;10:103–9.
5. Paglioli E, Palmini A, Paglioli E, daCosta JC, Portuguez M, Martinez JV, et al. Survival analysis of the surgical outcome of temporal lobe epilepsy due to hippocampal sclerosis. Epilepsia 2004;11:1383–91.
6. Paglioli NE, Palmini A, Portuguez M, Paglioli E, Azambuja N, daCosta JC, et al. Seizure and memory outcome following temporal lobe surgery: selective compared with nonselective approaches for hippocampal sclerosis. J Neurosurg 2006;1:70–8.
7. Chu K, Kim M, Jung KH, Jeon D, Lee S-T, Kim J, et al. Human neural stem cell transplantation reduces spontaneous recurrent seizures following pilocarpine-induced status epilepticus in adult rats. Brain Res 2004;1023:213–21.
8. Shetty AK, Hattiangady B. Prospects of stem cell therapy for temporal lobe epilepsy. Stem Cells 2007;25:2396–407.
9. Krampera M, Pizzolo G, Aprili G, Franchini M. Mesenchymal stem cells for bone, cartilage, tendon and skeletal muscle repair. Bone 2006;39:678–83.
10. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson MS, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–5.
11. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002;109:1291–302.
12. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000;290:1779–82.
13. Bonilla S, Alarcón P, Villaverde R, Aparicio P, Silva A, Martínez S. Haematopoietic progenitor cells from adult bone marrow differentiate into cells that express oligodendroglial antigens in the neonatal mouse brain. Eur J Neurosci 2002;15:575–82.
14. Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. Transplanted bone marrow generates new neurons in the human brains. Proc Natl Acad Sci USA 2003;100:1364–9.
15. Bonilla S, Silva A, Valdés L, Geijo E, García-Verdugo JM, Martínez S. Functional neural stem cells derived from adult bone marrow. Neuroscience 2005;133:85–95.
16. Iihoshi S, honmou O, Houkin K, Hashi K, Kocsis JD. A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. Brain Res 2004;1007:1–9.

17. Baker AH, Sica V, Work LM, Williams-Ignarro S, de Nigris F, Lerman LO, et al. Brain protection using autologous bone marrow cell, metalloproteinase inhibitors, and metabolic treatment in cerebral ischemia. Proc Natl Acad Sci EUA 2007;104:3597–602.
18. Kamiya N, Ueda M, Igarashi H, Nishiyama Y, Suda S, Inaba T, et al. Intra-arterial transplantation of bone marrow mononuclear cells immediately after reperfusion decreases brain injury after focal ischemia in rats. Life Sci 2008;83:433–7.
19. Giraldi-Guimarães A, Rezende-Lima M, Bruno FP, Mendez-Otero R. Treatment with bone marrow mononuclear cells induces functional recovery and decreases neurodegeneration after sensorimotor cortical ischemia in rats. Brain Res 2009;1266:108–20.
20. de Vasconcelos, dos Santos A, daCosta Reis J, Diaz Paredes B, Moraes L, Jasmin. et al. Therapeutic window for treatment of cortical ischemia with bone marrow-derived cells in rats. Brain Res 2009;1306:149–58.
21. Brenneman M, Sharma S, Harting M, Strong R, Cox Jr CS, Aronowski J, et al. Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. J Cereb Blood Flow Metab 2010;30(September):140–9.
22. Chopp M, Li Y. Treatment of neural injury with marrow stromal cells. Lancet Neurol 2002;1:92–100.
23. Hu DZ, Zhou LF, Zhu JH. Marrow stromal cells administrated intracisternally to rats after traumatic brain injury migrate into the brain and improve neurological function. Chin Med J 2004;117:1576–8.
24. Liu N, Chen R, Du H, Wang J, Zhang Y, Wen J. Expression of IL-10 and TNF-alpha in rats with cerebral infarction after transplantation with mesenchymal stem cells. Cell Mol Immunol 2009;6(3):207–13.
25. Mezey E. Bone marrow-derived stem cells in neurological diseases: stones or masons? Regenerative Med 2007;2:37–49.
26. Parr AM, Tator CH, Keating A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. Bone Marrow Transplant 2007;40(7):609–19.
27. Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, et al. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. J Neurosci Res 2003;73(6):778–86.
28. Deng YB, Ye WB, Hu ZZ, Yan Y, Wang Y, Takon BF, et al. Intravenously administered BMSCs reduce neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. *Neurol Res* 2009 (May), in press, doi:10.1179/174313209X414434.
29. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369–77.
30. Crain BJ, Tran SD, Mezey E. Transplanted human bone marrow cells generate new brain cells. J Neurol Sci 2005;233:121–3.
31. Shyu WC, Lee YJ, Liu DD, Lin SZ, Li H. Homing genes, cell therapy and stroke. Front Biosci 2006;11:899–907.
32. Leite JP, Cavalheiro EA. Effects of conventional antiepileptic drugs in a model of spontaneous recurrent seizures in rats. Epilepsy Res 1995;20:93–104.
33. Clifford DB, Olney JW, Maniotis A, Collins RC, Zorumski CF. The functional anatomy and pathology of lithium-pilocarpine and high-dose pilocarpine seizures. Neuroscience 1987;23:953–68.
34. Racine RJ. Modification of seizure activity by electrical stimulation. I. After-discharge threshold electroencephalograph. Clin Neurophysiol 1972;32:269–79.
35. Dalbem A, Silveira CV, Pedroso MF, Breda RV, Werne Baes CV, Bartmann AP, et al. Altered distribution of striatal activity-dependent synaptic plasticity in the 3-nitropropionic acid model of Huntington's disease. Brain Res 2005;1047:148–58.
36. Alvares OL, Genro BP, Vaz BR, Pedroso MF, daCosta JC, Quillfeldt JA. AM251, a selective antagonist of the CB1 receptor, inhibits the induction of long-term potentiation and induces retrograde amnesia in rats. Brain Res 2006;1075:60–7.
37. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. New York: Academic; 1998.
38. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984;134:127–36.
39. Rodrigues AL, Arteni NS, Abel C, Zylbersztejn D, Chazan R, Viola G, et al. Tactile stimulation and maternal separation prevent hippocampal damage in rats submitted to neonatal hypoxia-ischemia. Brain Res 2004;1002:94–9.
40. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987;147:229–63.
41. Amaral DG, Witter MP. Hippocampal formation. In: Paxinos G, editor. The rat nervous system. 2nd ed., San Diego: Academic; 1995. p. 443–94.
42. Priel MR, dos Santos NF, Cavalheiro EA. Developmental aspects of the pilocarpine model of epilepsy. Epilepsy Res 1996;26:115–21.
43. Roch C, Leroy C, Nehlig A, Namer IJ. Magnetic resonance imaging in the study of the lithium-pilocarpine model of temporal lobe epilepsy in adult rats. Epilepsia 2002;43:325–35.
44. Roch C, Leroy C, Nehlig A, Namer IJ. Predictive value of cortical injury for development of temporal lobe epilepsy in 21-day-old rats: an MRI approach using the lithium-pilocarpine model. Epilepsia 2002;43:1129–36.
45. Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol 1998;56:149–71.
46. Andersson PB, Perry VH, Gordon S. The kinetics and morphological characteristics of the macrophage-microglial response to kainic acid-induced neuronal degeneration. Neuroscience 1991;42:201–14.
47. Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J Pharmacol Exp Ther 2003;304:1–7.
48. Soares MB, Lima RS, Rocha LL, Takyia CM, Pontes-de-Carvalho L, de Carvalho AC, et al. Transplanted bone marrow cells repair heart tissue and reduce myocarditis in chronic chagasic mice. Am J Pathol 2004;164:441–7.
49. Berezovskaya O, Maysinger D, Fedoroff S. The hematopoietic cytokine, colony-stimulating factor 1, is also a growth factor in the CNS: Congenital absence of CSF-1 in mice results in abnormal microglial response and increased neuron vulnerability to injury. Int J Dev Neurosci 1995;13:285–99.
50. Akiyama y, Radtke C, Honmou O, Kocsis JD. Remyelination of the spinal cord following intravenous delivery of bone marrow cells. Glia 2002;39:229–36.
51. Sasaki M, Honmou O, Akiyama Y, Uede T, Hashi K, Kocsis JD. Transplantation of an acutely isolated bone marrow fraction repairs demyelinated adult rat spinal cord axons. Glia 2001;35:26–34.
52. Clusmann H, Nitsch R, Heinemann U. Long lasting functional alterations in the rat dentate gyrus following entorhinal cortex lesion: a current source density analysis. Neuroscience 1994;61:805–15.
53. Zhou W, Raisman G, Zhou C. Transplanted embryonic entorhinal neurons make functional synapses in adult host hippocampus. Brain Res 1998;788:202–6.
54. Anderson P, Sundberg SH, Sveen O, Swann JW, Wigström H. Possible mechanisms for long-lasting potentiation of synaptic transmission in hippocampal slices from guinea pigs. J Physiol 1980;302:463–82.
55. Izquierdo I, Cammarota M, Da Silva WC, Bevilaqua LR, Rossato JI, Bonini JS, et al. The evidence for hippocampal long-term potentation as a basis of memory for simple tasks. Anais da Academia Brasileira de Ciências 2008;80:115–27.
56. Bliss TV, Lomo T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 1973;232:331–56.
57. Engel J. So what can we conclude – do seizures damage the brain? Progr Brain Res 2002;135:509–12.
58. Engel J. Models of focal epilepsy. Suppl Clin Neurophysiol 2004;57:392–9.